Calmodulin activation of the reductase domain of mammalian neuronal nitric oxide synthase

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To my Mum and Dad and to Evelyn
Acknowledgments

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Abstract

Mammalian neuronal nitric oxide synthase (nNOS) is a homodimeric enzyme that catalyses oxidation of L-Arg to produce the neurotransmitter nitric oxide (NO). Each monomer consists of a reductase domain (nNOSrd), which binds FAD and FMN stoichiometrically, and the oxygenase domain, which contains a P450-like Cys-ligated heme and a tetrahydrobiopterin molecule. nNOSrd is linked to the oxygenase domain of the enzyme by a calmodulin (CaM) binding peptide and acts as the electron supplier during NO synthesis. Within the nNOSrd, the FAD receives electrons from NADPH via hydride transfer and then sequentially passes them to the air stable semiquinone form of the FMN cofactor. Ultimately, the 2-electron reduced FMN hydroquinone transfers one electron at a time to the ferric heme of the adjacent nNOS oxygenase domain. NO synthesis is activated by CaM / Ca\(^{2+}\) binding. This activation is believed to rely on a large scale structural rearrangement that increases the electron transfer from the nNOSrd to the heme and within the reductase domain itself. The substrate (NADP(H)) binding represses the nNOSrd electron transfer by stabilising it in a "locked" conformation that restricts the FMN sub-domain accessibility by the heme. In order investigate the CaM activation mechanism of nNOS, the effect of the conformational changes of nNOSrd and the binding of NADP(H) on the redox potential of the flavins cofactors were assessed for the isolated FAD and FMN sub-domains by OTTLE potentiometry. The results showed that the presence of the FAD/FMN sub-domain interface does not alter the thermodynamic properties of the redox couples involved in the catalysis. This is consistent with the fact that CaM binding has a small effect on the flavins reduction potentials. Only the FMN/FMNH\(^{+}\) redox couple was found to be stabilised by the presence of the FAD sub-domain (increase of 80 mV). The same redox couple was also kinetically stabilised toward oxidation. The isolated FAD sub-domain was found to have similar redox potentials to the isolated nNOSrd. In the presence of NADP\(^{+}\), both the FAD sub-domain and the nNOSrd formed the charge-transfer complex with a long-wavelength absorption band centred at 780 nm. Formation of this complex was found to stabilise the FADH\(^{+}\)/FADH\(^{+}\) redox couple by approx. 30 mV. This small stabilisation is likely to be due to the fraction of bound NADP\(^{+}\) molecules adopting the π-stacked conformation. Therefore, it is possible that in the CaM-free enzyme, the conformation of...
the bound NADP⁺ may control both electron transfer between the FAD and FMN and from FMN to heme by modulating the potential of the FAD hydroquinone. The accessibility of the FMN cofactor to the heme was assessed by comparing the pre-steady-state rates of cytochrome c reduction for the CaM-free and the CaM-bound nNOSrd and for the isolated FMN sub-domain. The results showed that if the FMN, in the isolated FMN domain, is assumed to be fully accessible, then it is 100 % accessible in the CaM-bound enzyme, 45 % accessible in the uncomplexed enzyme and only 3 % accessible in the NADPH-bound nNOSrd in the absence of CaM. This suggests that the binding of CaM is responsible for a structural reorganisation of nNOSrd that “unlocks” the conformation of the enzyme and enables the FMN sub-domain motion in order to shuttle an electron from FAD cofactor to the heme.

The specificity of NADP(H) in repressing the electron transfer from the reductase domain to cytochrome c was studied by using NADP(H) analogues. Results showed that the specificity of NADPH in inducing nNOSrd conformational change relies upon the interaction of both the tightly-bound ADP substituent and the labile nicotinamide substituent and that the tightly-bound ADP substituent is essential to position the nicotinamide moieties for full electron transfer repression. It appears that the “locked” conformation of the enzyme, believed to inhibit the electron transfer to the heme, is specific for the NADP(H).

Comparison of the nNOSrd flavin reduction for a fully oxidised and a one-electron reduced (blue semiquinone form) starting enzyme, in the absence of CaM, showed that the first hydride transfer is faster (≈ 25-fold) for the fully oxidised enzyme compared to the one-electron reduced enzyme. It is interesting to note that the same hydride transfer to the fully oxidised nNOSrd was CaM independent, but that the formation of stable blue semiquinone consecutive to the first hydride transfer was enough to recover CaM dependency. In the fully oxidized nNOSrd, the loss of the hydrogen-bond formed between the FMN semiquinone and the protein backbone is likely to disturb the interaction between the FMN and FAD domains causing the lack of CaM dependency of hydride transfer. In the case of the native nNOS (one-electron reduced), the rate of hydride transfer in the presence of CaM underwent a 20-fold increase.
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### Abbreviations

#### Amino acids

<table>
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<tr>
<th>Alanine</th>
<th>Ala</th>
<th>A</th>
<th>Leucine</th>
<th>Leu</th>
<th>L</th>
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<tbody>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
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<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
<td>Phenylalanine</td>
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<td>F</td>
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<td>C</td>
<td>Proline</td>
<td>Pro</td>
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<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
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<td>Ile</td>
<td>I</td>
<td>Valine</td>
<td>Val</td>
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#### Mutations

Amino acid mutations are represented as a code-number-code, where the first code and number represents the original residue and its position on the polypeptide chain, and the second code represents the corresponding residue in the mutant enzyme. For example, the mutation of a phenylalanine, residue number 1395, to an alanine is represented by Phe1395→Ser, thus generating the mutant F1395S.

#### Kinetic parameters

- $K_m$: Michaelis constant
- $K_d$: Dissociation constant
- $K_i$: Inhibition constant
- $k_{cat}$: Rate constant at saturation
- $k_2$: Second-order rate constant
- $k_{ET}$: Rate constant for electron transfer
- $k_{obs}$: Observed rate constant
- $t$: Time

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### Standard units

<table>
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<tr>
<td>Å</td>
<td>angstrom</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
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### Textual abbreviations

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<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive NOS</td>
</tr>
<tr>
<td>CPR</td>
<td>Cytochrome P450 reductase</td>
</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenolindophenol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial NOS</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mono-nucleotide</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin: NADP⁺ reductase</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>H4B</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nNOS</td>
<td>Nueronal NOS</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOHA</td>
<td>N-Hydroxyl-L-Arginine</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOSFAD</td>
<td>NOS FAD sub-domain</td>
</tr>
<tr>
<td>NOSFMN</td>
<td>NOS FMN sub-domain</td>
</tr>
<tr>
<td>NOSox</td>
<td>NOS oxidised</td>
</tr>
<tr>
<td>NOSrd</td>
<td>NOS reduced</td>
</tr>
<tr>
<td>OTTLE</td>
<td>Optically transparent thin layer electrode</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDZ</td>
<td>Postsynaptic density-95/disks large/zonula occludens-1</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
</tr>
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</table>
Chapter 1

Introduction
1.1 Introduction

Nitric oxide (NO) is a widespread intracellular and intercellular signalling molecule in mammals. It is involved in the regulation of physiological processes including regulatory functions in the central nervous, cardiovascular, and immune systems. It is also found to exert control in platelet inhibition, programmed cell death, and host responses to infection. Its physiological significance was first discovered in 1980 by Robert Furchgott and John Zawadski who were attempting to identify the substance that mediates blood vessel relaxation and regulates the vascular tone. This agent, defined as endothelium-derived relaxing factor (EDRF), was initially assumed to be a protein, like most other neurotransmitters (Furchgott and Zawadski, 1980). However, in 1986, Furchgott (1987) and Louis Ignarro (1986) independently proposed that EDRF was nitric oxide.

The discovery that the gaseous radical NO was produced by living systems and utilized as a genuine intra- and extra-cellular signal molecule generated great interest. In addition to various groups of hormones, signalling peptides and classical neurotransmitters, this distinct class of gaseous species offers unique types of signalling mechanism. The particularity of NO is to be produced as needed without requiring any special storage and transfer mechanism. Because it is a small, neutral molecule, it can diffuse rapidly across cell membranes in all directions and, depending on the conditions, can reach targets located more than several hundred microns away. Being a free radical, NO is chemically very active and its half life can vary from milliseconds to minutes or even hours depending on factors like its concentration, the redox state of the environment and the presence of scavengers. Its unpaired electron makes it suitable for reacting with other radicals and particularly superoxide which can combine with NO to form the highly reactive and toxic radical peroxynitrite, used by immune system. The reactivity of NO makes it a potential effector molecule to kill tumours and pathogens. The combined properties of its ability to regulate enzymes across long distances as well as its high reactivity with other molecules give NO its unique dual role of a powerful signalling molecule and lethal effector molecule.
Chapter 1. Introduction

The NO activity *in vivo* can be separated into two basic categories of "direct" and "indirect" effects. Direct chemical reactions defines the reactions were NO interacts with biological targets. Such reactions are generally rapid and require low concentrations of NO. They account for the majority of the physiological effects of NO with the most common involving its binding to heme-containing proteins like guanylyl cyclase. Indirect effects involve the product of NO and either O₂ or O₂⁻, rather than NO itself. The reactive intermediates that are responsible for indirect effects of NO can undergo various chemical reactions that results in either nitrosative or oxidative stress depending on the species involved. For example, the formation of peroxynitrite, hydrogen peroxide, and dinitrotrioxide all have been linked to cell death through protein nitration and increased mutagenesis (Bonfoco *et al.*, 1995). It appears that NO produced at low concentrations for short periods of time tends to generate direct effects where NO controls vital functions, such as neurotransmission or vascular tone; whereas higher and sustained NO concentrations induce indirect effects used to destroy parasites and tumours cells by inhibiting iron-containing enzymes or by directly interacting with the DNA of these cells. The best established target for NO, when it is produced at low concentration, is guanylyl cyclase. NO binds to heme at the active site of the enzyme, altering its conformation and resulting in the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. Cyclic GMP is a signaling molecule that activates protein kinases. Some examples of the effect of the release of cGMP are the relaxation of smooth muscle via the activation of protein kinase G.

1.2. Nitric oxide synthases

NO is synthesized in mammalian cells by a family of three nitric oxide synthases (NOS) that are known as inducible NOS, endothelial NOS and neuronal NOS. A simple classification of the NOS isoforms associates the NO produced by nNOS and eNOS to the direct effect, whereas iNOS is more involved in the indirect effect. The overall amino acid sequence identity within the three types of NOS is generally well conserved. For example, the three human NOS isoforms share about 55% sequence
homology, with particularly strong sequence conservation noted in regions of the proteins involved in catalysis. The overall catalytic scheme of NO synthesis via oxidation of L-Arginine and the requirement for homodimerization is also shared by the three NOS isoforms. The main difference, in terms of regulation, observed between the 3 different isoforms is that both nNOS and eNOS are expressed constitutively in the cell and are activated by the reversible binding of calmodulin in presence of Ca$^{2+}$, whereas iNOS, most often, is synthesized in the cell in response to inflammatory or pro-inflammatory mediators. The two isoforms eNOS and nNOS are called constitutive NOS (cNOS) to distinguish them from the inducible isoforms (iNOS) that binds CaM irreversibly in immunoactivated cells.

As denoted by its prefix, nNOS was originally purified and cloned from neuronal tissues. However, the expression level of nNOS is, now, known to be greater in skeletal muscle. The inducible type of NOS was originally purified from an immunoactivated macrophage cell line, although, it has since been identified in many different mammalian tissues like cardiac myocytes, glial cells, and vascular smooth muscle cells. Finally, eNOS was the last type of NOS to be isolated. It was originally found in the vascular endothelium, but has since been discovered in cardiac myocytes, blood platelets, brain.

In addition to their fundamental characteristics, NOS isoforms may play entirely distinct biological roles when expressed in different tissues with for example, a differential tissue-specific splicing of nNOS generating distinct proteins when the enzyme is expressed in neurons versus skeletal muscle (Silvagno et al., 1996).

1.2.1 NOS localisation

NO is implicated in a multitude of functions in mammalian organisms. Its beneficial role as a messenger in synaptic transmission or as a host defence molecule to kill target cells such as bacteria or tumour cells can be greatly affected when in excess or deficient. As such, NO can be the source of pathological states as diverse as septic shock, hypertension, stroke, and neurodegenerative diseases. Therefore, the gaseous properties as well as the reactivity of nitric oxide require a severe control in the cells where it is expressed.
Chapter 1. Introduction

Shaul et al., (1996) and Garcia-Cardena et al., (1996) showed that eNOS is localized in small invaginations, termed caveolae, found on the surface of the endothelial cell. This localization is the result of co-and post-translational acylations of the N-terminal region of enzyme. The interactions between eNOS and the caveolae are mediated by membrane proteins called caveolin. eNOS has been shown to bind the caveolin type 1 and 3 in endothelial cells and it appears that this interaction inhibits NO production (Ju et al., 1997). However, the interaction between eNOS and caveolin is reversed in presence of calmodulin suggesting that the intracellular calcium concentration governs eNOS activity by allowing binding of CaM and disposition of caveolin (Michel et al., 1997).

Cellular localization of nNOS also determined whether the effect of NO produced is being detrimental or beneficial for the surrounding environment. Neuronal nitric oxide synthase (nNOS) differs from the two other NOS family members in having an N-terminal segment that presents a PDZ motif (Cho et al., 1992) responsible for the cell membrane targeting of nNOS. The PDZ motif is a 80-120 amino acid protein domain attached to nNOS, that plays a role in targeting the protein to sub-membranous cytoskeleton or in regulating the intrinsic activity of the enzyme. PDZ motifs exist in many different types, most of which are involved in signal transduction events or organisation of cytoskeletal structures, all at or close to the plasma membrane.

In muscle cells, nNOS can be targeted to the dystrophin glycoprotein complex that anchors the cytoskeleton to the plasma membrane called sarcolemma. Membrane association of nNOS in skeletal muscle is mediated by direct interaction with α1-syntrophin, a component of the dystrophin complex. Binding of nNOS to syntrophin occurs through direct interaction between the two PDZ protein motifs present near the NH2-termini of both NOS and the syntrophin protein (Brennan et al., 1995).

In brain, a dystrophin glycoprotein complex is absent but in this case the nNOS PDZ extension targets the enzyme to postsynaptic sites where it specifically interacts with N-methyl-D-aspartic acid (NMDA) receptors (Wang et al., 1995). These mediate the Ca2+ influx into neuronal cells to activate nNOS (Figure 1.1).

Unlike other NOSs isoforms, iNOS is biosynthetically induced, always active and remains predominantly soluble in the cells. NO produced by iNOS exerts its effects
directly or by combining with superoxide to form peroxynitrite, both of which are free radicals that harm proteins and DNA to kill the invaders in case of inflammation. This activity requires massive NO production with an observed turn over rate of 185 min\(^{-1}\) for iNOS compared to 45 min\(^{-1}\) and 20 min\(^{-1}\) for nNOS and eNOS respectively (Roman et al., 2000a; Roman et al., 2000b). In the case of the inducible NOS, NO down-regulates its own production by formation of an inactive iron nitrosyl complex (Abusoud et al., 1995).

**Figure 1.1** Neuronal nitric oxide synthase is associated with the post-synaptic density protein (PSD-95) in the neuronal membrane (www.sigmaaldrich.com). In response to increased intracellular Ca\(^{2+}\), nNOS interacts with CaM. The Ca\(^{2+}\)-CaM complex, in combination with BH\(_4\), binds to nNOS and induces its translocation from the plasma membrane to the cytoplasm. The dephosphorylation of nNOS by calcineurin initiates the production of NO. NO activates guanylyl cyclase (GC) and activates the various cGMP-regulated signaling pathways. nNOS is inactivated on phosphorylation by protein kinase A (PKA) or protein kinase C (PKC) (Wang et al., 1995).
1.2.2 NOS organisation

The oxygenase domain (from amino acid 221 to 724 in nNOS) binds heme, O₂, tetrabiohydropterin (H₄B) and L-Arg to form the active site of the enzyme where the NO synthesis takes place. The N-terminal region of the oxygenase domain varies in length among the NOS isoforms with notably the existence of a PDZ-binding N-terminal extension (from residue 1 to 221) present in nNOS. The C-terminal reductase domain starts at the end of the CaM binding linker and varies in length depending on the isoforms (676 residues in nNOS). The reductase domain binds flavin mononucleotide cofactor (FMN), flavin adenine dinucleotide (FAD) and NADPH from its N-terminal to its C-terminal end respectively. (Figure 1.2)

The role of the reductase domain is to transfer the electrons acquired from NADPH dehydrogenation to the heme of the oxygenase domain in order for it to bind and activate molecular oxygen and, ultimately to generate NO from L-Arg. In the constitutive NOS, the electron transfer through the reductase domain is controlled by the reversible binding of CaM in presence of Ca²⁺, whereas the inducible isoform,
which also requires CaM to be active, binds the CaM irreversibly even in absence of free Ca\(^{2+}\).

### 1.2.3 NOS oxygenase domain

The three-dimensional structure of the oxygenase domains (NOSox) of iNOS (Crane et al., 1997; Fischmann et al., 1999), eNOS (Raman et al., 1998), and nNOS (Li et al., 2003) (Figure 1.3) have already been determined. The core structure, common to all NOS isoforms, is formed by one continuous fold made up of several overlapping or winged L sheets (Crane et al., 1997) that make the binding site for L-Arg, H\(_4\)B, and heme. Active site residues are located throughout the oxygenase domain, rather than being arranged in a linear series of subdomains along the polypeptide sequence. The structures obtained from the three isoforms are very similar to one another but show divergence with the structure of the cytochromes P-450, a large family of related Cys-ligated hemoproteins, in which the heme is surrounded by helical protein elements.

![Figure 1.3 Crystal structure of neuronal NOS oxygenase domain dimer (2x 67kDa) with L-Arg and H\(_4\)B bound, solved at 2.6Å resolution (Li et al., 2002). Both monomers (Green and Blue) contain heme cofactor (Red), L-Arg (Yellow), H\(_4\)B (Magenta) and Zinc (Brown).](image-url)
Previous experiments have shown that the heme iron in a cytokine-inducible NOS (iNOS) monomer is exposed to solvent and as a result is predominantly six-coordinated low spin, which in cytochrome P-450 is associated with a decrease in heme iron reduction potential. The only way, for the full length enzyme to shift heme iron spin equilibrium back towards high spin and thus enable electron transfer by increasing the reduction potential of the heme iron, is to form a homodimer (Ghosh et al., 1996b). Furthermore, it has been suggested that the iNOS subunit dimeric interaction only involves the oxygenase domains of each subunit, with the reductase domain existing as independent monomeric extensions (Ghosh et al., 1995; Ghosh et al., 1996a) (Figure 1.4). Later, Sagami et al (1993) demonstrated using chimeric nNOS dimers that only electrons transferred from the reductase domain of one subunit to the heme of the adjacent subunit mediate NO formation and that dimer formation does not require L-Arg in any of the NOS isoforms (Siddhanta et al., 1998).

Figure 1.4 Diagram of NOS dimerisation (Siddhanta et al., 1998). The two monomer (Light and dark grey) are represented by boxes corresponding to the different subunit of the reductase domain NADPH, FAD, FMN, CaM binding domain. The oxygenase domain contains (L-Arg, Fe, H4B). The red arrows represent the Path of electron transfer in nNOS dimer.
A positively charged surface patch neighbouring the back face of the heme is predicted to be the docking site of the negatively charged cofactor on the exposed face of the FMN binding sub-domain during the transfer of electrons (Crane et al., 1998). This hypothesis was supported by pulse radiolysis experiments on the K423E mutant where Lys423 was suggested to form a key contact in the electron transfer route from the FMN of the reductase domain to the heme of the oxygenase domain (Shimanuki et al., 1999). Molecular movements associated with dimerisation also expose a heme edge on the solvent-accessible convex side of the protein for possible electron transfer.

Each oxygenase domain of the NOS dimer binds one iron protoporphyrin IX per subunit. The iron is bound by Cys419 is thiolate in nNOS and corresponding residues in eNOS and iNOS. In the dimeric enzyme, two identical substrate-binding channels lead L-Arg to the heme pockets. L-Arg binds in an elongated conformation with only its guanidine nitrogens held close to the heme iron, in order for the heme to activate O₂ and to oxidise the substrate (Figure 1.5.13).

The dimer also contains two identical H₄B binding sites. H₄B binds near the dimer interface and is anchored by protein structural elements involved in the dimeric interaction (Figure 1.5.A). This explains why H₄B can promote NOS subunit dimerisation or stabilize an already existing dimeric structure (Stuehr and Abu-Soud, 1997). The H₄B rings are positioned to the side of the heme, almost perpendicular to the heme plane. The residues that participate in the binding of H₄B are also linked to L-Arg binding through an integrated network of hydrogen bonding, which may explain why these molecules bind in a cooperative manner to NOS.

The oxygenase domain of the different NOS isoforms contains eight conserved cysteine residues involved in the heme binding. Although these cysteines are characteristic of the thiol-ligated hemeproteins, their arrangement in NOS differs from cytochrome P-450. The N-termini of all NOSs also contains a highly conserved dual Cys motif that generates a tetrahedral coordination of Zn²⁺ in the enzyme at the interface of the heme dimer (Raman et al., 1998). The crystal structure obtained suggests that the binding of Zn²⁺ stabilises the two monomers with respect to each other, helping the binding of H₄B in the local structure of the dimer.
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Figure 1.5 Substrate and pterin binding sites in iNOS. A, pterin site highlighting some key interactions. The pterin is sandwiched between Trp463 in one subunit and Phe476 in the other. B, interactions between L-Arg and protein functional groups. One guanidinium nitrogen atom is positioned about 4 Å from the heme iron in position for hydroxylation by an iron-linked oxygen atom. (Adapted from Crane et al (1998))
1.2.4 NO Synthesis

Unlike cytochromes P-450, NOSs catalyse a multi-electron, rather than a two-electron, oxidation and therefore perform two separate oxidation cycles, one mono-oxygenase reaction to form N-hydroxy-L-Arginine from L-Arg and the second mono-oxygenase reaction to convert it to NO. The electrons acquired from NADPH dehydrogenation at the FAD site are transferred sequentially via the FMN to the heme. The FMN cycles between the hydroquinone and semiquinone oxidation states during catalysis in order to transfer one electron at time to the oxygenase domain at an optimum rate. In order for the NOS oxygenase domain to convert L-Arg to NO, NOS must generate reactive oxygen for each of the two steps in the reaction (Figure 1.6). During each cycle of NO production, two equivalents of dioxygen and one and a half equivalents of NADPH are consumed.

The first part of the reaction consists of L-Arg hydroxylation to N-Hydroxy- L-Arg (NOHA) (Figure 1.7). To accomplish this mono-oxygenation, a first electron is provided to the ferric heme by the flavins in the presence of molecular oxygen, to form the ferrous-dioxy intermediate. The ferrous-dioxy compound is particularly unstable in NOS and must rapidly incorporate a second electron to form the ferrous-peroxo species required for L-Arg hydroxylation. Adak et al (Adak et al., 2000) demonstrated that the rate of electron transferred from the flavin is too slow to prevent the ferrous dioxy intermediate from breaking down to superoxide and ferric heme. At this stage of the reaction, it has been shown that the electron is supplied by
the pterin co-factor (Wang et al., 2002). The advantage of H\textsubscript{4}B is an about 3–30 times faster rate of electron delivery compared to NOS flavoprotein, and this difference is sufficient to ensure that ferrous dioxy formation stays coupled to NO formation (Wei et al., 2003).

Figure 1.7 Model for NO biosynthesis (Stuehr et al., 2004)

The second mono-oxygenation reaction is a three-electron oxidation that converts the intermediate N-hydroxy-L-Arg to the free radical NO and L-citrulline. The reaction consumes only one electron and, as seen with the first mono-oxygenation, this electron is provided by the flavoprotein to the ferric heme. In the second step, the enzyme uses H\textsubscript{4}B to quickly incorporate second electron to create the oxidant that reacts with bound NOHA. The radical H\textsubscript{4}B\textsuperscript{+} is reduced back in a later stage of the reaction by accepting an electrons from the ferrous-NO complex in order to form the ferric-NO complex and facilitate NO dissociation.

NO binds with high affinity to the heme protein in the ferric or ferrous state. Single turn over (Boggs et al., 2000; Wang et al., 2001) and flash photolysis (Scheele et al., 1999) studies have shown that almost all NO generated binds to the NOS heme before leaving the enzyme. However, at this stage, the ferric heme-NO can either dissociate and release NO or can become reduced by the flavoprotein to form the ferrous heme complex. Because NO dissociation from the ferrous-NO complex is very slow (Ost and Daff, 2005), it instead reacts with dioxygen to generate the ferric...
enzyme and a higher oxide of nitrogen. This catalytic cycle is futile as it does not lead to the production of NO (Santolini et al., 2001b) (Figure 1.8).

\[
\begin{align*}
\text{Fe}^{3+} & \xrightarrow{k_r} \text{Fe}^{2+} + \text{O}_2 \\
\text{Arg, } k_{\text{cat}1} & \quad \text{NOHA, } k_{\text{cat}2} \\
\text{Fe}^{3+} + \text{NO} + \text{Citrulline} & \quad \text{Fe}^{2+} + \text{NO} \\
\text{kd} & \quad e^- \\
\text{kd} & \quad e^- \\
\text{NO}_3^- & \quad k_{\text{ox}} \\
\text{O}_2 & \quad k_{r'} \\
\text{Fe}^{2+} + \text{NO} & \quad \text{extremely slow} \\
\text{NO} & \quad \text{NO}
\end{align*}
\]

**Figure 1.8** Kinetic model for NOS catalysis. The NOS ferric heme binds newly formed NO before it can leave the enzyme active site. Depending on how fast NO is released, NOS catalysis can produce NO (Productive cycle) or release an oxidised form of NO (Futile cycle) (Stuehr et al., 2004)

The most representative structural and functional difference between cytochrome P-450 and the NOS oxygenase domain is probably the presence of the pterin co-factor in NOSs. The binding of H_{4}B to NOS has been demonstrated to be essential to reach the full catalytic activity of the enzyme (Gorren et al., 1996). The NOS enzyme has evolved towards the use of H_{4}B that not only prevents the consumption of NO in the futile cycle by maintaining an appropriate ferric heme reduction rate, but also to minimize the uncoupled consumption of NADPH by conducting efficient oxygen activation.
nNOSrд belongs to a small family of structurally related dual-flavin reductases that also includes cytochrome P450 reductase (Bredt et al., 1991), methionine reductase (Olteanu and Banerjee, 2001) and novel reductase-1 (Paine et al., 2000). These proteins are made of the juxtaposition of the FMN and FAD/NADPH modules attached by a flexible hinge region (Garcin et al., 2004). It is believed that these reductases are the product of gene fusion because their FMN and FAD/NADPH subdomains show a high similarity to flavodoxins (Watenpaugh et al., 1973) and ferredoxin NADP⁺ reductases (Karplus et al., 1991), respectively. The N-terminal part of the reductase domain is linked to the oxygenase domain through an approximately 20 amino acids peptide that binds CaM in presence of Ca²⁺ whereas, the C-terminal end binds NADPH (Figure 1.9).

Figure 1.9 Crystal structure of nNOS reductase domain solved at 2.3Å. The NADPH/FAD binding domain (Blue) contains the NADP cofactor (Magenta), FAD cofactor (Yellow) and the C-terminal extension (Red). The FMN-binding domain (Green) contains the FMN cofactor (Orange), the inhibitory insert (Black). The CaM linker extends from the N-terminal extremity but is not observed. Adapted from Garcin et al (2004).
Figure 1.10 Redox states of the flavin ring. The top line shows the oxidised (quinone) form, the middle line the semiquinone and the bottom line the reduced (quinol) form. The proton equilibria are shown from left to right, pKₐ values for deprotonations are shown. In the semiquinone form, the neutral and anionic forms are shown in blue and red respectively. The midpoint reduction potential for the two-electron reduction of the non-protein coordinated flavin is –200 mV. However, this value can vary greatly in flavoenzymes from –400 mV to +60 mV (Fraaije et al., 1999; Ghisla and Massey, 1989). This is an indication of the ability of the protein to modulate the reduction potential via the presence or absence of nearby charges, and the degree of solvent accessibility of the flavin (Ghisla and Massey, 1989). In the case of nNOS, the flavins are tightly bound to the protein through an extensive network of hydrogen bond and hydrophobic stacking that favour depending on the redox state at pH 7.5 the neutral oxidised form, the blue semiquinone form and the anionic reduced form (Represented in the dashed black boxes). A recurrent feature of flavoenzymes is the situation of the flavin N5 ~3.5 Å from the site of either hydrogenation or dehydrogenation (Fraaije and Mattevi, 2000). In nNOS, the protein backbone (Gly 810) stabilises the protonated N5 of FMN cofactor to prevent it from oxidising in normal enzyme turnover.

The physiological role of the reductase domain is to transfer electrons acquired from NADPH-dehydrogenation to the heme in order to catalyse NO synthesis. The characteristic diflavin system allows a two-electron donor (NADPH) to donate
electrons to a one-electron acceptor (heme), by forming stable semiquinone radical intermediates (Figure 1.10). Characterisation of FMN-deficient mutants of nNOS reveals that the electrons are transferred from NADPH to FAD and then to FMN cofactor (Daff et al., 1999). The pair of electrons derived from NADPH dehydrogenation at the FAD is sequentially transferred, via the FMN domain, to the heme. The FMN oscillates between the hydroquinone and semiquinone oxidation states during catalysis and functions as a single electron donor (Masters, 1994). The FMN to heme electron transfer appears to be the critical rate determining event for NO synthesis and is also the step activated by CaM (Abusoud and Stuehr, 1993).

1.3.1 External electron acceptors

Cytochrome c and ferricyanide reduction are both commonly used tools to assess and compare CaM activation of the electron transfer in isolated reductase domain (nNOSrd) and native nNOS. Ferricyanide is a one-electron acceptor that shunts the electron flow between FAD and FMN cofactors. Because of its small size, it is freely accessible to the FAD cofactor and is used to assess the rate of FAD reduction by NADPH. Cytochrome c is a one-electron acceptor that can only reacts with the FMN cofactor due to charge repulsion with FAD domain. (Klatt et al., 1992; Sheta et al., 1994). This catalytic activity was demonstrated to be activated on CaM binding, to a similar extent, for either native nNOS or isolated reductase domain (Gachhui et al., 1996b). Matsuda and Iyanagi (1999) showed that cytochrome c reacts only very slowly with the air stable FMN semiquinone and that the electron transfer to cytochrome c occurs from the FMN hydroquinone. This transfer of an electron is essentially unidirectional. During the catalytic turnover with NADPH and cytochrome c, the FMN alternates between the hydroquinone and semiquinone redox states, receiving electrons from FAD and passing them to cytochrome c (Scheme 1.1). Because cytochrome c is a protein of over hundred amino acids, its access is restricted to the FMN cofactor for the enzyme in its locked conformation (Craig et al., 2002). Therefore, the rate of cytochrome c reduction can be used as a probe for FMN accessibility and enzyme conformation.
Scheme 1.1 Cytochrome c reduction by nNOSrd. It involves hydride transfer from NADPH to FAD cofactor (A→B) and subsequent interflavin one-electron transfer (B→C & D→E). The hydroquinone form of the FMN cofactor donates electrons, one at time, to perform cytochrome c reduction (C→D & E→A) (Craig et al., 2002). Chemically reduced nNOSrd (F) can also be used to study cytochrome c reduction. CaM-binding to nNOSrd increases the turn over rate of cytochrome c reduction (Gachhui et al., 1996b).

1.3.2 Structure of nNOSrd

Recently the structure of NADP-bound nNOSrd, solved at 2.3Å resolution, was published by Garcin et al (2004). The structure displays nNOS FAD- and FMN-binding domain with NADP bound, the C-terminal extension and autoinhibitory insert, but no CaM binding linker. Although the lack of crystallographic data for the CaM binding region prevents a full picture of the electron-transfer regulating-element, considerable clarification of the conformation of the bound-NADP and FAD/FMN interface is brought.
1.3.2.1 FAD/NADP binding domain

The FAD sub-domain of NOS (NOSFAD) catalyses the reverse reaction of ferredoxin-NADPH reductase, by accepting two electrons from NADPH in order to reduce the oxygenase domain. The N-terminal extremity of NOSFAD is linked via a hinged region to NOSFMN and to the NADPH binding domain on its C-terminal extremity.

![Flavin Mononucleotide (FMN) and Flavin Adenine Dinucleotide (FAD)](image)

**Figure 1.11** Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). In orange is represented the isoalloxazine ring system that is shared by both flavins. It is responsible for the flavins redox properties. The two flavin forms differ in the identity of the side chain.

NOSFAD binds the FAD cofactor (Figure 1.11) in an elongated conformation and it is held tightly to the protein through hydrophobic stacking with Phe1395 at its re-face, Tyr1175 at the si-face and by an extensive network of hydrogen bonds. A water molecule might play the role of a general acid/base in the protonation/deprotonation of the N1 atom of the flavin in NOS and is also proposed to be important for catalysis in CPR (Hubbard *et al.*, 2001) (Figure 1.12). FAD N5 makes a long hydrogen bond (3.6Å) with the hydroxyl side chain of Ser1176. This H-bonding network enhances the stabilisation of the one-electron reduced form of the FAD in order to facilitate the transfer of one electron at time to the FMN semiquinone (Hubbard *et al.*, 2001). The exposure of the xylene ring side of FAD to the surface of
the molecule and the strategic location of NOSFAD within the reductase domain enables the FAD cofactor to directly react with both NADPH and FMN cofactors.

Figure 1.12 Residues in the vicinity of the FAD isooalloxazine ring (Zhang et al., 2001).

Figure 1.13 On the left is represented the NADP$^+$ molecule (oxidised substrate). On the right, the nicotinamide is the redox active part in the NADP(H).
The substrate NADPH (Figure 1.13) is bound to the NADPH binding domain through an extensive network of hydrogen bonds that anchor the negatively charged phosphate group of its ADP substituent to the protein. Once bound, NADPH is in equilibrium between productive and unproductive conformations (Figure 1.14). In the productive conformation, the nicotinamide ring of NADPH, after displacing the FAD shielding residue (Phe1395), stackes with the isoalloxazine ring in order to hydride transfer to the FAD. The non-productive conformations are all the other conformations where NADP is not stacked and cannot react with FAD. Because the nicotinamide and Phe1395 stacking with the FAD are mutually exclusive, the energy necessary for one to displace the other controls the ratio of productive and non-productive conformations of the NADPH and therefore regulates the inlet of electrons into the NOS reductase domain. Although molecular modelling to determine the percentage time of NADPH in the different conformations hasn’t been done yet, the difference spectra at 510 nm between the NADP⁺-bound and free-forms for the mutant F1395S and wild type determines ≈ 35 % of the NADP⁺ bound to the wild type nNOSrd in the productive conformation.

A particularity of the Phe1395 residue is to be located just prior to an additional C-terminal domain (21 to 42 amino acids in NOSs) that forms a regulatory element characteristic of NOS (Roman et al., 2000b). Crystallographic data has shown that the C-terminal extension extends from the end of the NADP-binding site and forms a right angle turn at Arg1400 followed by an α-helix. It is interesting to note that Arg1400 interacts with NADP via a salt bridge, and also that the entire C-terminal tail interacts with the NADP-binding and FMN-binding domains. Thus, the displacement of Phe1395 on nicotinamide stacking is likely to affect the position of the C-terminal extension and, by consequence, the conformation of the reductase domain. The presence of a phosphorylation site (Ser1412) also suggests that interactions between the reductase domain and the C-terminal tail can be disrupted on phosphate binding as part a regulatory mechanism of the enzyme (Roman et al., 2000b).
Figure 1.14 The active site of nNOSFAD (A) (Zhang et al., 2001) and ferredoxin reductase Y308S mutant (B) (Deng et al., 1999) with NADP⁺ bound in unproductive and productive conformations respectively.

1.3.2.2 FMN-binding domain

The FMN domain is linked to the FAD domain via its C-terminal end and to the oxygenase domain via the CaM binding region at its N-terminal. The role of the FMN domain is to shuttle electrons from NOSFAD, one at a time and at an optimum rate, to the oxygenase domain in the native enzyme. The FMN domain contains FMN cofactor (Figure 1.10) that is tightly bound and quasi-buried in a pocket formed by the protein. Two residues, Tyr889 and Phe809, control the reactivity of the cofactor by stacking each side of the isoalloxazine ring. The N5 of the FMN cofactor forms an hydrogen bond (3.6Å) with the peptide carbonyl of Gly810 (Figure 1.15). This structural characteristic may stabilise the semiquinone form and prevents the full oxidation of the cofactor. The FMN cofactor can only transfer one electron at time by oscillating between its semiquinone and hydroquinone redox states.

The crystallographic data demonstrates that the FAD/FMN interface is created by the interaction between the positively charged concave face of NOSFAD and the negatively charged exposed face of NOSFMN. In this conformation, the two cofactors are completely buried inside the protein. At the centre of this interface, the FAD- and FMN-binding domain interact through a double salt bridge between
residues Glu762, Glu816 and Arg1229 that are conserved in cNOSs, and also hydrophobic contacts and hydrogen bonds. The connecting domain located on the top of the FAD/FMN domain holds the two domains together in order to align the two flavin cofactors. The 4.8Å distance that separates the FAD and FMN cofactors in this conformation suggests that direct electron transfer is possible.

![Figure 1.15](image)

**Figure 1.15** Tyr889 and Phe809, located within the FMN, domain sandwich the FMN cofactor by stacking each side of the isoalloxazine ring (Garcin *et al.*, 2004). The peptide carbonyl of Gly810 forms a 3.6Å length hydrogen bond with N5 of the FMN isoalloxazine.

*In vivo*, all NOSs are active as dimers. Dimerisation is primarily mediated through the interaction of NOSox and electron transfer operates *in trans*, from the NOSrd of one subunit to the NOSox of the other subunit. Modelling experiments have shown that according to the length of the CaM binding region that links NOSrd and NOSox, a conceivable 70Å could separate the FMN cofactor, in its electron accepting position, from the heme. In order for the electron to move such a distance, the FMN domain would have to swing back and forth between an electron accepting position and an electron donating position (Garcin *et al.*, 2004). Conformation movement of NOSFMN could bring the electron within 15Å of the heme and enable direct electron transfer. Mutation studies on a sequence conserved patch of positively charged residues close to the back face of the heme reinforces the hypothesis of a direct interaction between the NOSFMN and NOSox (Adak *et al.*, 1999; Crane *et al.*, 1999; Kobayashi *et al.*, 2001). The CaM-binding region (20-25 amino acids) located at the N-terminal end of NOSFMN binds CaM in presence of Ca$^{2+}$. The binding of CaM reorients NOSrd with respect to NOSox to enable efficient inter-domain electron transfer.
transfer. In eNOS, the presence of an autoinhibitory insert (42-45 amino acid) within NOSFMN interferes with CaM binding as part of regulation of electron transfer to the heme. Crystallographic data shows that this insert is sequestered into a hydrophobic pocket formed between the FMN-binding domain and the NADPH binding domain. This suggests that any conformational change of the insert induced on CaM binding can affect the conformation of the whole NOSrd and its subsequent ability to transfer electrons.

The electron transfer from the electron source NADPH to the heme in all NOSs is thought to be gated by the rate limiting conformation change of NOSFMN. In eNOSs, the binding of CaM regulates the mobility of the FMN domain possibly by interacting with the C-terminal tail and NOSFMN insert.

1.3.4 Regulatory effect of the control elements

NOSrd presents additional complexities with respect to its electron transfer mechanism that are not found in the related flavoprotein cytochrome P450 reductase (CPR). Besides CaM binding, other components involved in controlling NOSrd electron transfer include the C-terminal extension (Roman et al., 2000a; Roman et al., 2000b), an auto-inhibitory insert on the FMN domain (Chen and Wu, 2000; Daff et al., 1999; Montgomery et al., 2000; Nishida and de Montellano, 2001; Salerno et al., 1997b) and a kinase dependent phosphorylation site (Adak et al., 2001; Butt et al., 2000; Fulton et al., 1999; Hayashi et al., 1999). Independent mutations of each of these control elements have shown that they all contribute to impede the electron transfer into, within and out of the reductase domain in the CaM-free enzyme and that the binding of CaM relieves this electron transfer repression.
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1.4 NOSrd control elements

1.4.1 Calmodulin binding

The putative CaM-binding region in NOS isozymes, encompassing 20 – 25 amino acids is located between the NOS oxygenase and reductase domains. It was first identified based on primary and secondary structure analyses (Bredt et al., 1991; Stuehr et al., 1991; Xie et al., 1992). This local affinity of the NOS protein for CaM was further demonstrated by Sheta et al (1994), who showed, using limited trypsinolysis, that the full length NOS possesses one site of cleavage at the junction between the oxygenase and reductase domain that is protected on CaM binding. The CaM linker of NOS possesses conserved hydrophobic residues which are spaced according to the classical Ca$^{2+}$-dependent “1-5-8-14” canonical motif (Rhoads and Friedberg, 1997).

1.4.1.1 Calmodulin description

The most commonly used calmodulin in the study of NOSs is the synthetic form of the bovine brain CaM (Newton et al., 1998). It is a polypeptide which is constituted of a chain of 148 amino acids leading to a mass of 16706.6 Da (Shirran et al., 2005). It is a ubiquitous, small, acidic protein that is predominantly deprotonated in physiological conditions, pH range 6-8. Apo CaM is comprised of two globular domains connected by a flexible linker (Figure 1.16). Each domain consist of two helix - loop - helix Ca$^{2+}$ binding regions referred to as EF hand structures (Vandonselaar et al., 1994). Upon exposure to calcium, CaM undergoes a major structural change; the two anti parallel helices in each EF hand of apo CaM become perpendicular. The resultant movement exposes methionine-rich hydrophobic surfaces that form critical Van der Waals interactions with the hydrophobic face of the target recognition site (Ikura et al., 1992; Meador et al., 1992) (Figure 1.17).
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Figure 1.16 Crystal structure of recombinant *Paramecium tetraurelia* calmodulin at 1.68 Å resolution. Unbound calmodulin showing the four Ca$^{2+}$ ions in green (1osa.pdb) (Ban et al., 1994).

Figure 1.17 Multidimensional NMR structure of Calmodulin (*Drosophila melanogaster*) complexed with rabbit skeletal myosin light chain kinase (calmodulin-binding domain) (cyan). (2bbn.pdb) The four Ca$^{2+}$ ions are green. (Ikura et al., 1992)

1.4.1.2 NOSs/CaM interaction

The requirement for calmodulin in NO synthesis is an essential characteristic of all the three NOS isoforms. However, each type of NOS binds CaM with important...
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differences in Ca\textsuperscript{2+} dependency. The affinity of CaM toward peptides derived from the CaM binding site of the different NOS isoforms follows a general order of iNOS $\gg$ eNOS $\gg$ nNOS with the respective $K_D$ values of 0.1; 4 and 1-2 nM. However, the Ca\textsuperscript{2+} dependence of NO synthesis is 300 and 150 nM for nNOS and eNOS respectively and that iNOS is independent of free Ca\textsuperscript{2+} (Cho et al., 1992). In fact, in iNOS, the binding of CaM occurs at intracellular Ca\textsuperscript{2+} levels and is essentially irreversible without destruction of the enzyme (Venema et al., 1996), whereas only the 4 Ca\textsuperscript{2+}/Calmodulin complex binds to cNOS (Shirran et al., 2005). CaM is constituted of two globular domains or lobes which can bind two Ca\textsuperscript{2+} each. Spectroscopic studies performed by Zhang et al (1995) demonstrated that the nNOS-derived peptide binds in an antiparallel manner (the peptide N-terminus interacts with the CaM C-terminal lobe; the peptide C-terminus interacts with the CaM N-terminal lobe). Another characteristic of CaM is that the C-terminal lobe displays a 6-fold higher affinity toward Ca\textsuperscript{2+} than its C-terminal homologue and that CaM can bind the constitutive NOS CaM linker with zero, one, or two globular domains (Linse et al., 1991). Persechini et al (1996) demonstrated that in nNOS, the C-terminal globular domain of CaM binds first, probably when the Ca\textsuperscript{2+} concentration is low. The C-terminal globular domain of CaM is essential for the nNOS/CaM complex stabilisation, but cannot activate the enzyme. Activation of the enzyme relies on the binding of the N-terminal globular domain.

Peptide swapping experiments where the CaM binding sequences of eNOS or nNOS were replaced by the one of iNOS still displayed a Ca\textsuperscript{2+} dependence for CaM binding indicating that the Ca\textsuperscript{2+}-dependence of CaM binding doesn’t only rely on the peptide sequence. The Ca\textsuperscript{2+} insensitivity characteristic of iNOS must be conferred by several disparate structural features throughout the protein (Lee et al., 2000).

1.4.1.3 Calmodulin activation of NOS

CaM binding has been demonstrated to be required for triggering heme reduction by the NADPH reduced flavin domain Abu-Soud and Stuehr (1993). In the absence of CaM, nNOS is able to catalyse only slow NADPH oxidation (13.6 nmol/min per mg). This consumption of NADPH is heme independent reduction of O$_2$ via the reductase domain alone. In contrast, on CaM binding, the rate of NADPH oxidation
increases by 60-fold (906 nmol/min per mg) corresponding to an optimum rate of heme reduction essential to a normal rate of NO production (660 nmol/min per mg) when L-Arg is present. Interestingly, in the absence of L-Arg, the consumption of NADPH by CaM bound nNOS drives the heme-catalysed reduction of \( \text{O}_2 \) to superoxide (1450 nmol/min per mg). Thus CaM activates electron transfer in the neuronal isoform independently of L-Arg binding.

The observed effect of CaM binding in triggering NO production in nNOS was demonstrated by Abu-Soud et al. (1994a). CaM was also showed to be a direct activator of the electron transfer in the isolated reductase domain (Gachhui et al. (1996a)). The recombinant nNOSrd, expressed in yeast, was similar to the native NOS with respect to flavin content, fluorescence properties, stabilization of a flavin semiquinone radical, kinetics of flavin reduction by NADPH, and rates of electron transfer to cytochrome \( \text{c} \) and ferricyanide in the presence or absence of bound CaM, indicating that CaM action is independent of electron transfer to the oxygenase domain. The exception was that the reductase domain alone does not catalyse the reduction of \( \text{O}_2 \) or the production of NO consistent with the fact that these reactions require NOS heme iron to be present. On CaM binding, the same increase in protein tryptophan and flavin fluorescence could be monitored in the isolated NOS reductase domain as in full-length nNOS. This indicates that the fluorescence increases observed in the native enzyme are not due to a repositioning of the reductase domain relative to the oxygenase domain or of the flavins relative to the heme but instead is the result of structural changes within the reductase domain itself. This was the first evidence that calmodulin binding to the nNOS reductase domain causes a conformational change that opens up the domain structure. The activation role of calmodulin in NO production seems to be the consequence of an inter-domain conformation change that facilitates the electron transfer from the reductase domain to the oxygenase domain and also the consequence of a conformation change within the reductase domain that increases the electron transfer. Studies have shown that nNOSrd activation is not dependent on the presence of the oxygenase domain (Gachhui et al., 1996b). Therefore, the CaM effect can be studied using the isolated nNOSrd instead of the more complex full length enzyme. Common methods for this purpose monitor the rate of flavin reduction and also the rate of ferricyanide and
cytochrome c reduction. Different studies have been carried out to assess which particular nNOSrd electron transfer step is activated on CaM binding. Abu-Soud et al (1994a) demonstrated that NOS reductase domain undergoes a direct activation on CaM binding leading to a 20-fold increase in flavin reduction by NADPH. Matsuda and Iyanagi (1999) investigated the rates of nNOSrd reduction by NADPH, in the absence or presence of Ca\(^{2+}\)/CaM by using stopped-flow and rapid scan spectrophotometry. The results of their studies indicated that the reduction of the oxidized reductase domain by NADPH proceeds by hydride transfer mechanism and subsequent interflavin one-electron transfer due to the air-stable semiquinone form of the FMN that gates the electron from FAD to the heme \textit{in vivo} (Table 1.1). Based on the fact that in the CaM-free enzyme, the ferricyanide reduction at the FAD is faster than cytochrome c reduction at the FMN, they suggested that the intra-molecular one-electron transfer between the two flavins is rate limiting, but that on CaM binding, the flow of electron between the two flavins becomes unrepressed making the hydride transfer from NADPH to FAD the rate limiting step of the reduction process.

Further studies done by Roman et al (2001) demonstrated a separate effect of CaM on each of the two phases of the flavins reduction. In their conditions, the initial hydride transfer phase of the flavin reduction underwent a 6-fold increase in rate on CaM addition, whereas the second step that consisted in interflavins electron transfer displayed a 2-fold rate enhancement (Table 1.1). Interestingly, the steady-state rate, per electron transferred, is higher for cytochrome c in the absence of CaM (6 s\(^{-1}\)) than for the NO synthesis that occurs only in presence of CaM (3 s\(^{-1}\)) and so is potentially fast enough to support heme reduction and NO synthesis; however, electrons simply are not transferred efficiently to the heme in the absence of CaM (Roman \textit{et al.}, 2002). Studies using chimeras of NOS have established that the maximal rate of NO synthesis by native NOS was determined by the maximum intrinsic ability of the reductase domain to deliver electrons to the heme (Nishida and de Montellano, 1998).
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<tr>
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<th>Rate (min⁻¹)</th>
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<tr>
<td></td>
<td>NO synthesis</td>
</tr>
<tr>
<td></td>
<td>-CaM</td>
</tr>
<tr>
<td>nNOSWT</td>
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<tr>
<td></td>
<td>660¹b</td>
</tr>
<tr>
<td>nNOSrd</td>
<td>-</td>
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</tbody>
</table>

¹ (Roman et al., 2000b); ² (Daffet al., 1999); ³ (Matsuda and Iyanagi, 1999)

- Hydride transfer rate;  b - Interflavin electron transfer rate

Table 1.1 Rate of steady-state NO synthesis, cytochrome c reduction and ferricyanide reduction for wild type and isolated reductase domain forms of nNOS. Rate of flavin reduction by NADPH was obtained by rapidly mixing the isolated nNOSrd in the absence of external electron acceptor with NADPH using stopped flow apparatus in anaerobic condition.

1.4.2 Autoinhibitory insert

The differences of reactivity and regulation between the constitutive and the inducible isoforms of NOS cannot only be explained by the variations in the amino acid sequences of their CaM binding regions. Additional elements must also be involved either in iNOS to increase the CaM affinity or in nNOS and eNOS to decrease the CaM affinity as part of their regulatory mechanism. Alignment of the NOS reductase sequences with the homologous proteins CPR and the small electron carrier flavodoxins demonstrates the presence of a 45 amino acid insert only present in nNOS and eNOS (Figure 1.18). Interestingly, all NOS isoforms require the binding of CaM to produce NO. The main difference between the isoforms of NOS lies in their CaM affinities, which is greater for the inducible NOS. In fact, CaM binds to iNOS at a Ca²⁺ concentration well below the intracellular Ca²⁺ resting concentration whereas, CaM only binds to constitutive NOS during transitory increases in intracellular Ca²⁺ concentration.
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Introduction

Autoinhibitory loop insert

<table>
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<tr>
<th>Protein (Species)</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYPOR (human)</td>
<td>WL.QE...TDVD</td>
<td>164</td>
</tr>
<tr>
<td>iNOS (murine)</td>
<td>SLFNL...REL</td>
<td>609</td>
</tr>
<tr>
<td>eNOS (bovine)</td>
<td>AL.MENSGPYNSSPFQEQHKSXIKRFNSVSCSDPLVSSWRRKKESSNNTDSAGA</td>
<td>643</td>
</tr>
<tr>
<td>nNOS (rat)</td>
<td>AL.MEMRHP.NS...VQEERKSYKVRFSVSSYSDSRKSSGDGPDLRDNFESTGP</td>
<td>873</td>
</tr>
</tbody>
</table>

Figure 1.18 Sequence alignment of CPR and three NOS isoforms showing the putative autoinhibitory loop insert (Brunner et al., 1998) from Roman et al (2000b).

The most sequence-conserved portion of the insert in eNOS encompasses the residues 836 to 849 in rat nNOS. The N-terminal sequence includes a phosphorylation site at Ser847 and folds into an α-helix. Tyr841, Phe845 and Val848 are three conserved hydrophobic residues preceded by two basic residues Arg838 and Lys 839. Together these amino acids form a motif that is similar to the canonical "1-5-8-14" Ca²⁺-dependent CaM binding motif (Rhoads and Friedberg, 1997). Previous studies have suggested that an additional non-conserved C-terminal patch of basic residues in the eNOS insert interferes with CaM binding by interacting with the CaM binding linker (Salerno et al., 1997a). Because none of the NOS structures published so far contain both the autoinhibitory insert and the CaM binding peptide; it is difficult to establish a clear role for the autoinhibitory insert versus CaM binding in the interaction of the different sub-domain within the reductase domain as well as the interaction that controls the electron transfer between the reductase and the oxygenase domain. However, experiments carried out by Sheta et al (1994) showed that limited trypsinolysis leads to a single preferred cut site in rat nNOS at Arg 727 within the CaM binding sequence in the CaM-free enzyme. On CaM binding, they observed that the CaM binding site became protected from trypsin degradation, but that a novel cleavage site was revealed at the dibasic site (Arg855-Lys856) within the FMN insert of nNOS. They concluded that the exposure of this tryptic site after the binding of CaM was the result of a displacement through binding domain overlap.

Salerno et al (1997b) demonstrated that chimeric eNOS and nNOS, which have had their CaM binding sequences replaced with the corresponding sequence from iNOS, still require Ca²⁺ for full activity. Because regulation of enzyme systems by Ca²⁺/CaM typically involves displacement of an intrinsic autoinhibitory polypeptide,
they hypothesized that the binding of Ca\(^{2+}\)/CaM to cNOSs may similarly trigger activation by displacing a control element. To confirm this hypothesis, they synthesized polypeptides based on the sequence of the eNOS insert. The binding of radiolabelled CaM analogues \((^{125}\text{I} \text{CaM})\) and L-Arg analogue \(([^3\text{H}]\text{G}-\text{nitro-L-Arginine})\) was assayed to determine whether the synthetic peptides interfere or not with the binding of CaM and L-Arg onto NOSs. The addition of 50-100\(\mu\)M of each of these polypeptides were tested in catalytic assays for NO formation and cytochrome c reduction in all three NOS isoforms. They concluded that in cNOS, the insert derived peptides interfere with the CaM binding site but not with the arginine binding site and trigger inhibition of NO formation and cytochrome c reduction. Under the same conditions, the insert derived peptide did not significantly inhibit iNOS as it binds CaM more tightly. This inhibition was fully reversible on addition of an excess of CaM indicating a competitive inhibition mode. Interestingly, the inhibition of CaM binding by peptide could not be overcome by excess Ca\(^{2+}\). From the different peptides they used, the hexapeptide WRRKRK synthesized from the eNOS insert was the most efficient in term of inhibitory effect of the cNOS enzyme, but displayed only minimal inhibition of iNOS. The insertion within the FMN domain represents the single most prominent difference between cNOSs and iNOS amino acid sequences, considered over their entire length. This correlates with the CaM-dependent NO synthesis for cNOS and may explain why iNOS has the highest NO production. It is to note that nNOS which contains a weak inhibitory insert is only 3-fold less active than iNOS and that eNOS which contains the most potent insert is 20-fold less active than iNOS.

On the basis of the ability of the inserts to form an autoinhibitory domain designed to destabilize CaM binding and increase its Ca\(^{2+}\) dependence, Daff \textit{et al} (1999) investigated the role of the insert by constructing two deletion mutants of nNOS. The nNOS\textDelta{40} and \textDelta{42} were designed to remove the main part of the loop for comparison with the loopless iNOS. They observed that both mutants were able to sustain maximal NO production at lower Ca\(^{2+}\) concentration threshold compared to wild type enzyme. In absence of CaM, the loop deletion of nNOS\textDelta{40} construction was found to retain 30% activity compared to a total absence of NO synthesis in the case of the wild type enzyme. NADPH addition also leads to spontaneous heme
reduction in the case of CaM-free nNOSΔ40, whereas this reaction could only be carried out in presence of CaM in the wild type enzyme. However, the eNOSΔ43 and eNOS Δ45 investigated by Nishida and Ortiz de Montellano (1999) and Chen and Wu (2000) respectively demonstrate that in eNOS the insert strongly inhibits the CaM-free enzyme and that, unlike nNOS, its repression on NO synthesis is not fully relieved on CaM binding. In fact, the result of its suppression in eNOSΔ43 lead to an increase of activity by 2-fold in the CaM-bound native eNOS and a 2-fold enhancement in cytochrome c reduction in the CaM-bound eNOS reductase domain. The eNOSΔ45 studied by Chen and Wu (2000) was the first insert deleted mutant to possess an endogenous CaM bound insensitive to EGTA. This similarity with iNOS is responsible for full reduction activity toward cytochrome c and ferricyanide in absence of free-Ca2+ and free-CaM.

In the case of nNOS reductase, the investigation of a combined removal of both the CaM binding domain and the putative inhibitory insert did not result in calmodulin-independent reductase activity but in an enzyme showing similar activity to the CaM-free wild type nNOS (Montgomery et al., 2000). This suggests that CaM is essential to align the reductase domain for optimal electron transfer through the flavins even in the absence of the insert.

1.4.3 C-terminal extension

Another significant difference between NOS and CPR is that all NOS isozymes, including iNOS, contain additional amino acids at the C terminus beyond the residues homologous to the C-terminus of CPR (Figure 1.19). This extension consists of 21 amino acids for the iNOS isoform, 33 and 42 amino acids for nNOS and eNOS respectively.
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Introduction

Tail region

<table>
<thead>
<tr>
<th>CYPOR (human):</th>
<th>RYSLDVWS</th>
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<tbody>
<tr>
<td>iNOS (murine):</td>
<td>RYHEDIFG AVFSYGAKKGSALBBPKATRL (+21)</td>
</tr>
<tr>
<td>nNOS (rat):</td>
<td>RYHEDIFG VTLRTEVTNSRELSAFEESKDADEVFSS (+33)</td>
</tr>
<tr>
<td>eNOS (bovine):</td>
<td>RYHEDIFG LTLRTQEVTSRIRTSQFSLQERHLRGAVFWAFDPGPDTGP (+42)</td>
</tr>
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</table>

Figure 1.19 Sequence alignment of the C-terminal residues of cytochrome P450 oxidoreductase (CYPOR) and three NOS isoforms showing a highly conserved region in NOS (RYHEDIFG) just prior the tail region of the NOSs (Nishida and de Montellano, 1998) from Roman et al (2000b).

In the early crystal structure of the nNOS NADPH/FAD binding site obtained by Zhang et al (2001), limited trypsinolysis was used to generate the isolated FAD domain, which lacked the C-terminal extension. However, in the structure of nNOSrd reported by Garcin et al (2004), 17 of 33 amino acid residues of the C-terminal tail (1397-1413) were resolved. This shows that the C-terminal tail extends from the NADPH-binding domain toward the CaM-binding region. Accordingly, the C-terminal extension was suggested to stabilise the FMN- and NADPH-binding domains in the electron-accepting position via the binding of NADP(H) anchored by a network of hydrogen bonds and ionic interactions.

Studies carried out by Roman et al (2000a) demonstrated that the deletion of 21 amino acids from the C-terminus of iNOS results in a mutant with a 10-fold faster cytochrome c reduction and 20% faster NO synthesis activity. The kinetic consequences of this truncation in term of NADPH oxidation, measured under cytochrome c reduction conditions, were 2-fold rate increase of the hydride transfer to the FAD and a 5 fold faster interflavins electron transfer. The rate of electron transfer from the reductase domain to the heme was also increased by 20%. Interestingly, the air–stable semiquinone of the mutant displayed an extra sensitivity to oxygen compared to the intact iNOS suggesting that in the absence of the tail, the flavins become more solvent exposed resulting in a faster rate of FMN oxidation. Faster rate of electron transfer into and between the flavins in absence of the C-terminal extension as well as a more solvent exposed FMN cofactor suggested that C-terminal extension is necessary to maintain a conformation of the enzyme that repress electron transfer.

Similar studies were also carried out with cNOS in order to investigate the interaction between the C-terminal extension, autoinhibitory loop and CaM binding.
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site (Roman et al., 2000b). Characterisation of the truncated nNOS and eNOS revealed unstable FMN semiquinone toward oxygen as it was previously seen with the truncated iNOS. They observed that the cytochrome c reduction in the absence of CaM was increased by 21 and 7-fold for nNOS and eNOS respectively. The truncation appears then to enhance the rate of cytochrome c reduction to a greater extent than does the addition of CaM in the wild type enzyme, where only a 10-fold increase is observed. Surprisingly, on CaM addition, the mutants activity dropped back to the rate of the CaM-bound wild type. Therefore, these results demonstrate that in the CaM-bound wild type cNOS, the C-terminal extension does not limit the rate of cytochrome c reduction but that CaM does.

Stopped flow experiments were also carried out by the same group to monitor the rate of flavin and heme reduction by the truncated eNOS and nNOS. The results revealed that the absence of C-terminal extension, in the absence of CaM, leads to a 7-fold increase of the fast phase of the flavins reduction followed by a 2-fold increase of the slow phase. The same experiment performed in the presence of CaM showed a similar rate of flavins reduction for both phases compared to the wild type enzyme. Therefore, it appears that the removal of the C-terminal extension is not sufficient to relieve the electron transfer repression in the CaM-free enzyme since the binding of CaM enhanced flavin reduction in the truncated enzymes. However, the side chain of the FAD-stacking residue Phe1395, located at the beginning of the C-terminal extension, must move away from the FAD isoalloxazine ring to allow a productive binding interaction with the nicotinamide ring of NADPH that is essential for hydride transfer to FAD (Figure 1.19 & 1.20).

Adak et al (2002) suggested that this side chain movement would act in conjunction with and influence the C-terminal and autoinhibitory control elements to triggers repression of electron transfer from CaM-free nNOS. In order to understand better how Phe1395 helps to regulate NOS electron transfer and its NO catalysis, Konas et al (2004) designed and characterised the F1395S mutant of nNOS. By looking at the UV-visible properties at 510 nm of nNOSrd in the presence of NADP(H), they determined that F1395S presents 65% more nicotinamide ring in productive conformation with FAD for hydride transfer compared to wild type nNOS. Accordingly, they also monitored, using stopped flow kinetic apparatus, an 8-fold
slower rate of NADP\(^+\) dissociation for the mutant regardless of the presence of CaM. Previous experiments carried out with the wild type enzyme showed that the rate of flavin reduction by NADPH is stimulated on CaM binding, whereas F1395S only displayed a rate of flavin reduction similar to the CaM-free wild type. This suggests that slower NADP\(^+\) release could eventually limit the flavin reduction process. Characteristically, the turn-over rate of cytochrome \(c\) reduction by wild type nNOS is 10-fold slower for the CaM-free enzyme. In this case, the same experiment revealed that F1395S is not repressed in absence of CaM and displays an intermediate turn over rate insensitive to CaM binding. Therefore, they concluded, that the CaM enhancement of flavin reduction rate, characteristic of the native NOS, is not responsible for the increased steady-state cytochrome \(c\) reductase activity of the CaM-free F1395S. Further pre-steady state kinetic studies of cytochrome \(c\) reduction and EPR measurement demonstrated that F1395S is characterized by a more accessible FMN in absence of CaM compared to the wild type nNOSrd. Konas et al (2004) concluded that Phe1395 regulates nNOSrd catalysis by facilitating NADP\(^+\) release to prevent this step from being rate limiting, and also enables NADP(H) to properly regulate a conformational equilibrium involving FMN sub-domain that controls reactivity of the FMN cofactor in electron transfer.

In order to understand better the role of NADP(H) binding in the control of the conformational equilibrium of the enzyme, Tiso et al (2004) hypothesises that Arg1400 enables an interaction between bound NADP(H), the C-terminal tail, and the FMN module that could conceivably link NADP(H) binding to repression of FMN electron transfer in CaM-free nNOS. For that they constructed R1400E and R1400S mutants for the nNOSrd and full length enzyme. The characteristics of these mutations were to either eliminate the charge interaction between Arg1400 and 2'-Pi of NADPH, or to switch it to a charge-repelling interaction. It is to note that the mutations didn’t fundamentally affect the affinity and the selectivity of the enzyme for NADP(H) compared to NAD(H).

Stopped-flow kinetic studies of flavins reduction revealed that R1400E is less repressed than the wild type enzyme and that the mutant R1400E totally lacks repression in the absence of CaM and that both mutants have a rate of flavin reduction similar to wild type in the presence of CaM. Single turn-over experiments
also demonstrated a loss of NADPH-repression for cytochrome c reduction catalysis in the absence of CaM suggesting that Arg1400 is involved in the mechanism that causes the FMN shielding in the presence of NADPH for the CaM-free enzyme.

In the CaM-free state, the full length mutants weren’t able to synthesize NO despite a 4-fold faster NADPH consumption rate compared to wild type. The greater portion of NADPH oxidation was uncoupled to NO release and suggests that Arg1400 is involved in electron transfer to nNOS ferric heme. The CaM bound state was characteristic of a faster rate of ferric heme reduction and a faster rate of ferrous heme-NO complex formation leading to a lower steady state rate of NO synthesis.

Native soybean CaM protein and CaM-cardiac troponin c chimeras designed to support different rates of ferric heme reduction were used to slow down the inter-domain electron transfer in order to recover NO synthesis activity in steady state. The result of this experiment suggests that R1400E mutation causes CaM to support a rate of ferric heme reduction that is beyond the optimal value for the steady state NO-synthesis activity of nNOS.

Tiso et al (2004) concluded that Arg1400 assists NADPH to repress the transfer of electrons into and out of the CaM-free nNOSrd and that the interaction R1400/NADPH regulates the mobility of the FMN module that controls both the CaM-bound and CaM-free enzyme (Figure 1.20).

Based on the remarkable sequence homology between CPR and NOS isoforms, Jáchymová et al (2005) studied the evolutionary origin of these two enzyme families through the addition of the NOSs characteristic C-terminal peptides to CPR. They observed that NOS C-terminal extension inhibits the rate of reduction of cytochrome c and DCPIP that both react with FMN cofactor, but does not affect the reduction of ferricyanide that reacts with the FAD cofactor. They concluded that these results fully agree with the fact that the C-terminal tail interrupts electrons flux between FMN and FAD by altering the distance and alignment of the two flavins (Roman et al., 2000b). Furthermore, they observed a greater fluorescence quenching in presence of the tail suggesting a conformation of the enzyme that favours the shielding of the cofactor as seen with NOSs.
1.4.4 Phosphorylation sites

In addition to intrinsic protein controls on NOS activity, extrinsic elements such as phosphorylation sites have been found to play a major role in the regulation of NO production. Fulton et al (1999) and Dimmeler et al (1999) demonstrated that specific phosphorylation serine residue of eNOS C-terminal extension results in an increase of NO production as well as a lower Ca\(^{2+}\)/CaM binding threshold. Moreover, directed mutagenesis of the same serine for a negatively charged glutamate lead to the same activation as the phosphorylation. These results obtained with eNOS are consistent with the model in which the C-terminal tail inhibits the electron flow through the flavins domain in the absence of CaM. A similar phosphorylation site
was also found at Ser1412 in nNOS (Adak et al., 2001). Unlike the results obtained with eNOS, for which phosphorylation of the C-terminal extension corresponded to an NO synthesis activation, phosphorylation of the residue Ser1412 in nNOS lowers NO production as a consequence of a too high electron flow through the reductase domain. The positive effect observed consecutively to the phosphorylation of eNOS may be explained by a sub-optimum rate of heme reduction for the dephosphorylated enzyme, whereas nNOS heme reductase already occurs at a near optimal rate. Chen et al (1999) also demonstrated that in eNOS the absence of CaM revealed a phosphorylation site (Thr495 in eNOS) within the CaM binding linker that inhibits CaM binding and the resulting activation of the enzyme. Multiple kinases and phosphatases were found to target specifically on, only one or both sites of the CaM linker and C-terminal tail in order to modulate NO production by eNOS (Michell et al., 2001).

Hayashi et al (1999) demonstrated that the phosphorylation of Ser847 within the autoinhibitory insert of nNOS results in large inhibition of NO synthesis and cytochrome c reduction consecutive to suppression of CaM binding. Similar phosphorylation site have been also reported in eNOS (Butt et al., 2000).

1.4.5 Concerted action of the regulatory element in nNOS

Isolated nNOS reductase domain and full length enzyme present similar CaM dependent steady state activity for cytochrome c reduction and ferricyanide in presence and absence of bound CaM (Gachhui et al., 1996b). This indicates that all the intrinsic and extrinsic regulatory elements required for the control of the electron flow to NOSox are either contained in nNOSrd (Abusoud et al., 1994a) or targeted to act on it. All NOSs require the binding of CaM for NO synthase activity or to activate the electron transfer into and out of the isolated reductase domain. It is by rearranging the autoinhibitory insert within the FMN (Salerno et al., 1997b), and also the FAD stacking residue Phe-1395 (Konas et al., 2004) and C-terminal extension (Roman et al., 2000b), that the binding of CaM and NADPH modulates the flavins alignment with respect to each other and the FMN cofactor accessibility (Craig et al., 2002) to enable or disable the electron transfer to external electron acceptors. CaM
binding is also implicated in realignment of nNOSrd/nNOSox to ensure efficient inter-subunit electron transfer in the full length enzyme (Abusoud and Stuehr, 1993). The autoinhibitory insert was shown to interact with the NADPH- and FMN-binding domain (Garcin et al., 2004) and to destabilise the binding of CaM (Sheta et al., 1994). This suggests that the insert may help to stabilise the locked conformation of the NOSrd (Craig et al., 2002) that impedes the electron transfer through the enzyme. The crystallographic data obtained suggests that the C-terminal extension extends across the FMN domain toward its N terminus (Garcin et al., 2004), interfering with CaM binding (Lane and Gross, 2002). Adak et al (2002) and Konas et al (2004) suggested that the Phe1395 side chain movement would act in conjunction with and influence the C-terminal and autoinhibitory control elements to trigger repression of electron export from CaM-free nNOS. The implication of the N-terminal part of the tail in hydrogen bonding together the 2'-phosphate part of NADPH (Arg1400 in nNOS), the NADPH binding site and the FMN domain seems to be agreement with the nNOS lock-conformation generated by NADP(H), but not NAD(H), kinetically observed in the absence of CaM.

Roman et al (2000b) and Adak et al (2001) demonstrated that specific phosphorylation of Ser1412 of the C-terminal extension results in an increase of the inter-domain electron transfer in nNOSrd as well as a lower Ca\(^{2+}/\)CaM binding threshold. Hayashi et al (1999) demonstrated that the phosphorylation of Ser847 within the autoinhibitory insert of nNOS results in large inhibition of NOS synthesis and cytochrome c reduction consecutive to suppression of CaM binding.

At low Ca\(^{2+}\) concentration, the CaM binding energy is too low to displace the FMN autoinhibitory insert from the CaM linker which disables the binding of CaM to nNOS. It is probable that in absence of bound CaM, the binding of NADPH reorients C-terminal extension within nNOSrd and contributes to stabilise the FMN domain in a conformation where the FMN cofactor is not accessible for electron transfer to NOSox. However, at sufficient Ca\(^{2+}\) concentration, the CaM binding energy displaces the FMN insert with a possible subsequent reorientation of the C-terminal tail. Such rearrangement is likely to destabilise and relieve, at least partly, the locked conformation of the enzyme. The gain in NOSFMN mobility may induce a realignment of FMN with respect to FAD in order to enable efficient interflavin
electron transfer. Moreover, the mobility equilibrium of NOSFMN, between an electron-accepting and an electron-donating position, would also allow it to shuttle electrons from FAD to heme. Furthermore, the existence of the hydrogen bond between the FMN cofactor and Gly810 permit to transfer only one electron at time from the 2 electrons donor NADPH to the one electron acceptor heme. All the intricate regulatory mechanisms not only act as a switch for the electron transfer but also modulate the rate of the electron transfer in order to ensure electrons transfer at optimum rate to the heme for efficient NO production.
1.5 Aims of the project

The aim of this work is to further understand the mechanisms involved in the activation of nNOS by calmodulin.

The main questions to be addressed are:

- **How does CaM interact with nNOS?**
  Structures have only been obtained for CaM bound to peptides and for CaM-free nNOSrd. Here, the aim is to crystallise nNOSFMNCaM to clarify the interactions between FMN domain, CaM linker and CaM.

- **Do conformational changes alter the redox potential of the flavins?**
  Although the redox potentials determined for CaM-free and CaM-bound nNOSrd have shown no CaM dependency, there is no information on the effect of the substrate (NADP+) binding on the redox potentials. In this study, the substrate dependence of the nNOSFAD redox potential is determined by spectroelectrochemical analysis for the enzyme in the presence and absence of NADP+.

- **How does CaM trigger electron transfer to the heme?**
  Previous work has shown that electron transfer in NOS is gated by conformational changes in the reductase domain and that in order to overcome this effect CaM must bring the FMN closer to the heme. Here, the aim is to measure the accessibility of FMN by the heme in the presence or absence of CaM. For that cytochrome c is used as an external electron acceptor to mimic the nNOSox and the accessibility of the FMN is measured by the rate of stopped flow cytochrome c reduction.
- **How specific is the NADP(H) inhibitory effect of the nNOSrd electron transfer to the heme?**

The substrate (NADPH) has been shown to induce a conformational change in the nNOS reductase domain that represses the electron transfer through the enzyme by locking the conformation of the reductase domain preventing its access to the heme. The inhibition of electron transfer from nNOSrd by substrate is examined here for specificity, using substrate analogues.

- **What is the CaM dependency of nNOSrd flavin reduction by NADPH?**

The conformation of nNOSrd determines the probability of NADPH to stack and reduce the enzyme by hydride transfer, depending on the mobility of the Phe1395 residue and the C-terminal extension. In order to determine the effect of the conformational changes on nNOSrd, induced by CaM binding, stopped flow reduction of nNOSrd by NADPH is compared in the presence and absence of CaM.
Chapter 2

Experimental
2.1 Strains and plasmids

Recombinant rat nNOS reductase domain (nNOSrd) was expressed in *E. coli* strain BL21 (DE3) using plasmid pCRNNR (Newton *et al.*, 1998) (Figure 2.1). This plasmid coexpresses the rat nNOS reductase domain residues 695 to 1429 (including the CaM binding site) and bovine brain calmodulin domain. The same expression vector was used for the expression of the FMN sub-domain residues 695 to 946 (Newton *et al.*, 1998) (Figure 2.2).

![Figure 2.1 Physical maps of the vectors created to express nNOSrd (Newton *et al.*, 1998).](image)

The map is drawn to scale with the total size derived from the sum of the exact size of the DNA fragment used in the construction. The rat nNOSrdCaM+ (residues 695 to 1429 including the CaM binding site) is expressed by the plasmid pCRNNR. The CaM+ reductase domains is coexpressed with the gene for bovine CaM using a polycistronic messenger RNA. The vector uses a LacZ promoter with stop codons in the reading frames located between the promoter and the start of the gene. The plasmid has an upstream tac promoter and a strong rrmB (ribosomal RNA promoter) ribosomal terminator downstream of the gene. In order to express the nNOSFMNCaM+, the gene for nNOSrd was excised using EcoR1 and a fragment corresponding to the FMN-binding domain (residues 695-946). The resultant gene fragment was cloned back into the EcoR1 site of the original vector in place of the nNOSrd sequence (vector pCRNNFMN).
2.1.2 Plasmid DNA purification

Plasmid DNA from 5 ml cultures was purified by using the QIAprep Spin miniprep kit (QIAGEN) as per the manufacturer’s instructions.

2.1.3 Cell transformation

30 µl of competent cells BL21 (DE3) *E.coli* (Invitrogen™) was thawed on ice prior to adding and mix by tapping gently 5 to 10 ng of plasmidic DNA, in a volume of 1 to 5 µl. The mix was incubated 30 minutes on ice before being heat shocked for exactly 30 seconds in a water bath at 42°C and then removed the vial from the bath and quickly place on ice. 250 µl of pre-warmed SOC medium (See paragraph 2.1.) were added before incubating the vial at 37°C for exactly 1 hour in a shaker at 225 rpm. Finally, 10 and 100 µl of the transformation reaction were plated onto two LB plates containing 25 mg/ml of carbenicillin. The plates were inverted and incubated at 37°C overnight.
Chapter 2. Materials and Methods

2.2 Cell growth techniques

2.2.1 Media

2.2.1.1 Lysogeny Broth medium (LB)
The Lysogeny Broth medium was prepared by mixing 10 g/l of bacto-tryptone, 5 g/l of bacto-yeast extract and 5 g/l of NaCl in water.

2.2.1.2 Terrific Broth medium (TB)
The Terrific Broth medium was prepared by mixing 20 g/l of Bacto-tryptone, 10 g/l of yeast extract, 4 ml/l of glycerol, 2.6 g/l of KH₂PO₄ and 4.3 g/l of Na₂HPO₄ in water.

2.2.1.3 Super Optimal Catabolite Medium (S.O.C).
The S.O.C medium was prepared by mixing 5 g/l of bacto-tryptone, 5 g/l of bacto-yeast extract, 10 mM of NaCl, 2.5 mM of KCl, 10mM of MgSO₄, 10mM of MgCl₂ and 20mM of glucose in water.

(All Media were prepared with Millipore Q Ultrapure water and autoclave sterilised at 121 °C)

2.2.2 Antibiotic
Carbenicillin was used at a working concentration of 50 μg/ml (Stock solution 50 mg/ml made up in dH₂O)

2.2.3 LB-agar Plates
15 g/l of agar powder was dissolved in LB and sterilised. LB-Agar solution was allowed to cool to the touch prior to addition of carbenicillin (25 μg/l).
2.3 Protein expression and purification

2.3.1 Starter cultures
10 ml starter cultures of bacteria were grown in TB media at 37°C overnight in presence of 50 µg/ml of Carbenicillin. Cultures were inoculated with single fresh-transformed colony using a sterile wire loop.

2.3.2 Protein Expression
nNOSrdCaM and nNOSFMNCaM were expressed in the optimised conditions described by (Newton et al., 1998). For that, E. coli strains containing the respective plasmid were grown in 1 litre baffled shake flasks (350 ml of media / flask) at 37°C (120 rpm) on Terrific-Broth media (TB) containing 20 g/l Yeast extract, 10 g/l bacto-tryptone, 4 ml/l glycerol, 2.6 g/l KH₂PO₄ and 4.3 g/l Na₂HPO₄. Carbenicillin was added to a final concentration of 25 µg/ml. Cells were grown until the A₆₀₀ was 1, then isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce expression of protein and the temperature was lowered to 30°C (shake 110 rpm). The cells were harvested 24 hours after induction by centrifugation (16000 g, 10 min).

2.3.3 Protein Extraction
The cells expressing nNOSrdCaM or nNOSFMNCaM proteins were lysed by sonication on ice in 50 mM Tris-HCl, pH 7.5, 0.1 M KCl and 1 mM CaCl₂ (Buffer A) with 1 ml Protease Inhibitor Cocktail for non-His-tagged proteins per 30g E. coli (Sigma) and 1 mM PMSF. The lysate was centrifuged at 20000g for 1hr to isolate the soluble protein in the supernatant.
2.3.4 Protein Purification

2.3.4.1 Purification of recombinant nNOS FMN domain plus calmodulin (nNOSFMNCaM).

The supernatant isolated from the centrifugation of the lysate was passed through a DEAE Sepharose column (2.5 x 15cm) and eluted with Buffer A. The nNOSFMNCaM fractions were loaded on to a Q-Sepharose FPLC column (Pharmacia) and eluted with a gradient of 0.1-0.4M KCl in Buffer A. Enzyme containing fractions were concentrated to approx. 100 μM and stored at -80 °C. To remove excess CaM, enzyme fractions were further purified by Superdex S200 FPLC chromatography in 50 mM Tris-HCl, pH 7.5, 0.2M KCl, 1 μM CaCl₂.

2.3.4.2 Purification of recombinant nNOSrd and nNOS FAD domain (nNOSFAD).

Recombinant rat nNOS reductase domain (nNOSrd) and synthetic bovine brain calmodulin contained in the isolated supernatant from the centrifugation of the lysate were purified as described previously (Craig et al., 2002) on 2',5'-ADP-agarose (Sigma) and CaM-agarose (Sigma).

2.3.4.3 Purification of nNOS FAD domain (nNOSFAD)

nNOS FAD domain was generated by digesting 10 ml x 20 μM nNOSrd with 1 ml immobilized trypsin (Sigma) in 50 mM Tris-HCl, pH 7.5, 0.1M KCl, (Buffer B) at room temperature for 2 hours with gentle stirring (Zhang et al., 2001). The suspension was centrifuged at 2000g for 10 min and filtered to remove the trypsin, and the supernatant was applied to a 2', 5'-ADP-agarose column (1 x 10 cm). The yellow band was washed with 100 ml Buffer B and eluted with 20 ml 1 mM 2',5'-ADP in Buffer B plus 0.3 M KCl. Enzyme containing fractions were concentrated to approx. 200 μM and stored at -80 °C. It should be noted that nNOS FAD domain generated in this way is truncated by 22 amino acids from the C-terminus and consists of residues 963-1407 of rat nNOS (Zhang et al., 2001).
2.3.4.4 Purification of CaM.

The CaM-rich fraction corresponding to the flow-through of the ADP-, CaM-sepharose and DEAE-sepharose chromatography columns collected during nNOSrd and nNOS FMN domain purification respectively, was calcified with CaCl$_2$ to an approximate concentration of 10mM before being boiled to remove the excess of unwanted proteins. These were loaded onto a 200 ml phenyl sepharose chromatography column, washed with 800 ml buffer C ($50 \text{mM Tris-HCl, pH 7.5, 1 mM CaCl}_2$), 1 litre Buffer C + 0.5 M NaCl, and 1 litre of buffer C. The CaM was eluted using buffer 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and the fractions containing CaM were identified with SDS-PAGE, concentrated with an evaporator (BUCHI Rotavapor R-114), dialysed in buffer C, freeze dried (Edward Super Modulyo) and stored at -20°C.

2.4 Protein characterisation

2.4.1 SDS-PAGE

Pre-poured NuPAGE® 4-12 % Bis-Tris Gel (Invitrogen™) was used to verify the purity of the protein. The gel was run at 200 V for 1 hour at 50 W. The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS (pH 8.3). Samples were mixed with SDS-PAGE loading buffer 50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol and boiled for 3 minutes to denature the polypeptide chain of protein prior to loading. The molecular weight of proteins was estimated by comparison with prestained broad range marker, SeeBlue Plus 2® Invitrogen™.
Chapter 2.  
Materials and Methods

<table>
<thead>
<tr>
<th>Proteins marker</th>
<th>Molecular weight (in kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>188</td>
</tr>
<tr>
<td>Phosphorylase B</td>
<td>98</td>
</tr>
<tr>
<td>BSA</td>
<td>62</td>
</tr>
<tr>
<td>Glutamic Dehydrogenase</td>
<td>49</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase</td>
<td>38</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>28</td>
</tr>
<tr>
<td>Myoglobin Red</td>
<td>17</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>6</td>
</tr>
<tr>
<td>Insulin, B chain</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.1 Approximate Molecular Weights (in kDa) for Molecular Weight Standards, SeeBlue Plus 2®, in NuPAGE MES Buffer for Molecular Weight Standards, SeeBlue Plus 2®, in NuPAGE MES Buffer Approximate molecular weights for the markers.

2.4.1.1 Coomassie staining

The gels were stained in a solution of 0.25% (w/v) Coomassie Brillant Blue, 45% (v/v) methanol, 45% (v/v) glacial acetic acid for up to 1 hour. For the destain solution, the dye was omitted.

2.4.1.2 Western blotting

Proteins were separated on a NuPAGE® Bis-tris electrophoresis system (Invitrogen™) using a gradient gel ranging from 4 to 12% acrylamide concentration. Proteins were transferred to a nitrocellulose membrane by electroblotting for 90 minutes at 30 V in transfer buffer. Transfer buffer consisted of 500 mM Bicine, 500 mM Bis-tris, 20.5 mM EDTA and 1 mM chlorobutanol (pH 7.2). Membranes were Coomassie stained and the protein band of interest was cut out for N-terminal sequencing.
2.4.2 N-terminal sequencing
(With Dr. A. Cronshaw, ICMB sequencing service)
Immobilised protein samples were solubilised for Edman degradation and degradation products were analysed on an Applied Biosystems Procise 4HT microsequencer using the method of Hayes et al. (1989).

2.4.3 LC-MS
(With Dr. R. Smith, University of Edinburgh)
Samples were diluted with buffer 50mM Tris-HCl pH7.5, 100mM KCl, 5% glycerol to the concentration of approximately 100μM and 4μl of Formic acid was added. A Jupiter C5 column was used to desalt the sample completely. Proteins were eluted with an acetonitrile gradient (5%-95% in water) in presence of 1% formic acid. A UV-detector and a mass spectrometer were used in series to monitor the eluted proteins. The Micromass® platform II Mass Spectrometer was controlled by the software Masslynx V 3.5. The instrument was operated using the electrospray positive ionisation mode (cone voltage 70V).

2.4.4 Extinction coefficient
The flavin cofactors of 1ml of 10 μM nNOS FAD or FMN sub-domain in Tris-HCl, pH 7.5, 50mM, 100mM KCl 7.5 were released by addition of 20 μl lauryl sulfate (anionic detergent) (Sigma) 10% m/v at 60°C. Spectral analysis was performed on a Shimadzu UV-1501 UV-vis spectrophotometer at 60°C. The extinction coefficient was worked out by comparison of the spectrum of the native protein with that of the cofactor released.
(ε445nm FMN = 12500 M⁻¹.cm⁻¹, ε450nm FAD = 11100 M⁻¹.cm⁻¹)

2.4.5 Quantitation
The protein concentration was determined by spectrophotometric Bradford assay (Bradford and Williams, 1976) using a calibration curve absorption versus concentration (at 595 nm) (Figure 2.3) established with bovine serum albumin in the
same buffer Tris/HCl pH 7.5 (50mM), KCl (100mM) as the protein of interest. The absorbances were fitted with a linear function. The concentrations for the nNOSFMNCaM and nNOSFAD samples that fit between the absorbance values of the standard dilutions were calculated using the linear equation.

\[ A = 0.06279 + 0.01818 \times [\text{prot}] \]

\[ R^2 = 0.99762 \]

**Figure 2.3** Standard Bradford curve with bovine serum albumine using a UV-vis spectrophotometer Shimadzu UV-1501.

### 2.4.6 Crystallography assays

(With Dr. C. Mowat, University of Edinburgh)

nNOS FMN sub-domain crystallographic assays were performed using the method of hanging drop vapour diffusion method in Linbro trays (24 containers) at 4°C and 18°C in the dark.

The final volume of prepared solution (salt, buffer, precipitant, water) in the container was 1ml. A micropipette (Pipetman P-10 Gilson) was used to apply and mix 2 µl of nNOS FMN sub-domain CaM bound (20 mg/ml) plus 2 µl of prepared solution on the coverslip. The coverslip was settled upside down and sealed on the top of container using white soft paraffin.

Calmodulin activation of the reductase domain of mammalian neuronal nitric oxide synthase
2.5 Spectroelectrochemistry

Spectroelectrochemical analysis of nNOSFMNCaM, nNOSrd and nNOSFAD was conducted in an OTTLE cell (Optically Transparent Thin Layer Electrode) constructed from a modified quartz EPR cell with a 0.3 mm path length, containing a Pt/Rh (95/5) gauze working electrode (wire diameter 0.06 mm, mesh size 1024 cm\(^{-1}\), Engelhardt, UK), a platinum wire counter electrode and a Ag/AgCl reference electrode (model MF2052, Bioanalytical Systems, IN 47906, USA) (Figure 2.4)(Ost et al., 2003).

Figure 2.4 Optically transparent thin layer electrochemical (OTTLE) cell

Enzyme samples (1 ml x 200 \(\mu\)M) were eluted through a G25 column pre-equilibrated with 0.1 M Tris pH 7.5, 0.5 M KCl, concentrated to 300 \(\mu\)M and stored overnight in an anaerobic glovebox at 0 °C. The following mediators: pyocyanine (10 \(\mu\)M), 2-hydroxy-1,4-napthoquinone (20 \(\mu\)M), FMN (5 \(\mu\)M), benzyl viologen (10 \(\mu\)M) and methyl viologen (10 \(\mu\)M) were then added. Spectroelectrochemical titrations were performed at 25 ± 2 °C using an Autolab PGSTAT10 potentiostat and a Cary 50 UV/Vis spectrophotometer.
Table 2.2 Mediator and their respective concentrations used in potentiometry assays.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Em vs SHE (mV)</th>
<th>For FMN sub-domain (μM)</th>
<th>For FAD sub-domain (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxy-1,4-naptoquinone (HNQ)</td>
<td>-154</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>benzyl viologen (BV) (μM)</td>
<td>-311</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>Flavin mononucleotide (FMN) (μM)</td>
<td>-200</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>methyl viologen (MV) (μM)</td>
<td>-430</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Phenazine ethosulfate (PES) (μM)</td>
<td>+55</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Pyocyanine (PYO) (μM)</td>
<td>-34</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

The potential of the working electrode was typically decreased in 30 mV steps until the enzyme was fully reduced and increased step-wise until re-oxidation was complete. After each step the current and UV/Vis absorption spectrum was monitored until no further change occurred. This equilibration process typically lasted 15 mins. The Ag/AgCl reference electrode employed in the OTTLE cell was calibrated against indigotrisulfonic acid (E0 = -99 mV vs. SHE) and FMN (E0 = -220 mV vs. SHE) in the same buffer conditions and found to be +205 ± 2 mV relative to the standard hydrogen electrode (SHE). All electrode potentials have been corrected accordingly. Absorbance changes were plotted against the potential of the working electrode and analyzed by fitting absorbance data at one or more wavelengths simultaneously to a modified Nernst equation (Equation 1) using Origin 7.0 (Microcal). nNOSFAD redox titrations were also conducted in the presence of equimolar amounts of ADP+ and NADP+. In the latter case the data were analyzed used Equation 2, which incorporates the additional NADP+/NADPH 2e redox couple (E3). The nNOSrd data were analyzed as described previously.
Chapter 2. Materials and Methods

Equation 1

\[
a \log_{10}\left(\frac{2E - E'_1}{59}\right) + b + c \log_{10}\left(\frac{E'_2 - E}{59}\right)\\
1 + \log_{10}\left(\frac{E - E'_1}{59}\right) + \log_{10}\left(\frac{E'_2 - E}{59}\right)
\]

Where \(a, b\) and \(c\) are the absorbances of oxidized flavin, flavin semiquinone and hydroquinone respectively. \(E\) is the potential of the working electrode and \(E'_1\) and \(E'_2\) are the midpoint potentials of the oxidized/semiquinone and semiquinone/hydroquinone redox couples [28]. \(a, b, c, E'_1\) and \(E'_2\) are variables determined by least-squares fitting.

Equation 2

\[
a \log_{10}\left(\frac{2E - E'_1 - E'_2}{59}\right) + b \log_{10}\left(\frac{E - E'_2}{59}\right) + c + d \log_{10}\left(\frac{E'_3 - E}{29.5}\right)\\
1 + \log_{10}\left(\frac{2E - E'_1 - E'_2}{59}\right) + \log_{10}\left(\frac{E - E'_2}{59}\right) + \log_{10}\left(\frac{E'_3 - E}{29.5}\right)
\]

Where \(a, b, c\) and \(d\) are the absorbances of oxidized flavin, flavin semiquinone, flavin hydroquinone:NADP+ charge-transfer complex and flavin hydroquinone:NADPH complex respectively. \(E\) is the potential of the working electrode and \(E'_1, E'_2\) and \(E'_3\) are the midpoint potentials of the oxidized/semiquinone, semiquinone/hydroquinone-NADP CT complex and hydroquinone-NADP+ CT complex/hydroquinone-NADPH complex redox couples respectively. \(a, b, c, d, E'_1, E'_2\) and \(E'_3\) are variables determined by least-squares fitting.

2.6. Steady state kinetic

All steady state experiments were performed at 25.0°C in 50 mM Tris-HCl, pH 7.5, 100 mM KCl buffer using UV-visible spectrometry (Shimadzu UV-1610 spectrophotometer).

1ml aliquots were prepared with 1 mM NADPH and 2-100 μM cytochrome c or 0.2 mM-12 mM ferricyanide in buffer and incubated at 25°C prior to use. The reaction was initiated by adding the enzyme to the mix at a final concentration of 3-20 nM.
The reduction of cytochrome \( c \) or ferricyanide was also performed in the same conditions in presence of a 10 fold excess of CaM/Ca\( ^{2+} \) (3mM, 30mM).

\( K_m \) values for cytochrome \( c \), ferricyanide with or without CaM were determined from experiments in which the concentration of these molecules was varied and by reciprocal analysis of the velocity \textit{versus} concentration data.

### Extinction coefficients for electron acceptors

<table>
<thead>
<tr>
<th>Electron Acceptor</th>
<th>( \lambda ) (nm)</th>
<th>( \Delta \varepsilon ) (M(^{-1}).cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferricyanide (K salt, BDH)</td>
<td>420</td>
<td>1010</td>
</tr>
<tr>
<td>Cytochrome ( c ) (horse heart, Sigma)</td>
<td>550</td>
<td>22,640 (Hazzard \textit{et al.}, 1986)</td>
</tr>
</tbody>
</table>

### Extinction coefficients for enzyme (determined Section 3.2.3)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( \lambda ) (nm)</th>
<th>( \Delta \varepsilon ) (M(^{-1}).cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOSFAD</td>
<td>456</td>
<td>10540</td>
</tr>
<tr>
<td>nNOSFMN</td>
<td>456</td>
<td>10400</td>
</tr>
<tr>
<td>nNOSrd</td>
<td>456</td>
<td>20940</td>
</tr>
</tbody>
</table>

### 2.7 Anaerobic Stopped-flow Spectrophotometry

Stopped-flow experiments were performed on an SX.18MV stopped-flow spectrophotometer (Applied Photophysics) at a temperature of 25 ± 1 °C contained within an anaerobic glove box (Belle Technology) to prevent the reaction of reduced nNOSrd with molecular oxygen. Oxygen levels were maintained below 5 ppm in a nitrogen atmosphere at all times. Data were analyzed using Origin 7 (Microcal). Averaged traces from four or more measurements were used for analysis. For all stopped-flow experiments, the buffer was 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, supplemented with 2 mM CaCl\(_2\) when CaM was present, unless otherwise stated. Buffer was made anaerobic by bubbling with nitrogen for 2 h before being left to equilibrate in the anaerobic box overnight. NADPH, NADP\(^+\), NADH, NAD\(^+\), 2',5'-ADP, cytochrome \( c \) (horse heart, type I; Sigma), and dithionite were brought into the anaerobic box in powder form and dissolved in anaerobic buffer.
Concentrations were determined by absorption spectroscopy on a Cary 50 Biospectrophotometer within the anaerobic box using the extinction coefficients 29,500 M\(^{-1}\) cm\(^{-1}\) at 550 nm for cytochrome c (dithionite-reduced sample), 6,200 M\(^{-1}\) cm\(^{-1}\) at 340 nm for NADPH, 18,000 M\(^{-1}\) cm\(^{-1}\) at 260 nm for NADP\(^+\), 6,220 M\(^{-1}\) cm\(^{-1}\) at 340 nm for NADH, 18,000 M\(^{-1}\) cm\(^{-1}\) at 260 nm for NAD\(^+\), and 15,400 M\(^{-1}\) cm\(^{-1}\) at 260 nm for 2',5'-ADP. Reduced nNOSrd protein was generated by titration with a concentrated solution of NADPH or dithionite until no further spectral change occurred. One electron reduced protein was generated by titration with a concentrated solution of dithionite until the ratio of the absorbances at 592 nm and 456 nm was within 0.25-0.3. Enzyme (reduced or oxidized) was made anaerobic and free of excess reductant by passage down a 1.5 x 20-ml Sephadex G-25 (Sigma) size separation column immediately prior to use. CaM was brought into the box in a 3 mM solution containing 30 mM CaCl\(_2\) and added to the nNOSrd to achieve a 2:1 concentration ratio of CaM to nNOSrd. Concentrations of components of stopped-flow reaction mixtures are given as final concentrations after mixing.
Chapter 3

Protein Characterisation
3.1 Protein preparation

3.1.1 Preparation of nNOSFMNCaM
Neuronal NO synthase FMN-binding domain was co-expressed with calmodulin in *E. coli* and purified with CaM bound throughout.

Purification of nNOSFMNCaM from 25 g *E. coli* was performed in 4 steps. In the first step of purification, crude extracts were loaded on to a DEAE anionic exchange column, the yellow fractions were collected and gathered, ¼ diluted in buffer B and the protein was loaded on to a Q-sepharose (pharmacia) chromatography column run by an FPLC system (ACTA) coupled with a 220 nm UV-vis detector (Figure 3.1). The initial lysate was dark grey/blue, indicating the air stable semiquinone form of the enzyme. As the purification progressed, the FMN turned yellow as oxidation occurred (confirmed by SDS-PAGE, data not shown). The blue-grey semiquinone was easily regenerated by addition of dithionite and remained in this oxidation state for several hours in the presence of air. Inclusion of 10 mM DTT in the buffers during purification was found to prevent oxidation; however, this was not necessary for the stability of the enzyme or its purification.

![Fractions collected](image)

**Figure 3.1** nNOS FMN sub-domain first Q-sepharose elution in presence of a linear gradient of 0-400mM KCl. The graph shows the absorbance at 220 nm of protein eluting versus volume of eluant.
The yellow fractions eluted between 350-400mM KCl and containing the nNOSFMNCaM (determined by SDS-PAGE, data not shown) were collected and gathered, diluted in buffer B and loaded on to a second Q-sepharose (Figure 3.2).

![Fractions collected](image)

Figure 3.2 nNOS FMN sub-domain second Q-sepharose elution in presence of a linear gradient of 0-400mM KCl. The graph shows the absorbance at 220 nm of protein eluting versus volume of eluant.

The fractions containing the nNOSFMNCaM (determined by SDS-PAGE, data not shown) were collected, mixed together, concentrated using centrifugal concentrators Centriprep 10 (Amicon) and eluted through a superdex 200 column (2.5 cm x 60 cm) (pharmacia) (2ml/min) (Figure 3.3). The concentration of CaCl₂ was kept low in order to avoid calcium sulphate crystal formation in presence of MgSO₄ commonly used as salt to crystallise the protein. The $K_m$ of fully Ca²⁺ bound CaM is known to be $K_m \leq 0.3$ μM (Nishida et al 1999). The 10 μM CaCl₂ used at the last purification step represents at least 30 $K_m$, which is enough to keep the CaM bound on the enzyme and ensure 1:1 stoichiometry. Also it is much less than the final concentration of enzyme, so most of the Ca²⁺ in the sample is bound.
Figure 3.3 nNOS FMN sub-domain gel filtration (superdex 200) elution equilibrated in Tris/HCl 50mM, pH 7.5, 10 µM CaCl$_2$ and 100mM KCl. The graph shows the absorbance at 220 nm of protein eluting versus volume of eluant.

The main peak was collected, concentrated up to 20 mg / ml and stored at -80°C. In order to check the purification progress, SDS PAGE was performed after each step (Figure 3.4).
3.1.2 Preparation of nNOSrd

nNOS reductase domain was prepared as described earlier in materials & methods (Noble et al 1999). A 6-litre nNOS reductase domain culture gave us after purification 70 mg protein of 80% estimated purity determined by SDS-PAGE (Figure 3.5).
3.1.3 Preparation of nNOSFAD

The nNOS FAD sub-domain was produced from nNOSrd by trypsin digestion. In order to control the degradation process, we ran an electrophoresis gel at different times during the digestion (Figure 3.5).

After the last purification step, 30mg nNOSFAD were obtained with 90% estimated purity determined by SDS-PAGE (Figure 3.5). The purity is estimated suitable for further experiments, and the enzymatic digestion process from nNOSrd gave a 67% molarity yield.

3.1.4 Purification of CaM

Usually, 3 litres of flow-through collected from nNOSrd purification were boiled before being calcified with CaCl₂. The CaM containing fractions eluted from the phenyl sepharose column were determined by SDS-PAGE, concentrated and freeze-dried. The yield of the purification was commonly 100mg of CaM per litre of culture and the purity was estimated to be close to 90%. The quality of the newly purified
Chapter 3. 

Protein characterisation

CaM was tested by looking at its activation of the steady-state cytochrome c reduction by nNOSrd.

![Image of a denaturing electrophoresis gel with a band marked as CaM (16.7 KDa)]

**Figure 3.6** CaM eluted fraction from the phenyl sepharose column. Analysis of CaM purity using a denaturing electrophoresis gel (acrylamide 12%)

### 3.2 Protein characterisation

#### 3.2.1 UV-vis spectrum

The following spectra are UV-visible spectra of nNOSFMNCaM (Figure 3.7), nNOSrd (Figure 3.8) and nNOSFAD (Figure 3.9) in their oxidised (yellow), one electron reduced (Blue) and reduced (black dotted) redox states.
3.2.1.1 Characteristic spectra of nNOSFMNCaM

Figure 3.7 Characteristic spectra of nNOSFMNCaM (6μM). The yellow spectrum corresponds to the oxidised form of the enzyme with an absorbance maxima at 375 nm and 457 nm. The blue spectrum corresponds to the semiquinone form of the enzyme with an absorbance maxima at 350 nm and 592 nm and the black dotted spectrum corresponds to the electrochemically generated fully reduced enzyme with no characteristic absorbance bands.

3.2.1.2 Characteristic spectra of nNOSrd

Figure 3.8 Characteristic spectra of nNOSrd (80μM). The yellow spectrum corresponds to the oxidised form of the enzyme with an absorbance maxima at 457 nm. The blue spectrum corresponds to the one-electron-reduced form (FADoxidised/FMNssemiquinone) of the enzyme with an absorbance maxima at 457 nm and 592 nm and the black dotted spectrum corresponds to the electrochemically generated fully reduced enzyme with no characteristic absorbance bands.
3.2.1.3 Characteristic spectra of nNOSFAD

Figure 3.9 Characteristic spectra of nNOSFAD (11μM). The yellow spectrum corresponds to the oxidised form of the enzyme with an absorbance maximum at 457 nm. The blue spectrum corresponds to the semiquinone form of the enzyme with an absorbance maximum at 592 nm and the black dotted spectrum corresponds to the electrochemically generated fully reduced enzyme with no characteristic absorbance bands.

3.2.2 Protein stability

nNOSFMNCaM, nNOSrd and nNOSFAD were stable in conditions of use that are 4°C during purification process and 25°C for experiments done using stopped-flow apparatus, spectrophotometer and ottole potentiometry devices. The buffer used for all the experiments was 50 mM Tris, pH 7.5 to which was added 0 to 0.5 M KCl or 0 to 100 mM NaCl.

3.2.2.1 nNOSrd and nNOSFAD

Both nNOSrd and nNOSFAD proteins were found to be stable in their NADP-bound form as well as their uncomplexed form. nNOSrd was found to be stable regardless the presence of bound CaM. Finally, both enzymes were found to be stable for any of their redox state.
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3.2.2.2 Stability of nNOSFMNCaM

Attempts to isolate the CaM-free nNOSFMN were made in order to study the CaM binding effect on redox and kinetics properties of the FMN sub-domain of nNOS. However, removal of CaM at any stage during the purification procedure by addition of EGTA led to proteolysis of the domain and/or precipitation of the protein. The following conditions refer to detergents (Tween 20 and deoxycholate) and salts (KCl or NH₄⁺) were added to nNOSFMNCaM solutions, which did not prevent protein precipitation (Table 3.1).

<table>
<thead>
<tr>
<th>[Protein] (µM)</th>
<th>Buffer (M)</th>
<th>Salt (M)</th>
<th>Glycerol (%)</th>
<th>Detergent (%)</th>
<th>Protein stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.5</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>Fast precipitation</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.5</td>
<td>-</td>
<td>0.5</td>
<td>Fast precipitation</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>Fast precipitation</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.5</td>
<td>-</td>
<td>1</td>
<td>Fast precipitation</td>
</tr>
<tr>
<td>60</td>
<td>0.05</td>
<td>0.5</td>
<td>20</td>
<td>-</td>
<td>Slow precipitation</td>
</tr>
</tbody>
</table>

Table 3.1 nNOSFMNCaM stability was tested for 20µM of protein at 10°C using UV-vis spectrophotometry in order to detect spectral changes indicating precipitation.

3.2.3 Determination of the extinction coefficients

3.2.3.1 Extinction coefficient of nNOSFMNCaM and nNOSFAD

The flavin cofactors of 1ml of 10 µM nNOS FAD or FMN sub-domain in Tris-HCl, pH 7.5, 50 mM, 100 mM KCl 7.5 were released using 20 µl lauryl sulfate 10% m/v at 60°C. The temperature remained constant while the UV-vis spectra were being recorded at 60°C in order to keep the lauryl sulfate soluble (Figures 3.10 and 3.11).
Based on the fact that the primary structure of the denatured protein does not display any light absorption in the range of visible wavelength and that the folded protein, interacts, shifts and decreases the maximum of absorbance of the bound flavin, it is possible to define whether all of the flavin cofactor is really no longer bound after

Calmodulin activation of the reductase domain of mammalian neuronal nitric oxide synthase
Chapter 3. Protein characterisation
denaturation. The results demonstrates that the $\lambda_{\text{max}}$ corresponding to the spectra of the denatured enzyme matches exactly those of the free flavin cofactors. This indicates a full release of the FMN cofactor by the protein allowing calculation of the extinction coefficients by comparison of the $\lambda_{\text{max}}$ value under native and denaturing conditions.

$$\text{Abs}_{\text{max nNOS Flavoprotein}} \times \varepsilon_{\text{max Flavin}} = \varepsilon_{\text{max n NOS Flavoprotein}}$$

The extinction coefficient obtained for nNOSFMNCaM is $\varepsilon_{\text{max}} = 10400 \text{ M}^{-1} \text{cm}^{-1}$ at 456 nm and $\varepsilon_{\text{max}} = 10560 \text{ M}^{-1} \text{cm}^{-1}$ at 456 nm nNOSFAD.

3.2.3.2 Extinction coefficient of nNOSrd

nNOSrd is made of both nNOSFMNCaM and nNOSFAD. The large scale conformational change on CaM binding does not affect the spectral characteristics of the nNOSrd suggesting that the extinction coefficient can be determined as the sum of the extinction coefficients of the isolated nNOSFMNCaM and nNOSFAD (Gachhui et al., 1996b; Sheta et al., 1994). Therefore, the calculated extinction coefficient obtained for nNOSrd is $\varepsilon_{\text{max}} = 20960 \text{ M}^{-1} \text{cm}^{-1}$ at 456 nm.

3.2.4 Cofactor quantitation

3.2.4.1 FMN and FAD cofactor quantitation

In order to confirm the reliability of our expression system, i.e. to test whether the produced protein is complete and properly folded, it was essential to determine the proportion of enzyme with bound FMN. For that the concentration of an enzyme sample was calculated from the ratio of the holoenzyme concentration, using its specific UV-vis absorbance, and the total protein concentration (holoenzyme + apoenzyme). The total protein concentration was determined by the method of Bradford. The results showed that the purified enzyme contains 69% of holoenzyme.
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Due to the inaccuracy of the Bradford assay (± 20%), this result was considered as a qualitative result concluding that the protein produced is representative of the nNOSFMNCaM.

3.2.4.2 FAD cofactor quantitation

Results using the same method for FAD quantitation reveals that all nNOSFAD enzyme contain FAD cofactor bound. It is important to note that all the concentrations determination for potentiometry and kinetic experiment were calculated using UV-vis absorbance of the oxidised enzyme at 456 nm as in this case only the holoenzyme concentration matters.

3.2.4.3 Proportion of FAD/FMN cofactor in nNOSrd

Work done in the laboratory by Kitamura et al (not published) using fluorescence spectroscopy revealed that purified wild type nNOSrd used in this work contains on average a stoichiometric amount of FAD cofactor and a ratio FMN/FAD of 0.8. These results are in agreement with previous work (Gachhui et al., 1996a; Matsuda and Iyanagi, 1999). See protocol used by Kitamura et al to determine the nNOSrd flavin content in Appendix 8.1.

3.2.5 Mass spectrometry

(Done in collaboration with Dr R. Smith, University of Edinburgh)

The molecular weight of the nNOSrd was determined by electrospray mass spectrometry. A mass spectrometer directly linked to HPLC was used in order to lower the time the enzyme spent out of Tris buffer and so prevent its precipitation. Two sets of mass spectrometry experiments were carried out in order to compare the mass of an enzyme accidentally proteolysed during purification (Figure 3.12) and the native one (Figure 3.13). The difference between the two enzymes in term of activity, is a lower CaM response for the proteolysed enzyme.
Figure 3.12 Mass spectrum of proteolysed nNOSrd. Actual mass is 79514.8 ± 1 Da. From the amino acid sequence in Appendix 8.2.3, the predicted mass of nNOSrd is 83431 Da. This gives a discrepancy of 3920 Da due to proteolysis.

Figure 3.13 Mass spectrum of nNOSrd. Actual mass is 80698.0 ± 1 Da. From the amino acid sequence in Appendix 8.2.3, the predicted mass of nNOSrd is 83431 Da. This gives a discrepancy of 2730 Da due to proteolysis.
Chapter 3. Protein characterisation

Samples were injected into the mass spectrometer (Micromass Platform electrospray mass spectrometer) and analysed under the following conditions: ESI-ve, cone voltage = 70 V, acquired range 500 – 2500 Da. The raw data were baselined substractioned and smoothed, then centred to extract the charged ion series and transformed to give the total mass of the protein sample (MassLynx software).

3.2.6 Edman sequencing
(Done in collaboration with Dr A. Cronshaw, University of Edinburgh)

CaM binding to the nNOSrd has been shown to activate the electron transfer through the enzyme (Abusoud et al., 1994b; Craig et al., 2002; Gachhui et al., 1996b) with notably a 10-fold enhancement in the steady-state rate of cytochrome c reduction (Figure 5.1). However, previous experiments have demonstrated that nNOSrd was sensitive to degradation at both its C-terminus and N-terminus extremities leading to the loss of CaM / Ca^{2+} dependency in term of electron transfer regulation (Knight and Scrutton, 2002; Roman et al., 2000b). In order to determine the level of degradation at both the N-terminus and the C-terminus of the proteolyzed nNOSrd with low CaM response (Section 3.2.5, Figure 3.12), N-terminal sequencing was carried out. N-terminal sequencing was also done for nNOSFMNCaM to determine the integrity of the protein to ensure the validity of its redox properties determined in Section 4.2.

N-terminal sequencing was carried out on both nNOSFMNCaM and nNOSrd protein by automated Edman degradation on a Applied Biosystems Procise 4HT microsequencer connected with an on-line PTH-amino acid analyzer. For that, the nNOSFMNCaM and nNOSrd protein samples were further purified by SDS-PAGE before being transferred to a nitrocellulose membrane by electroblotting and Coomassie stained (Figure 3.14). Finally, the band corresponding to the respective proteins were cut out to be loaded into the sequencer.
Chapter 3. Protein characterisation

The gene for nNOSrd corresponds to a protein fragment that encompass the residues 695 to 1429 giving an theoretical average mass of 83431 Da (Determined using Expasy ProtParam tool software). The N-terminal sequencing carried out by Edman degradation demonstrates that 50% of the low-CaM response nNOSrd protein tested is missing 21 amino acids at the N-terminus. The first seven amino acids detected from the HPLC elution profile were W22KGTVGT28 (Appendix 8.2.1). The mass spectrometry determined mass for this batch of enzyme was 79515 Da (Figure 3.12). This discrepancy of 3920 Da suggests that ≈12 amino acids are missing at the C-terminus in addition to the 21 amino acid missing in the N-terminus. The enzyme was purified using CaM affinity chromatography column suggesting that the protein obtained binds CaM in a qualitative way. Previously, Matsuda and Iyanagi (1999) studied the nNOSrd electron transfer activation by CaM. The enzyme they used was also truncated by 20 - 21 amino acid compared to the theoretical length of the presently studied nNOSrd. However they observed normal CaM-dependent activity suggesting that the poor CaM response of the current proteolysed enzyme is mostly the consequence of a damaged C-terminal extension.
Chapter 3. Protein characterisation

The recombinant nNOSFMN corresponds to the nNOS protein fragment from 695 to 946 (Appendix 8.2.2). The N-terminal sequence obtained demonstrates that more than 70% of the nNOSFMN had the expected length with no degradation happening during the protein production. The five first residues were determined to be M_{695}LNYR_{699} as expected. The retention volume for Met_{595} indicates that the methionine was in its sulphone form probably resulting of post-transcriptional modification carried out during its production in _E.coli_.

\[
\begin{align*}
R - S - R & \xrightarrow{\text{Oxidation}} R - S^+ - R \\
\text{Thioether} & \quad \text{Sulphone}
\end{align*}
\]

3.2.7 Crystallography assays
(Done in collaboration with Dr C. Mowat, University of Edinburgh)

As none of the nNOS crystal structures solved so far contains the CaM-binding site, little information is known about CaM/nNOSrd interaction. Following this idea, the aim of this work was to get the crystal structure of the nNOSFMN complexed with CaM. The main advantage of considering nNOSFMN\textit{CaM} over nNOSrd\textit{CaM} is the loss of the protein mobility at the FMN/FAD domain interface and also a smaller molecular weight which increases the chance of crystallization. The first conditions tried were the commercial screens: Hampton Crystal screen 1, Molecular Dimensions Clear Strategy screen 1&2 (Na cacodylate buffer pH6.5) and Hampton Research Membfac screen. Further trials were conducted based upon the results of these. In addition, general screens involving varying pH and precipitant (PEGs, ammonium sulphate) were also carried out. The best crystallisation results were obtained for 20mg/l of protein concentration. Although the results were reproducible, most of the conditions led to the formation of tiny clusters of poor quality crystals making it difficult to optimise the conditions (Figure 3.15). N-terminal sequencing and mass spectrometry characterisation determined that nNOSFMN protein was mostly degradation-free (Section 4.). Moreover the enzyme purity revealed by SDS-PAGE
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Protein characterisation

(Paragraph 1.1 Figure 3.4) appeared to be suitable for crystallography. The crystallisation difficulties encountered were probably the fact of inhomogeneity resulting from FMN cofactor binding instability encountered when the oxidised enzyme remains in solution for a long period of time. A possible solution is to carry out crystallisation process in anaerobic conditions in presence of DTT in order to stabilise the cofactor in the semiquinone state through hydrogen bonding between the N5 of the cofactor and the Gly810.

Figure 3.15 FMN sub-domain crystals in a drop of protein in 0.4 M MgSO₄, 0.1M Tris-HCl pH 8.5, 0.1 M KNa tartrate.
Chapter 4

Thermodynamics
4.1 Introduction

*In vivo*, NO synthesis by neuronal NOS is regulated by the reversible binding of CaM in response to intracellular elevation of Ca$^{2+}$ concentration (Abusoud *et al.*, 1994a). Studies on CaM activation have shown that NO synthesis correlates with the rate of electrons delivered by nNOSrd (Nishida and de Montellano, 1998) and that the activating function of CaM with respect to nNOS is complete within the reductase domain itself (Gachhui *et al.*, 1996b). CaM binding to nNOSrd activates its reduction by NADPH, interflavin electron transfer and electron transfer to external electron acceptors, nNOS heme or cytochrome c. The direction of the net electron transfer between NADP, FAD and FMN is determined by the reduction potentials of electron donor and acceptor. The flux of electrons is orientated from the species with a more negative reduction potential, the electron donor, to the species with a more positive reduction potential, the electron acceptor. The difference in values between redox potentials across the system determines the driving force for electron transfer between species and therefore the reactivity of this system. The redox properties of the bound flavin cofactors, in nNOSrd, are affected by the interaction of the isoalloxazine ring with amino acid residues of the isoalloxazine binding site. Protein conformation changes can also modify the environment of the flavins and affect its redox properties. The formation of hydrogen bonds between the protein and the flavin often involve stabilising preferentially one redox form of the flavin cofactor. In addition to the interactions with amino acids, the NADP nicotinamide stacking can alter the redox properties of FAD isoalloxazine. This has been previously demonstrated with synthetic dinucleotides with linkers of various lengths (Blankenhorn, 1975). CaM activation of the nNOSrd has been suggested to be the consequence of conformational changes (Noble *et al.*, 1999) that enhance flavin to flavin and flavin to heme electron transfer.

The aims of this chapter are to investigate the regulatory mechanism of CaM and particularly the effect of the nNOSrd conformational change on the reduction potentials of the flavins. The effect of substrate (NADP$^+$) binding on the reduction potentials of the FAD cofactor is directly related to this.
All of the redox processes of nNOSrd are reversible. The equilibrium for any of these catalytic steps is dynamic, which means that at equilibrium electrons are transferred in both directions from reduced to oxidized cofactors with equal rates, so that no net electron transfer occurs. Then, at equilibrium, the values of the redox potentials characteristic of the four redox couples FAD/FADH, FADH/ FADH₂, FMN/FMNH, and FMNH/FMNH₂ of nNOSrd determines the electron population for each of these redox couples.

In the present work, the reduction potentials of the nNOSrd flavin cofactors at equilibrium were measured by anaerobic titration using an OTTLE potentiometry cell in order to obtain information on the electron transfer mechanism and electron repartition in nNOSrd. The presence of both FAD and FMN cofactors in nNOSrd makes redox potential determination difficult especially in the presence of NADPH due to the presence of an additional redox couple. In order to simplify redox potential determination, the FAD and FMN domains were separated and studied on their own. In this way, it was also possible to investigate perturbation of flavin redox potential consecutive to nNOSrd conformational change upon CaM binding. The FAD and FMN redox potentials were compared for nNOSrd and for the isolated domains where the FAD/FMN interface is absent. The effect of the substrate (NADP⁺) binding on the electron repartition in nNOSrd was also investigated. In order to carry this out, the midpoint potentials of the oxidized/semiquinone and semiquinone/hydroquinone redox couples of the nNOSFAD were determined in the presence and absence of stoichiometric amounts of NADP⁺. An NADP analogue, ADP, was used to further assess the effect of nicotinamide stacking in affecting the redox potential of the FAD cofactor.

In order to compare the redox potentials obtained for the isolated domains and for nNOSrd, electrochemical titration of nNOSrd was carried out. The changes in UV/Vis spectrum observed during the OTTLE cell potentiometry titration (Figure 4.1) for nNOSrd closely matched those reported previously (Noble et al., 1999) using chemical reduction/oxidation in 0.05M Tris/HCl pH 7.1, 0.1M KCl. The data (Table 4.1) were fitted with the Nernst equation, using absorption coefficients calculated for the separate domains. Comparison with previous data obtained by Noble et al (1999)
Chapter 4. Thermodynamics

shows a broad shift in potential of 30-60 mV largely attributable to the increase in pH.

<table>
<thead>
<tr>
<th>Redox Couple (mV)</th>
<th>FMN/H⁺</th>
<th>FMNH₂⁻</th>
<th>FAD/H⁺</th>
<th>FADH₂⁻</th>
<th>NADP/H⁺</th>
<th>Heme⁴⁺FeIII²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOSrd + CaM⁵</td>
<td>-98 ± 5</td>
<td>-300 ± 8</td>
<td>-296 ± 6</td>
<td>-320 ± 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nNOSFMNCaM</td>
<td>-179 ± 3</td>
<td>-314 ± 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nNOSFAD</td>
<td>-</td>
<td>-291 ± 3</td>
<td>-326 ± 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nNOSFAD + ADP⁶</td>
<td>-</td>
<td>-297 ± 6</td>
<td>-323 ± 7</td>
<td>-</td>
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</tr>
<tr>
<td>nNOSFAD + NADP⁷</td>
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<td>-304 ± 5</td>
<td>-290 ± 5</td>
<td>-356 ± 3</td>
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<td>-</td>
</tr>
<tr>
<td>Free NADP⁷</td>
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<td>-</td>
<td>-</td>
<td>-332⁸</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nNOS Heme domain⁹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-306d</td>
</tr>
</tbody>
</table>

Table 4.1 Reduction potentials of the FMN, FAD and NADP⁺ cofactors of nNOSrd, nNOSFMNCaM and nNOSFAD. Measurements were taken at 25 ± 1 °C in 100 mM Tris/HCl buffer pH 7.5, 0.5 M KCl.

- Reduction potential of the flavin oxidized/semiquinone redox couple
- Reduction potential of the flavin semiquinone/hydroquinone redox couple
- Reduction potential of the NADP⁺/NADPH 2-electron redox couple adjusted by 30 mV per pH unit (Clark, 1960).
- Reduction potential of the isolated nNOS oxygenase domain heme Fe³⁺Fe²⁺ redox couple in the presence of L-Arginine (Presta et al., 1998) repeated under above conditions.
- In the presence of stoichiometric amounts of bound CaM, ADP and NADP⁺ respectively.

Figure 4.1 UV/Vis absorbance spectra of nNOSrd during electrochemical reduction. The yellow and heavy dotted spectra correspond to the fully oxidised (with stable FMN semiquinone) and fully reduced (from electrochemical reduction) nNOSrd respectively. The blue spectra and the light dotted spectra correspond to the NADP⁺-bound nNOSrd with maximum charge-transfer band and the nNOSrd without NADP⁺ at a similar stage of reduction respectively. All spectra were recorded in 100 mM Tris/HCl pH 7.5, 0.5 M KCl at 25 °C.
All redox titrations for the reductase and the isolated domains were carried out in similar conditions. The enzyme and ligand concentrations in these experiments were 300-500 μM, which is well in excess of the $K_i$ values of these inhibitors (Wolthers and Schimerlik, 2001) ensuring 1:1 complex formation (Daff, 2004).

The proteins, nNOSrd and separated domains nNOSFMN and nNOSFAD, used for the following experiments were obtained as described in Chapter 3.3. During the expression and purification, all precautions were taken to ensure that nNOSrd remained in its air-stable FMN semiquinone form in order to prevent loss of FMN cofactor and C-terminal extension degradation. $Ca^{2+}$ was also present throughout the purification to keep the CaM bound at all times in order to prevent any degradation of the CaM binding linker.

The presence of CaM was found to be necessary for the nNOSFMN domain stability preventing the determination of the midpoint potentials for the CaM-free enzyme. In the presence of CaM, the FMN cofactor formed a stable blue semiquinone, which oxidized slowly in air, much like the FMN semiquinone formed in nNOS holoenzyme and in the reductase domain.

### 4.2 Spectroelectrochemical analysis of nNOSFMNCaM

Figure 4.2 shows the UV-visible spectral change monitored for the stepwise reduction of the enzyme by electrons in the OTTLE potentiometry cell. The oxidised nNOSFMNCaM is a two electron acceptor which has absorbance maxima at 457 nm and 375 nm. The determination of the midpoint potentials for FMN/FMNH and FMNH/FMNH$_2$ redox couples of nNOSFMNCaM was carried out using OTTLE potentiometry. The first step of enzyme reduction leads to the formation of a neutral blue semiquinone typical of flavodoxin with absorbance maxima at 350 nm and 592 nm (Mayhew, 1992). The second step is the formation of the hydroquinone with no characteristic visible absorbance. Data obtained for the electrochemical reduction of the enzyme show that the two redox couples have different behaviour (Figures 4.3
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Thermodynamics

and 4.4). Providing that the concentration of enzyme and mediators are sufficient, the relaxation rate is limited by the conductivity of the solution as determined by the concentration of electrolyte present and the arrangement/dimensions of the cell. Typically 10-15min are necessary for the current to equilibrate after each 30 mV potential step as observed for FMNH/FMNH₂ redox couples whereas a longer equilibration process is required in both reduction and oxidation for the first redox couple FMN/FMNH.

Figure 4.2 UV/Visible spectra of nNOS FMN domain with bound CaM during spectroelectrochemical redox titration. 275 μM nNOSFMNCaM was reduced electrochemically in 0.1 M Tris/HCl, pH 7.5, 0.5 M KCl at 25 °C with steps of -30 mV between each spectrum. The yellow spectrum corresponds to the fully oxidised enzyme and the blue spectrum corresponds to the maximum amount of semiquinone formed during the titration. Arrows indicate how the spectra change as reduction proceeds.
Figure 4.3 Determination of redox potentials for nNOS FMN domain with bound CaM. Panel A, spectroelectrochemical titration with 15 min. intervals between 40 mV redox steps in the reductive (4) and oxidative (−) directions; Panel B, spectroelectrochemical titration with full equilibration after each redox step. Data are shown fitted to Equation 1, Chapter 2.5. The broken lines mark the midpoint potentials of the two FMN redox couples for the data in panel B. The value determined for $E_1$ (FMN/FMNH) and $E_2$ (FMNH/FMNH$_2$) are -179 mV and -314 mV respectively.

Figure 4.3 plots formation of semiquinone at 592 nm versus the potential of the working electrode. The enzyme, in the first part of the experiment, was fully reduced by 30 mV increments in potential (red trace) and then reoxidised in the same manner (Blue trace). In panel A, the absorbance of the semiquinone was recorded after 20 min equilibration time for each potential step, whereas panel B shows the data after full potential equilibration according to the time-course plot (Figure 4.4).
Figure 4.3 panel A shows the free reversibility of the semiquinone/hydroquinone couple whereas the oxidised/semiquinone shows a large hysteresis phenomenon with a gap of 80 mV separating the forward and reverse redox transition. Figure 4.4 shows that the equilibration time for the first redox couple was up to 20 times longer than for the second one.

![Figure 4.4 Spectroelectrochemical time-course for nNOS FMN domain with bound CaM. The current flowing between working and counter electrodes (I) is plotted against time. The potential of the working electrode was stepped down by 30 mV after each equilibration. Data relate to Fig. 3, Panel B.](image)

The data plotted in Figure 4.3 panel B were used to calculate the equilibrium reduction potentials of the two FMN redox couples; these were -179 mV for FMN/FMNH and -314 mV for FMNH/FMNH₂ (Table 4.1). The reduction potential for the FMN oxidized/semiquinone redox couple for the isolated domain is 80 mV lower than that observed for the FMN domain in nNOSrd, indicating that the FAD/FMN interface stabilises the FMN semiquinone thermodynamically. It is of interest to note that this effect was not observed with the related enzymes human CPR (Munro et al., 2001) and methionine synthase reductase (Wolthers et al., 2003). The same redox couple was also found to exhibit strong hysteresis during electrochemical reduction and oxidation, as shown in Figures 4.3 and 4.4. Analogy with related flavodoxins suggests that the protonated N5 of the FMN semiquinone hydrogen-bonds the peptide carbonyl of Gly810 (Figure 4.5) and that this
stabilisation, absent in the oxidised form, creates the hysteresis phenomenon observed Figure 4.3 A (Mayhew, 1992).

The result is a kinetically stable semiquinone, which does not oxidize during the normal catalytic turnover of nNOS. This is in agreement with the slow oxidation of FMN semiquinone in air and its very slow cytochrome c reduction capabilities despite it being thermodynamically favourable. The F MNH/F MNH₂ redox couple is, however, the same in both nNOSFMNCaM and nNOSrd. This redox couple is freely reversible and is utilized in the transfer of electrons from FAD to heme in nNOS. The FAD-domain, therefore, appears to have little influence on the potential of the viable electron on the FMN cofactor. Given this, it also seems unlikely that the conformation of the FAD domain could affect the reduction potential of the FMN sq/hq couple. The kinetic and thermodynamic stability of the FMN semiquinone toward oxidation ensures that the FMN cofactor acts only as a one-electron donor in order to deliver single electrons to the heme at an optimum rate. Transfer of electrons pairwise to the heme would result in a build up of stabilized ferrous NO complex during turnover, which would ultimately slow the overall rate of catalysis (Santolini et al., 2001b).
4.3 Spectroelectrochemical analysis of nNOSFAD

4.3.1 Uncomplexed nNOSFAD

Figure 4.6 panel A shows the UV-visible spectral change monitored for the stepwise reduction of nNOSFAD by electrons in the OTTLE potentiometry cell. The oxidised nNOSFAD is a two-electron acceptor which has an absorbance maximum at 460 nm. The reduction of the enzyme leads to the absorbance decaying at 460 nm, accompanied by an absorbance increase at 597 nm characteristic of the FAD neutral blue semiquinone. Further reduction leads to the disappearance of these bands. In Figure 4.6 panel B, the absorbance at 460 nm is plotted, as well as the difference in absorbance at 650 nm and 700 nm versus the corrected potential of the working electrode. The two sets of data were fitted simultaneously to a Nernst 1+1 electron equation (Chapter 2.5, Equation 1) to give reduction potentials of -291 mV and -326 mV for the FAD/FADH* and FADH*/FADH2 couples respectively (Table 4.1).
Figure 4.6 Determination of redox potentials for nNOSFAD. Panel A, UV/Vis spectra collected during spectroelectrochemical titration (reduction and reoxidation) of 300 μM enzyme in 100 mM Tris/HCl pH 7.5, 0.5 M KCl at 25 °C. The yellow spectrum corresponds to the fully oxidised enzyme and the blue spectrum corresponds to the maximum amount of semiquinone formed during the titration. Panel B, plot of electrode potential v. absorbance at 460 nm (-) (FAD reduction and oxidation) and the difference in the absorbances at 650 nm and 700 nm (4) (Semiquinone build up and decay) respectively, fitted simultaneously to Equation 1, Chapter 2.5. The broken lines mark the midpoint potentials of the two FAD redox couples, at equilibrium, for the data in panel B. The value determined for $E_1$ (FAD/FADH) and $E_2$ (FADH/FADH₂) are -291 mV and -326 mV respectively. Both redox couples were found freely reversible in the conditions of the experiment.

4.3.2 nNOSFAD complexed with NADP⁺

The same experiment was carried out for nNOSFAD in the presence of NADP⁺, but unlike NADP-free nNOSFAD, there were significant differences in both the UV/Vis spectra of intermediates formed during reduction (Figure 4.7) and in the final reduction potentials.
Figure 4.7 panel A shows the UV-visible spectral changes during the stepwise reduction of nNOSFAD in presence of NADP⁺ in the OTTLE potentiometry cell. In addition to the spectra observed for the reduction of the uncomplexed enzyme, the reduction of the NADP⁺-nNOSFAD complex presents an extra band at long wavelength. This broad absorbance, at 780 nm, is characteristic of an FADH₂-NADP⁺ charge-transfer complex.

Figure 4.7 Determination of redox potentials for nNOSFAD in the presence of NADP⁺. Panel A, UV/Vis spectra collected during spectroelectrochemical titration (reduction and reoxidation) of 300 µM enzyme and NADP⁺ (1:1 complex) in 100 mM Tris/HCl pH 7.5, 0.5 M KCl at 25 °C. The yellow spectrum corresponds to the fully oxidised enzyme and the black spectrum corresponds to the maximum amount of charge transfer complex formed during the titration. Panel B, plot of electrode potential v. absorbance at 355 nm (?) (NADP reduction and oxidation), 460 nm (–) (FAD reduction and oxidation), 780 nm (8) (Charge transfer complex build up and decay) and the difference in the absorbances at 650 nm and 700 nm (4) (FAD semiquinone build up and decay) respectively, fitted simultaneously to Equation 2, Chapter 2.5. The broken lines mark the midpoint potentials of the two FAD redox couples and the NADP⁺/NADPH redox couple, at equilibrium, for the data in panel B. The value determined for E₁ (FAD/FADH), E₂ (FADH/FADH₂) and E₃ (NADP⁺/NADPH) are -304 mV, -290 mV and -356 mV respectively. All three redox couples were found freely reversible in the condition of the experiment.
NADP⁺ is the product of NADPH dehydrogenation at the FAD site of nNOS. This species is known to form a charge-transfer complex with the FAD hydroquinone as the nicotinamide and isoalloxazine rings stack above one another (Batie and Kamin, 1986; Daff, 2004; Wolthers and Schimerlik, 2001). This extra band was also seen for nNOSrd with bound NADP⁺ as seen in Figure 4.7 panel A and Figure 4.1 respectively. The reduction of the NADP⁺/FADH₂ complex led to the disappearance of the absorbance at 780 nm as the FADH₂:NADPH was generated. Formation of NADPH was accompanied by an increase in absorbance at 355 nm (Figure 4.7 panel A). The absorbance at 355 nm is the approximate position of an isosbestic point in the nNOSFAD redox titration and is mainly influenced by changes in the relative concentrations of NADP⁺ and NADPH. The amount of FAD in the oxidised form was followed by monitoring the absorbance change at 460 nm. The contribution of the charge transfer absorbance at 780 nm on the semiquinone absorbance at 597 nm was minimized by looking at the difference in the absorbances at 650 nm and 700 nm (ΔA₆₅₀₋₇₀₀). All of the wavelength or differences in wavelength described are plotted in Figure 4.7 panel B against the corrected potential of the working electrode. Together they indicate how the concentrations of each of the four redox states (FAD:NADP⁺, FADH:NADP⁺, FADH₂:NADP⁺, FADH₂:NADPH) vary with potential. The data were fitted simultaneously to Nernst 1+1+2 electron equation (Chapter 2.5, Equation 2) to give reduction potentials of -304 mV, -290 mV, -356 mV for the FAD/FADH⁺, FADH⁺/FADH₂ and NADP⁺/NADPH couples respectively (Table 4.1). The extinction coefficient of the FADH-NADP⁺ charge-transfer complex at 780 nm was also calculated using the molar absorption coefficient at 456 nm for the oxidized isolated FAD domain (10560 M⁻¹cm⁻¹) and the value obtained was 970 M⁻¹cm⁻¹.

To further investigate the effect of NADP⁺ on changing the redox properties of the nNOSFAD and to demonstrate that these changes can be attributed to the NMN part of NADP, the same experiment was carried out in the presence of an NADP analogue, ADP. ADP was chosen because it shares the negatively charged phosphate group in position 2’ of the ribose adenine part of NADP. This phosphate is essential
for NADP/NAD discrimination as its absence (in NAD), there is a 1000-fold increase in the $K_m$ (Murataliev et al., 2004).

### 4.3.3 nNOSFAD complexed with ADP

![Figure 4.8 Determination of redox potentials of nNOSFAD in the presence of ADP. Panel A, UV/Vis spectra collected during spectroelectrochemical titration of 300 μM enzyme and ADP (1:1 complex) in 100 mM Tris/HCl pH 7.5, 0.5 M KCl at 25 °C. The yellow spectrum corresponds to the fully oxidised enzyme and the blue spectrum corresponds to the maximum amount of semiquinone formed during the titration. Panel B, plot of electrode potential v. absorbance at 460 nm (\(\lambda\)) (FAD reduction and oxidation) and the difference in the absorbances at 650 nm and 700 nm (\(\bullet\)) (FAD semiquinone build up and decay) respectively, fitted simultaneously to Equation 1, Chapter 2.5. The broken lines mark the midpoint potentials of the two FAD redox couples, at equilibrium, for the data in panel B. The value determined for $E_1$ (FAD/FADH) and $E_2$ (FADH/FADH$_2$) are -297 mV and -323 mV respectively. Both redox couples were found freely reversible in the conditions of the experiment.
Figure 4.8 panel A shows the UV-visible spectral change monitored for the stepwise reduction of nNOSFAD in the presence of ADP by electrons in the OTTLE potentiometry cell. In Figure 4.8 panel B the absorbance at 460 nm and the difference in absorbance at 650 nm and 700 nm are plotted against the potential of the working electrode. The data were fitted to a Nernst 1+1 electron equation (Chapter 2.5, equation 1). ADP corresponds to a fragment of NADP⁺ in which the NMN moiety is missing. In order to compare the spectral changes characteristic of NADP⁺, and particularly of the NMN part of the NADP⁺, the semiquinone absorbance parameter calculated for the uncomplexed enzyme was also used in the presence of ADP and NADP⁺ as their presence is unlikely to affect the extinction coefficient greatly. As expected, no formation of a charge-transfer species was observed during the titration. The presence of ADP did not affect the UV/Vis spectra of the different nNOSFAD redox species to the same extent as that observed in the presence of NADP⁺ (Figure 4.8 panel A). The proportion of semiquinone generated during the redox titration of the ADP:nNOSFAD complex was less than for the uncomplexed enzyme, but was more than in the presence of NADP⁺. Although it is clear that there is slightly less semiquinone formed during this redox titration in the presence of ADP, the shifts in potential observed are similar to the experimental errors (Table 4.1).

The nNOSFAD redox titrations were all freely reversible, with no hysteresis observed in any of the redox couples. However the NADP⁺ binding was found to stabilise the hydroquinone form of the FAD hydroquinone by increasing the reduction potential of semiquinone/hydroquinone redox couple by 30-40 mV. ADP-binding to nNOSFAD did not lead to a potential shift suggesting that the NMN part of NADP is involved in this effect. NMN is also responsible for the extra absorption band formation at 780 nm seen for NADP⁺ binding charge transfer complex. NADP⁺ stacking has been shown to shift the redox potential of FNR (Batie and Kamin, 1986) in similar experiments. Note that in the case of FNR, the potentials of the NADP-FAD system are such that electron transfer operates in the reverse direction toward NADP reduction (Batie and Kamin, 1986).
Recently, Adak et al (2002) reported that Phe1395 is involved in rat nNOS regulation. Phe1395 is representative of a conserved group of aromatic amino acids that shield the *re* face of the FAD isoalloxazine ring in NOS, CPR (Hubbard et al., 2001) and FNR (Deng et al., 1999). The aromatic side chains of these shielding residues must move away from the FAD isoalloxazine ring to allow productive binding interaction with the nicotinamide ring of NADPH that is essential for hydride transfer to FAD (Zhang et al., 2001). Figure 4.9 illustrates the conformational change required for NADPH to initiate hydride transfer.

![Figure 4.9](image-url)  
**Figure 4.9** A: Representation of the crystal structure of nNOSFAD active site where the residue Phe1395 is stacked above the isoalloxazine ring and disfavors the conformation in which NADP binds productively (Zhang et al., 2001). The presence of an aromatic residue shielding the access to the FAD cofactor is characteristic of diflavin reductase protein one of which is the ferredoxin reductase (FNR). B: The mutation of this aromatic residue, in the Y308S FNR mutant (Deng et al., 1999), stabilizes the conformation where NADPH is stacked with the flavin.

Experiments carried out by Konas et al (2004) revealed that in absence of the aromatic side chain of Phe1395 in nNOS, NADP⁺ binds with a significantly higher percentage of its nicotinamide ring occupying a productive conformation relative to wild type enzyme. They also determined that the absence of the aromatic side chain enables ≈100% occupancy of the productive binding mode in oxidised nNOSrd,
whereas only $\leq 35\%$ of the NADP$^+$ bound to oxidised wild type is found in the productive conformation. Phe1395 is representative of a conserved group of aromatic amino acids that shield the re face of the FAD isoalloxazine ring in NOS, related flavoproteins, and simpler FNR proteins (Deng et al., 1999; Hubbard et al., 2001; Zhang et al., 2001). The aromatic side of these shielding residues must move away from the FAD isoalloxazine ring to allow a productive binding interaction with the nicotinamide ring of NADPH that is essential for hydride transfer to FAD (Zhang et al., 2001). The phenyl side chain of Phe 1395 appears to have distinct roles in regulating rat nNOS. It was suggested to regulate the proportion of the NADP bound in productive conformation. By destabilising the NADP$^+$ affinity, the aromatic residue also prevents NADP$^+$ release from limiting the catalytic activity of the enzyme (Konas et al., 2004). Phe1395 has also been demonstrated to be involved in the stabilisation of the FMN shielded conformation when NADP binds in the absence of CaM. In this conformation, the inaccessibility of the FMN cofactor represses the electron transfer to external electron acceptors such as nNOS heme domain or cytochrome c (Craig et al., 2002). Here, the stabilisation of the FADH/FADH$_2$ redox couple in the presence of NADP$^+$ suggests that conformation of NADP$^+$ is also likely to affect the equilibrium distribution of electrons in the FAD and FMN cofactors of the 3-electron reduced nNOSrd. Although the potential shift observed for FAD sq/hq appears too small (30-40 mV) to affect the overall catalytic rate-constants, it is the result of an average over all the conformations existing at equilibrium and takes no account of the proportion of enzyme molecules with NMN and FAD in the stacked conformation. Craig et al (2002) demonstrated that the addition of NADPH to fully reduced nNOSrd causes the enzyme to adopt a conformation in which the FMN hydroquinone is inaccessible to the external electron acceptor, cytochrome c. It is likely that the NADPH adopts an unstacked conformation in this case, since formation of a charge-transfer complex between the two reduced nucleotides is improbable. If the $\pi$-stacked NADP(H) conformation corresponds with the open enzyme conformation, the redox potential shift observed for the NADP-bound nNOSrd is likely to impede the electron transfer by stabilising the FAD hydroquinone. Therefore, all the conditions are gathered to repress the electron
transfer from the CaM-free nNOSrd. Since CaM-binding relieves conformational restriction of nNOSrd, this would activate electron transfer through the enzyme.

4.4 Conclusions

The reduction potentials of the FAD/FADH, FADH/FADH₂, FMNH/FMNH₂ and heme Fe³⁺/Fe²⁺ redox couples for the isolated nNOS domains all lie within a 30 mV range, in the presence or absence of substrates. This facilitates efficient electron transfer through the enzyme and ensures that all these redox states are populated at equilibrium.

The electrochemical study of both the isolated FAD- and FMN-domain reveals that none of the redox couples involved in the enzyme catalytic turnover is affected by the absence of the FAD/FMN-interface. This confirms previous results suggesting that CaM activation does not control the electron transfer by altering redox potentials of the flavins. Only the oxidised to semiquinone redox couple of the FMN cofactor was found to be 80 mV more negative. However this redox couple is not catalytically involved and its kinetic and thermodynamic stability towards oxidation ensures that in the native enzyme, the FMN cofactor acts only as a one-electron donor to maintain appropriate ferric heme reduction in native nNOS and ensure efficient NO production.

The binding of NADP⁺ was found to have a modest effect on the redox potential (-326 to -290 mV) of the fully reduced FAD. However, the potential shift observed is likely to be the result of only productively bound NADP⁺ forming a π-stacking interaction with the FAD cofactor. Depending on the fraction of NADP⁺ bound in productive conformation, it is possible that in the CaM-free enzyme, the conformation of the bound NADP⁺ may control both electron transfer between the FAD and FMN and from FMN to heme by modulating the potential of the FAD hydroquinone.
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5.1 Introduction

NO production is correlated with the rate at which nNOSrd delivers electrons to the heme (Nishida and de Montellano, 1998; Santolini et al., 2001a). Both this and the rate of electron transfer through the reductase domain of nNOS is activated by the binding of CaM (Abusoud et al., 1994a). Complex interactions of intrinsic inhibitory features like the C-terminal extension and the autoinhibitory insert compete with CaM binding to regulate and maintain NO production at a physiological level. The aim of this chapter is to determine how CaM activates electron transfer in nNOSrd. The different redox states of nNOSrd have characteristic UV-visible spectra that can be monitored by UV-vis spectrophotometry. This property was used to assess the rate of electron transfer during reduction and oxidation of the enzyme. The ability of nNOSrd to transfer electrons to external electron acceptors was investigated by monitoring cytochrome c reduction (Klatt et al., 1992).

5.2 Kinetics of cytochrome c reduction

5.2.1 Steady-state cytochrome c reduction by nNOSrd

The catalytic turn-over with NADPH and cytochrome c (Scheme 1.1), by either native nNOS or nNOSrd, proceeds by the transfer of one electron at time from the FMN hydroquinone and is essentially unidirectional (Adak et al., 1999; Daff et al., 1999) (Figure 5.1). As described in Chapter 4, the FMN semiquinone is kinetically stable and does not oxidize during the normal catalytic turnover of NOS. Only the FMNsq/hq redox couple is involved in the transfer of electrons from FAD to cytochrome c enabling nNOSrd to receive electrons from the two-electron donor NADPH and pass them one at time to the one electron acceptor cytochrome c, or to nNOS heme in the case of the native enzyme. CaM binding to the enzyme triggers a 10-fold enhancement in the rate of cytochrome c reduction (Figure 5.1). The affinity of cytochrome c for the enzyme was approximately 6 μM regardless of the presence or absence of CaM (Table 5.1).
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These two parameters were obtained by fitting the rate of cytochrome c reduction versus the cytochrome c concentration (to the Michaelis-Menten equation) and are consistent with results from the full-length enzyme (Daff et al., 1999; Gachhui et al., 1996b).

Figure 5.1 Steady-state turn-over rate of cytochrome c reduction by nNOSrd fitted to the Michaelis Menten equation using Origin 7 (Microcal).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CaM/Ca\textsuperscript{2+}</th>
<th>$k_{\text{cat}}$ (s\textsuperscript{-1})</th>
<th>$K_m$ (\textmu M)</th>
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</thead>
<tbody>
<tr>
<td>Native nNOS</td>
<td>+</td>
<td>59.2±4.2</td>
<td>NA</td>
</tr>
<tr>
<td>(Daff et al 1999)</td>
<td>-</td>
<td>6.0±0.3</td>
<td>NA</td>
</tr>
<tr>
<td>nNOSrd</td>
<td>+</td>
<td>60.0±3.3</td>
<td>6.0±1.2</td>
</tr>
<tr>
<td>(Montgomery et al 2000)</td>
<td>-</td>
<td>4.5±1.6</td>
<td>2.3±1.4</td>
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<tr>
<td>nNOSrd</td>
<td>+</td>
<td>49.6±4.5</td>
<td>7.2±1.5</td>
</tr>
<tr>
<td>(Presently studied)</td>
<td>-</td>
<td>4.7±1.3</td>
<td>5.9±2.1</td>
</tr>
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</table>

NA: not available

Table 5.1 Steady-state cytochrome c reduction rates for wild type and reductase domain of rat nNOS. (Measured in similar but non identical conditions).

5.2.2 Pre-steady state cytochrome c reduction by nNOSrd

Pre-steady-state cytochrome c reduction by pre-reduced nNOSrd is a method to assess the ability of the FMN to transfer an electron to external electron acceptors. The rate of cytochrome c reduction directly depends on the FMN cofactor’s accessibility, with a more accessible FMN leading to faster cytochrome c reduction. Since both cytochrome c and nNOSox exclusively accept electron equivalents from...
the FMN cofactor, cytochrome c can be used to mimic nNOSox in order to probe the degree of FMN accessibility to nNOSox, in the presence or absence of CaM, in the native enzyme.

5.2.2.1 CaM effect on nNOSrd conformation

Previous experiments have shown that β-NADPH, the physiological substrate of nNOSrd, paradoxically represses the electron transfer through nNOSrd by locking the enzyme in a conformation where the FMN cannot access and donate electrons to cytochrome c (Craig et al., 2002). The purpose of the experiment reported in Figure 5.2 was to investigate the effect of CaM on the overall accessibility of the FMN cofactor. For that, the pseudo-first order rate constants for cytochrome c reduction were measured by mixing pre-reduced enzyme with a substoichiometric concentration of cytochrome c in a stopped flow spectrophotometer. The rate constants were fitted to a single exponential function and were found to be linearly dependent on the enzyme concentration. Second order rate constants were calculated by linear regression and compared to the value obtained for the CaM-free uncomplexed enzyme (Figure 5.2). The comparison indicates a 5-fold enhancement of the second order rate constant after CaM addition and a 6-fold drop in the presence of NADPH. The addition of CaM, in the presence of NADPH, leads to a 27-fold increase in the second order rate constant for nNOSrd. The binding of CaM to the enzyme does not only overcome the NADPH inhibitory effect but also activates cytochrome c reduction suggesting that CaM enables the FMN to be accessed by cytochrome c to a greater extent than for the uncomplexed enzyme. Interestingly, the second order rate constant obtained for cytochrome c reduction by the isolated FMN domain was similar to that obtained for CaM-bound nNOSrd (Table 5.2). The results indicate that the FMN accessibility to external electron acceptors, in the case of CaM-bound nNOSrd, is similar to that of the isolated FMN domain. If the FMN in the isolated FMN domain is assumed to be fully accessible, then by comparison, it is 45% accessible in the uncomplexed enzyme and only 3% accessible when NADPH binds to this.
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<table>
<thead>
<tr>
<th></th>
<th>nNOSrd</th>
<th>nNOSFMNCaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CaM</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>$k_{2nd}$ ($\mu M^{-1}.s^{-1}$)</td>
<td>$6.5 \pm 0.5$</td>
<td>$1.1 \pm 0.1$</td>
</tr>
</tbody>
</table>

Table 5.2 Second order rate constants ($k_{2nd}$) for cytochrome c reduction by nNOSrd & nNOSFMN.

Figure 5.2 Determination of pre-steady-state second order rate constants for cytochrome c reduction by nNOSrd and nNOSFMN. Shown are pseudo-first order rate constants for the reduction of 2μM cytochrome c (at 25°C) on mixing with excess nNOSrd or nNOSFMN prereduced by an excess of dithionite. The analysis was carried out using Origin 7 (Microcal). The rate constants, derived from fitting a single exponential function, were linearly dependent on enzyme concentration. The second order rate constants were calculated by linear regression analysis (Table 5.2).

5.2.2.2 Specificity of NADPH inhibitory effect

In order to understand how β-NADPH acts to inhibit electron transfer from the reductase domain, the following substrate analogues were investigated: 2',5'-ADP, 2',5'-ATP-ribose, nicotinamide, β-NMN, α-NADPH, β-NAADP$^+$ (Figure 5.3). The pre-steady state rate of cytochrome c reduction by nNOSrd was evaluated for each of these inhibitors and compared with that of the uncomplexed and the β-NADPH-bound nNOSrd. All the inhibitors studied, apart from nicotinamide and β-NMN, contain the 2'-adenosine phosphate extremity as it has been shown to be essential for the tight binding of the physiological substrate (Zhang et al., 2001).
Figure 5.3 Structures of the inhibitors used to demonstrate the specific inhibitory role of β-NADPH in the pre-steady state reduction of cytochrome c.

Figure 5.4 Inhibition of cytochrome c reduction by nNOSrd. Effect of 100μM inhibitor (Listed in the Figure 5.3) on pre-steady state reduction of 6μM cytochrome c (at 25°C) by 10μM prereduced nNOSrd, compared to the uncomplexed and the CaM-bound form of the enzyme. The graphs show the cytochrome c absorbance change at 550 nm versus time.
The control reactions using β-NADPH with and without CaM and the uncomplexed enzyme without CaM shows that nNOSrd contains all the regulatory elements necessary to cause a 30-fold increase in the first order rate constant for cytochrome c reduction when CaM binds to the β-NADPH-bound enzyme (Craig et al., 2002). The stopped flow experiments show that 2',5'-ADP, and α-NADPH all induce a 50% drop in the cytochrome c reduction first order rate constant compared to uncomplexed enzyme despite the fact that the 5'-phosphoribose is missing from 2',5'-ADP. 2',5'-ATP-ribose shares a very close resemblance with β-NADPH, by only lacking the nicotinamide, which makes it responsible for the 75% decrease in cytochrome c reduction. β-NMN induces 60% inhibition in cytochrome c reduction that may be explained by a network of hydrogen bonds with the residues Thr1251, Arg1010 and probably Ser1196 ensuring tight binding. This strong effect is even more unexpected because the β-NMN moiety does not contains the 2'-phosphate...
required for discrimination between β-NADH and β-NADPH in catalysis and observed in these pre-steady-state reactions. Another striking effect of β-NMN is that the addition of CaM only restores 30% of the rate of cytochrome c reduction. α-NADPH binds similarly to 2',5'-ADP. It appears that the alpha orientation of the nicotinamide disrupts further inhibition. Surprisingly, β-NAADP⁺ fails to inhibit cytochrome c reduction, despite the close structural homology between it and β-NADPH. It appears that modification of the nicotinamide amide group to a carboxylic acid is enough to completely disrupt the effect seen with β-NADPH. Although the 2',5'-ATP-ribose part of β-NADPH has the greatest affinity for nNOSrd, by virtue of electrostatic interactions between its phosphate groups and basic amino-acid residues, it can not induce full conformational locking of nNOSrd. In β-NADPH, the weakly bound nicotinamide substituent clearly plays an important role which cannot be reconstituted by addition of nicotinamide and 2',5'-ATP-ribose together or even 2',5'-ADP and β-NMN, demonstrating a role of the 2',5'-ATP-ribose part of β-NADPH to position the nicotinamide in the active site of the enzyme. Furthermore, inhibition appears to be specific for this group, a phenomenon that must result from the interaction between it and the protein in the conformationally locked state. The structure of nNOSrd shows β-NADPH bound with the nicotinamide group interacting with the adenosine group of FAD, the guanidinium group of Arg1010 and the protein backbone from amino acids 1194-1196 (Figure 5.5) (Garcin et al., 2004). Currently it is difficult to say if any of these interactions are significant.
5.3 Kinetics of nNOSrd electron transfer

CaM activates NO synthesis by facilitating the electron transfer to the oxygenase domain and also by activating the reductase domain itself. Comparison of CaM activation of the cytochrome c and ferricyanide reduction by reduced nNOSrd and full length enzyme has shown that the effect of CaM is complete within the reductase domain itself (Gachhui et al., 1996b). Studies using cytochrome c reveal that nNOSrd can deliver electrons at rates potentially fast enough to sustain NO synthesis even in the absence of CaM activation (Roman et al., 2000b). The CaM requirement for NO synthesis suggests that in the absence of CaM, the electrons are not transferred efficiently to the heme and that CaM-binding facilitates the electron transfer by triggering large-scale structural protein reorganization. However, CaM binding has also been shown to increase the rate of electrons coming into and out of nNOSrd as well as the inter-flavin electron transfer (Matsuda and Iyanagi, 1999). Studies of CaM activation on the different steps of electron transfer within nNOSrd
have been carried out for the one-electron reduced native state and the fully oxidized form of the enzyme. Matsuda and Iyanagi (1999) proposed that in absence of CaM, the interflavin electron transfer gates electrons going out to the nNOSrd and that on CaM binding the hydride transfer becomes rate limiting. Previous experiments carried out by Matsuda and Iyanagi (1999), Guan et al (2003) have compared the kinetics of NADPH reduction of the fully oxidized to the FMN semiquinone form of the enzyme by fitting the data using a double exponential function. Fully oxidized enzyme reduction by NADPH has also been characterized using multiple wavelength fitting and a quadruple exponential function to assign in more detail the different steps involved in the reduction (Knight and Scrutton, 2002; Konas et al., 2004). Here, the last fitting method is used to underline the implication of the air-stable semiquinone of the native enzyme in the control of the reduction process and to assign rates for the different steps of the reduction mechanism for the fully oxidized and the one-electron-reduced nNOSrd. For that, the absorbance changes associated with nNOSrd flavin reduction by NADPH were recorded by rapidly mixing a solution of nNOSrd in the presence or absence of CaM with an excess of NADPH. Similar experiments were also carried out to assess the effect of NADP⁺ on the rate of reduction of FAD by NADPH and to assess the inhibitory effect of ADP, nicotinamide, NMN, and NADP⁺ on the rate of nNOSrd reduction by NADPH. The effect of CaM on the rate of nNOSrd re-oxidation was studied by monitoring the absorbance change obtained for the rapid mixing of the ADP/nNOSrd complex with NADP⁺ in the stopped flow apparatus.

5.3.1 Flavin reduction

The reduction of the flavins of nNOSrd by NADPH was observed by recording the visible spectrum change as time progressed (Figure 5.6). In these experiments, the spectral changes, in the presence or absence of CaM, were compared for the fully oxidised (Figure 5.6 I) and the one-electron-reduced form of nNOSrd (Figure 5.6 II). The fully oxidised form was generated by incubating the stable semiquinone purified protein with an excess of ferricyanide that was removed by gel filtration. The one-
electron reduced enzyme was generated by anaerobic titration with dithionite until the 592 nm / 457 nm absorbance ratio was between 0.26 and 0.3. Global fitting of the spectra obtained by photodiode array revealed that the best fit for the fully oxidised nNOSrd data is obtained using a four-step model (Appendix 8.3.1 & 8.3.2), whereas a three-step model suited the one-electron-reduced nNOSrd data (Appendix 8.3.3 & 8.3.4). The presence or absence of CaM did not affect the number of steps involved in the reduction of either of these enzymes (Figure 5.6 insets). Figure 5.6 shows the spectra of intermediates formed during reduction. On mixing with an excess of NADPH, the FAD of nNOSrd is reduced to the hydroquinone form by hydride transfer. If the FMN is oxidised then both electrons are transferred to it from the FAD, allowing the FAD to be reduced by a second equivalent of NADPH leading to the fully reduced form of the enzyme (FADhq/FMNhq) that contains 4 electrons (Figure 5.6 I & Scheme 5.1). The one-electron reduced enzyme (FADox/FMNsq) can only react once with the two electron donor NADPH, forming the stable three-electron-reduced enzyme in equilibrium between the two species FADhq/FMNsq and FADsq/FMNhq (Figure 5.6 II & Scheme 5.2). All the rates corresponding to the reduction of fully oxidised and one-electron-reduced nNOSrd are gathered Table 5.4.
Figure 5.6 Reduction of nNOSrd by NADPH. 14μM of nNOSrd was rapidly mixed with 0.2mM of NADPH in the stopped-flow instrument at 25°C and the absorbance change was monitored using diode array spectrophotometry (See data in Appendix 8.3). Data were fitted to A→B→C→D→E model for fully oxidised nNOSrd and to α→β→γ→δ model for one-electron-reduced nNOSrd using Global Analysis (Applied Photophysics). The rates obtained are gathered in Table 5.4. Panels I & II show the fitted spectra in the absence of CaM and the insets are in the presence of CaM. The dotted spectra correspond to nNOSrd before reaction with NADPH.
Scheme 5.1 Stopped-flow reduction of the fully oxidized nNOSrd by NADPH. The red dots represent the electrons loaded in each flavin.

Scheme 5.2 Stopped-flow reduction of the nNOSrd in the FMN semiquinone state by NADPH. The red dots represent the electrons loaded in each flavin.
Figure 5.6 I corresponds to fully oxidised nNOSrd. In the absence of CaM, two major absorbance changes, from spectra A→C and C→D, can be observed. These spectral changes can be attributed to the first and the second hydride transfer respectively (Scheme 5.1 steps 1 & 3). Interestingly, little semiquinone at 592 nm accumulates during the two step reaction. The first hydride transfer appears to be a biphasic process. The absorbance change corresponding to the first step (A→B) is fast (180 s⁻¹) and concerns the FAD reduction at 457 nm without any semiquinone formation, whereas, the second step (B→C) is slower (50.5 s⁻¹) and displays some semiquinone accumulation due to interflavin electron transfer happening during FAD reduction. The decrease in rate between A→B and B→C suggests that the NADP⁺/FAD species is in equilibrium with NADPH/FAD species and that full reduction of the FAD cofactor is rate limited by NADP⁺ release and/or interflavin electron transfer. In the presence of CaM, it appears that the 1ˢᵗ hydride transfer happens in one step (A'→B') of absorbance amplitude similar to the 1ˢᵗ hydride transfer in CaM-free conditions. From Table 5.4, it appears that the rates of the step A'→B' in presence of CaM is equivalent to the rate of the step A→B in absence of CaM. Furthermore, regarding the absorbance change, it seems that the presence of CaM accelerates and so removes the step B→C, perhaps by enhancing interflavin electron transfer. This is in agreement with previous work carried out by Matsuda and Iyanagi (1999) suggesting that interflavin electron transfer is activated on CaM binding. Note that regardless of the presence of CaM, the same absorbance change is lost during the stopped flow dead time (Table 5.4).

From Figure 5.6 I, it is apparent that the 1ˢᵗ hydride transfer is faster (180 s⁻¹) than the 2ⁿᵈ hydride transfer (7 s⁻¹) and that CaM accelerates the 2ⁿᵈ hydride transfer in particular (123 s⁻¹). The 2ⁿᵈ hydride transfer appears to be a monophasic process and may corresponds to the step C→D in the absence of CaM and C'→D' in the presence of CaM. The rate of the 2ⁿᵈ hydride transfer undergoes an approximatively 18-fold enhancement in the presence of CaM.

The one-electron-reduced enzyme can react once by hydride transfer to the FAD which may then transfer a single electron to the FMN. Figure 5.6 II shows that in the absence of CaM, the semiquinone band at 600 nm remains largely unchanged on hydride transfer suggesting no net change of the semiquinone concentration. The
hydride transfer mainly results in absorbance change at 457 nm and appears to be a monophasic step regardless of the presence of CaM and can be attributed to the step $\beta \rightarrow \gamma$ for the CaM-free nNOSrd and to the step $\alpha' \rightarrow \beta'$ for CaM-bound nNOSrd (Scheme 5.2 step 1'). For the CaM-free enzyme, the hydride transfer process is slow (8.9 s$^{-1}$), although the trace shows a short fast phase (Figure 8.9 & Table 5.4) probably resulting from the contamination with oxidised or damaged enzyme. On CaM binding, the rate of hydride transfer undergoes similar enhancement (approx. 17-fold) as for the 2nd hydride transfer in the case of the fully oxidised enzyme. The CaM-free enzyme remains in three-electron reduced state after reaction with NADPH (Figure 5.6 II), whereas the CaM-bound enzyme undergoes further reduction, resulting in the loss of the semiquinone spectrum. This process can only be initiated by disproportionation of flavin radicals on different enzyme molecules, and is dependent on intermolecular electron transfer. The inability of the CaM-free enzyme to undergo disproportionation is likely a result of the three-electron reduced enzyme forming a "locked" complex in which the FMN is inaccessible to other molecules. This supports the observation that cytochrome $c$ reacts slowly with the NADPH-bound CaM-free enzyme due to conformational locking (Section 5.1 & 5.2).

For the fully oxidised and the one-electron-reduced nNOSrd, the nature of the steps following the hydride transfer is unclear. However, these steps represent further NADP$^+$ dissociation and electron reorganisation leading to full reduction of the enzyme.
### Table 5.4 Rates of anaerobic flavin reduction by excess NADPH

In the table are gathered the rates and percentage of absorbance change corresponding to the fitted data displayed Figure 5.6.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$k^\alpha$ (s$^{-1}$) (%) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised -CaM</td>
<td>Dead time</td>
</tr>
<tr>
<td>Oxidised +CaM</td>
<td>Dead time</td>
</tr>
<tr>
<td>1 e$^\alpha$ reduced -CaM</td>
<td>Dead time</td>
</tr>
<tr>
<td>1 e$^\alpha$ reduced +CaM</td>
<td>Dead time</td>
</tr>
</tbody>
</table>

$^a$ Percentage of the total absorbance change occurring in the instrument dead time.

$^b$ Individual rate constants are reported as follows: Calculated rate (s$^{-1}$) (% of the total absorbance change for this process).

It is intriguing that the first hydride transfer to the oxidized enzyme is considerably faster than the first hydride transfer to the one-electron reduced enzyme. Both follow a similar mechanism and occur in structurally identical FAD domains. The only difference is the redox state of the FMN. In forming the stable blue neutral semiquinone, the newly protonated FMN N5 must form a hydrogen-bond with the peptide carbonyl of Gly810 to stabilize itself (Figure 1.15). This may result in a difference in the structure of the domain-domain interface. If domain motion is important to the catalytic functioning of nNOSrd, this small perturbation could be significant. This implies that the FMN redox state directly affects the rate of hydride transfer to the FAD in the neighbouring domain. Consistent with this, the second NADPH to FAD hydride transfer to the originally fully oxidised nNOSrd occurs at a
similar rate to the primary reaction with the one-electron reduced enzyme. The second reaction must occur after FMN reduction and protonation of the isoalloxazine ring at the N5 position.

5.3.2 Inter-flavin electron transfer

Experiments involving the reduction of the air-stable FMN semiquinone form of nNOSrd by NADPH have shown that in the absence of CaM, the enzyme forms a stable three electron reduced species. However, on CaM addition, full reduction is reached by a disproportionation mechanism described in section 5.2.1. The crystal structure published by Garcin et al (2004) demonstrates that the distance between the FAD and FMN cofactors in the FMN electron accepting position is not greater than 5Å which suggests a direct and rapid electron transfer. Experiments carried out by Craig et al (2002) have shown that in the absence of CaM, NADPH-binding leads to a locked conformation of the nNOSrd where the FMN is not accessible to external electron acceptors. The aims of the following experiments are to probe the disproportion mechanism in the CaM-free enzyme using the mediator methyl viologen, in order to determine whether thermodynamics can control electron transfer on the stopped-flow timescale.

Figure 5.7 presents the results obtained for CaM-free and CaM-bound (inset) one-electron-reduced nNOSrd on reaction with NADPH in the presence or absence of methyl viologen. In panels A and B, the reduction of CaM-free enzyme is shown in the absence or presence of methyl viologen respectively. The plots of the absorbance change at 457 nm for nNOSrd reduction (Panel C) were fitted to a single exponential function. The observed rates gathered in Table 5.5 show that the addition of methyl viologen does not affect the observed rate of nNOSrd reduction regardless of the presence of CaM. However, in the absence of CaM, methyl viologen destabilises the semiquinone leading to the full reduction of the enzyme (Panel B). Otherwise, nNOSrd remains three-electron-reduced for an extended period, even in the presence of excess NADPH. CaM-bound nNOSrd disproportionation may be facilitated by the mobility of the FMN domain that is equilibrium between an electron accepting conformation where the FMN cofactor is buried at the interface of the FAD and
FMN domain and a more solvent-exposed conformation. The CaM-free enzyme is trapped in a locked conformation where the cofactors are not accessible to large external electron acceptors. Methyl viologen acts in this case as a mediator enabling disproportionation (Scheme 5.3).

Figure 5.7 CaM activates radical disproportionation during nNOSrd reduction. 35μM 1 electron-reduced nNOSrd was mixed with an excess of NADPH (0.2mM) in the stopped-flow instrument at 25°C. The visible spectrum of the enzyme was followed using diode array spectrophotometry. Panel A shows a sample of spectra collected over 4s for nNOSrd without CaM; panel B is in the presence of methyl viologen. The inset of panel B shows the same experiment in the presence of CaM without methyl viologen. The red and green dotted lines in panel C plot the absorbance change at 457 nm in the presence or the absence of CaM respectively. In panel D, the change in absorbance at 592 nm is plotted in red, green and black corresponding to enzyme in the presence or the absence of CaM and in absence of CaM with methyl viologen respectively.
Table 5.5 Rates of nNOSrd reduction by NADPH at 457 and 592 nm obtained as described in Figure 5.6. The rates are the result of fitting at 457 and 592 nm to triple and single exponential functions respectively using Origin 7 (Microcal). The abbreviation MV stands for methyl viologen.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Dead time (%)</th>
<th>( k_{457\text{ nm}} \text{ (s}^{-1}) ) (%)</th>
<th>( k_{592\text{ nm}} \text{ s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CaM -MV</td>
<td>13</td>
<td>80 ± 1.1 (25)</td>
<td>0.8 ± 0.01</td>
</tr>
<tr>
<td>+MV</td>
<td>6</td>
<td>55 ± 1.5 (27)</td>
<td>0.8 ± 0.1 (58)</td>
</tr>
<tr>
<td>+CaM -MV</td>
<td>26</td>
<td>143 ± 0.3 (61)</td>
<td>0.46 ± 0.01</td>
</tr>
</tbody>
</table>

A similar rate of semiquinone decay is observed in both CaM-free enzyme with methyl viologen and CaM-bound enzyme (Table 5.5, Figure 5.7 D). Although their behaviour toward the stability of the semiquinone is similar, the observed rate at 457 nm for the CaM-free enzyme has not changed on addition of methyl viologen suggesting that the rate of hydride transfer is dependent on the presence of CaM. The full reduction of the CaM-free nNOSrd is facilitated by the presence of methyl
viologen that transfers electrons between different enzyme molecules leading to full FMN reduction. Methyl viologen acts as a mediator of electrons which suggests that the reduction of the FMNH/FMNH redox couple is not thermodynamically gated in the CaM-free enzyme. Hydride transfer from NADPH to FAD in CaM-free one-electron reduced nNOSrd was described to be a slow process \((8.9 \text{s}^{-1})\) in section 5.2.1. Here, the results show that interflavin electron transfer does not limit the rate of hydride transfer (Table 5.5).

### 5.3.3 FAD reduction in the isolated nNOSFAD

In these experiments, FAD reduction by NADPH was compared for the oxidised nNOSFAD with and without a stoichiometric amount of bound NADP\. The aim of these experiments was to determine whether NADP\(^+\) activates the reduction of nNOSFAD by NADPH. In both cases, the global fitting of the spectra obtained by photodiode array revealed that the data are best fitted to a double exponential function. The three limiting spectra separating the two steps of reduction acquired for NADP\(^+\)-bound FAD and NADP\(^+\)-free FAD are presented in Figure 5.8 I and Figure 5.8 II respectively.

![Figure 5.8 Kinetics of nNOSFAD reduction by NADPH. 50µM of nNOSFAD was rapidly mixed with 200µM NADPH in the stopped-flow instrument at 25°C. The absorbance change of the enzyme was followed using diode array spectrophotometry. The time-dependent spectral modifications consecutive to the reduction of NADP\(^+\)-bound nNOSFAD and NADP\(^+\)-free nNOSFAD were fitted to \(A \rightarrow B \rightarrow C\) model by global analysis (Applied photophysics) and the fitted spectra are shown in panels I & II respectively (See data in Appendix 8.4). Insets correspond to the absorbance decrease at 457 nm](image-url)
(Dotted curve). The red line in the inset corresponds to 457 nm absorbance decrease fitted to a double exponential function using Origin 7 (Microcal).

The presence or absence of NADP\(^+\) does not affect the overall spectral change of the FAD reduction by NADPH. Furthermore, and unlike Guan \textit{et al} (2003), no significant differences were observed in the reduction rate (Table 5.6), or absorbance change contribution for each of these rates. The nNOSFAD used in this experiment was obtained by limited trypsinolysis as describe in chapter 2 (3.4.3), whereas Guan \textit{et al} (2003), used recombinant FAD/NADP domain that theoretically contains the C-terminal extension. According to the mass of the digestion product, evaluated by SDS-PAGE, the cleavage happens between the connecting domain and the FMN domain at the N-terminus and at the base of the C-terminal extension (Zhang \textit{et al}., 2001). nNOSFAD reduction by NADPH was expected to be monophasic due the reaction of a stoichiometric amount of NADPH with FAD cofactor. Instead the reduction process appears to be biphasic, with half of the absorbance change corresponding to each step (Figure 5.8).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Reduction(^a) % after 1s</th>
<th>(k_1) s(^{-1}) (%)</th>
<th>(k_2) s(^{-1}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOSFAD</td>
<td>91</td>
<td>26.7 ± 0.09 (46)</td>
<td>7.9 ± 0.02 (45)</td>
</tr>
<tr>
<td>+NADP</td>
<td>92</td>
<td>38.1 ± 0.14 (54)</td>
<td>8.6 ± 0.01 (38)</td>
</tr>
</tbody>
</table>

Table 5.6 Rates of anaerobic nNOSFAD reduction by excess NADPH. In the table are gathered the rates and percentage of absorbance change corresponding to the fitted data displayed Figure 5.8.

\(^a\): Percentage of the total reduction completed after 1s of reaction.

\(^b\): Individual rate constants are reported as follows: Calculated rate (s\(^{-1}\)) (% of the total absorbance change for this process).

In these experiments, NADPH has to displace NADP\(^+\) in order to bind and react with nNOSrd making NADP\(^+\) a competitive inhibitor for the binding of NADPH. Similar rates of reduction for the NADP\(^+\)-bound and uncomplexed nNOSFAD suggests that NADP\(^+\) dissociation does not slow down FAD reduction. This is in agreement with the fact that NADP\(^+\) is not tightly bound to the enzyme and can dissociate quickly (\(K_d = 4 \, \mu\text{M}\)) (Guan and Iyanagi, 2003). From fitted spectra, it appears that similar
events happen in the 2 steps of FAD reduction with a notable decrease of the absorbance at 457 nm and charge transfer complex band formation at 700 nm. The slow formation of a charge-transfer band on addition of NADPH suggests that NADP+/FADH species mainly contributes to this broad band absorbance and that the hydride transfer is the rate limiting step of FAD reduction (Scheme 5.4 step 2).

Scheme 5.4 FAD reduction by excess of NADPH.

Because NADPH was not in large excess in the conditions of the experiment, NADP+/FADH is favoured over NADPH/FADH at the end of the reaction (Scheme 5.4 step 3).

5.3.4 Inhibition of flavin reduction by NADPH analogues

The aim of this experiment was to investigate the binding specificity of NADPH with nNOSrd. For that, the rate of flavin reduction was monitored in the presence of NADPH analogues in order to probe the region of NADPH involved in recognition and binding. In Figure 5.9 is plotted the spectral change at 457 nm for the reduction of the CaM-free FMN semiquinone form of nNOSrd by NADPH in the presence of inhibitors. The uncomplexed enzyme gave a triple exponential time-course, whereas the inhibitor-bound enzymes gave double exponentials. The calculated rates obtained for different concentrations of each ADP, NMN and nicotinamide are gathered in Table 5.7.

Figure 5.9 (A) shows that the addition of ADP inhibits hydride transfer from NADPH to the enzyme. 2',5'-ADP is a fragment of NADP containing the 2'-phosphate on the adenosine region essential for the NAD/NADP discrimination in
the enzyme. Binding studies carried out with CPR have shown that ADP has a $K_d$ only 10-fold higher than NADP, making it a good competitive inhibitor of enzyme reduction (Murataliev and Feyereisen, 2000). An inhibition of flavin reduction is observed suggesting that ADP acts as a competitive inhibitor that prevents NADPH binding and subsequent hydride transfer.

Pre-incubation of the enzyme with oxidized nicotinamide (Figure 5.9 (B)) leads to a small concentration dependent increase in the rate of reduction by NADPH. However, the effect is very small and probably due to ionic strength as the result of the very high concentration of nicotinamide used.

According to Figure 5.9 (C), a stoichiometric amount of NMN triggers a 10-fold inhibition of the flavin reduction activity. Interestingly, this inhibition is not concentration dependent for an excess of NMN which suggests that NMN acts as a non-competitive inhibitor of NADPH. Steady state cytochrome $c$ reduction by CPR is strongly inhibited by ADP, but no detectable inhibition was observed for NMN up to 20mM (Murataliev and Feyereisen, 2000), whereas the inhibition of nNOSrd reduction is greater with NMN compared with ADP. It is possible that NMN binds to nNOSrd where the NMN part of NADPH, in the non-productive conformation, binds to nNOSrd. Once bound the NMN might interact with residues (Thr1398, etc.) of C-terminal extension which would lead to the stabilisation of the FAD-stacked residue (F1395) in a conformation that impedes NADPH stacking.
Figure 5.9 Kinetics of nNOSrd reduction by NADPH in the presence of inhibitors. 1 electron reduced nNOSrd (20μM) was incubated for 10min., with: nicotinamide (A), ADP (B) and NMN (C). The mixture was rapidly mixed with 0.2mM NADPH in a stopped-flow instrument at 25°C and the absorbance of the enzyme was followed using diode array spectrophotometry. The absorbance changes at 457 nm are shown versus time for different inhibitor concentrations. Traces were fitted to double exponential functions using Origin 7 (Microcal). The rates obtained are gathered in Table 5.7.
Table 5.7 Rates of anaerobic nNOSrd reduction by excess NADPH in the presence of inhibitors ADP, nicotinamide and NMN. The absorbance decreases at 457 nm displayed in Figure 5.9 for different concentrations of inhibitors were fitted with a double exponential function using Origin 7 (Microcal). Rates in presence of inhibitors are compared with the rates corresponding to the three-step mechanism of uncomplexed nNOSrd reduction.

5.3.5 Kinetics of flavin re-oxidation

In order to study the reversibility of flavin reduction by NADP⁺, nNOSrd was pre-reduced with dithionite in anaerobic conditions. The excess of dithionite as well as the secondary products of dithionite were removed using a G25 gel filtration column. Therefore at the start of the reaction, the enzyme was four-electron reduced. In these experiments, fully reduced nNOSrd was mixed with a stoichiometric amount of ADP. Excess NADP⁺ was then rapidly mixed with the ADP-enzyme complex and the absorption change at 457 nm indicated oxidation of the flavins. Contrary to what is observed during the reduction of the enzyme, a large increase in absorbance at 720 nm was detected. In fact, it appears that during the re-oxidation, a charge transfer complex resulting from the stacking of NADP⁺ and FAD hydroquinone (170 - 200 s⁻¹) can accumulate due to the slow hydride transfer step that is controlled by the release of NADPH.
Figure 5.10 Kinetics of re-oxidation of ADP/nNOSrd complex by NADP⁺. A single equivalent of ADP was mixed with reduced nNOSrd and incubated for 10min. to form the nNOSrd/ADP complex. 55μM of nNOSrd/ADP complex was rapidly mixed with an excess of NADP⁺ (0.2mM) in the stopped-flow instrument at 25°C and the absorbance of the enzyme was followed using diode array spectrophotometry. The panels A and B show a sample of the spectra collected in the absence and in the presence of CaM respectively. In both panels, the spectrum corresponding to the maximal amount of charge transfer observed is represented by the red dotted line. The absorbance change corresponding to the charge transfer complex at 720 nm (panels C & D) and corresponding to the oxidized form of the enzyme at 457 nm (insets in panels C & D) are were plotted in a black dotted line against time and the red lines correspond to the single exponential fit of their formation and decay, and formation respectively. Data were fitted using Origin 7 (Microcal).

The observed rate of enzyme oxidation followed at 457 nm (15 s⁻¹ for CaM-free and 12 s⁻¹ for CaM-bound nNOSrd) corresponds to the rate of NADPH release indicated by the charge transfer band decay. The insets located in panels C and D of the Figure 5.10 represent the flavin oxidation at 457 nm for the CaM-free and CaM-bound enzyme respectively. In both cases, the re-oxidation mediated by hydride transfer follows a monophasic process limited by NADPH release. Interestingly, this process is CaM independent whereas the forward reaction was extremely CaM-dependent.
(Section 5.2.1). According to the insets C and D, the increase at 457 nm tails off after 0.5 second for both CaM-free and CaM-bound enzyme. However, the spectra shown in panels A and B show that more oxidation takes place in absence of CaM. The values for the ratios of the absorbance at 592 nm and 457 nm are 0.27 for the CaM-free enzyme and 0.37 for the CaM-bound enzyme suggesting that the CaM-free enzyme is fully one-electron reduced whereas the CaM-bound enzyme is more than one-electron reduced. A possible explanation of the slow and partial re-oxidation of the enzyme in the presence of CaM is that the conformation induced by the binding of CaM lowers the shielding competition between the aromatic residue Phe1395 and the nicotinamide. This increase of NADP affinity could lead to a slower and partial release of NADPH preventing full nNOSrd oxidation (Konas et al., 2004).

Other experiments involving the re-oxidation of uncomplexed nNOSrd were carried out. In these cases, the spectral change at long wavelength corresponding to charge transfer formation mostly occurred in the stopped-flow dead time preventing the measurement of an accurate rate (Data not shown). Thus, the rate at which NADP$^+$ stacks with the FAD hydroquinone is very fast (>200 s$^{-1}$).

### 5.4 Conclusion

It is clear that, upon NADPH binding, the FMN cofactor becomes less accessible to the external electron acceptor cytochrome c. The equilibrium between the shielded and the deshielded conformations of the FMN cofactor is displaced from 45% FMN accessible in the uncomplexed enzyme to only 3% FMN accessible when NADPH is bound (Section 5.2.2.1). Experiments show that the specificity of NADPH in inducing nNOSrd conformation change relies on the interaction of both the tightly-bound ADP substituent and the labile nicotinamide substituent (Figure 5.11). However, isolated ADP and nicotinamide fails to fully repress the enzyme and the absence of a nicotinamide inhibitory effect suggests that the tightly-bound ADP substituent is essential to position nicotinamide moieties for full electron transfer repression. When in place, the substrate binding energy is enough to restrict the
normal motion of the FMN domain, favouring the "locked" conformation of the enzyme.

Figure 5.11 Crystal structure of nNOSrd showing the unproductive binding of NADP to the active site (Garcin et al., 2004). It should be noted that there is no crystal structure available for nNOSrd with NADPH in productive conformation. However, productively bound conformation of NADPH has been shown for the Y308S mutant of the related enzyme ferredoxin-NADP reductase (Deng et al., 1999).

During the catalytic turnover, nNOSrd is in equilibrium between a productive conformation, where nicotinamide is stacked to the FAD isoalloxazine, and a non-productive conformation. The productive conformation of NADPH requires the displacement of the FAD-stacking residue F1395 located at the base of the C-terminal extension suggesting that there is also a connection between NADPH to FAD hydride transfer and domain motion (Garcin et al., 2004; Konas et al., 2004). Phe1395 and the C-terminal extension controls the proportion of NADPH stacking to
the FAD. By controlling the probability of NADPH stacking, the nNOSrd conformation is likely to control the rate of hydride transfer and subsequent FAD reduction. This is the likely origin of the CaM-dependency of the reduction reaction. It is clear from the reduction traces that for the CaM-free enzyme, the first hydride transfer step is faster when the starting enzyme is fully oxidized than when the FMN is one-electron reduced (stable blue sq). Previous experiments have shown that the "locked" conformation of nNOSrd is highly sensitive to structural perturbation (Daff et al., 1999; Roman et al., 2000b; Tiso et al., 2004). Therefore, in the case of the fully oxidized nNOSrd, it is possible that loss of the hydrogen-bond formed between the FMN semiquinone and the protein backbone is enough to disturb the interaction between the FMN and FAD domains causing an increase in the rate of hydride transfer. Accordingly, the rate of hydride transfer does not show any CaM dependency. However, it appears that the formation of the stable FMN semiquinone is enough to recover the CaM dependency of hydride transfer. Interestingly, the one-electron reduced nNOSrd shows that the rate of formation of the charge transfer complex and the hydride transfer are CaM-dependent for the reduction of the enzyme by NADPH but that the reverse process is CaM-independent for both events.
Chapter 6

Conclusion

&

Future work
6.0 Conclusions and Future work

NO production by mammalian neuronal NOS is tightly regulated in order to participate in normal synaptic transmission. NOS is unique in that its reductase activity can be modulated by CaM. The aim of this work was to understand more about the CaM activation mechanism of the reductase domain of nNOS.

Crystal structures of the NADP+/FAD domain and NADP+-bound reductase domain of nNOS have been solved recently. This has permitted further understanding about the positioning of the tightly bound FAD and FMN cofactors and the labile NADPH substrate within the protein and with respect to each other. The structure and localization of control elements such as the C-terminal extension and auto-inhibitory loop have allowed investigation of interactions and motion occurring during the enzyme turn-over. However, the two crystal structures available for the C-terminal part of the enzyme are both lacking the CaM binding linker and bound CaM making it difficult to understand the crucial interactions involving CaM, the reductase domain and particularly the autoinhibitory insert of the FMN sub-domain. In order to obtain information on these interactions, the aim was to obtain the crystal structure of nNOSFMNCaM. Unfortunately, crystallization of nNOSFMNCaM was unsuccessful which prevented resolution of these issues. However, protein crystallisation of nNOSFMNCaM could be pursued in anaerobic conditions in the presence of reductant (DTT) and extra FMN to create homogenous one-electron-reduced enzyme and increase the chance of getting protein crystals.

The conformation of nNOSrd has been shown to be dependent on NADP(H) binding. In order to investigate the possibility that the conformation of the reductase domain and the presence of NADP(H) binding influences the redox properties of the flavin cofactors, the potentials of the isolated FAD and FMN sub-domains of nNOS were determined by OTTLE potentiometry. Results showed that the redox couples involved in catalysis are unchanged in the isolated domains compared to nNOSrd suggesting that the conformation of nNOSrd does not control the electron transfer by altering the redox potential of the flavins. This result is consistent with the fact that...
CaM binding has a small effect on the flavin reduction potentials. Only the FMN/FMNH redox couple was stabilised (increase of 80 mV) by the presence of FAD sub-domain. However, the same redox couple was also found to be kinetically stabilised due to a hydrogen bond existing between Gly810 and the protonated N5 of the FMN cofactor in order to ensure that FMN acts as a one-electron donor to the heme. Midpoint-potential determination for nNOSFAD shows that NADP(H) is responsible for a small stabilisation (30-40 mV) of the semiquinone/hydroquinone redox couple. This effect is likely to be due to the fraction of bound NADP\(^+\) molecules adopting the \(\pi\)-stacked conformation. Therefore, it is possible that in the CaM-free enzyme, the unproductive conformation of NADP(H) locks the enzyme in a conformation where FMN is not accessible to external electron acceptors and that in the productive conformation, the electrons are trapped as a charge-transfer complex. CaM binding to the enzyme overcomes NADP(H) repression by releasing the "locked" conformation. In order to assess the effect of the productive conformation of NADP(H) on the redox potential of FAD semiquinone/hydroquinone redox couple, similar experiments could be carried out on the mutant of nNOSFAD as this mutant of nNOSrd has been shown to binds NADP essentially in productive conformation.

In nNOS, the rate of electron transfer to the heme has been shown to be dependent on protein conformational changes. In the presence of substrate (NADP(H)), CaM-free nNOSrd has also been suggested to adopt a conformation that restricts the access of the FMN cofactor and slows down the heme reduction. In order to assess the accessibility of the FMN cofactor to external electron acceptors, pre-steady-state rates of cytochrome \(c\) reduction were compared for the CaM-free and the CaM-bound nNOSrd and for the isolated FMN sub-domain. The results showed that, if the FMN in the isolated FMN domain is assumed to be fully accessible, then it is 100\% accessible in the CaM-bound enzyme and only 3\% accessible in the NADPH-bound nNOSrd in the absence of CaM. This suggests that the binding of CaM is responsible for a large scale structural reorganisation of nNOSrd that "unlocks" the conformation of the enzyme and enables FMN sub-domain motion in order to shuttle an electron from FAD cofactor to the heme.
Crystal structures solved for both substrate bound nNOSFAD and nNOSrd show that the NADP\(^+\) is bound to the enzyme in a non-productive conformation. This conformation of NADPH has been suggested to stabilize nNOS in a "locked" conformation that inhibits electron transfer from nNOS to the heme. In order to investigate the specificity of NADP(H) in inhibiting the electron transfer from the reductase domain to the heme, the inhibitory effect of substrate analogues were evaluated. Results show that the specificity of NADPH in inducing nNOSrd conformational change relies upon the interaction of both the tightly-bound ADP substituent and the labile nicotinamide substituent and that the tightly-bound ADP substituent is essential to position nicotinamide moieties for full electron transfer repression. It appears that the "locked" conformation of the enzyme, believed to inhibit the electron transfer to the heme, is specific for the NADP(H).

Results showed that NMN induces 60% inhibition in cytochrome c reduction and that the addition of CaM only restores 30% of the original activity. Moreover, stopped-flow reduction of nNOSrd suggests that NMN acts as a non-competitive inhibitor of NADPH. Experiments such as evaluating the dependence of the pre-steady-state rate constant for reduction of cytochrome c by nNOSrd on the concentration of NMN in the presence and absence of CaM needs to be done to investigate the NMN binding site. Stopped-flow reduction of NMN-bound nNOSrd could be repeated for more NMN concentrations.

Previous attempts to assess the CaM activation of nNOSrd flavin reduction by NADPH were made by a different laboratory. However, controversial rates of hydride transfer were obtained. Depending on the length of the protein construct, integrity of the CaM linker, degree of protein degradation and redox state of the enzyme prior to reacting, the number of steps involved for the nNOSrd reduction as well as the CaM activation were different. In order to bring some clarification to the effect of the redox state of nNOSrd before reaction, the CaM dependency of nNOSrd flavin reduction was compared for a fully oxidised and a one-electron reduced (blue semiquinone form) starting enzyme. The results showed that the first hydride transfer is faster (\(\approx 25\)-fold) for the fully oxidised enzyme compared to the one-electron
reduced enzyme. It is interesting to note that the rate of the first hydride transfer for
the fully oxidised enzyme was CaM independent, but that the formation of stable
blue semiquinone consecutive to the first hydride transfer was enough to recover the
hydride transfer CaM dependency. In the case of the fully oxidized nNOSrd, it is
possible that loss of the hydrogen-bond formed between the FMN semiquinone and
the protein backbone is enough to disturb the interaction between the FMN and FAD
domains causing the lack of CaM dependency of hydride transfer. In the case of the
native nNOS (one-electron reduced), the rate of hydride transfer in the presence of
CaM underwent a 20-fold increase.
Ferricyanide reduction is used to assess the ability of FAD cofactor to transfer
electrons from NADPH. However, the midpoint potential of ferricyanide is such that
it is likely to oxidize the nNOSFMN semiquinone (\( E_{0}^{FMN_{ox}/FMN_{sq}} = -98 \text{ mV} \))
preventing the study of FAD electron transfer in the native nNOSrd. Previous results
show that on CaM binding, the rate of interflavin electron transfer consecutive to the
first hydride transfer to the fully oxidized nNOSrd is increased by 2- to 3-fold which
is in agreement with the CaM enhancement for ferricyanide reduction. Because the
presence of air-stable semiquinone is likely to affect the rate at which FAD transfers
electrons, the use of a mediator like Phenosafranine (\( E_{0}=-252\text{mV}, \varepsilon_{520nm}=21200 \)) that
would accept electrons from FAD hydroquinone without oxidising FMN
semiquinone could be envisaged to assess the rate of interflavin electron transfer in
the native nNOSrd.
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Appendix 8.1

Protocol of flavin content determination in nNOSrd used by Kitamura et al

Buffer in use for Flavin Fluorescence

Buffer G: 25 mM potassium phosphate, pH 7.7

Standard solutions of pure FAD and FMN at 1μM were made up fresh in assay Buffer G. To establish the calibration curve, fluorescence was measured with standard solutions first. 2 ml of Buffer G and 0.5 ml of FAD was transferred to a 1 cm path-length fluorescence cuvette. The fluorescence was measured with excitation set at 447 nm and emission at 525 nm. Then 0.15 ml of 0.4 M HCl was added to the cuvette to adjust the pH to 2.6, and the fluorescence was remeasured. Readings were taken for another different concentration. The process is repeated with FMN at pH 7.7 and 2.6.

A constant for the fluorescence of FAD at pH 7.7 was calculated from data using:

\[ F_{7.7} = D_{7.7}[\text{FAD}] \]

where \( F_{7.7} \) was the fluorescence value measured for pure FAD at pH 7.7 and \( D_{7.7} \) is the “fluorescence constant” (in units of M-1 cm-1) for FAD at this pH.

This process was repeated to determine fluorescence constants for FMN at pH 2.6 (\( N_{2.6} \)) and for FAD at pH 7.7 (\( D_{7.7} \)), and pH 2.6 (\( D_{2.6} \)).

Samples of the enzymes were diluted with assay buffer to the final concentrations of 1μM. Aliquots of the flavoprotein sample were placed into eppendorf tubes, covered with aluminium foil and transferred to a bath of boiling water for 3 min. The samples were then centrifuged at 10000 g for 15 min at 4°C to precipitate denatured protein. 0.5 ml of supernatant and 2 ml of buffer was measured with the same method as above.

For the samples, the fluorescence values at pH 7.7 and 2.6 were given by:

\[ F_{7.7} = N_{7.7}[\text{FMN}] + D_{7.7}[\text{FAD}] \]

and by \( F_{2.6} = N_{2.6}[\text{FMN}] + D_{2.6}[\text{FAD}] \).

The values of the fluorescence constants for FMN/FAD were known and hence the concentrations of FMN and FAD in the samples could be determined.
Appendix 8.2

8.2.1 N-term sequencing elution profile:nNOSrd

Figure 8.1 N-terminal sequencing elution profile of nNOSrd.
8.2.2 N-term sequencing elution profile: nNOSFMN

Figure 8.2 N-terminal sequencing elution profile of nNOSFMN.
### Appendix

#### 8.2.3 Amino acid sequence of nNOSrd and nNOSFMN

<table>
<thead>
<tr>
<th>nNOSrd &amp; nNOSFMN N-terminus (695)</th>
</tr>
</thead>
<tbody>
<tr>
<td>670-680-690-700-710-720</td>
</tr>
<tr>
<td>HMENEYRCNG GCPADWVWIV PPMGSITPV FHQMHLNYRL TPSFEYQPFM WNTHVWKGTN</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CaM binding region</th>
</tr>
</thead>
<tbody>
<tr>
<td>730-740-750-760-770-780</td>
</tr>
<tr>
<td>GTPTERRAIG FEKLAEIKFS SKRLGQAMG KVRHATILEY TETGKSKAYA KTLCEIFKHA</td>
</tr>
</tbody>
</table>

| 790-800-810-820-830-840        |
| FDAMAMSMEEE YDIVHLEHEAA LVLL[VSTFG NGDFPENGEK FGCALEMREH PNSQVPEREKS |

| 850-860-870-880-890-900         |
| YKVRFNSVSS YSDSRKSSGD GPDLRDMFES TGPLANVRFS VFGLGSAYFP HFCAPGHAVD |

| 910-920-930-940-950-960         |
| TLEEELGER ILKMREGDEL CQQAEEAFRTW AKIVFKEACD VFCVGDVNI EKPNNLISN |

...  

<table>
<thead>
<tr>
<th>nNOSFMN C-terminus (946)</th>
</tr>
</thead>
<tbody>
<tr>
<td>970-980-990-1000-1010-1020</td>
</tr>
<tr>
<td>QQRQFDIQHK GMNPCPMVLV FGCRQSKIDH IYREETLQAK NKGVFERELY AYSRESQDRPK</td>
</tr>
</tbody>
</table>

| 1030-1040-1050-1060-1070-1080 |
| KYVQDVLOQEQ LASEVYRLAK EQQGHYVCG DVTMAADVILK AIQRINTQQQ KLSEEDACVF |

| 1090-1100-1110-1120-1130-1140-1150 |
| ISRLRDDNRY HEDIFGVTLL TPEVTNRLGS ESIAFPEGSK KDADEVFSS |

Figure 8.3 nNOSrd and nNOSFMN amino acid sequence. The amino acids in blue represent the amino acids missing for the proteolysed nNOSrd.
Appendix 8.3

8.3.1 Stopped-flow reduction of the oxidised CaM-free nNOSrd by NADPH

Figure 8.4 Reaction of the CaM-free oxidised nNOSrd with NADPH monitored by stopped-flow photodiode array spectroscopy. Plot A: Time-dependent spectral changes on rapidly mixing the 14 μM CaM-free oxidised nNOSrd with an excess of NADPH (0.2 mM) at 25°C. The experiment was performed over 4 s after the initial mixing event. Plot B: For a clarity purpose, only selected spectra (black trace) from the experiment shown in A are represented with the corresponding time at which they were recorded. The first spectrum was recorded at 1.28 ms after mixing. Data were fitted using the multi-wavelength fitting software, Global Analysis (Applied photophysics), to a four steps model (A→B→C→D→E) with a quadruple exponential function. The calculated variance of the fitting is $7.237 \times 10^{-4}$. The rates obtained are gathered in Table 5.4. The red overlays are the fit corresponding to each of the selected spectra.
Figure 8.5 Single wavelength absorption transients for the reactions of CaM-free nNOSrd with NADPH. The single wavelength data were extracted from data presented in Plot A, and the traces were obtained by plotting the absorbance at 457 nm (Plot C) and 592 nm (Plot D) vs time. The fit shown in red is the result of the multi-wavelength fitting process described in Figure A, but it is in this case displayed for only one wavelength (457 nm in Plot C and 592 nm in Plot D) vs time. The overall residual of the fitting is displayed in the frame below the corresponding absorbance vs time plot.
8.3.2 Stopped-flow reduction of the oxidised CaM-bound nNOSrd by NADPH

Figure 8.6 Reaction of the CaM-bound oxidised nNOSrd with NADPH monitored by stopped-flow photodiode array spectroscopy. Plot A: Time-dependent spectral changes on rapidly mixing the 14 μM CaM-bound oxidised nNOSrd with an excess of NADPH (0.2 mM) at 25°C. The experiment was performed over 4 s after the initial mixing event. Plot B: For a clarity purpose, only selected spectra (black trace) from the experiment shown in A are represented with the corresponding time at which they were recorded. The first spectrum was recorded at 1.28 ms after mixing. Data were fitted using the multi-wavelength fitting software, Global Analysis (Applied photophysics), to a four steps model (A→B→C→D→E) with a quadruple exponential function. The calculated variance of the fitting is 4.338e⁻⁴. The rates obtained are gathered in Table 5.4. The red overlays are the fit corresponding to each of the selected spectra.
Figure 8.7 Single wavelength absorption transients for the reactions of CaM-free nNOSrd with NADPH. The single wavelength data were extracted from data presented in Plot A, and the traces were obtained by plotting the absorbance at 457 nm (Plot C) and 592 nm (Plot D) vs time. The fit shown in red is the result of the multi-wavelength fitting process described in Figure A, but it is in this case displayed for only one wavelength (457 nm in Plot C and 592 nm in Plot D) vs time. The overall residual of the fitting is displayed in the frame below the corresponding absorbance vs time plot.
8.3.3 Stopped-flow reduction of the FMN semiquinone CaM-free nNOSrd by NADPH

Figure 8.8 Reaction of the CaM-free FMN semiquinone nNOSrd with NADPH monitored by stopped-flow photodiode array spectroscopy. Plot A: Time-dependent spectral changes on rapidly mixing the 14 μM CaM-free FMN semiquinone nNOSrd with an excess of NADPH (0.2 mM) at 25°C. The experiment was performed over 4 s after the initial mixing event. Plot B: For a clarity purpose, only selected spectra (black trace) from the experiment shown in A are represented with the corresponding time at which they were recorded. The first spectrum was recorded at 1.28 ms after mixing. Data were fitted using the multi-wavelength fitting software, Global Analysis (Applied photophysics), to a four steps model (A→B→C→D→E) with a quadruple exponential function. The calculated variance of the fitting is 9.659e-4. The rates obtained are gathered in Table 5.4. The red overlays are the fit corresponding to each of the selected spectra.
Figure 8.9 Single wavelength absorption transients for the reactions of CaM-free nNOSrd with NADPH. The single wavelength data were extracted from data presented in Plot A, and the traces were obtained by plotting the absorbance at 457 nm (Plot C) and 520 nm (Plot D) vs time. The fit shown in red is the result of the multi-wavelength fitting process described in Figure A, but it is in this case displayed for only one wavelength (457 nm in Plot C and 520 nm in Plot D) vs time. The overall residual of the fitting is displayed in the frame below the corresponding absorbance vs time plot.
8.3.4 Stopped-flow reduction of the FMN semiquinone CaM-bound nNOSrd by NADPH

Figure 8.10 Reaction of the CaM-bound FMN semiquinone nNOSrd with NADPH monitored by stopped-flow photodiode array spectroscopy. Plot A: Time-dependent spectral changes on rapidly mixing the 14 μM CaM-free FMN semiquinone nNOSrd with an excess of NADPH (0.2 mM) at 25°C. The experiment was performed over 4 s after the initial mixing event. Plot B: For a clarity purpose, only selected spectra (black trace) from the experiment shown in A are represented with the corresponding time at which they were recorded. The first spectrum was recorded at 1.28 ms after mixing. Data were fitted using the multi-wavelength fitting software, Global Analysis (Applied photophysics), to a four steps model ($A \rightarrow B \rightarrow C \rightarrow D \rightarrow E$) with a quadruple exponential function. The calculated variance of the fitting is $6.637e^{-4}$. The rates obtained are gathered in Table 5.4. The red overlays are the fit corresponding to each of the selected spectra.
Figure 8.11 Single wavelength absorption transients for the reactions of CaM-free nNOSrd with NADPH. The single wavelength data were extracted from data presented in Plot A, and the traces were obtained by plotting the absorbance at 457 nm (Plot C) and 520 nm (Plot D) vs time. The fit shown in red is the result of the multi-wavelength fitting process described in Figure A, but it is in this case displayed for only one wavelength (457 nm in Plot C and 520 nm in Plot D) vs time. The overall residual of the fitting is displayed in the frame below the corresponding absorbance vs time plot.
Appendix 8.4

**Stopped-flow reduction of nNOSFAD by NADPH**

Figure 8.12 Kinetic of isolated uncomplexed nNOSFAD domain reduction by NADPH. 50µM of isolated uncomplexed nNOSFAD domain was rapidly mixed with 0.2mM NADPH in the stopped-flow instrument at 25°C and the absorbance of the enzyme was followed using diode array spectrophotometry. Here is represented the time-dependent spectral modifications consecutive to the reduction of uncomplexed nNOSFAD (Panel I) and NADP⁺-bound nNOSFAD (Panel II).
Appendix 8.5

8.5.1 Meetings

Enzyme mechanism. A structural perspective, Saint Andrews, UK
   January 12 - 14th, 2003

7th Firbush Redox Enzymes meeting, Perthshire, UK
   June 4 - 6th, 2003

The UK NO Forum, Nottingham, UK, (Speaker)
   December 15th, 2003

The Inorganic Biochemistry Discussion Group, London, UK
   January 8 - 9th, 2004

8th Firbush Redox Enzymes meetings, Perthshire, UK
   June 2 - 4th, 2004

15th International Symposium on Flavins and Flavoproteins, Shonan
   village center, Japan (Poster)
   April 17 - 22nd, 2005

9th Firbush Redox Enzymes meetings (Speaker)
   June 8 - 10th, 2005
8.5.2 Publications


Redox Properties of the Isolated Flavin Mononucleotide- and Flavin Adenine Dinucleotide-Binding Domains of Neuronal Nitric Oxide Synthase†

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Received April 7, 2004; Revised Manuscript Received June 15, 2004

ABSTRACT: Electron transfer through neuronal nitric oxide synthase (nNOS) is regulated by the reversible binding of calmodulin (CaM) to the reductase domain of the enzyme, the conformation of which has been shown to be dependent on the presence of substrate, NADPH. Here we report the preparation of which has been shown to be dependent on the presence of substrate, NADPH. Here we report the preparation of the isolated flavin mononucleotide (FMN)-binding domain of nNOS with bound CaM and the electrochemical analysis of this and the isolated flavin adenine dinucleotide (FAD)-binding domain in the presence and absence of NAPDH and ADP (an inhibitor). The FMN-binding domain was found to be stable only in the presence of bound CaM/Ca2+, removal of which resulted in precipitation of the protein. The FMN formed a kinetically stabilized blue semiquinone with an oxidized/semiquinone reduction potential of —179 mV. This is 80 mV more negative than the potential of the FMN in the isolated reductase domain, that is, in the presence of the FAD-binding domain. The FAD semiquinone/hydroquinone redox couple was found to be similar in both constructs. The isolated FAD-binding domain, generated by controlled proteolysis of the reductase domain, was found to have similar FAD reduction potentials to the isolated reductase domain. Both formed a FAD—hydroquinone/NADP+ charge-transfer complex with a long-wavelength absorption band centered at 780 nm. Formation of this complex resulted in thermodynamic destabilization of the FAD semiquinone relative to the hydroquinone and a 30 mV increase in the FAD semiquinone/hydroquinone reduction potential. Binding of ADP, however, had little effect. The possible role of the nicotinamide/FADH2 stacking interaction in controlling electron transfer and its likely dependence on protein conformation are discussed.

Nitric oxide (NO) is produced physiologically to fulfill a range of signaling functions and as an immune response agent (1, 2). The mammalian NO synthases (NOS) generate NO by catalyzing the monoxygenation of L-arginine to N-hydroxy-arginine and the subsequent conversion of this NO by catalyzing the monooxygenation of L-arginine to citrulline. NO synthases are homodimers with each subunit arranged in a dimer interface in the enzyme (3, 4). During each cycle, 2 equiv of dioxygen and 1.5 equiv of NADPH are consumed. Four cofactors are required for activity: a cysteine-ligated heme, bound by the oxygenase domain, around which the active site is constructed (5, 6), tetrahydrobiopterin, an electron donor/acceptor required during oxygen activation, and one each of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), bound by the reductase domain. The mammalian NO synthases are homodimers with each subunit arranged such that the reductase domain of one subunit supplies the oxygenase domain of the other with electrons (7, 5). There are three isoforms, two of these are constitutively expressed (neuronal, nNOS), and endothelial, eNOS) and are activated by the reversible binding of calmodulin (CaM) at elevated concentrations of intracellular calcium. The third is known as the inducible isoform; it was first discovered in macrophages and binds CaM even at negligible Ca2+ concentrations and is regulated at the transcriptional level. The NO synthase oxygenase domain is structurally unique and forms the main dimer interface in the enzyme (5, 6), whereas the reductase domain is closely related by sequence and structure to mammalian cytochrome P450 reductase (CPR) (9–11). For both CPR and NO synthase, the reductase domains are composed of a FAD-binding domain, related to ferredoxin reductase (FNR), which is fused to a FAD-binding domain, related to flavodoxin. Electrons derived from NADPH dehydrogenation at the FAD site are transferred sequentially via the FNM to the heme. The FNM oscillates between the hydroquinone and semiquinone oxidation states during catalysis and functions as a single electron donor (12). The FNM to heme electron transfer appears to be the critical rate-determining event for NO synthesis and is also the step activated by CaM (13). Electrons can also be transferred...
specifically from the FMN to the external electron acceptor, cytochrome c. This process was also found to be CaM-dependent in both the holoenzyme and the isolated nNOS reductase domain (14, 15). The redox properties of the cofactors are largely independent of CaM, suggesting that the activation mechanism involves a structural rearrangement of the reductase domain (16, 17).

The nNOS and eNOS reductase domains both contain a series of additional protein inserts/extensions, which have been shown to control their calmodulin dependence (18). These include the CaM binding site, which is located at the N-terminus and consists of approximately 20 amino acids, an autoinhibitory insert of 42–45 amino acids located in the middle of the FMN-binding domain (19–21), and an autoinhibitory C-terminal extension of 20–30 amino acids (21, 22). The sequences of the inserts are all isoform-specific and help to define the three different enzyme groups. The autoinhibitory domains appear to suppress the reductase and NO-synthesis activity of the enzyme, and CaM binding relieves this effect. Recently the substrate, NADPH, was also shown to inhibit electron transfer from reduced nNOSrd to cytochrome c by stabilizing a conformation in which the FMN is inaccessible to electron acceptors (24). The mechanism by which NADPH exerts conformational control may involve the FAD stacking residue F1395 (25), which lies at the start of the autoinhibitory C-terminal extension, and may also be dependent on the redox state of the FAD.

This study focuses on the redox properties of the isolated FMN- and FAD-binding domains, particularly the effect of substrate (NADP+) binding on the reduction potentials of the FAD cofactor.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant nNOS FMN Domain Plus Calmodulin (nNOSFMNCaM).** The nNOS-FMNCaM (pCRNNFMN) expression plasmid was constructed from plasmid pCRNNR, the expression plasmid for rat nNOS reductase domain (nNOSrd) residues 695–1429 (including the CaM binding site) and synthetic bovine brain calmodulin (26). The gene for nNOSrd was excised using EcoRI, and a fragment corresponding to the FMN-binding domain (residues 695–946) was amplified using PCR. The following PCR primers were used to introduce EcoRI sites at either end of the sequence: 5'-gactcattgctacatcagcttctg-3' (forward) and 5'-tcaggatccgaattctcaatcccccacgcagaac-3' (reverse). The resultant gene fragment was cloned back into the E. coli strain JM109 (DE3) using plasmid pCRNNR (26) and purified as described previously on 2',5'-ADP-agarose (Sigma) and CaM-agarose (Sigma) (24). nNOS FAD domain was generated by digesting 10 mL of 20 μM nNOSrd with 1 mL of immobilized trypsin (Sigma) in 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, (buffer B) at room temperature for 2 h with gentle stirring (10). The suspension was centrifuged at 2000g for 10 min and filtered to remove the trypsin, and the supernatant was applied to a 2',5'-ADP-agarose column (1 cm × 10 cm). The yellow band was washed with 100 mL of buffer B and eluted with 20 mL of 1 mM 2',5'-ADP in buffer B plus 0.3 M KCl. Enzyme-containing fractions were concentrated to approximately 200 μM and stored at −80 °C. It should be noted that the nNOS FAD domain generated in this way is truncated by 22 amino acids from the C-terminus and consists of residues 963–1407 of rat nNOS (10).

**Spectroelectrochemistry.** Spectroelectrochemical analysis of nNOSFMNCaM, nNOSrd, and nNOS FAD was conducted in an OTTLE cell constructed from a modified quartz paramagnetic resonance (EPR) cell with a 0.3 mm path length containing a Pr/Rh (95/5) gauze working electrode (wire diameter 0.06 mm, mesh size 1024 cm−1), Engelhardt, U.K.), a platinum wire counter electrode, and an Ag/AgCl reference electrode (model MF2052, Bioanalytical Systems, West Lafayette, IN 47906) (27). Enzyme samples (1 mL × 200 μM) were eluted through a G25 column prequillibrated with 0.1 M Tris pH 7.5, 0.5 M KCl, concentrated to 300 μM, and stored overnight in an anaerobic glovebox at 0 °C. The following mediators were then added: pyocyanine (10 μM), 2-hydroxy-1,4-naphthoquinone (20 μM), FMN (5 μM), benzyl viologen (10 μM), and methyl viologen (10 μM). Spectroelectrochemical titrations were performed at 25 ± 2 °C using an Autolab PGSTAT10 potentiostat and a Cary 50 UV/vis spectrophotometer. The potential of the working electrode was typically decreased in 30 mV steps until the enzyme was fully reduced and increased stepwise until reoxidation was complete. After each step, the current and UV/vis absorption spectrum was monitored until no further change occurred. This equilibration process typically lasted 15 min. The Ag/AgCl reference electrode employed in the OTTLE cell was calibrated against indigotrisulfonic acid (E'f = −99 mV vs SHE) and FMN (E'f = −220 mV vs SHE) in the same buffer conditions and found to be ±205 ± 2 mV relative to the standard hydrogen electrode (SHE). All electrode potentials have been corrected accordingly. Absorbance changes were plotted against the potential of the working electrode and analyzed by fitting absorbance data at one or more wavelengths simultaneously to a modified Nernst equation (eq 1) using Origin 7.0 (Microcal). nNOSFAD redox titrations were also conducted in the presence of equimolar amounts of ADP+ and NADP+. In the latter case, the data were analyzed with eq 2, which incorporates the additional NADP+/NADPH 2 e− redox couple (E'f). The nNOSrd data were analyzed as described previously.
The Isolated FMN and FAD Domains of nNOS

\[
\text{a} \log_{10} \left[ \frac{E - E_1'}{59} \right] + b + c \log_{10} \left[ \frac{E_2' - E_2}{59} \right] + \log_{10} \left[ \frac{E_2' - E_2}{29.5} \right] + \log_{10} \left[ \frac{E_2' - E_2}{59} \right]
\]

(1)

where \(a\), \(b\), and \(c\) are the absorbances of oxidized flavin, flavin semiquinone, and hydroquinone, respectively, \(E\) is the potential of the working electrode, and \(E_1'\) and \(E_2'\) are the midpoint potentials of the oxidized flavin/semiquinone and semiquinone/hydroquinone redox couples (28). Parameters \(a\), \(b\), \(c\), \(E_1'\), and \(E_2'\) are variables determined by least-squares fitting.

\[
\begin{align*}
\text{a} \log_{10} \left[ \frac{E - E_1'}{59} \right] + b \log_{10} \left[ \frac{E - E_2'}{59} \right] + c + \\
d \log_{10} \left[ \frac{E_1' - E_1}{29.5} \right] + \log_{10} \left[ \frac{E_2' - E_2}{29.5} \right] + \\
\log_{10} \left[ \frac{E_2' - E_2}{59} \right] \end{align*}
\]

(2)

where \(a\), \(b\), \(c\), and \(d\) are the absorbances of oxidized flavin, flavin semiquinone, flavin hydroquinone—NADP+ charge-transfer complex, and flavin hydroquinone—NADPH complex, respectively, \(E\) is the potential of the working electrode, and \(E_1'\), \(E_2'\), and \(E_3'\) are the midpoint potentials of the oxidized flavin/semiquinone, semiquinone/hydroquinone—NADP complex, and hydroquinone—NADP complex redox couples, respectively. Parameters \(a\), \(b\), \(c\), \(d\), \(E_1'\), \(E_2'\), and \(E_3'\) are variables determined by least-squares fitting.

RESULTS

Preparation of nNOSFMNCaM. Neuronal NO synthase FMN-binding domain was coexpressed with calmodulin in E. coli and purified with CaM bound throughout. Removal of CaM at any stage during the purification procedure by addition of EGTA led to proteolysis of the domain, precipitation of the protein or both. Addition of the detergents Tween 20 and deoxycholate did not prevent this. Purification by DEAE and Q-sepharose anion-exchange chromatography and Superdex S200 gel filtration resulted in a 1:1 complex of nNOS FMN domain and Ca\(^{2+}\)-bound CaM with a purity of >90% as judged by SDS–PAGE (Figure 1). Typical yields were 10 mg of pure protein from 30 g of E. coli, wet weight. In the initial lysate, the nNOSFMNCaM complex was dark gray/blue, indicative of the stable semiquinone form. As the purification progressed, the FMN turned yellow as oxidation occurred. Inclusion of 10 mM DTT in the buffers during purification was found to prevent oxidation; however, this was not necessary for the stability of the enzyme or its purification. As the purification progressed, the FMN turned yellow as oxidation occurred. The blue-gray semiquinone was easily regenerated by addition of dithionite and remained in this oxidation state for several hours in the presence of air. Inclusion of 10 mM DTT in the buffers during purification was found to prevent oxidation; however, this was not necessary for the stability of the enzyme or its purification. Figure 1 shows an SDS–PAGE gel of purified nNOS FMN domain with bound CaM at different stages of purification: lane 1, cell lysate; lane 2, after DEAE Sephadex; lane 3, after Q-Sepharose FPLC; lane 4, after Superdex S200 FPLC.

Spectroelectrochemical Analysis of nNOSFMNCaM. The midpoint potentials of the FMN/FMNH and FMNH/FMNH\(^2\) redox couples of nNOSFMNCaM were determined using OTTLE potentiometry. Figure 2 shows the UV/vis spectrum of nNOSFMNCaM as it is reduced and oxidized stepwise in an OTTLE cell. The oxidized enzyme has absorbance maxima at 457 and 375 nm. These decay on reduction to the semiquinone form, which has absorbance maxima at 350 and 592 nm typical of the neutral blue FMN semiquinone formed by flavodoxin (31). Further reduction results in the disappearance of these bands. The two FMN redox couples were found to have fundamentally different spectroelectrochemical characteristics; this is illustrated in Figures 3 and 4. Typically,
and oxidative (O) directions; (B) spectroelectrochemical titration with 20 min intervals between 40 mV redox steps in the reductive (TA) domain with bound CaM: (A) spectroelectrochemical titration with full equilibration after each redox step. Data are shown fitted to eq 1; the broken lines mark the midpoint potentials of the two FMN redox couples for the data in panel B.

FIGURE 3: Determination of reduction potentials for nNOS FMN domain with bound CaM: (A) spectroelectrochemical titration with 20 min intervals between 40 mV redox steps in the reductive (O) and oxidative (C) directions; (B) spectroelectrochemical titration with full equilibration after each redox step. Data are shown fitted to eq 1; the broken lines mark the midpoint potentials of the two FMN redox couples for the data in panel B.

The current flowing between working and counter electrodes (I) is plotted against time. The potential of the working electrode was stepped down by 30 mV after each equilibration. Data relate to Figure 3, panel B.

after the potential of the working electrode is stepped by 30 mV, the resulting surge in current relaxes back to the base level within 10–15 min. Providing the concentration of enzyme and mediators are sufficient, the relaxation rate is limited by the conductivity of the solution as determined by the concentration of electrolyte present and the arrangement/dimensions of the cell. During both reduction and oxidation of the FMN through the oxidized flavin/semiquinone redox couple, a significant slowing of the equilibration process occurred. The semiquinone/hydroquinone redox couple, on the other hand, exhibited normal relaxation behavior. Figure 3 shows the results of two different spectroelectrochemical titrations. In Figure 3A, the absorbance due to semiquinone generated is plotted against reduction potential as the working electrode was stepped down by 30 mV at 15 min intervals until the FMN was fully reduced and then the process was reversed. While the semiquinone/hydroquinone couple was freely reversible, the oxidized flavin/semiquinone couple was observed to be severely hysteretical under these conditions with some 80 mV separating the forward and reverse redox transitions. In Figure 3B, a similar plot is shown in which the solution was left to equilibrate fully after each redox step. At the higher potentials, equilibration took place over as much as 5 h, that is, 20-fold more slowly than at low potentials. This is illustrated in Figure 4. The protracted relaxation of the current shown was accompanied by a slowly changing visible spectrum. The data plotted in Figure 3B were used to calculate the equilibrium reduction potentials of the two FMN redox couples; these were —179 mV for FMN/FMNH and —314 mV for FMNH/FMNH2 (Table 1).

Spectroelectrochemical Analysis of nNOSFAD. The midpoint potentials of the oxidized flavin/semiquinone and semiquinone/hydroquinone redox couples of the nNOS FAD-binding subdomain were determined in the absence and presence of stoichiometric amounts of ADP and NADP+.

The enzyme and ligand concentrations in these experiments were 300–500 μM, which is well in excess of the Kd values of these inhibitors (32). They should therefore form 1:1 complexes (33). Figure 5A shows how the UV/visible spectrum of nNOSFAD changes during electrochemical reduction. The absorbance maximum at 460 nm decays rapidly during the early stages of reduction and a band at 397 nm appears, characteristic of a neutral blue semiquinone. This ultimately decays away as the FAD hydroquinone is formed. In Figure 5B, the absorbance at 460 nm and the difference in the absorbances at 650 and 700 nm are plotted against the corrected potential of the working electrode. The two sets of data were fitted simultaneously to eq 1 to give reduction potentials of —291 and —326 mV for the FAD/FADH and FADH/FADH2 couples, respectively.

In the presence of NADP+, there were significant differences in both the UV/vis spectra of intermediates formed during reduction (Figure 6) and the final reduction potentials determined. As the nNOSFAD—NADP+ complex was reduced, the 460 nm band disappeared and the neutral, blue FAD semiquinone appeared (at 697 nm), as observed for the uncomplexed enzyme (Figure 6A). However, further reduction resulted in the appearance of a broad absorbance band at 780 nm, characteristic of a FADH2—NADPH charge-transfer complex. NADP+ is the product of NADPH dehydrogenation at the FAD site of nNOS and is known to form a charge-transfer complex with the FAD hydroquinone as the nicotinamide and isoaxalazine rings stack above one another (32–34). At lower potentials, the charge-transfer complex was reduced, resulting in the formation of the FADH2—NADPH complex and loss of the charge-transfer band. Formation of NADPH was accompanied by an increase in absorbance at 355 nm (Figure 6). The absorbance at 355 nm is the approximate position of an isosbestic point in the nNOSFAD redox titration and is mainly influenced by changes in the relative concentrations of NADP+ and NADPH. Absorbance changes at 460 nm are mainly due to the disappearance of oxidized FAD. The ΔAsos—700 (difference in the absorbances at 650 and 700 nm) indicates the formation and decay of FAD semiquinone (the subtraction minimizes contributions from the charge-transfer complex), and the absorbance at 780 nm is due to the FADH—NADP+ charge-transfer complex. In Figure 6B, all these values are plotted against the potential of the working electrode. Together they indicate how the concentrations of each of the four redox states (FAD—NADP+, FADH—NADP+, FADH2—NADPH) vary with potential.
Table 1: Reduction Potentials of the FMN, FAD, and NADP⁺ Cofactors of nNOSrd, nNOSFMNCaM, and nNOSFAD⁺

<table>
<thead>
<tr>
<th>redox couple</th>
<th>nNOSrd + CaM⁺</th>
<th>nNOSFMNCaM</th>
<th>nNOSFAD</th>
<th>nNOSFAD + ADP⁺</th>
<th>nNOSFAD + NADP⁺⁺</th>
<th>free NADP⁺⁺/CaM⁺/ADP⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMNH/H⁺</td>
<td>-98 ± 5</td>
<td>-179 ± 3</td>
<td>-291 ± 6</td>
<td>-304 ± 5</td>
<td>-297 ± 6</td>
<td>-306 ± 8</td>
</tr>
<tr>
<td>FADH/H⁺</td>
<td>-300 ± 8</td>
<td>-314 ± 3</td>
<td>-326 ± 3</td>
<td>-314 ± 3</td>
<td>-326 ± 3</td>
<td>-323 ± 7</td>
</tr>
<tr>
<td>NADP⁺/H⁺</td>
<td>-320 ± 10</td>
<td>-320 ± 10</td>
<td>-320 ± 10</td>
<td>-320 ± 10</td>
<td>-320 ± 10</td>
<td>-320 ± 10</td>
</tr>
<tr>
<td>heme Fe⁺⁺/Fe⁺⁺</td>
<td>-356 ± 3</td>
<td>-356 ± 3</td>
<td>-356 ± 3</td>
<td>-356 ± 3</td>
<td>-356 ± 3</td>
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*Measurements were taken at 25 ± 1 °C in 100 mM Tris/HCl buffer, pH 7.5, 0.5 M KCl. *Reduction potential of the flavin oxidized/semiquinone redox couple. *Reduction potential of the flavin semiquinone/hydroquinone redox couple. *Reduction potential of the NADP⁺/NADPH two-electron redox couple adjusted by 30 mV per pH unit (29). *Reduction potential of the isolated nNOS oxygeanse domain heme Fe⁺⁺/Fe⁺⁺ redox couple in the presence of L-arginine (30) repeated under above conditions. *In the presence of stoichiometric amounts of bound CaM, ADP, and NADP⁺⁺, respectively.

The data were fitted simultaneously to eq 1 to give equilibrium reduction potentials for the three transitions (Table 1). The data were also used to calculate the molar absorption coefficient for the FADH⁺—NADP⁺ charge-transfer complex at the absorption maximum of 780 nm to be 970 M⁻¹ cm⁻¹. The molar absorption coefficient for the oxidized isolated FAD domain used in this calculation was determined to be 10 560 M⁻¹ cm⁻¹ at the absorption maximum of 456 nm.

The presence of ADP had much less influence on the UV/vis spectra of the different nNOSFAD redox species (Figure 7). The proportion of semiquinone generated during the redox titration was less than that for the uncomplexed enzyme but was more than that in the presence of NADP⁺. No charge-transfer species were observed. In Figure 7B, the absorbance at 460 nm and the difference in absorbance at 650 and 700 nm are plotted against the potential of the working electrode. The data were fitted to eq 1. In this case, the semiquinone absorbance parameter was fixed to the same value obtained in the absence of ADP (which was the same as that obtained in the presence of NADP⁺). Fixing this parameter is likely to give better consistency because the extinction coefficient is unlikely to change much in the presence of the inhibitor (it is otherwise spectroscopically identical). The error margins given in Table 1 reflect the uncertainty in this value. ADP appeared to affect the oxidized FAD/semiquinone redox couple, disfavoring semiquinone formation slightly. Although it is clear that there is slightly less semiquinone formed during this redox titration, the shifts in potential observed are similar to the experimental errors.

The nNOSFAD redox titrations were all freely reversible with no hysteresis observed in any of the redox couples.

*Spectroelectrochemical Analysis of nNOS Reductase Domain.* OTTLE cell potentiometric titration of nNOSrd (not shown) was conducted under the same conditions as used for the subdomains above (0.1 M Tris/HCl, pH 7.5, 0.5 M KCl). The changes in UV/vis spectrum observed closely...
FIGURE 7: Determination of reduction potentials of nNOSFAD in the presence of ADP: (A) UV/vis spectra collected during spectroelectrochemical titration of 300 μM enzyme and ADP (1:1 complex) in 100 mM Tris/HCl, pH 7.5, 0.5 M KCl at 25 °C; (B) plot of electrode potential vs absorbance at 460 nm (O) and the difference in the absorbances at 650 and 700 nm (●) fitted simultaneously to eq 1.

matched those reported previously (17) using chemical reduction/oxidation in 0.05 M Tris/HCl, pH 7.1, 0.1 M KCl. The data (see Table 1) were fitted in a similar way, using absorption coefficients calculated for the separate domains (above) and show a broad shift in potential of 30–60 mV largely attributable to the increase in pH. The results are useful for comparison with the subdomains and show how their separation affects the properties of the FMN and FAD cofactors. The only significant difference is in the potential of the FMN/FMNH redox couple, which is 80 mV higher in nNOSrd than in the isolated FMN–CaM domain. This indicates that contacts between the two subdomains are important for controlling the environment of the FMN cofactor. The experiment with nOSFAD was repeated in the presence of a stoichiometric amount of NADP+, which produced a charge-transfer complex with similar spectral characteristics (i.e., band position and intensity) to that observed with the isolated FAD domain (Figure 8). Fitting of the electrochemical data obtained was abandoned due to the complexity of the system, which has five separate redox couples. However, the data appeared to be consistent with the behavior of the NADP+–free enzyme and NADP+–bound nOSFAD in the analogous experiments.

DISCUSSION

Electron transfer through neuronal NO synthase is controlled by a remarkable series of structural features and ligands. These include autoinhibitory domains and the reversible ligation of calmodulin (CaM) and inhibitor proteins (13, 14, 18–23, 37). The reductase domain of nNOS is considered to be catalytically repressed in the absence of CaM with both reduction and oxidation of the flavins being affected. However, attempts to determine which individual catalytic steps repress electron transfer through nNOSrd have resulted in controversy with electron transfer (36, 38, 39), hydride transfer (15), and product dissociation (24, 36) all being suggested.

It has recently been shown that the conformation of nNOSrd is dependent on NADP/H binding (24) and that in the holoenzyme, the FAD-stacking residue (F1395) helps to regulate electron transfer from the reductase domain to heme (25). The possibility that the conformation of the reductase domain, NADP/H binding, or both influence the redox properties of the flavin cofactors and therefore the electron transfer steps was assessed by studying the separate FMN- and FAD-binding subdomains. The FMN domain (nNOS-FMNCaM) was found to be stable only in the presence of bound CaM/Ca2+, preventing analysis of the CaM-free form. The FMN cofactor formed a stable blue semiquinone, which oxidized slowly in air, much like the FMN semiquinone formed in nNOS holoenzyme and in the reductase domain (nNOSrd). However, the reduction potential for the FMN oxidized/semiquinone redox couple was 80 mV lower than that in nNOSrd. This indicates that the FAD-domain has a large influence on the environment of the FMN and acts to stabilize the semiquinone thermodynamically. This effect was not observed with the related enzymes human CPR (44) and methionine synthase reductase (45). The same redox couple was also found to exhibit strong hysteresis during electrochemical reduction and oxidation, as shown in Figures 3 and 4. By analogy with related flavodoxins, the cause of this effect is likely to be a hydrogen bonding interaction between the peptide carbonyl of Gly810 and the flavin N5, which is protonated in the semiquinone form but not in the oxidized form (31). The result is a kinetically stable semiquinone, which does not oxidize during the normal catalytic turnover of nNOS. The FMN sq/hq redox couple is, however, the same in both nNOSFMNCaM and nNOSrd. This redox couple is freely reversible and is utilized in the transfer of electrons from FAD to heme in nNOS. The FAD-domain, therefore, appears to have little influence on the potential of the viable electron on the FMN cofactor. Given this, it also seems unlikely that the conformation of the FAD domain...
could affect the reduction potential of the FMN sq/hq couple. The kinetic and thermodynamic stability of the FMN semiquinone toward oxidation ensures that the FMN cofactor acts only as a one-electron donor. This is likely to be important to the mechanism of nitric oxide synthesis, which requires the delivery of single electrons at an optimum rate. According to the kinetic model defined by Santolini et al., (40) transfer of electrons pairwise to the heme would result in a build up of stabilized ferrous NO complex during turnover, which would ultimately slow the overall rate of catalysis.

The reduction potentials of the FAD cofactor were also found to be unchanged in the isolated nNOS FAD domain, indicating that the FMN domain has little effect on the redox properties of this cofactor. It seems unlikely, therefore, that the conformation of the reductase domain alone could control the driving force for electron transfer through the flavins. This is consistent with the fact that CaM binding has little effect on the flavin reduction potentials (17). Substrate (NADP⁺) binding, however, was found to stabilize the hydroquinoline form of the FAD, by increasing the reduction potential of the sq/hq couple by 30–40 mV. Some destabilization of the semiquinone also occurred, as indicated by a decrease in the potential of the ox/sq couple. The inhibitor ADP did not cause stabilization of the FAD-hydroquinone, suggesting that interactions between the nicotinamide substituent (NMN) and the reduced enzyme are responsible for the effect. The most likely cause is a favorable interaction between the oxidized NMN and the FAD hydroquinone, which form a π-stacked charge-transfer complex. Similar complexes are formed by the nNOSFAD and nNOSrd constructs as shown in Figures 6 and 8 and have been characterized for the related enzymes P450 reductase (CPR) (41) and ferredoxin reductase (FNR) among others (34). For FNR, it has also been shown that the two-electron reduction potential of the FAD/NADP⁺ system increases on formation of a charge-transfer complex, although in this case the NADP⁺ is preferentially reduced rather than the FAD. This is due to a more negative FAD reduction potential in FNR, which generally operates in the reverse direction to nNOS-FAD, catalyzing NADPH formation rather than dehydrogenation (34). The X-ray crystal structure of the nNOSFAD construct (10) shows the FAD π-stacked with the F1395 residue (Figure 9); this must move for hydride transfer to occur from NADPH, which is shown bound in a nonproductive conformation. The analogous residues in FNR and CPR are Y308 and W677, respectively. The X-ray structures of the Y308S mutant of FNR (35) and the W677G mutant of CPR (43) show the NMN substituent stacked with the FAD in a productive conformation. Figure 9 illustrates the conformational change required for the NADPH to initiate hydride transfer. Substantial movement of the active site residues must also occur during this process; however, these cannot be predicted from the mutant structure. The nonproductive conformation of bound NADP⁺ appears to be dominant for NOS, FNR, and CPR. This stems from the high affinity of the enzymes for the ADP substituent of the substrate, which guarantees a low Kₐ, and negative binding affinity for the NMN group, as demonstrated for CPR (42). It has therefore been proposed that the stacking residue acts to dislodge the NMN group from the FAD as part of the catalytic process. Mutants lacking a bulky aromatic at this position bind NADP(H) more tightly and are catalytically limited by product dissociation. The charge-transfer complex formed by the CPR FAD domain W676 mutant and NADPH was found to be particularly stable, releasing NADP⁺ at a much lower rate (41). Recent evidence also suggests a link between the conformation of bound NADP(H), the FAD-stacking residue, and the accessibility of the FMN to external electron acceptors (e.g., the oxygenase domain and cytochrome c) in nNOS. We can now add that the conformation of the NADP⁺ is also likely to affect the reduction potentials of the FAD, assuming the π-stacking interaction is responsible for the shifts observed in the presence of NADP⁺. Therefore, with bound NADP⁺, the conformation of the three-electron reduced nNOS reductase domain may influence the equilibrium distribution of electrons in the FAD and FMN cofactors and vice versa. The shift in the FAD sq/hq potential observed is only 30–40 mV, which would be too small to have a significant effect on overall catalytic rate constants. However, this shift is averaged over all the conformations existing at equilibrium and takes no account of the proportion of enzyme molecules with NMN and FAD in the stacked conformation.

Scheme 1 shows a simple model describing the NADP⁺-bound FAD sq/hq redox couple. The nNOSFAD is assumed to exist in two conformations, which are roughly assigned to the π-stacked and unstacked conformations depicted in

FIGURE 9: The active sites of nNOS FAD domain (A) (10) and ferredoxin reductase Y308S mutant (B) (32) with NADP⁺ bound.
The unstacked reduction potential is given by

$$E_0 = -323 \text{ mV},$$

i.e., ADP-bound nNOS-FAD, and the reduction potential of the fully \( \pi \)-stacked enzyme is given by

$$E_N.$$  

The reduction potential shift observed experimentally (33 mV) may all be involved in defining the energy of interaction.

The high value for the hydroquinone compared to the

FAD stacking in the nNOS FAD domain on the \( \text{sq/\text{hq}} \) reduction potential as the ADP-bound enzyme

The three unknowns can be modeled using the Nernst equation and Scheme I to produce the plot in Figure 10. However, if the FAD semiquinone is unable to form energetically favorable \( \pi \)-stacked complexes with the NADP, it is possible that \( E_N \) lies in the \(-200 \) to \(-250 \) mV range. This would influence the equilibrium distribution of electrons between the FAD and FMN, particularly for the three-electron reduced enzyme form, such that when the NADP\(^+\) is in the stacked conformation, the FAD hydroquinone would be favored, preventing formation of FMN hydroquinone. In the unstacked conformation, the reverse would be true. Addition of NADPH to fully reduced nNOSrd causes the enzyme to adopt a conformation in which the FMN hydroquinone is inaccessible to the external electron acceptor, cytochrome \( c \). It is likely that the NADPH adopts an unstacked conformation in this case, since formation of a charge-transfer complex between the two reduced nucleotides is improbable. If the \( \pi \)-stacked NADP\((H)\) conformation corresponds with the open enzyme conformation, it may be that electron transfer from nNOSrd is inhibited by a FAD—NADPH reduction potential switch in this conformation and by inaccessibility of the FMN in the other conformation (with unstacked NADP\(^+\)). Since CaM binding relieves conformational restriction of nNOSrd, this would activate electron transfer through the enzyme.

In a recent report by Dunford et al. (46), the potentials of the FAD in an isolated FAD-domain construct were reported to be markedly different: \(-177 \) mV for the oxidized FAD/semiquinone and \(-310 \) mV for the semiquinone/hydroquinone couples (at pH 7). However, the data are of poor quality and appear to have been analyzed inappropriately. These potentials would result in almost total FAD semiquinone formation at the reduction midpoint, yet it is clear from the spectra presented that this is not the case. Mutation of the FAD-stacking residue F1395 to serine is also reported to be markedly different: \( -323 \) mV for the ADP-bound enzyme \( (E_0 = -323 \text{ mV}) \), \( E_N \) is the reduction potential of the stacked form (as in Scheme I) and \( E_{\text{NADP-bound}} \) is the experimentally determined reduction potential of the substrate-bound enzyme.

**CONCLUSIONS**

The reduction potentials of the FAD/FADH, FADH/

FADH\(_2\), FMNH/FMNH\(_2\), and heme Fe\(^{II}/Fe^{III}\) redox couples for the isolated nNOS domains all lie within a 30 mV range in the presence or absence of substrates. This facilitates efficient electron transfer through the enzyme and ensures that all these redox states are populated at equilibrium. The FMN semiquinone was found to be kinetically and thermo-
The Isolated FMN and FAD Domains of nNOS dynamically stabilized toward oxidation, although the thermodynamic stability was greater in the presence of the FAD domain. NADP⁺ was found to induce a modest shift in the FADH/FADH₂ redox couple from −326 to −290 mV, probably induced by the formation of a r-stacked charge-transfer complex. The significance of this effect is dependent on the relationship between the enzyme conformation and the fraction of bound NADP⁺ molecules adopting the r-stacked conformation. It is possible that electron transfers between both FAD and FMN and FAD and heme are influenced by the conformation of bound NADP⁺, which may act to inhibit electron flow through nNOS in the absence of CaM. Further studies are required to determine whether the shift in the FAD reduction potential is tightly coupled to conformational changes and whether the resulting electron redistribution is enough to inhibit electron transfer from FMN to heme in the CaM-free enzyme under turnover conditions.

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REFERENCES


The formation of a complex between calmodulin and neuronal nitric oxide synthase is determined by ESI-MS

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Calmodulin (CaM) is an acidic ubiquitous calcium binding protein, involved in many intracellular processes, which often involve the formation of complexes with a variety of protein and peptide targets. One such system, activated by Ca\(^{2+}\) loaded CaM, is regulation of the nitric oxide synthase (NOS) enzymes, which in turn control the production of the signalling molecule and cytotoxin NO. A recent crystallographic study mapped the interaction of CaM with endothelial NOS (eNOS) using a 20 residue peptide comprising the binding site within eNOS. Here the interaction of CaM to the FMN domain of neuronal nitric oxide synthase (nNOS) has been investigated using electrospray ionization mass spectrometry (ESI-MS). The 46 kDa complex formed by CaM–nNOS has been retained in the gas-phase, and is shown to be exclusively selective for CaM.4Ca\(^{2+}\). Further characterization of this important biological system has been afforded by examining a complex of CaM with a 22 residue synthetic peptide, which represents the linker region between the reductase and oxygenase domains of nNOS. This nNOS linker peptide, which is found to be random coil in aqueous solution by both circular dichroism and molecular modelling, also exhibits great discrimination for the form of CaM loaded with 4\([\text{Ca}^{2+}\)]. The peptide binding loop is presumed to be configured to an \(z\)-helix on binding to CaM as was found for the related eNOS binding peptide. Our postulate is supported by gas-phase molecular dynamics calculations performed on the isolated nNOS peptide. Collision induced dissociation was employed to probe the strength of binding of the nNOS binding peptide to CaM.4Ca\(^{2+}\). The methodology taken here is a new approach in understanding the CaM–nNOS binding site, which could be employed in future to inform the specificity of CaM binding to other NOS enzymes.

Keywords: ESI-MS; calmodulin; CaM–nNOS complex

1. INTRODUCTION

Electrospray ionization mass spectrometry (ESI-MS) has rapidly become an established and important technique with which to analyse non-covalent complexes in a solvent free environment. The ‘softness’ of desolvation and ionization allows complexes present in solution to resist the dissociation process and survive intact into the gas-phase. Several non-covalently bound systems have been studied by ESI-MS including DNA–protein (Xu et al. 1999), protein–metal (Taylor et al. 2001), protein–ligand (Bruce et al. 1998) and protein–protein (Robinson 2002). In addition the technique has been used to screen ligands from combinatorial libraries (Wigger et al. 2002) and for competitive binding of inhibitors (Smith & Whitesides 1995). Whether these gas-phase complexes relate to solution phase complexes and structures is still a matter of debate. Both results that correlate between gas-phase and solution phase (McLafferty et al. 1998) and results that contrast (Rogniaux et al. 1999) have been published. However it is generally agreed that ESI-MS is a powerful tool that can provide information on complexes which at the very least, compliments data achieved by other structural methods.

ESI-MS of biological samples typically employs solution conditions, comprising an organic solvent and low pH, to aid desolvation and protonation of the sample. However, these conditions can destroy the tertiary, and to some degree secondary structure of the protein and non-covalent complexes are unlikely to be preserved (Jarrold 2000). Furthermore, these conditions are far removed from most physiological environments, and therefore results cannot be readily correlated or compared to what may be happening in vivo. Therefore, to analyse non-covalent complexes, the sample is introduced from an aqueous solution, buffered to a relevant physiological pH.

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ESI-MS has been used here to study the ubiquitous intercellular protein Calmodulin (CaM). CaM interacts with an impressive number of proteins, with a wide physiological diversity (Hoeftich & Ikura 2002), which have a broad biological role in the body. CaM binding proteins can be categorized into six groups (Chin & Mearns 2000).

(i) Proteins which bind irreversibly to CaM irrespective of Ca$^{2+}$. An example is phosphorylase kinase, an enzyme which requires denaturing conditions to dissociate CaM but is activated in the presence of Ca$^{2+}$.

(ii) Proteins which bind to apo CaM but dissociate reversibly in the presence of Ca$^{2+}$. Examples of this group are neuromodulin and neurogranin which might serve as intercellular reservoirs for CaM at resting concentrations of Ca$^{2+}$, but liberate Ca$^{2+}$ activated CaM in response to elevated Ca$^{2+}$.

(iii) Proteins which form low affinity, inactive complexes with CaM at low concentrations of Ca$^{2+}$, when the Ca$^{2+}$ sites of CaM are empty or partially occupied. At greater concentrations of Ca$^{2+}$, these proteins engage in high affinity complexes with and are activated by CaM. Calcineurin and smooth muscle light chain kinase belong to this group.

(iv) Proteins which bind to CaM in the presence of Ca$^{2+}$, which then inhibits their function. This group includes enzymes such as select members of the G protein receptor kinases and well as the C-terminal reductase domain and the two domains are presumed to be largely independent of calcium concentration (Daff 2001). nNOS consists of an N-terminal oxygenase domain and a C-terminal reductase domain and the two domains are linked by a peptide. It is to this peptide that CaM can bind reversibly when it becomes activated by calcium (Vorherr et al. 1993). CaM binding activates nNOS (and eNOS), providing them with a rapid response mechanism during their participation in signalling cascades. In contrast iNOS, which produces NO to then engage in protein and DNA destruction, is expressed with CaM bound and this has been shown to be largely independent of calcium concentration (Daff 2003). Here ESI-MS is employed to study the interaction of calmodulin and calcium, and the subsequent binding of the FMN domain of nNOS. Specifically, we examine the interactions between CaM and the proposed CaM binding region of nNOS (Aoyagi et al. 2003). This approach has been taken before using urca gel electrophoresis and fluorescence spectroscopy (Vorherr et al. 1993) and for the CaM–eNOS system, with X-ray crystallography (Aoyagi et al. 2003), but never by ESI-MS. The dissociation of the complex by collision induced dissociation (CID) of CaM and the nNOS peptide yields insights into the structure and modes of binding of this biologically important system. The methodology taken here could be applied to site directed mutants of the nNOS–CaM binding interface to further probe the specific interactions.

2. EXPERIMENTAL PROCEDURES

2.1. Protein and peptide synthesis

Bovine calmodulin was prepared from Escherichia coli cells expressing the protein as described previously (Craig et al. 2002) and stored as a freeze dried solid. Calmodulin was dissolved in ultrapure water at 20 mg ml$^{-1}$. Approximately 100 μl was dialysed prior to analysis against 41 ammonium acetate (10 mM, pH 6.8) using Slide-a-Lyser dialysis cassettes from Pierce (Pierce Biotechnology, Rockford, IL). To chelate calcium from CaM, 0.4 M EGTA was added to the dialysed fractions in the presence of 10 mM ammonium acetate. The sample was then passed along a 10DG column (Biorad Laboratories), and protein containing fractions pooled and concentrated on a spin cartridge concentrator. All glassware and plasticware were acid treated.
washed with 2 M HCl and rinsed with ultrapure water to prevent calcium contamination.

The nNOS FMN domain comprising residues 695-946 of rat nNOS with bound CaM (CaM-nNOS) was obtained as 50 μl of 20 mg ml⁻¹ in tris buffer (Garnaud et al. 2004). The sample was diluted in half with ultrapure water, to 350 μM. Prior to analysis 100 μl was dialysed against 41 ammonium acetate cassettes from Pierce.

Fmoc-based solid-phase peptide synthesis of the proposed calmodulin binding region of nNOS, the linker peptide, Ac-KRRAIGFKKLAEAVKFSAKLM-NH₂ (Aoyagi et al. 2003) was conducted manually, on a 0.1 mmol scale, using Rink amide resin (200–400 mesh) and employing 0.5 mmol (5 equiv.) of each Fmoc amino acid per coupling reaction and HBTU/HOBt as coupling reagents. Resin and standard Fmoc protected amino acids were purchased from Novabiochem. The average coupling time was 4 h and the reaction progress was monitored using LC-MS and the Kaiser ninhydrin test. After synthesis the N-terminal amino acid was capped with acetic anhydride (1 mmol) in the presence of catalytic N,N-dimethylanilinopyridine (0.01 mmol). The peptide was then cleaved from the solid-support by treatment with 95% TFA, 2.5% H₂O, 2.5% ethanedithiol for 3 h and purified by reversed-phase HPLC and lyophilized to afford the product as white ‘fluffy’ solid. LC-MS was performed using a Phenomenex Luna C18 LC-MS column (2.1×50 mm) and a gradient of 5–95% acetonitrile containing 0.1% TFA over 25 min (flow rate of 0.2 ml min⁻¹). Semi-preparative HPLC was performed using a Phenomenex LUNA C18 column and a gradient of 10–90% acetonitrile containing 0.1% TFA over 50 min (flow rate of 3.0 ml min⁻¹).

2.2. Mass spectrometry

Measurements were taken on a Micromass Q-ToF instrument (Micromass UK Ltd, Manchester, UK) equipped with a Z-spray nanoflow source. Gold/palladium plated borosilicate glass nanoelectrospray needles (Proxeon Biosystems A/S, Odense, Denmark) were used to spray the sample into the mass spectrometer. Source conditions were kept constant for all measurements and consisted of; capillary (needle) voltage 800–1200 V, cone voltage 50 V, at 80°C. Q-ToF instruments mass spectrometers incorporate both a quadrupole and an orthogonal time of flight detector. When measuring the full m/z range of the sample, the quad was used in the RF-only mode as a wide bandpass filter. Ions then pass through the hexapole collision cell, which is pressurized to 10 psi with argon gas and pass into the ToF, where they are detected on a multichannel plate set at 2.9 kV. For CID, the injection voltage into the collision cell was increased in 5 V increments.

Data were collected using MASSLYNX software version 3.2. A Savitzky–Golay smoothing algorithm was applied to raw data spectra at peak width at one-half height. Data was then deconvoluted to represent true mass using the transform package supplied by MASSLYNX version 3.1. The instrument was calibrated daily prior to experiments using horse heart myoglobin (Sigma Aldrich).

2.3. Circular dichroism

Circular dichroism (CD) experiments were carried out on a Jasco J-810 Circular Dichroism Spectrometer. Samples of the nNOS peptide were diluted to 100 μM in the required solvent and analysed in a 0.5 mm cell. Spectra were acquired from 190 to 500 nm, at a scan rate of 5 nm min⁻¹.

2.4. Molecular modelling using the AMBER force field (Case et al. 2005)

The nNOS linker peptide was built using X-Leap (Case et al. 2005) according to the sequence given above. All amino acids were held at their physiological ionization states, generating a peptide with net charge +6. A simulated annealing procedure was employed for an initial gas-phase energy minimization of the structures. High temperature dynamics was performed at 800 K, followed by dynamics at decreasing temperatures according to an exponential cooling curve. At 0 K the candidate structure is subjected to minimization using a steepest descent approach followed by a conjugate gradient algorithm. The minimized structure is then used as the seed for the next run of high temperature dynamics. Candidate structures of 300 were generated of the nNOS peptide. The lowest energy structure obtained was then subjected to another simulated annealing cycle with the use of an implicit solvent model (Tsui & Case 2000). Both the lowest energy conformations achieved were subjected to dynamics at 300 K for 1 ns. All calculations were performed on an SGI ORIGIN 200 which forms part of the EPIC biocomputing facility. The lowest energy structures from this run are those discussed within the text. These structures were viewed and analysed using visualizing molecular dynamics (Humphrey et al. 1996).

3. RESULTS AND DISCUSSION

3.1. Denaturing and buffered conditions with and without calcium addition

Calmodulin was initially analysed under ‘standard’ acidic electrospray mass spectrometry conditions, in a 1:1 methanol:water solution with 1% acetic acid. The predominantly bi-nodal mass spectrum of positive ions is shown in figure 1a, where ions are observed in the range 20<z<6. This exhibits a clear distribution centred at z=+15, a second at z=+9 and a third series around z=+11 is also apparent. This multinodal distribution of charges suggests several different structures existing in equilibrium in the solution and illustrates how ESI-MS can be used to assess protein conformations as a function of solvent conditions, as shown by several researchers (Lafitte et al. 1995; Jarrold 2000; Dobo & Kaltashov 2001). The deconvoluted mass obtained over several charge states was

1. www.epic.ed.ac.uk.
suggests that the number of conformations now present is 16 706.6 Da, which is in excellent agreement with the theoretical average solution mass 16 706.45 Da.

The same sample was analysed in ammonium acetate (10 mM, pH 6.8) and water and the charge distributions acquired under these differing solvent conditions are shown in figure 1a, respectively. The predominantly single distribution, centred on z = +9 for the buffered solution, and at z = +12 in water, suggests that the number of conformations now present in solution has reduced compared to that obtained in acidic electrospray conditions (figure 1e). This is likely to be a narrow distribution around the folded native conformation, where less of the basic residues are available for protonation. Closer inspection of figure 1b,c shows more evidence for preservation of such a native structure. Deconvolution of all charges results in not showing more evidence for preservation of such a native structure. Deconvolution of all charges results in not showing more evidence for preservation of such a native structure.

Ammonium acetate buffer was made with ultrapure water, but our calmodulin appears to have a limited and variable source of calcium ions, which despite careful acid washing of all glassware was difficult to eliminate totally. This inconsistency in background calcium levels is apparent by a comparison of the insets in figure 1b,c where the higher charge state series obtained when spraying from pure water actually yields an increased level of Ca\(^{2+}\) bound CaM, in contrast to the argument given above. This may also be due to buffer salts ligating at the Ca\(^{2+}\) binding sites up until desolvation is complete, thus preventing Ca\(^{2+}\) addition. The protein was subsequently analysed in 10 mM ammonium acetate with the addition of 100 μM calcium acetate, to provide a controlled level of calcium (figure 1d). Under these conditions the spectrum obtained has a similar charge distribution to figure 1c, tending towards lower charge states, again indicative of a more compact and folded structure (Jarrold 2000). Overall five species of calmodulin are found, corresponding to apo calmodulin and calmodulin complexed with a bound calcium ion.

The work of several other researchers, including that reported in Lafitte et al. (1995), Nousiainen et al. (2003) also finds the stoichiometry of calcium binding to CaM in the gas-phase to be effectively similar to that existing in solution. However, there exists some controversy about the maximum number of calcium ions that may be bound to the protein, for example Lafitte et al. (1995) describe binding of up to 17 calcium ions, Gross and co-workers report up to 7 (Nemirovskiy et al. 1999), while Veenstra et al. (1997) observe a maximum of four. It is likely that this is due to the presence of varying concentration of free calcium in solution, and as stated on the website in footnote 1, the effect of source conditions on retention of auxiliary bound calcium ions.

To investigate this further, all calcium were chelated from the CaM sample using EGTA. Mass spectra were obtained with controlled and increasing amounts of calcium present. Figure 2 shows the number of calcium ions found bound to the calmodulin protein at a range of concentrations up to and including stoichiometric levels of CaM–Ca\(^{2+}\). The CaM was present at 25 μM in all of these experiments; the points shown are taken from deconvolutions over the whole charge state envelope. As the concentration of calcium is increased the number of bound calcium ions also increases, until at a concentration ratio of 1 : 1 CaM–Ca\(^{2+}\), CaM.4Ca\(^{2+}\) is the dominant species present. This is a somewhat surprising result, which suggests that a significant portion of the

![Figure 1. ESI-MS spectrum of 50 μM CaM in (a) 1 : 1 methanol : water and 1% formic acid, (b) ultrapure water, (c) 10 mM ammonium acetate (pH 6.8) and (d) 10 mM ammonium acetate pH 6.8 with addition of 100 μM calcium acetate. Insets show deconvoluted data obtained from each spectrum.](image-url)
The population of CaM.nCa$^{2+}$ species as a function of changing calcium ion concentration. Spectra were acquired in negative ionization mode, with the concentration of calmodulin maintained at 25 μM. The first spectrum were obtained post EGTA treatment of the protein, and then at the following μM concentrations of calcium acetate: 5; 10; 25; 100. The points represent the intensity of the CaM.nCa$^{2+}$ species, each normalized to the most intense peak in the spectrum.

Figure 2. The population of CaM.nCa$^{2+}$ species as a function of changing calcium ion concentration. Spectra were acquired in negative ionization mode, with the concentration of calmodulin maintained at 25 μM. The first spectrum were obtained post EGTA treatment of the protein, and then at the following μM concentrations of calcium acetate: 5; 10; 25; 100. The points represent the intensity of the CaM.nCa$^{2+}$ species, each normalized to the most intense peak in the spectrum.

Another explanation for this is that our protein concentration is not accurate; to measure it we have used an absorption coefficient of CaM of 2900 M$^{-1}$ cm$^{-1}$ recorded at 276 nm, which we believe to be correct for human CaM which possess two tyrosine residues (Hans J. Vogel, Calgary University, private communication). Other values have been quoted the use of which, would alter the concentrations recorded here. From negative ionization conditions appear appropriate, although appears from the binding stoichiometry determined via ESI-MS as presented in figure 2, that this does not represent a full picture of the populations of CaM which must exist in solution.

Another explanation for this is that we are presenting data here in negative ionization mode. We have avoided looking at the CaM.nCa$^{2+}$ system in positive ionization mode, because in addition to CaM.nCa$^{2+}$ species we also detect significant adduct sodium and potassium ions adducting to the calmodulin, presumably leached from the nanospray needles. This point has been made by Loo and co-workers (Hu et al. 1994), who also advocate examining CaM in negative ionization mode. Calmodulin is an acidic protein and over the pH range of 6–8 will be predominantly deprotonated. This is the state which then configures to calcium ions and/or to targets and so protein present in solution is not being detected. One explanation for this is that we are presenting data here in negative ionization mode. We have avoided looking at the CaM.nCa$^{2+}$ system in positive ionization mode, because in addition to CaM.nCa$^{2+}$ species we also detect significant adduct sodium and potassium ions adducting to the calmodulin, presumably leached from the nanospray needles. This point has been made by Loo and co-workers (Hu et al. 1994), who also advocate examining CaM in negative ionization mode. Calmodulin is an acidic protein and over the pH range of 6–8 will be predominantly deprotonated. This is the state which then configures to calcium ions and/or to targets and so protein present in solution is not being detected. One explanation for this is that we are presenting data here in negative ionization mode. We have avoided looking at the CaM.nCa$^{2+}$ system in positive ionization mode, because in addition to CaM.nCa$^{2+}$ species we also detect significant adduct sodium and potassium ions adducting to the calmodulin, presumably leached from the nanospray needles. This point has been made by Loo and co-workers (Hu et al. 1994), who also advocate examining CaM in negative ionization mode. Calmodulin is an acidic protein and over the pH range of 6–8 will be predominantly deprotonated. This is the state which then configures to calcium ions and/or to targets and so protein present in solution is not being detected. One explanation for this is that we are presenting data here in negative ionization mode. We have avoided looking at the CaM.nCa$^{2+}$ system in positive ionization mode, because in addition to CaM.nCa$^{2+}$ species we also detect significant adduct sodium and potassium ions adducting to the calmodulin, presumably leached from the nanospray needles. This point has been made by Loo and co-workers (Hu et al. 1994), who also advocate examining CaM in negative ionization mode. Calmodulin is an acidic protein and over the pH range of 6–8 will be predominantly deprotonated. This is the state which then configures to calcium ions and/or to targets and so protein present in solution is not being detected. One explanation for this is that we are presenting data here in negative ionization mode. We have avoided looking at the CaM.nCa$^{2+}$ system in positive ionization mode, because in addition to CaM.nCa$^{2+}$ species we also detect significant adduct sodium and potassium ions adducting to the calmodulin, presumably leached from the nanospray needles. This point has been made by Loo and co-workers (Hu et al. 1994), who also advocate examining CaM in negative ionization mode. Calmodulin is an acidic protein and over the pH range of 6–8 will be predominantly deprotonated. This is the state which then configures to calcium ions and/or to targets and so
Complex between calmodulin and nitric oxide

Figure 4. ESI-MS spectrum of 50 μM CaM and 50 μM of nNOS in 10 mM NH₄OAcM. In this spectrum, the open diamonds correspond to CaM with 0-4Ca²⁺, the open triangles to the FMN domain of the nNOS complex, the black triangles to the FMN domain of the nNOS–FMN complex, the black stars to the FMN domain of the nNOS–FMN–CaM.4Ca²⁺ complex and the black stars to the FMN domain of the nNOS–FMN–CaM.4Ca²⁺ complex. In the inset which shows the undeconvoluted spectrum, the peaks corresponding to the calmodulin charged ion series are shown by open circles. Other peaks in this inset correspond to charged ion series from the protein nNOS, both with and without the FMN co-factor and also from the ensuing complex calmodulin. The high intensity low mass peaks are due to buffer ions and other sample impurities.

The effect of calcium ions on the conformation(s) of calmodulin can also be seen in the ratio of calmodulin to calcium as a function of charge state. Figure 3 plots the different net charge states obtained for the different amounts of calcium incorporated into the protein at the ratio 1:2 CaM–Ca²⁺ which limits the uptake of calcium to 4 bound as discussed above. With no calcium ions the charge distribution is centred on —16, indicative of an open structure accessible to charging. However incorporating two calcium ions reduces this to —15, indicating the conservation into the gas-phase of a tighter structure than the apo form. This phenomenon continues up to 4 calcium ions where the distribution centres on z = —11, which coupled with the fact that the maximum number of specific calcium ions are bound suggests a much tighter structure. Narrowing charge states distributions as a function of Ca²⁺ addition is also indicative of a more compact and less dynamic structure for the fully calcium loaded form. Such observations are in line with that reported by others investigating CaM using ESI-MS (Lafitte et al. 1995; Veenstra et al. 1997; Nemirovskiy et al. 1999; Nousiainen et al. 2001).

The effect of Ca²⁺ binding at each of the 4 sites in CaM has been investigated by several researchers (Cox et al. 1988). It is evident that there are two pairs of sites, which demonstrate ‘paired co-operativity’. Those in the C-terminal domain have an affinity for calcium which is 10–15 times higher than that in the N-terminal domain, although absolute and even relative calcium affinities will vary according to the ionic strength of the liquid. The charge states series obtained for apo 1 and 2 calcium ions centres at z = —15 while those for 3 and 4 calcium ions at z = —11 to —12. It is tempting to speculate that this is symptomatic of calcium binding (or being retained) in the EF hands in the C-terminal domains in the higher charge states, and again this illustrates the structural information which can be gathered from analysis of ESI-MS data.

3.2. Complex with nNOS

Once configured with calcium, CaM is able to bind specifically to target proteins. Figure 4 shows the deconvoluted spectrum data of calmodulin with the nNOS protein, and the complex formed between them when sprayed at a ratio of 1:1. Raw data are shown in the inset in this figure. Calmodulin is here present in apo, and nCa²⁺ forms where n = 1, 2, 3 and 4 and nNOS is observed at masses of 28 228 Da and with its bound cofactor (FMN) at 28 682 Da. All five species of CaM are available, however not all 5 species form complexes with nNOS, and only a single peak is seen at 45 090 Da. This corresponds to the mass of the complex with CaM.4Ca²⁺. A peak is also seen for nNOS complexed to CaM.4Ca²⁺ with its bound cofactor (FMN) where the ratio's are the same as that obtained for the nNOS protein alone—the co-factor having no significant effect on the relative binding affinity. It is conceivable that the cofactor is lost from nNOS during the electrospray process, or that nNOS is present in solution with and without FMN, but nevertheless it does not appear critical to gas-phase complex stability.

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That the protein binds exclusively to the 4Ca$^{2+}$ form of calmodulin, suggests highly specific binding exists between CaM.4Ca$^{2+}$ and nNOS which has here been successfully retained in the gas-phase. The abundances of the isolated CaM isoforms are altered with respect to those shown in figure 1d (see lower right-hand side inset in figure 4). Remarkably there is very little free CaM.4Ca$^{2+}$, apparently it has been scavenged by the nNOS peptide, and correspondingly the abundance of CaM.2Ca$^{3+}$ is also low, due to the cooperativity of binding two calcium ions leading to fully calcified CaM.4Ca$^{2+}$. To our best knowledge this is the first CaM–enzyme complex that has been observed intact by ESI-MS.

### 3.3. Complex with nNOS peptide

The study of the complex was simplified by analysing only the CaM binding region of nNOS peptide. This allows for a more detailed analysis of the binding affinity in the gas-phase on our instrument. Figure 5 shows the raw data spectrum of 50 μM Calmodulin with 50 μM of nNOS peptide in 100 μM Ca(OAc)$_2$ and 1 mM NH$_4$OAc. Alongside the main series assigned to calmodulin, a second series, corresponding to the CaM–nNOS complex can clearly be seen.$^2$ As the concentration of the nNOS peptide is varied substoichiometrically, the amount of complex observed also decreases, at a ratio of 1:2 CaM–nNOS no increase in the intensity of complex peaks is apparent, suggesting a specific binding. This second series only corresponds to peptide addition to the CaM.4Ca$^{2+}$ form of the protein, as is apparent in the deconvoluted spectrum (inset in figure 5). Although all 5 species of calmodulin are present, a complex forms only with CaM.4Ca$^{2+}$. Furthermore, in the absence of calcium no complex was seen. The observed charge state distributions of the complex are different from that of the free CaM, as shown in figure 5, where the complex is observed in positive ionization mode, the peptide is only observed bound to CaM in charge states $z=+7$–10, whereas CaM is seen in a range of charge states from $z=+7$–18. In negative ionization mode (not shown) the complex is observable over the range $z=-6$–10, which as may be seen from figure 3 is significantly narrower for uncomplexed CaM.4Ca$^{2+}$.

These results may be contrasted with those obtained by Gross et al. who examined the CaM–melittin complex using ESI-MS (Nemirovskiy et al. 1997). For low Ca$^{2+}$ concentration they obtained melittin binding to CaM.2Ca$^{2+}$, which was never here observed for the CaM–nNOS system either with the protein or the linker peptide. However with higher Ca$^{2+}$ concentrations, they show a distinct preference for CaM.4Ca$^{2+}$, which indicates that melittin to CaM binding is less specific that that found here for the nNOS linker peptide, although their experimental source conditions are somewhat different. This is also confirmed by the narrower charge state distribution that we observe here for the CaM–nNOS peptide complex than that reported by Gross et al. for CaM–melittin. A recent study by Schulz et al. (2004) has also examined the CaM–melittin complex with mass spectrometry, but the resolution of the MALDI-ToF instrument they employed is not sufficient to elucidate stoichiometry of Ca$^{2+}$ binding. Their cross-linking studies provide evidence for two modes of peptide binding to CaM, described as one parallel and one antiparallel. These findings are interesting and the experimental methodology novel, their analysis relies on comparisons to NMR structure data for (Ikura et al., 1992), and the MS studies per se do not discern the number of calcium ions present within the complex.

Structures have been obtained for calmodulin complexed with a peptide which constitutes the CaM binding site in the enzyme myosin light chain kinase (MLCK). Both the smooth muscle (Meador et al., 1992; crystal) and the skeletal muscle variants (Ikura et al., 1992; NMR) have been reported and these studies present the complex only in the presence of four calcium ions. Derrick and co-workers have examined this system via ESI FT–1CR-MS (Hill et al., 2000; Nousiaen et al., 2001, 2003) and were able to form a complex between apo calmodulin and the MLCK peptide. They suggest that the CaM–MLCK complex forms first and that calcium ions bind subsequently, perhaps cooperatively, Derrick’s findings differ somewhat from the detailed biophysical work of Bayley et al. (1996) who have found the complex CaM.2Ca$^{2+}$, wherein the C-terminal EF hands have first been occupied is a necessary intermediate state prior to binding the target MLCK. Persechini et al. (1994), taking a similar approach to Bayley, investigate the binding of CaM domains to MLCK and nNOS. They find that the activity of MLCK enzymes is retained at 80% by binding C-terminal domains at the sites normally occupied by the C and N terminus, whereas nNOS activity under similar conditions is only 50% of normal levels. This signifies the relative importance of both CaM domains being available and in their calcium loaded form for stable nNOS complex formation.

Our findings illustrates the power of gas-phase techniques in interrogating the dynamics of complex formation, but also underlies the specificity of the CaM–nNOS complex, which was only observable in the presence of calcium and only formed a complex with CaM.4Ca$^{2+}$, both in its ‘abbreviated’ form as the linker peptide and with a substantial portion of the nNOS enzyme.

### 3.4. Collision induced dissociation of CaM–nNOS: positive ionization

Using the quadrupole as a mass selective analyser, single charge states of the complex were isolated for CID using argon in the collision cell; fragments were measured using the ToF analyser. The most dominant complex peaks here present at $z=+7$ and $+8$ were selected.

Figure 5a,b show isolation of these charge states and their dissociation arising as the collision voltage is increased. Voltages of 45 V were required to produce a

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$^2$Since all observed CaM complex ions are found with 4 calcium ions, we will simplify complex-CaM.4Ca$^{2+}$ to CaM–complex.
significant amount of cleavage of nNOS peptide from the complex, higher injection energy than this resulted in complete loss of transmission of the complex or its dissociated constituents. Figure 5a shows the +8 charge state of CaM-nNOS losing the +3 charge state nNOS peptide and (for higher injection energies) the +2 charge state nNOS peptide. Similarly the +7 charge state dissociates with loss of +2 charge state nNOS peptide. In both cases this suggests that the +5 charge state of the protein is retained. However, this is
Figure 7. Products of dissociation of the isolated charge states ions of the CaM–nNOS peptide complex. In each figure the top spectrum shows the isolation of the respective charge state and the bottom figure the related dissociation products at 45 collision volts with 10 psi argon in the collision cell. (a) +8 charge state, (b) +7 charge state, (c) top: -10 charge state and (d) -9 charge state.

3.5. Collision induced dissociation of CaM–nNOS: negative ionization

The experiment described above was also performed on deprotonated species and similarly the most intense peaks (here -10 and -9) were selected for isolation. The fact that the protein in each case is also carrying +8 charges from the four Ca$^{2+}$ ions suggests that the complex ions observed with $z = -9$ and -10, the complex possesses -17 and -18 deprotonated acidic residues, some of which may be present at the binding site. Figure 5c,d shows the isolation of -10 and -9 charges states, respectively, and the dissociation products observed as the collision voltage is increased. Compared to the positive ionization CID the complexes here appear much more stable and less resistant to dissociation, even at high injection energies which suggests a different structural form to the complex ion formed from positive ESI compared with negative ESI. The dissociation of the -10 complex shows -9 and -8 protein, implying a corresponding peptide product with $z = -1$ or -2. The same occurs with dissociation of the -9 complex which dissociates to -8 and -7 protein, again suggesting loss of the peptide with a -1 and -2 charge. In both cases the protein retains all of its bound calcium ions, which is consistent with the findings of Derrick and co-workers (Nousiainen et al. 2003) for low energy in source dissociation of the CaM–MLCK complex, although at higher collision energies they do observe breakup of the CaM-4Ca$^{2+}$ complex.

No signals are seen corresponding to negatively charged nNOS peptide product ions. The peptide is very basic with only one acidic side chain, and it is difficult to observe as a deprotonated ion. The effective decrease in the charge state of the complex to form CaM products must be due to proton transfer from the nNOS peptide product, potentially forming a neutralized carboxylic group on CaM. For nNOS peptide this leaves an unobservable neutral species implying that binding at the CaM–nNOS interface is via at least one salt bridge interaction in the gas-phase. This observation has been made by Getzoff and co-workers (Aoyagi et al. 2003) for the eNOS linker peptide, where strong interactions are present between the basic residues at the N terminus and several glutamic acid residues in CaM. In addition they describe a hydrophobic pocket created around methionine residues in CaM which is configured by specific residues in the (NOS) linker peptide binding loop. To examine the likely configuration of the nNOS peptide we have employed molecular mechanics based calculations.

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3.6. nNOS peptide conformation via molecular modeling and circular dichroism

The low energy structures of nNOS peptide obtained using the Amber force field are shown in figure 6a (gas-phase) and figure 6b (born solvent). These are both representative snapshots taken from 1 ns dynamics runs. nNOS peptide is signified by the peptide backbone, with residues 7, 10, 12, 18 (Phe, Ala, Val, Leu) which are those thought to be primarily involved in the interaction with CaM (Aoyagi et al. 2003) depicted by liquorice representations. The gas-phase low energy structures were all elongated with separated N and C termini, while the Born solvent model structures tended to more compact globular conformations. As shown in figure 6a, gas-phase structures exhibited some helicity, presenting the first three residues proposed (according to the generalized calcium dependent 1-5-8-14 motif; Rhoads & Friedberg 1997) to interact with the central linker α-helix of CaM along one face and the fourth residue on the opposite side. This binding motif is analogous to that found with MLCK (Ikura et al. 1992; Nousiainen et al. 2003), and is identifiable by (ideally) 12 amino acid residues flanked by two bulky hydrophobic residues. The nNOS peptide examined here also exhibits this classic motif, where the first hydrophobic residue is phenylalanine (1) and the last leucine (14). The additional hydrophobic amino acids found at positions five and eight further stabilize binding to CaM. If these are presented in a plane within the peptide/protein target they will form favourable hydrophobic interactions with CaM.

Figure 6c shows the eNOS peptide from the crystal structure of the CaM-eNOS complex as solved by Getzoff and co-workers (Aoyagi et al. 2003), again the residues mapped to interact with CaM are illustrated by liquorice representations. While our gas-phase molecular model of nNOS peptide does not overlay to the eNOS, it is apparent that the peptide in the FMN CaM binding domain that we synthesized, has the propensity to form a helix, which in turn offers the correct proposed residues for the hydrophobic interactions with CaM. We believe, while unstable in solution, this peptide will not be solvated by water and would essentially be in a hydrophobic environment with a significantly lower dielectric constant. To mimic this, the linker peptide was analysed in methanol where it did indeed form a helical structure (data not shown). The caveat to this finding is that many peptides of this length will form random coil structures in solution and could then form helical structures in less aqueous media, or in the gas-phase. Formation of this secondary structure by the nNOS peptide compliments the findings of Getzoff and co-workers (Aoyagi et al. 2003) for the eNOS linker peptide which is shown to be helical in complex formation with CaM (figure 6c). These physical investigations imply that this region of the FMN domain of nNOS contains a peptide loop with a helical propensity. While unstable in solution, this helix is stabilized on complex formation with CaM4- Ca2+ as shown from our mass spectrometry studies.

4. CONCLUSIONS

This work has shown that more information can be taken from mass spectrometry than simply molecular mass. The degree of charging seen on the protein and its complexes has provided insights into the structure which correlate well with information from other analytical techniques. Our use of CID and complementary techniques have further interrogated this system allowing us to outline a number of important observations about the CaM-nNOS complex, as follows.

(i) Solution conditions alter the range of conformations available to the protein; analysis of the charge state distributions indicates that mass spectrometry can be employed to examine cooperative binding of metal ions at different sites within the protein.

(ii) The specificity of the technique has been highlighted by the targeted binding of nNOS peptide to CaM4Ca2+.

(iii) We have confirmed the proposed CaM binding site in nNOS peptide as residues Lys730-Met750 via the specific binding of the synthesized nNOS peptide to CaM4Ca2+.

(iv) CID yields charged products of both the protein and peptide which indicate that gas-phase binding is partly mediated via electrostatic salt bridge interactions, although we expect hydrophobic interactions to be dominant.

(v) This peptide is found to be helical in hydrophobic conditions such as those presented on CaM binding. Molecular modelling of the peptide in the gas-phase produces a helix which corresponds to that found in the crystal structure of eNOS-CaM.

If the CaM recognition and binding by nNOS is similar to that postulated by Getzoff for eNOS, then the residues responsible for interaction will align along one side of the linker peptide. Our model of the nNOS peptide certainly displays this. Additionally our experimental work demonstrates that this peptide exhibits...
exclusive selectivity for the fully calcium loaded form of CaM which is the configured conformation required to accept the binding domain presented by nNOS. It appears likely that nNOS exhibits the classic 1–5–8–14 Ca$^{2+}$ dependent binding motif (Rhoads & Friedberg 1997) where here 1 corresponds to residue 7 (Phe) in the nNOS peptide. Our observation (figure 6a) that the fourth residue in this motif does not lie along the same plane as the first three is also consistent with the findings of Getzoff et al. who state that this last key hydrophobic residue lies outside of the interior created by holo-CaM as it complexes.

We present evidence from CID that electrostatic salt bridge interactions are present at the binding site and are subsequently ruptured as the complex breaks up. This is proved by the charge reduction that occurs when the CaM product is detected as a negative ion (figure 7) which must correspond to proton transfer from nNOS peptide to the protein. Gas-phase complex binding will favour electrostatic above hydrophobic interactions (Robinson et al. 1996), although the hydrophobic crevice formed by the rearrangement of the helices of CaM on binding to the nNOS target (Aoyagi et al. 2003; Schulz et al. 2004) and the 1–5–8–14 motif presented by nNOS mean that hydrophobic interactions must play a dominant role in peptide binding. Since it is apparent that the N-terminal section of the nNOS peptide interacts with glutamic acid groups in the protein (Aoyagi et al. 2003), we conclude that proton transfer is likely to occur here, especially since this is likely to be not too deeply embedded in the protein. It is possible that the interactions involved in the complex obtained under positive ionization conditions are somewhat different, there may be less deprotonated glutamic acid residues available for the electrostatic interactions, and hence charge is retained on basic residues on the peptide during dissociation. Future work could investigate this by mutating out selective residues on the nNOS peptide or by taking the approach of Gachhui et al. (1997) and Kondo et al. (1999), who employ mutated calmodulin chimeras to determine the effect of altering the complex interface on NOS activity. Our findings differ from those of Derrick and co-workers for the peptide complex formed between CaM and the target site in MLCK which also follows the 1–5–8–14 paradigm (Hill et al. 2000; Nousiainen et al. 2003), and also from those of Gross et al. with mellitin (Nemirovskiy et al. 1997). Both of these systems have been shown by mass spectrometry to form complexes which are not dependent on four calcium ions sequestering to CaM, whereas in our hands the nNOS system is only observable bound to the 4Ca$^{2+}$ loaded form of calmodulin.

The work presented here shows for the first time an isolated gas-phase complex between the FMN domain of nNOS and calmodulin. We have used a variety of techniques to probe the structure and strength of binding of this biologically active system, and we believe that studies of this nature will have increased importance since they utilise relatively low amounts of protein and have the potential to interrogate binding interactions in microscopic detail.

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Proceeding title: Conformational Control of NO Synthase Reductase Domain
Abstract: (For web database reference)

The constitutive isoforms of mammalian NO synthase (eNOS and nNOS) are homodimeric flavocytochromes regulated by Ca$^{2+}$ and calmodulin (CaM), which binds to a hinge peptide linking the oxygenase and reductase domains of the enzyme. CaM activates electron transfer both across the domain interface and within the reductase domain itself. The NO synthase reductase domain, which is related to cytochrome P450 reductase, binds FMN and FAD cofactors and couples NADPH dehydrogenation to electron transfer. Rapid-reaction stopped-flow studies show that both steps are activated by CaM binding. The mechanism of activation seems to involve the release of the enzyme from an unproductive "locked" conformation induced by substrate binding, which restricts the motion necessary for catalytic action. This effect is dependent on numerous protein structural features, the redox states of the flavin cofactors and the structure of the substrate. The enzyme's conformational balance is thus easily tipped towards activation by modifying any of these, or by CaM binding.
Conformational Control of NO Synthase Reductase Domain

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Introduction
The constitutive mammalian nitric oxide synthases (e.g. neuronal NOS) are calcium/calmodulin (CaM)-dependent flavocytochromes, which bind one molecule each of heme, FMN, FAD and tetrahydrobiopterin per subunit. They catalyse the synthesis of NO and citrulline from L-arginine via two consecutive monooxygenation reactions [1]. Each NOS subunit consists of a heme/H4B-binding oxygenase domain and an FAD/FMN-binding reductase domain. The reductase domain is closely related to mammalian cytochrome P450 reductase and supplies the oxygenase domain of the other subunit with electron equivalents, generated by NADPH dehydrogenation at the FAD site. The neuronal (nNOS) and endothelial (eNOS) isoforms of NOS generate NO to transmit signals between cells via the guanylate cyclase-cGMP pathways involved in

Fig. 1. Crystal structure of the nNOSrd [5] with NADP (black sticks) bound. Left: The grey shaded background shows the FAD binding sub-domain with the c-terminal extension (black helix). The dots represent the space occupied by the FMN domain. Right: the NADP binding site showing the disposition of ADP vs. FAD and FMN cofactors and its interaction with Ser1196 and the amino-adenosine group of the FAD.
neurotransmission and the regulation of blood flow/pressure. Regulation of both enzymes is achieved by the reversible binding of calmodulin (CaM) to the inter-domain hinge region of nNOS and eNOS through changes in the intracellular Ca2+ concentration. CaM-binding activates FMN to heme electron transfer resulting in the turnover of arginine and oxygen at the heme site [2]. Interestingly, the isolated reductase domain of nNOS (nNOSrd) is also activated by CaM towards reduction of artificial electron acceptors such as cytochrome c [3], which is exclusively reduced by the FMN domain. Both activation mechanisms are thought to result from large-scale conformational changes on CaM binding, which has no effect on the redox properties of the enzyme’s flavin cofactors [4].

The structure of nNOSrd (without bound CaM) was recently determined [5]. A representation of this is shown in Fig 1. The structure shows the FAD and FMN bound in close proximity in their distinct domains. The FMN appears to be shielded from interactions with other proteins by the FAD-domain. A molecule of NADPH (the substrate) is bound to the FAD-domain, but is in a conformation unsuitable for transferring a hydride to the FAD without a large movement of its nicotinamide substituent. This motion is blocked by the FAD-stacking residue Phe1395. The structure also shows the positions and partial structures of two autoinhibitory inserts found within the amino-acid sequences of the constitutive NOSs. One is a loop of around 40 amino acids found in the FMN-domain and the other is an extension to the C-terminus of around 30 amino acids, continuing on from Phe1395 (shown as a black helix). Both inserts have been shown to repress the activity of NOS in the absence of CaM [6]. Modification of either can result in tighter CaM binding, activation of NO synthesis in the absence of CaM and an increase in cytochrome c reductase activity in the absence of CaM. Electron transfer activity from nNOSrd to external electron acceptors (e.g. cytochrome c) appears to model the FMN to heme electron transfer process required in the holoenzyme. Both require a movement of the FMN-domain from its position shown in Fig. 1, so as to increase the FMN accessibility for external electron transfer. A convenient way of monitoring the conformation of nNOSrd is via pre-steady state reduction of cytochrome c by an excess of reduced enzyme. The more accessible is the FMN, the faster the rate of cytochrome c reduction [7]. Surprisingly, the accessibility of the FMN in nNOSrd appears to be decreased considerably on substrate (NADPH) binding. As such, bound substrate seems to inhibit electron transfer from the enzyme by “locking” the FMN-domain into an inaccessible conformation (i.e. Fig. 1). This is released by CaM binding, which presumably induces conformational flexibility.

The inhibition of electron transfer from nNOSrd by substrate is examined here for specificity, using commercially available substrate analogues. The CaM-dependency of nNOSrd flavin reduction by NADPH is also discussed.

**Experimental Procedures**

**Materials and protein purification.**

The nNOSrd was expressed in *E. coli* cells and purified by ADP agarose and CaM sepharose affinity chromatography in the one-electron reduced form as previously reported [7]. Substrate (NADPH) and substrate analogues were all purchased from Sigma.
Flavin reduction
Reduction of the flavins of nNOSrd by NADPH was achieved by stopped-flow (Applied Photophysics) mixing of 7 μM fully oxidised or one-electron reduced enzyme in presence or absence of CaM/Ca$^{2+}$ with 100 μM NADPH. The change in absorbance at 457 nm was followed and the resultant traces fitted to a single exponential function. Complete spectra were also recorded at 2 ms intervals using a diode array detector. One-electron reduced enzyme was prepared in an anaerobic chamber under an N2 atmosphere and titrated against dithionite until the appropriate visible absorption spectrum was obtained. Oxidised enzyme was prepared by treatment with ferricyanide followed by size-exclusion chromatography to remove excess oxidant.

Inhibition of cytochrome c reduction
Stopped-flow cytochrome c reduction experiments were carried out as previously reported [7]. 2 μM cytochrome c was mixed with 10 μM nNOSrd (dithionite-reduced) with and without bound CaM and with and without a 10-fold excess of inhibitors (β-NADPH, 2', 5'-ADP, ATP-rib, α-NADPH, β-NAADP+ incubated with the enzyme 10 min prior to use). Cytochrome c reduction was monitored at 550 nm, and pseudo-first order rate constants were calculated by fitting the resultant traces to single exponential functions. Second order rate constants were determined by dividing the pseudo-first order constants by the enzyme concentration.

Results and Discussion

Kinetics nNOSrd reduction by NADPH
On mixing with an excess of NADPH, the FAD of nNOSrd is reduced to the hydroquinone form by hydride transfer. Subsequent events are dependent on the redox state of the FMN. If the FMN is oxidized then both electrons are transferred to it from the FAD, allowing the FAD to be reduced by a second equivalent of NADPH. This process is shown in Fig. 2A. Interestingly, little semiquinone accumulates during the two-step reaction. From Fig. 2C it is apparent that the first hydride transfer step is faster than the second and that CaM accelerates the second step in particular.

The one-electron reduced enzyme (containing stable FMN semiquinone) reacts by hydride transfer to the FAD, which may then transfer a single electron to the FMN. The electron transfer step results in no net change in the concentration of semiquinone, so this process results in little change to the visible spectrum. Consequently Fig 2B mainly shows a decrease in absorbance at 457 nm resulting from the hydride transfer reaction. The semiquinone band at 600 nm is largely unchanged. For the CaM-free enzyme the hydride transfer process is slow (approx. 9s$^{-1}$), although the trace in Fig. 2D does show a short fast phase probably resulting from contamination with oxidized or damaged enzyme. The CaM-free enzyme remains in the three-electron reduced state after reaction with NADPH (Fig. 2B), whereas the CaM-bound enzyme undergoes further reduction, resulting in the loss of the semiquinone spectrum (Fig. 2B inset). This process can only be initiated by disproportionation of flavin radicals on different enzyme molecules, and is dependent on intermolecular electron transfer. The inability of the CaM-free enzyme to undergo disproportionation is likely a result of the three-electron reduced enzyme forming a “locked” complex in which the FMN is inaccessible to other molecules.
This supports the observation that cytochrome c reacts slowly with the NADPH-bound CaM-free enzyme due to conformational locking.

It is intriguing that the first hydride transfer to the oxidized enzyme is considerably faster than the first hydride transfer to the one-electron reduced enzyme. Both follow a similar mechanism and occur in structurally identical FAD domains. The only difference is the redox state of the FMN. In forming the stable blue semiquinone, the newly protonated FMN N5 must form a hydrogen-bond with the peptide carbonyl of Gly810 to stabilize itself. This may result in a difference in the structure of the domain-domain interface. If domain motion is important to the catalytic functioning of nNOSrd, this small perturbation could be significant. This implies that the FMN redox state directly affects the rate of hydride transfer to the FAD in the neighbouring domain.

Consistent with this, the second NADPH to FAD hydride transfer to fully oxidised nNOSrd occurs at a similar rate to the primary reaction with the one-electron reduced enzyme. The second reaction must occur after FMN reduction and protonation of the isoalloxazine ring at the N5 position.
Inhibition of cytochrome c reduction by pre-reduced nNOSrd

Previous experiments have shown that β-NADPH, the physiological substrate of the enzyme, paradoxically represses the electron transfer through the enzyme by locking the conformation of the reductase domain enabling its access to the heme where the Nitric oxide formation take place [7]. The external electron acceptor cytochrome c can be used to mimic the oxygenase domain of the enzyme in order to study the kinetic behaviour of the nNOSrd on its own, since both accept electron equivalents exclusively from the FMN cofactor. In order to understand how β-NADPH acts to inhibit electron transfer out of the reductase domain, we investigated the following substrate analogues: ADP, ATP-rib, α-NADPH, β-NAADP+ by comparing the kinetic behaviour they induce with that of the uncomplexed and the β-NADPH bound nNOSrd (Fig. 3). All the inhibitors studied contain the 2'-adenosine phosphate extremity as it has been shown to be essential for the tight binding of the physiological substrate.

The control reactions using NADPH with and without CaM and the uncomplexed enzyme without CaM have shown that the nNOSrd contains all the regulatory elements necessary to cause a 30-fold increase in the first order rate constant for cytochrome c reduction when CaM binds to the NADPH-bound enzyme.

The stopped flow experiments show that ADP, ATP-rib and α-NADPH all induce a 50% drop in the cytochrome c reduction first order rate constant compared to uncomplexed enzyme despite the fact that the 5'-phosphoribose is missing from ADP.

The observed inhibition relative to β-NADPH indicates that α-NADPH binds similarly to ADP and that the alpha orientation of the nicotinamide disrupts further inhibition. Surprisingly, β-NAADP+ fails to inhibit cytochrome c reduction, despite the close structural homology between it and β-NADPH. It appears that modification of the nicotinamide amide group to a carboxylic acid is enough to completely disrupt the effect.
Fig. 3. Effect of the different inhibitors $\beta$-NADPH, ADP, ATP-rib, $\alpha$-NADPH and $\beta$-NAADP$^+$ on the rate of cytochrome c reduction by nNOSrd compared to the uncomplexed and the CaM-bound form of the enzyme.

**Table 1.** Second order rate constants ($k_{2nd}$) for reduction of cytochrome c by excess nNOSrd in the presence or absence of bound CaM (+/− CaM)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>none</th>
<th>$\beta$-NADPH</th>
<th>ADP</th>
<th>ATP-rib</th>
<th>$\alpha$-NADPH</th>
<th>$\beta$-NAADP$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOSrd (10 $\mu$M) mixed with cytochrome c (6 $\mu$M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaM (30 $\mu$M)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$k_{2nd}$ (s$^{-1}$)</td>
<td>6.2</td>
<td>27.3</td>
<td>1.1</td>
<td>30</td>
<td>3.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Although the ADP substituent of NADPH has the greatest affinity for nNOSrd, by virtue of electrostatic interactions between its phosphate groups and basic amino-acid residues, it cannot induce full conformational locking. The weakly bound nicotinamide substituent clearly plays an important role. Furthermore, inhibition appears to be specific for this group, a phenomenon that must result from the interaction between it and the protein in the conformationally locked state. The structure of nNOSrd shows NADP bound with the nicotinamide group interacting with the adenosine group of FAD, the guanidinium group of Arg1010 and the protein backbone from amino acids 1194-1196 [5]. Currently it is difficult to say if any of these interactions are significant.
Conclusions

It appears that the inhibition of nNOSrd cytochrome c reduction by NADPH stems from the formation of an inactive "locked" form of the enzyme which is specific for the substrate. This specificity is dependent on the chemical properties of both the tightly-bound ADP substituent and labile nicotinamide substituent. This suggests that NADPH is bound in a site in which both ends of the molecule are recognized. When in place, the substrate binding energy is enough to restrict the normal motion of the FMN domain, favouring the "locked" conformation. Recent results implicate motion of the FAD stacking residue (Phe1395) and the attached C-terminal extension with domain motion [8] this suggests that there is also a connection between NADPH to FAD hydride transfer and domain motion.

The rate of reduction of nNOSrd by NADPH is dependent initially on the hydride transfer step. The probability of hydride transfer is likely to depend on the stacking probability of the nicotinamide with the isoalloxazine ring system of the FAD. This is dependent on the lability of the Phe1395 residue and the C-terminal extension. Thus, there is a direct link between the conformation of the enzyme and the hydride transfer step. This is the likely origin of the CaM-dependency of the reduction reaction. It is clear from the reduction traces that for the CaM-free enzyme, the first hydride transfer step is faster when the starting enzyme is fully oxidised than when the FMN is one-electron reduced (stable blue sq). However, the high sensitivity of the "locked" enzyme form to structural perturbations (e.g. mutations, changes of substrate etc.) suggests that loss of a single hydrogen-bond (from the FMN to peptide backbone) on oxidation of the FMN could be enough to upset the interaction between the FMN and FAD domains – causing an increase in the rate of hydride transfer.

There is a compelling parallel between the acceleration of hydride and cytochrome c reduction on CaM binding to nNOSrd suggesting that conformational changes drive successive catalytic steps.

References