FLAVOCYTOCHROME $b_2$: ROLE OF THE INTERDOMAIN HINGE REGION.

R. Eryl Sharp

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UNIVERSITY OF EDINBURGH
OCTOBER 1994
To My Mum and Dad
For all the support and encouragement they have given me over the years
Declaration

The work presented in this thesis is the original work of the author, except where specific reference is made to other sources. It has not been submitted in part, or in whole, for any other degree. Some of the results presented have already been published.

R. Eryl Sharp
Acknowledgements

I would like to thank my first and second supervisors, Drs. Graeme Reid and Stephen Chapman for offering first rate supervision, in every respect. I really could not have asked for a better combination of personalities and expertise.

I must also extend my thanks to all the members of Graeme's and Steve's research groups, both past and present for being a great team to work and socialise with. Special thanks go to Dr. Patricia White and Dr. Forbes Manson, for showing me the 'ropes' and to Duncan Short, Dr. Patricia White, Dr. Sara Pealing and Euan Gordon for proof reading my thesis.

I would also like to acknowledge Dr. Graham Pettigrew for his help in determining redox potentials and Dr. John Parkinson for assisting me with nmr spectroscopy.
Abstract

The two distinct domains of flavocytochrome \( b_2 \) (L-lactate:cytochrome \( c \) oxidoreductase) are connected by a typical hinge peptide. Kinetic experiments have illustrated the importance of maintaining the structural integrity of the hinge for efficient interdomain electron transfer. To probe the role of the hinge in a more subtle manner, three mutant enzymes have been constructed; \( \Delta A3 \), \( \Delta A6 \) and \( \Delta A9 \) which have three, six and nine amino acids deleted from the hinge region respectively. Intra- (interdomain) and inter-protein (between flavocytochrome \( b_2 \) and cytochrome \( c \)) electron transfer was investigated by steady-state and stopped-flow kinetic analysis. All three hinge-deletion enzymes remained good L-lactate dehydrogenases as was evident from steady-state experiments with ferricyanide as electron acceptor (\( k_{cat} = 256 \text{ s}^{-1}, 276 \text{ s}^{-1} \) and \( 400 \text{ s}^{-1} \) for \( \Delta A3 \), \( \Delta A6 \), and \( \Delta A9 \), respectively, compared to \( 400 \text{ s}^{-1} \) for the wild-type enzyme) and from stopped-flow experiments monitoring flavin reduction (\( k_{cat} = 516 \text{ s}^{-1}, 520 \text{ s}^{-1} \) and \( 715 \text{ s}^{-1} \) for \( \Delta A3 \), \( \Delta A6 \) and \( \Delta A9 \), respectively, compared to \( 600 \text{ s}^{-1} \) for the wild-type enzyme).

The global effect of these deletions is to lower the enzymes' effectiveness as cytochrome \( c \) reductases. This property of \( \Delta A6 \) and \( \Delta A9 \) flavocytochromes \( b_2 \) is manifested at the interdomain electron-transfer step, where the rate of haem reduction is the same within experimental error as the steady-state rate of cytochrome \( c \) reduction: interdomain electron transfer has become rate limiting in the case of these two hinge-deletion enzymes, compared to the wild-type enzyme, where \( \alpha \)-H-abstraction from C-2 of L-lactate is rate-limiting. The situation for \( \Delta A3 \) is more complicated; the rate of haem reduction has fallen 35-fold compared with the wild-type enzyme (from \( 1500 \text{ s}^{-1} \) to \( 91 \text{ s}^{-1} \)) and, secondly, the steady-state rate of cytochrome \( c \) reduction has fallen 5-fold (from \( 207 \text{ s}^{-1} \) to \( 39 \text{ s}^{-1} \)). This implies that, for \( \Delta A3 \), interdomain electron transfer from fully reduced flavin to haem cannot be rate-limiting, as is the case for \( \Delta A6 \) and \( \Delta A9 \), but some other step, such as flavin semiquinone to haem electron transfer must be involved. These data, along with the measured kinetic isotope effects imply that complete structural integrity within the hinge region is essential for efficient interdomain communication.

Molecular modelling of the flavocytochrome \( b_2 \):cytochrome \( c \) complex indicates that the hinge region may form part of a binding site for cytochrome \( c \). To test this proposal, stopped-flow kinetics was used to obtain second order rate constants for the reduction of cytochrome \( c \) by pre-reduced hinge-deletion and wild-type
flavocytochromes $b_2$. The values for the wild-type enzyme were $4.7 \times 10^7$ and $1.4 \times 10^8$ M$^{-1}$s$^{-1}$, using horse heart and yeast cytochrome $c$ respectively. All the hinge-deletion mutants had second order rate constants which were lower than, but within an order of magnitude of the wild-type value for horse heart cytochrome $c$, with HΔ3 having the lowest value of $4.2 \times 10^6$ M$^{-1}$s$^{-1}$. For yeast cytochrome $c$, the values for all the hinge-deletion enzymes were the same within experimental error, with a value of $9 \times 10^7$ M$^{-1}$s$^{-1}$, only 1.5-fold less than that for wild-type. This implies that the hinge region of flavocytochrome $b_2$, contrary to the molecular modelling proposals, is not strongly implicated in complex formation with cytochrome $c$. This finding is reinforced by nmr experiments designed to investigate the affinity of cytochrome $c$ binding to wild-type and hinge-deletion flavocytochromes $b_2$. 

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Abreviations and Units

1. Amino acids have been denoted by both their single and three letter codes:

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Three letter abbreviation</th>
<th>One letter symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
</tr>
</tbody>
</table>

2. When referring to oligonucleotides, the following abbreviations are used:

A, Adenine; T, Thymidine; C, Cytosine and G, Guanine.
3. The following nomenclature has been adopted in referring to flavocytochrome b₂:

The hinge-deletion flavocytochromes b₂ are described by the abbreviation HA X, where; H, denotes the interdomain hinge region; Δ, denotes a deletion and X, denotes the number of amino acid residues deleted.

Point mutations are referred to as follows, considering a typical example, Y143F. This denotes an enzyme where tyrosine at position 143 in the amino acid sequence has been replaced by a phenylalanine residue, using site-directed mutagenesis.

4. Kinetic Parameters:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kₘ</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>kₖₐₜ</td>
<td>enzyme turnover number</td>
</tr>
<tr>
<td>Vₘₐₓ</td>
<td>limiting value for reaction rate</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>Kₖ</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>Kₛ</td>
<td>dissociation constant of the enzyme-substate complex</td>
</tr>
<tr>
<td>Sₜₜₒₜ</td>
<td>Substrate concentration at which enzyme turnover is maximal</td>
</tr>
</tbody>
</table>

5. Other Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-cyclohexyl-1-propanesulphonic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanidino</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine</td>
</tr>
<tr>
<td>ddATP</td>
<td>dideoxyadenosine</td>
</tr>
<tr>
<td>ddCTP</td>
<td>dideoxycytosine</td>
</tr>
<tr>
<td>ddGTP</td>
<td>dideoxyguanidino</td>
</tr>
<tr>
<td>ddTTP</td>
<td>dideoxythymidine</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DNA</td>
<td>dideoxynucleic acid</td>
</tr>
<tr>
<td>DNAse</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>EU</td>
<td>Enzyme unit</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>H. anomala</td>
<td>Hansenula anomala</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>I</td>
<td>ionic strength</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KIE</td>
<td>kinetic isotope effect</td>
</tr>
<tr>
<td>lactate</td>
<td>L-[2-H]-lactate (unless otherwise stated)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>Mr</td>
<td>relative molecular weight</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PMS</td>
<td>phenylmethylsulphate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAse</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>Tris</td>
<td>hydroxymethyl aminoethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>Vis</td>
<td>visible</td>
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6. Abbreviation of units (standard form):

<table>
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<th>Symbol</th>
<th>Unit Description</th>
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<tr>
<td>Da</td>
<td>Dalton units</td>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
<td>°C</td>
<td>degrees Centigrade</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
<td>K</td>
<td>Kelvin</td>
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7. Other unit abbreviations:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit Description</th>
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<tbody>
<tr>
<td>s⁻¹</td>
<td>first order rate constant</td>
</tr>
<tr>
<td>M⁻¹s⁻¹</td>
<td>second order rate constant</td>
</tr>
<tr>
<td>M⁻¹cm⁻¹</td>
<td>molar extinction coefficient</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
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<td>Page number</td>
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2.2.2 10 x TBE
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2.2.4 SDM buffer A
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2.2.6 0.1 M protein purification buffer
2.2.7 10 mM Tris/HCl buffer, pH 7.0 (0.10 M)
2.2.8 10 mM Phosphate buffer
2.2.9 20 mM Phosphate buffer, pD 7.0 in D$_2$O
2.2.10 10 mM CAPS buffer, pH 7.0 (0.10 M)
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2.2.13 4 x stacking buffer for SDS-PAGE
2.2.14 2 x SDS-PAGE loading buffer
2.2.15 10 % resolving gel for SDS-PAGE
2.2.16 5 % stacking gel for SDS-PAGE
2.2.17 5 x Tris-glycine electrophoresis buffer
2.2.18 10 x transfer buffer
2.2.19 Tris buffered saline

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Chapter 1

Introduction
1.1. GENERAL INTRODUCTION

Electron-transfer reactions are ubiquitous throughout biology and are indispensible for the homeostasis of all living organisms. In eukaryotic cells, the generation of most of the cellular demand for ATP occurs by the transfer of electrons from NADH through to oxygen, down a potential gradient of >1 Volt, via the respiratory complexes in the mitochondrial inner membrane. These complexes can be crudely thought of as being electrical wires (Slater, 1974). A similar situation occurs in the cell membrane of bacteria, where electrons can be transferred from a variety of donors down a potential gradient, via protein complexes, to a variety of electron acceptors. The nature of the donors and acceptors depends upon the type of bacterium and the physiological environment (Pettigrew & Moore, 1986). Additionally, in plant cells, ATP production can be driven by photoinitiated electron transfer from water through to NADP+, up a potential gradient, via the photosynthetic reaction complexes in the thylakoid membranes of chloroplasts (Barber & Anderson, 1994).

As well as being the underlying phenomenon responsible for energy generation in cells, it is becoming evident that electron-transfer reactions are involved in many other key cellular processes, examples of which are:

- Substrate activation in cytochromes P450, enzymes which catalyse the oxidation of organic molecules (Oraz de Montellano, 1986).
- Removal of the harmful by products of partial oxidation reactions, such as catalysis of hydrogen peroxide conversion to water by cytochrome c peroxidase (Everse et al., 1991) and conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase (Bannister et al., 1987).
- Electron-transfer reactions are now known to be involved in DNA metabolism, as exemplified by DNA photolyase (Sancar & Sancar, 1987).

All biological electron-transfer reactions occur between redox centres located in proteins. The redox centres can be organic molecules, such as flavin mononucleotide and nicotinamide adenine dinucleotide; metal ions coordinated to protein side chains, such as iron-sulphur clusters and copper, or coordinated to organic molecules, such as protoporphyrin. Redox proteins can either be membrane spanning, as is the case for the photosynthetic and respiratory complexes, which consist of a large number of polypeptides binding a whole host of different redox groups. Or, they can be soluble, as is the case for the more simple redox proteins such as the cytochromes c and blue copper proteins.
1.2. FACTORS GOVERNING PROTEIN MEDIATED ELECTRON TRANSFER

Biological electron-transfer reactions can occur over quite large distances, 10 to 30 Å and there are a number of factors which influence the probability and the rate of the electron-transfer event. This section will not attempt to give a detailed account of the theoretical basis for these factors, which have been extensively reviewed elsewhere (Marcus & Suttin, 1985), but a brief overview of the salient features will be described, along with their relevance to biological systems.

A simple redox system can be considered, where an electron is transferred from a reduced, $R'$ (electron rich) species to an oxidised, $O$ (electron deficient) species:

$$\text{R'} - e^- \rightarrow O'$$
$$O + e^- \rightarrow R$$

which combined, gives

$$\text{R'} + O \rightarrow R + O'$$

Typically, species $R'$ and $O$ are closely associated in a productive complex for electron transfer. Two qualitative assumptions can be made for the above system which are generally applicable for all biological electron-transfer reactions: the redistribution of electrons occurs in an isoenergetic system, i.e. the combined energy of the reactants and products must remain constant during the electron-transfer event; and that the Frank-Condon principle is obeyed (Kauzmann, 1957). The latter states that electron transfer occurs without any change in the nuclear configuration of the redox species. This implies that the rate of the transfer event must be rapid compared to the time scale of the changes in the nuclear configurations of the reactant and product species (in biological systems, the nuclear configuration depends upon a number of factors, such as bond lengths, solvent accessibility, etc), this is clearly an approximation, but it satisfactorily describes biological systems.

The above principles have been developed by Marcus into a powerful quantitative classical theory (Marcus, 1956, 1964, 1965 and 1968), whose applicability to electron-transfer reactions in biological systems has been demonstrated by many experimental investigations (McLendon & Hake, 1989; Farid et al., 1992). The main features of this theory are illustrated in Figure 1.2.1 for electron transfer between two different redox partners (where the free energy of the product species is lower than that of the reactants), the $x$- and $y$-axes represent the nuclear configuration of the reactant and product species, and the free energy of the system, respectively. The shape of the function for each species is typically
Figure 1.2.1. A Plot of Free Energy Against Nuclear Configuration For Electron Transfer Between Two Different Redox Centres

The diagram represents electron transfer between two different redox centres, R' and O. The left hand bowl is for the reactants (O + R') and the right hand bowl is for the products (R + O'). Electron transfer occurs at the intermediate configuration shown, via the route indicated by the bold arrows. The free energy vectors illustrate the sign and magnitude of: $\Delta G$, the free energy of the reaction; $\Delta G^*$, the activation energy and $\lambda$, the reorganisation energy (adapted from Chapman & Mount, 1995).
parabolic, as the free energy of either species will rise if perturbed by an external factor (such as collision with solvent molecules). The intersection of the two curves is the intermediate configuration that the reactant and product species must attain for electron transfer to occur. From Figure 1.4.1, $\Delta G^*$ is the activation energy of the reaction, $\Delta G$ is the difference in free energy between the two species (the driving force of the reaction) and $\lambda$ is the reorganisation energy (the total energy required for a Frank-Condon transition between the reactant and product species). The relationship between these parameters is given below:

$$\Delta G^* = \left[ (\Delta G + \lambda)^2 \right] / (4/\lambda)$$  \hspace{1cm} (1)

The electron-transfer rate depends upon the activation barrier and is given by the expression:

$$k' = k_0' \exp(-\Delta G^*/kT)$$  \hspace{1cm} (2)

Where, $T$ is the absolute temperature, $k$ is Boltzmann's constant, $k'$ is the observed electron-transfer rate and $k_0'$ is the electron-transfer rate when $\Delta G^*$ is zero. From equations (1) and (2), the variation in $k'$ with $\Delta G$ can be calculated. As $\lambda$ is always positive, the parameter $(\Delta G + \lambda)$ is the factor which governs the rate of reaction. Figure 1.2.2 shows three situations which can arise, depending upon the absolute value of $\Delta G$ compared to $\lambda$: When $\Delta G$ and $\Delta G^*$ are positive, a decrease in $\Delta G$ (an increase in the thermodynamic driving force) leads to a smaller, less positive $\Delta G^*$ and an increase in the electron-transfer rate, this is termed the normal region (Figure 1.2.2.A). At the point where $\Delta G = -\lambda$, $\Delta G^* = 0$ and the reaction is described as being activationless (Figure 1.2.2.B). If $\Delta G$ is decreased further still, then $(\Delta G + \lambda)$ becomes negative and $\Delta G^*$ becomes positive. Thus, in this region, as the thermodynamic driving force is increased, the activation barrier also increases and $k'$ falls off. This paradoxical behaviour occurs in the inverted region (Figure 1.2.2.C).

All the parameters discussed so far are thermodynamically controlled. For a true understanding of protein mediated electron transfer, the nature of the transfer medium, i.e. the protein matrix, must also be considered. The magnitude of the activationless electron-transfer rate constant, $k_0'$, in equation 2, depends upon the extent of electronic-wave function overlap between the donor and acceptors orbitals. For biological electron transfer, where the redox centres can be up to 30 Å apart, the intervening medium plays an important role in mediating electron tunnelling from the donor to acceptor redox centres. Since electronic wave functions decrease
Figure 1.2.2. A Plot of the Variation in Electron-Transfer Rate With Free Energy

The x- and y-axis denote the reaction free energy difference between reactants and products ($\Delta G$) and the logarithm of the electron-transfer rate constant ($k'$), respectively. The curve labelled (1), represents the relationship between these two parameters. Also shown are the three reactant and product free energy differences which give rise to the behaviour illustrated in curve 1: (A), the normal ($\Delta G + \lambda > 0$); (B), the activationless ($\Delta G + \lambda = 0$); and (C), the inverted ($\Delta G + \lambda < 0$), regions. The bold arrows denote the reaction pathway. The axes for plots (A) to (C) are as for figure 1.2.1 (adapted from Chapman & Mount, 1994).
exponentially with distance, it follows that the electron-transfer rate decreases in a similar manner between redox centres and \( k_0' \) is proportional to the electronic coupling strength between the two orbitals, which is defined by the tunnelling matrix element \( H_{AB} \):

\[
H_{AB} = H_{AB}^0 \exp[\beta/2(d-d^0)]
\]  (3)

Where, \( H_{AB}^0 \) is the electronic coupling at Van-der-Waals contact \( (d^0) \) and \( \beta \) is the rate of decay \( (\text{Å}^{-1}) \) of the coupling over a distance \( d \). In a vacuum, \( \beta = 2.8 \text{ Å}^{-1} \) and electron transfer only occurs over short distances, since at large distances, the electronic overlap is poor. From an extensive investigation of a number of intraprotein electron-transfer reactions primarily in the bacterial photosynthetic reaction centre, a value of \( \beta = 1.4 \text{ Å}^{-1} \) has been estimated for electron transfer through a protein matrix (Moser et al., 1992; Moser & Dutton, 1992). However, having a single value for \( \beta \) in proteins assumes that the protein matrix is homogeneous, which may be reasonable for systems that are optimised for efficient electron transfer. In such a system, electron transfer is proposed to occur by the shortest through space route between the donor and acceptor redox centres (Moser et al., 1992). However, many investigators believe that in some systems, the protein matrix is best described as being heterogeneous in nature and an alternative model based on a tunnelling pathway, has been devised for describing efficient long range electron transfer (Beratan & Onuchic, 1992; Beratan et al., 1990; Beratan et al., 1991). In this case a weighted pathway through the protein matrix is described, where the electron is transfered via a specific series of covalent bonds, hydrogen bonds and small through space jumps. From this, an effective pathway distance can be calculated, over which the electron has to traverse.

The next section deals with specific examples of biological electron-transfer reactions where the applicability of describing the electron-transfer event as occurring either through space or by tunneling pathways will be discussed.

### 1.3. SPECIFIC EXAMPLES OF PROTEIN MEDIATED ELECTRON TRANSFER

#### 1.3.1. Introduction

Over the last three decades, studies of biological electron transfer have concentrated upon correlating experimental findings with electron-transfer theory.
Both intraprotein (redox centres in the same protein) and interprotein (redox centres in different proteins) electron-transfer reactions have been extensively studied. For the latter systems, reactions between both physiological and non-physiological partners have been investigated to elucidate the role of molecular recognition in the specificity and control of electron-transfer events.

Recently, there have been significant advances in our understanding of protein mediated electron transfer. This has been due to a wide range of structural, spectroscopic, kinetic and site-directed mutagenesis (SDM) techniques and an ever increasing sophistication of analysis (Wuttke & Gray, 1993). The deduction of the crystal structure of the bacterial photosynthetic reaction centre has revolutionised our understanding of intraprotein electron-transfer reactions and the role of the protein environment in this process (Deisenhofer et al., 1984; Moser et al., 1992). In the last two years, crystal structures of physiological interprotein binary and ternary electron-transfer complexes have been reported; the cytochrome c:cytochrome c peroxidase (Pelletier & Kraut, 1992) and the methylamine dehydrogenase:amicyanin:cytochrome c₅₅₁I₂ (Chensef et al., 1994), complexes. This information should pave the way for increasing our knowledge of this rather intractable problem.

The next few sections will present a brief overview of our current understanding of both intra- and inter-protein electron transfer. In the latter case, however, as such a large number of systems have been investigated, I shall largely concentrate on the best studied systems to date, which is cytochrome c and its interaction with both a physiological (yeast cytochrome c peroxidase) and a non-physiological (cytochrome b₅) partner.

1.3.2. Intraprotein electron transfer

1.3.2.1. Bacterial Photosynthetic Reaction Centre: Dutton and co-workers have investigated the distance and thermodynamic driving force dependence of the rate of electron transfer in the bacterial photosynthetic reaction centre (Moser et al., 1992). The reaction centre has a number of redox groups situated within membrane spanning peptides (Michael & Diesenhofer, 1986; Figure 1.3.1). In this system, which is optimised for electron transfer, the rate of transfer falls off exponentially with increasing redox donor-acceptor distance. This implies that electron transfer can be thought of as occurring by a through-space process, with \( k_0' = 10^{13} \text{s}^{-1} \) at Van-der-Waals contact and \( \beta = 1.4 \text{ Å}^{-1} \) (section 1.2, equation 3). This is illustrated in Figure 1.3.2, which shows a plot of the logarithm of electron-transfer rate against distance, fitted to the above parameters of \( k_0' \) and \( \beta \). The plot shows an exponential decay dependence on the rate of electron transfer with through space donor-acceptor

...
Figure 1.3.1. Schematic Representation of the Bacterial Photosynthetic Reaction Centre

Key: P, bacteriochlorophyll dimer (BChl$_2$); B, bacteriochlorophyll monomer (BChl); H, bacteriopheophytin (Bph); QA and QB, quinones; Fe, iron. The approximate rate constants are given for the electron-transfer steps indicated by the arrows (Chapman & Mount, 1994).

Figure 1.3.2. Plot of the Logarithm of Electron-Transfer Rate Against Through Space Distance

This plot (from Van-der-Waals contact) shows—all the measured electron-transfer reactions in the bacterial photosynthetic reaction centre ($O =$ photosynthetic electron-transfer reactions). Also plotted are electron-transfer reactions for other protein systems ($A$). Numbers indicate electron-transfer reactions between designated prosthetic groups of the reaction centre; where, 1 = bacteriochlorophyll special pair, 2 = bacteriochlorophyll monomer, 3 = bacteriopheophytin, 4 = quinone A and 5 = quinone B. Values, $k_o' = 10^{13}$ s$^{-1}$ and $\beta = 1.4$ Å$^{-1}$ (adapted from Moser et al., 1992).
distance, the rate falls off by an order of magnitude from the maximum value $k_0$ (Moser et al., 1992).

1.3.2.2 Modified Redox Proteins: A recently developed technique has been exploited by Gray and co-workers for the investigation of intraprotein electron transfer. This involves the use of surface modified cytochromes (especially cytochrome c), which have had a redox active group chemically attached to a specific surface amino acid residue (Bowler et al., 1990). The most commonly used derivatives have ruthenium groups covalently attached to histidine or lysine, $(\text{NH}_3)_3\text{Ru-His-protein}$ (Winkler & Gray, 1992) and $(2,2'$-bipyridine)$_3\text{Ru-Lys-protein}$ (Durham et al., 1989). Photoexcitation of the ruthenium group results in the generation of a labile electron donor species, which can transfer electrons to the haem group. The main aim of such studies is to determine the nature of the electron-transfer event (elucidation of the reorganisation energy, $\lambda$ and the electronic coupling constant, $H_{AB}$) and whether it is best described as occurring by a through space or pathway model. Clearly, these are artificial situations when compared to the investigations of Dutton and co-workers, however, they still yield interesting semi-quantitative information regarding biological electron transfer.

Ruthenated cytochromes c from a variety of sources with a range of donor-acceptor redox group distances have been studied. These are: horse heart cytochromes c ruthenated at His33, 39, 62 and 72 (Wuttke et al., 1992; Figure 1.3.4); *Saccharomyces cerevisiae* iso-1 cytochromes c ruthenated at His62 and 72, the histidines being generated by SDM and semisynthesis, respectively (Bowler et al., 1989; Wuttke et al., 1992); and *Candida krusei* cytochrome c ruthenated at His39 (Therein et al., 1990). The rate of electron transfer in these systems was dependent upon the driving force ($\Delta G$) and the calculated values of $\lambda$ were all about 1.2 eV. However, the magnitude of $H_{AB}$ varied considerably for each ruthenated protein (Wuttke et al., 1992). Figure 1.3.3.A shows the dependence of electron-transfer rate on donor-acceptor distance for the above three systems (Wuttke et al., 1992; Chang et al., 1991). There is a much wider spread in the data than is the case for Figure 1.3.2, implying that fitting the data to a through-space, distance-dependent, electron-transfer process is clearly inadequate. However, if a pathways model is adopted, a much better description of the observed electron-transfer rates is obtained. This is illustrated in Figure 1.3.3.B (Wuttke et al., 1992) which is a similar plot to Figure 1.3.3.A, except the distance plotted is the effective $\sigma$-tunnelling pathway length and $\beta = 0.7$ Å⁻¹. Figure 1.3.4 shows the relative positions of ruthenated histidines on the
Figure 1.3.3. Plot of the Logarithm of Electron-Transfer Rate for Ruthenated Cytochromes c

(A) through space distance (from Van-der-Waals contact); \( k_0' = 3 \times 10^{12} \text{ s}^{-1} \) and \( \beta = 1.0 \text{ Å}^{-1} \) (solid line), 0.8 to 1.2 Å\(^{-1}\) (dashed lines), the best fit is obtained with \( k_0' = 1.6 \times 10^8 \text{ s}^{-1} \) and \( \beta = 0.66 \text{ Å}^{-1}\). (B) \( \sigma \)-tunnelling pathway distance, for ruthenated cytochromes c from horse heart; \( k_0' = 3 \times 10^{12} \text{ s}^{-1} \) and \( \beta = 0.71 \text{ Å}^{-1} \) (adapted from Wuttke et al., 1992).

Figure 1.3.4. The Proposed \( \sigma \)-Tunelling Pathway From Four Ruthenated Histidine Residues to the Haem of Horse Heart Cytochrome c

The numbers refer to the appropriate histidine residues. Solid lines indicate covalent bonds, dashed lines hydrogen bonds and dotted lines through space jumps (adapted from Wuttke et al., 1992).
surface of horse heart cytochrome c and the predicted electron tunnelling pathways to
the haem iron (Wuttke et al., 1992).

As well as addressing fundamental issues, these systems have been used to
study more subtle influences of the protein matrix on electron transfer (Winkler &
Gray 1992). One intriguing matter which has aroused considerable speculation, is
whether aromatic side chains situated between redox groups can facilitate electron
transfer, by acting as superexchangers of donor and acceptor wavefunctions (Liang &
Newton, 1992; Gruchus & Kuki, 1993). All the evidence collected so far, however,
indicates that the presence of aromatic side chains does not enhance the rate of
electron transfer (Casimiro et al., 1993).

The technique of photoexcitation of ruthenated proteins for studying
intraprotein electron transfer in redox proteins has expanded to include a wide range
of cytochromes, myoglobins, blue-copper proteins and iron-sulphur proteins
(Winkler & Gray, 1992 and references therein). Electron transfer in all these systems
is best described by pathway tunnelling approaches. Interestingly, however, and for
no obvious reasons, some of these systems appear far more facile at electron transfer
than others. Clearly, our current understanding of intraprotein electron transfer, is at
best, semiquantitative and this calls for a more detailed treatment of the process,
before quantitative, fundamental information can be attained.

1.3.3. Interprotein electron transfer

1.3.3.1. Cytochrome c-Cytochrome c Peroxidase: The interaction of
cytochrome c with yeast cytochrome c peroxidase is the subject of intense interest
and as such, is probably the best studied system to date (Moore & Pettigrew, 1990;
McLendon & Hake, 1992). Cytochrome c peroxidase catalyses the reduction of
hydrogen peroxide to water with the subsequent oxidation of two molecules of
ferrocytochrome c (Pettigrew and Moore, 1986). The reaction is illustrated in
Scheme 1.3.

**Scheme 1.3. Reaction catalysed by cytochrome c peroxidase.**

\[
\begin{align*}
\text{Ccp} + \text{H}_2\text{O}_2 & \rightarrow \text{Ccp(I)} + \text{H}_2\text{O} \\
\text{Ccp(I)} + \text{cyt c}_\text{red} & \rightarrow \text{Ccp(II)} + \text{cyt c}_\text{ox} \\
\text{Ccp(II)} + \text{cyt c}_\text{red} & \rightarrow \text{Ccp} + \text{cyt c}_\text{ox}
\end{align*}
\]
Abbreviations: Ccp, cytochrome \(c\) peroxidase (ferric state); Ccp(I), compound 1; Ccp(II), compound 2; cyt \(c_{\text{red}}\) and cyt \(c_{\text{ox}}\), reduced and oxidised cytochrome \(c\), respectively.

Oxidation of cytochrome \(c\) peroxidase by \(H_2O_2\) yields the compound I form, which contains an oxyferryl Fe(IV)=O haem (Yonenti & Ray, 1965, 1966), with a radical cation located on tryptophan 191 (Mauro \textit{et al.}, 1988; Sivavaja \textit{et al.}, 1989). This species is positioned about 4 Å away from the haem on the proximal side (Wang \textit{et al.}, 1990; Poulos & Kraut, 1980a) and is thought to be stabilised by extensive charge delocalisation onto neighbouring amino acid side chains (Liu \textit{et al.}, 1994). The first reduction of compound I by cytochrome \(c\), yields a semireduced form of cytochrome \(c\) peroxidase, termed compound II (Yonenti & Ray, 1965, 1966). This species exists as a pH dependent equilibrium mixture of the oxyferryl haem:'reduced' W191 and oxyferric haem:W191 radical cation (Coulson \textit{et al.}, 1971). Ferric cytochrome \(c\) peroxidase is generated by reduction from a second cytochrome \(c\) molecule. Elegant kinetic experiments, coupled with SDM and various forms of spectroscopy have shown that under certain pH conditions, electron transfer from cytochrome \(c\) to the haem in cytochrome \(c\) peroxidase always proceeds via W191 (Liu \textit{et al.}, 1994). So, this system can also give useful information regarding intraprotein electron transfer, coupled to interprotein electron transfer.

The crystal structures of both proteins are known to high resolution, along with the structures of a large number of site directed mutants (Poulos & Kraut, 1980; Fishel \textit{et al.}, 1987; Wang \textit{et al.}, 1990; Liu \textit{et al.}, 1994; Louie & Brayer, 1990; Mauk, 1991). Fifteen years ago, Poulos and Kraut (1980b) proposed a hypothetical, static model for a 1:1 interaction between these two proteins. The model was based on electrostatic interactions between four acidic side chains clustered together on the surface of cytochrome \(c\) peroxidase and four basic side chains surrounding the haem crevice in cytochrome \(c\) (Poulos & Kraut, 1980). The model has subsequently been refined by energy minimisation (Finzel \textit{et al.}, 1984). Initial attempts to co-crystallise cytochrome \(c\) and cytochrome \(c\) peroxidase were unsuccessful (Poulos & Kraut, 1987). However, recently, the separate crystal structures of cytochrome \(c\) peroxidase complexed to cytochrome \(c\) from horse heart and yeast \textit{iso}-I, have been determined (Pelletier & Kraut, 1992). It is interesting that although these structures show both forms of cytochrome \(c\) bound to the same general region of cytochrome \(c\) peroxidase, the orientation and precise nature of the interacting surfaces were different (the authors state that these differences are too large to be accounted for by the difference in the structures of yeast \textit{iso}-I and horse heart cytochrome \(c\), but may be due to the
different conditions under which the crystals were grown). Also, the nature of the interaction was quite different to that proposed in the hypothetical model; the interprotein contacts were largely hydrophobic, as opposed to electrostatic in nature. Since yeast iso-1 cytochrome c is the true physiological partner of cytochrome c peroxidase, the crystal structure of this complex will be considered here. The haem Fe-to-Fe distance between the two proteins is 26.5 Å and the angle of the haem planes is at 60° to one another.

Molecular Brownian dynamic simulations (Northrup et al., 1988) of cytochrome c docking with cytochrome c peroxidase, have identified three 'hot spots' for binding. One of these corresponds to the binding region in the crystal structure of the complex. Nuclear magnetic resonance (nmr) experiments, comparing the exchange rates of cytochrome c backbone amide protons in the free protein and when bound to cytochrome c peroxidase, indicate that the binding region on cytochrome c is much larger than that proposed in the crystal structure of the complex (Pelletier & Kraut, 1992). These simulations and solution studies of cytochrome c binding to cytochrome c peroxidase, clearly indicate that the interaction is not governed by a single static complex, but, by less well defined interaction regions on both proteins (This is a theme that will be developed throughout this chapter and will be discussed with respect to the interaction of flavocytochrome b2 with cytochrome c in chapter 4, section 4.5). A further investigation of complexation by nmr spectroscopy shows that about 90 % of the cytochrome c is bound with a 1:1 stoichiometry (Monech et al., 1992). The remaining complexes are of higher order stoichiometry implying that cytochrome c peroxidase can accommodate the binding of more than one cytochrome c molecule at a time.

The nature of the catalytic steps in cytochrome c peroxidase have been the subject of many kinetic experiments (Pettigrew & Moore, 1986; Summers & Erman, 1988; Kim et al., 1990). However, the reaction is very complicated, with the most recent scheme involving at least eight possible intermediates (Kim et al., 1990). Of more fundamental interest to this review, is the rapid-reaction kinetic studies, coupled with SDM, that have been performed to investigate the nature of the electron-transfer steps and the characterisation of the interacting surfaces.

The ionic strength dependence of the reaction has been extensively investigated and yields some interesting results. At low ionic strength, when a tight complex is formed, the rate of electron transfer from cytochrome c to cytochrome c peroxidase is lower than the rate measured at higher ionic strengths (Hazzard et al., 1988a,b). This behaviour is in complete contrast to that expected for an electrostatically stabilised electron-transferring complex, where the reaction rate
would be expected to decrease with increasing ionic strength. Also, charge reversal site-directed mutants in the proposed interaction regions of both proteins, under low ionic strength conditions, exhibited increased rates of electron transfer compared to that for the wild-type enzymes (Hazzard et al., 1988c; Corin et al., 1993). These results were interpreted as a requirement for a certain degree of mobility after initial complex formation, facilitating conformational exploration for the formation of an optimal geometry for electron transfer. Clearly, the most electrostatically stable complexes are not optimal for electron transfer.

The temperature dependent quenching of zinc porphyrin substituted cytochrome c peroxidase, by electron transfer from cytochrome c, has been investigated (Noeck et al., 1990 and 1991). In the preformed complex, at temperatures below 230 K, no electron transfer was observed. This implies that the proteins were 'locked' together in an inactive conformation for electron transfer and at higher temperatures, conformational gating was facilitated by thermal motion at the interprotein interface (Noeck et al., 1990 and 1991).

Recently, kinetic studies have been performed with cytochrome c derivatives that have ruthenium groups attached to various surface amino acids (Gerend et al., 1991; Hahm et al., 1992; Liu et al., 1994). Photoexcitation of the ruthenium results in rapid (> $10^6$ s$^{-1}$) intraprotein electron transfer from ruthenium to the cytochrome c haem, followed by a slower interprotein electron transfer from cytochrome c haem to cytochrome c peroxidase to generate compound I. The rate constants for the interprotein electron transfer were biphasic at low ionic strength and the fast phase rates were within an order of magnitude of one another ($5 \times 10^3$ to $5 \times 10^4$ s$^{-1}$), depending upon the location of the ruthenium group (Hahm et al., 1992). A similar situation occurred for the slow phase rate constants. However, at higher ionic strength, the magnitude of the rapid phase rate constants decreased, whereas those of the slow phase increased and, for certain derivatives the kinetics became monophasic (Hahm et al., 1992). This implies that there are two possible binding orientations for cytochrome c on cytochrome c peroxidase. The kinetics can be interpreted in two ways: either the binding orientation responsible for the slow phase is suboptimal for electron transfer; or this binding mode is inactive for electron transfer and the observed rate is due to a change in binding orientation to that of the optimised complex responsible for the fast phase of reduction. The authors tentatively suggest that this could be a conformational gating effect (Hahm et al., 1992). An important point to note is that even when the bulky ruthenium group (9 Å diameter) is placed near to the proposed interaction surfaces, it does not significantly impair interprotein
Figure 1.3.5. The Proposed Electronic Coupling Pathway Linking the Haem Groups of Cytochrome c and Cytochrome c Peroxidase (adapted from Pelletier & Kraut, 1992)
electron transfer. This suggests that the interaction region must be highly flexible, in order to accommodate these bulky groups.

The crystal structure of the cytochrome c:cytochrome c peroxidase complex shows a plausible σ-tunneling pathway for electron transfer. This pathway links the haem groups of each protein via the cytochrome c peroxidase residues W191, G192, A193, A194 (Figure 1.3.5), the latter two residues are proposed to form the major interaction region with cytochrome c (Poulos and Kraut, 1992). The validity of this pathway model for electron transfer and the nature of the interacting protein surfaces have been probed by rapid kinetics coupled with SDM (Corin et al., 1991, 1993; Miller et al., 1994). Mutation of Ala193→Phe, which is expected to alter both the binding region and the nature of the proposed electron-transfer pathway, had little effect on the association rate constant and the subsequent electron-transfer step. This implies that a large amount of 'plasticity' exists in the interaction, as the insertion of a bulky side chain can be accommodated in the interprotein region.

The reorganisation energy (λ) of this system has been estimated to be 1.4 eV, by temperature dependence and free energy perturbation methods (Conklin & McLendon; 1988). This is a relatively large value and is greater than the sum of calculated λ values for the redox changes in the separate proteins (uncomplexed). Redox linked interfacial dynamics are thought to provide a major contribution to λ in the complexed proteins. This is supported by the previously discussed findings, that limited surface diffusion within broadly defined interaction regions is necessary for efficient electron transfer. Indeed, charge reversal site-directed mutants in the proposed interaction region have lower values of λ than the wild-type complexes (eg, for cytochrome c peroxidase D37K derivative, λ = 1.0 eV for electron transfer) which gives further evidence for the key role played by dynamic motions (Conklin & McLendon; 1988).

**1.3.2.3. Cytochrome c-Cytochrome b5:** Cytochrome b5 is a membrane-bound protein abundant in the liver, where it is involved in stearyl CoA desaturation (Holloway & Katz, 1972) and cytochrome P450 reduction (Hildebrandt & Eastbrook, 1971). Another form is found in erythrocytes where it maintains haemoglobin in the reduced state (Mauk & Mauk, 1982). Although cytochrome c and cytochrome b5 are not physiological redox partners, they react readily *in vitro* via the formation of a tight binding complex, which is dominated by electrostatic interactions. Electron transfer occurs from b5-haem to cytochrome c haem through a potential difference (ΔE) of about +250 mV (Moore & Pettigrew, 1986). This is probably the simplest
interprotein redox system to study and because of this virtue, it has been extensively investigated.

The crystal structures of both proteins are known to high resolution, along with a large number of site directed mutants (Mathews et al., 1979; Takano & Dickerson, 1980; Mauk, 1991). The stoichiometry of the interaction has been probed by absorption spectroscopy (Mauk et al., 1982, 1986), pH titration (Mauk et al., 1991) and nmr spectroscopy (Eley & Moore, 1983; Burch et al., 1990; Whitford et al., 1990). All the data indicated that a 1:1 complex was formed, except for the nmr experiments of Whitford et al. (1990), who detected a higher order ternary complex, with 1:2 stoichiometry of cytochrome $b_5$ to cytochrome $c$. However, this second binding site was of much lower affinity than the primary one and was only detected in this case, because of the sensitivity of the technique (Whitford et al., 1990). The 1:1 association is quite tight and the stability of the complex is strongly dependent upon ionic strength; the dissociation constant ($K_d$) increases with increasing ionic strength, implying that the interaction is governed by electrostatics (Eltis et al., 1991).

In 1976, Salemme proposed the structure of a hypothetical complex for the solution interaction between cytochrome $c$ and cytochrome $b_5$ (Figure 1.3.6). This was the first model of its kind and it predicted a 1:1 interaction, with the haem crevices of the two proteins facing towards the interaction region. The complex was stabilised by electrostatic interactions between four acidic and four basic side chains around the haem crevices of cytochrome $b_5$ and cytochrome $c$ respectively. The haem groups were approximately coplanar with an edge-to-edge distance of about 8 Å (Salemme, 1976). This model has subsequently been refined by molecular dynamics (Wendoloski et al., 1987) and modified slightly by experimental data, but the essential features remain largely unchanged (Eltis et al., 1991). Unfortunately, attempts to obtain co-crystals of these two proteins have been unsuccessful, so, the results--of all-- the-SDM and -kinetic -studies are interpreted in terms of this model. Brownian dynamic simulations of the binding suggest that the interaction region is very similar to that proposed by Salemme (Wendoloski et al., 1987). However, the resultant complex is not static in nature, but is rather flexible, exploring a number of alternative geometries and molecular configurations, with differing favourabilities for electron transfer (Wendoloski et al., 1987; Eltis et al., 1991). This conclusion is supported by kinetic experiments investigating the rate of interprotein electron transfer with varying ionic strength (Meyer et al., 1993).

The role of individual amino acids proposed to form interprotein interactions has been extensively probed by SDM coupled with kinetic and biophysical
Figure 1.3.6. A hypothetical model for the interaction of cytochrome c with cytochrome b$_5$.

The model shows negatively-charged amino acid residues on cytochrome b$_5$ (red) interacting with positively-charged residues on cytochrome c (blue).
characterisation. These results are in general agreement with the model complex, if the interaction is regarded as being flexible (Northrup et al., 1993; Rodgers et al., 1988; Burrows et al., 1991). The effects of single mutations can be successfully predicted in a semi-quantitative manner by Brownian dynamic simulations of the mutant proteins (Northrup et al., 1993). Elegant experiments, measuring the changes in solvation volume, upon high pressure induced dissociation of the complex have been performed (Rodgers et al., 1988). These show that when each amino acid residue on cytochrome $b_5$ that is proposed to be involved in an interprotein interaction is mutated, the change in solvation volume accounts for the loss of an electrostatic interaction in the complex. The number of interactions 'lost' upon mutation agrees exactly with the model, with regard to the number and position of the interacting side chains (Rodgers et al., 1988).

Some controversy exists concerning the rate of electron transfer in the preformed complex. McLendon et al. (1985) measured a rate of 1600 s$^{-1}$ by pulse radiolysis, for the native system. The authors stated that the maximal observed rate for electron transfer (1600 s$^{-1}$) is well below the calculated theoretical rate. However, Willie et al. (1992), using cytochrome $b_5$ ruthenated at His65, at low ionic strength, measured a biphasic interprotein electron-transfer rate of $4 \times 10^5$ s$^{-1}$ and $3 \times 10^4$ s$^{-1}$, for the two phases. A similar situation occurred in this system as has been described for the cytochrome $c$:cytochrome $c$ peroxidase complex (when the ionic strength was increased, the observed rate constant became monophasic). This implies that the complex can adopt two different conformations with different electron-transfer properties (Willie et al., 1992). Considering the nature of the complex and the inter-haem distance, the rates measured by the photoexcitation induced electron transfer seem to be more reasonable values for the interprotein electron-transfer reaction.

A value of $\lambda = 0.8$ eV has been estimated by free energy perturbation methods (McLendon et al., 1985). This value is greater than the sum of the $\lambda$ values for the redox changes in the individual proteins (a similar situation was observed for the cytochrome $c$:cytochrome $c$ peroxidase system, as described earlier). Again, this suggests that changes in the interprotein interactions upon electron transfer are contributing to $\lambda$.

The roles of internal amino acid side chains in cytochrome $c$ have been studied in a number of systems, with regard to their influence upon protein structure and function. Of particular interest is Phe82, which is conserved in all cytochromes $c$ discovered to date (Mauk, 1991). As F82 is situated between the edge of the haem and the protein exterior, it has been proposed that its aromatic side chain could facilitate interprotein electron transfer between cytochrome $c$ and its redox partners.
Brownian dynamic simulations show that on the picosecond timescale, in the solvated cytochrome c:cytochrome b5 complex, F82 can flip away from the haem edge into the interprotein interaction region (Wendoloski et al., 1987). This possible conformational change of F82 could bring it into a suitable orientation to act as an electron 'bridge' between the two haems. However, kinetic experiments in the pre-formed complex, where F82 has been mutated to various other residues, show little change in the rate constants for electron transfer, compared to the native systems (Willie et al., 1993). Also, nmr experiments investigating the nature of the interaction region in the complex, show that on the nmr timescale, the environment of F82 does not change (Burch et al., 1990). These latter two studies correlate well with the results of section 1.3.2, concerning the absence of rate enhancement of electron transfer by aromatic residues (Casimiro et al., 1993).

1.3.3.3. Conclusions on interprotein electron transfer: It appears that interprotein electron transfer involves a rather nonspecific sequence of events. Evidence is accumulating that the idea of redox partners recognising and binding to one another via a single, well defined site, where they will possess the optimal geometry for subsequent electron transfer, is not a true description. Rather, an 'untidy' series of nonspecific binding, interfacial diffusion, followed by electron transfer occurs. The latter situation, which is apparently favoured by nature, clearly must be the best option. The probability of obtaining a precise alignment of small point charges in the random collision of large protein molecules is small. If however, the complexes can be electrostatically guided into a general interacting region, then most of the collisions will result in the formation of an initial complex. On the molecular timescale of dynamic motion at the protein interface, the subsequent redistribution of the complex (to form the optimal geometry for electron transfer) is fast (ps) compared to the rate of electron transfer (µs). A high degree of reaction specificity is thus assured by this conformational exploration.

1.3.4. Summary of protein mediated electron transfer

In this discussion of protein mediated electron transfer, a number of conclusions can be drawn and speculations made:

-Experimental results indicate that Marcus theory, in its simplest form, provides a reasonably accurate description of electron-transfer reactions in both intra- and inter-protein systems.

-For intraprotein electron transfer, where the donor-acceptor distance can be accurately determined, a good description of the transfer process can be obtained. In
systems where electron transfer is optimised, this can be best described by a through
space pathway. However, in certain situations (generally artificial ones) a through
bond tunnelling pathway is more appropriate. In many interprotein systems,
however, the donor-acceptor distances are ill defined and it is more difficult to
quantify the nature of the electron-transfer process.

-The key problem in the investigation of interprotein systems, is determining the
nature of the interacting surfaces of the redox partners and how biological specificity
is achieved through this molecular recognition. The bulk of this review has
concerned cytochrome c, which is a highly promiscuous protein, in that it has to
accept and donate electrons to a large number of redox partners. So, it is not
surprising that experiments indicate that its interaction with redox partners is
dynamic in nature. This will be further exemplified in the results and discussion
chapters of this thesis, where the interaction of cytochrome c with another of its
redox partners, flavocytochrome b₂ will be considered.

1.4. FLAVOCYTOCHROME b₂

1.4.1. Introduction

Flavocytochrome b₂ is an excellent paradigm for biological electron transfer:
the gene encoding the enzyme has been cloned and sequenced (Guiard et al., 1985),
allowing site-directed mutants to be constructed (Reid et al., 1988) and the protein
over-expressed in the bacterium E. coli (Black et al., 1989); the crystal structure of
the native wild-type (Xia & Mathews, 1990) and recombinant protein (Tegoni &
Cambillau, 1994) are known to high resolution; the protein is soluble and as will be
described, is an elegant model system for investigation of both intra-and inter-protein
electron transfer.

1.4.2. Physiological role of flavocytochrome b₂

As illustrated in Figure 1.4.1, flavocytochrome b₂ is a soluble component of
the intermembrane space of yeast mitochondria (Daum et al., 1982), where it
catalyses the dehydrogenation of L-lactate to pyruvate with subsequent electron
transfer to cytochrome c, i.e. it is an L-lactate:cytochrome c oxidoreductase. The fact
that flavocytochrome b₂ facilitates entry of electrons into the respiratory chain at the
level of cytochrome c, allows the yeast to respire on L-lactate (Pajot & Claisse,
1974). This can be demonstrated by inhibiting the respiratory chain with antimycin
which blocks electron transfer at the level of complex III (ubiquinol:cytochrome c
oxidoreductase).
Figure 1.4.1. Physiological Role of Flavocytochrome $b_2$

The topological arrangement of the short electron-transport chain involving flavocytochrome $b_2$ in yeast mitochondria. Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane; cyt, cytochrome; f.crt $b_2$, flavocytochrome $b_2$. (Chapman et al., 1991)
However, if the yeast are grown on L-lactate, respiration can still occur (Pajot & Claisse, 1974), implying that electrons from flavocytochrome $b_2$ can enter the respiratory chain at the level of cytochrome $c$.

### 1.4.3. Flavocytochrome $b_2$ structure

To date, flavocytochrome $b_2$ has been isolated and purified from two yeast species, *Saccharomyces cerevisiae* (Labeyrie *et al.*, 1978) and *Hansenula anomala* (Blazy *et al.*, 1976). Both proteins have been extensively characterised with regards to their chemical, physical and kinetic properties (Chapman *et al.*, 1991; Lederer, 1991). However, all the detailed structural information known about this enzyme is derived from the X-ray crystal structure of *S. cerevisiae* flavocytochrome $b_2$ (Xia & Mathews, 1990; Tegoni & Cambillau, 1994).

Initially, Xia & Mathews (1990) determined the crystal structure of the native enzyme to 2.4 Å resolution and more recently, Tegoni & Cambillau (1994) have solved the structure of the sulphite-bound recombinant enzyme to 2.6 Å resolution. The two forms of the enzyme were essentially isostructural, but in view of the greater resolution of the former structure and the nature of the ligand bound at the active site, this structure will be described here.

Flavocytochrome $b_2$ is a homotetramer, with a molecular weight of 57.5 kDa per subunit for the *S. cerevisiae* enzyme (Pajot & Groudinsky, 1970). Figure 1.4.2 shows that the crystallised enzyme has a pseudo-four-fold axis of symmetry and that each subunit is composed of two distinct domains (Xia & Mathews, 1990). The domain structure of one subunit is illustrated in Figure 1.4.3. The smaller N-terminal domain, termed the cytochrome $b_2$-core, comprises amino acid residues 1 to 100 and has a bis-histidine coordinated haem porphyrin IX prosthetic group (Figure 1.4.4). The $b_2$-core has extensive structural similarity to the cytochrome $b_5$ family of proteins, with the haem being bound in a cleft formed by a 'wall' of two $\alpha$-helices, from which the coordinating histidines are derived and the 'bottom' formed of mixed $\beta$-pleated sheet (Xia & Mathews, 1990). The $b_2$-core is connected by a single piece of polypeptide, termed the interdomain *hinge region*, to the larger flavin domain. The flavin domain consists of amino acid residues 101 to 487, with the flavin mononucleotide prosthetic group non-covalently bound at the active site for L-lactate dehydrogenation. The flavin domain has an $\alpha_8\beta_8$ barrel structure, with the FMN bound at the C- and N-terminal ends of the $\beta$-sheet barrels and $\alpha$-helices, respectively (Xia & Mathews, 1990). The tertiary structure of the flavin domain is very similar to that of the related flavoproteins (Figure 1.4.5), spinach glycolate oxidase (Lindqvist, 1989; Lindqvist *et al.*, 1991) and trimethylamine dehydrogenase.
Figure 1.4.2. Three Dimensional Crystal Structure of Flavocytochrome b$_2$.

The structure of *S. cerevisiae* flavocytochrome b$_2$ to 2.4 Å resolution (Xia & Mathews, 1990). View is down the pseudo four-fold axis of symmetry. Each subunit is shown in a different colour, with the prosthetic groups in dark blue.
Figure 1.4.3. The Domain Structure of a Single Flavocytochrome $b_2$ Subunit.

Illustration of the domain structure of a single subunit. Amino acid side chains are shown, along with the prosthetic groups in blue. The red region is the cytochrome $b_2$ core domain, the thick purple region is the interdomain hinge, the green region constitutes the flavin domain and the C-terminal tail.
Figure 1.4.4. Structure of the Cytochrome \( b_2 \) Domain

Tertiary structure of the \( b_2 \)-core domain. The \( \alpha \)-carbon backbone is represented as a ribbon. The haem is illustrated in bold wire, with the propionate oxygens and the haem nitrogens coloured in red and blue respectively.
Figure 1.4.5. Structural Comparison of the Flavocytochrome $b_2$ Flavin Domain with that of Spinach Glycolate Oxidase

Schematic illustrations of the tertiary and secondary structures of the flavin domain of a flavocytochrome $b_2$ subunit and the related flavoprotein spinach glycolate oxidase. Key: $\alpha$-helices are represented by rectangular boxes and circles; $\beta$-sheet by arrows and all other peptide by dashed or filled lines. The disordered loop on flavocytochrome $b_2$ is represented as a dotted line between helices E and F (Lederer, 1991).
Figure 1.4.6. The Active Site Structures in the Two Crystallographically Distinct Flavocytochrome $b_2$ Subunits

The active site structure in (A) subunit 1, where pyruvate is absent and (B) subunit 2, where pyruvate is present (Xia & Mathews, 1990).
from the methylotrophic bacterium W3A1 (Louis et al., 1986). Residues 488 to 511 comprise the C-terminal tail, which forms extensive intersubunit contacts. The structural similarity of the cytochrome and flavin domains of flavocytochrome b2 to the related proteins described above, strongly suggests that flavocytochrome b2 evolved by a gene fusion event between a cytochrome and a flavoprotein (Xia & Mathews, 1990).

In the flavocytochrome b2 structure, there are two crystallographically distinct subunits (Figure 1.4.6): in subunit one, the structure of the cytochrome b2 domain is resolved and no electron density attributed to either substrate or product is apparent at the active site and the flavin is in the reduced state; in subunit two however, the cytochrome b2 domain is not resolved due to positional disorder, but electron density attributed to pyruvate is located at the active site and the flavin is in the semiquinone state (Xia & Mathews, 1990). The significance of this will be described below and elaborated upon in Chapter 4, section 4.3.1.

In subunit 1, the interdomain contact area is about 400 Å² and a number of specific interdomain interactions occur: there is one salt bridge between K296 and a haem propionate; tyrosine 143, a key active site residue, forms a hydrogen bond to a haem propionate in subunit one, this is the most extensively studied interdomain interaction to date (Miles et al., 1992; Rouvière-Formy et al., 1994 and Figure 1.4.6). In addition, there are 5 main-chain hydrogen bonds and 12 hydrogen bonds to water from b2-core, some of these waters hydrogen bonding to the flavin domain.

Figures 1.4.6 and 1.4.15 shows the relative orientations of flavin and haem in flavocytochrome b2. The prosthetic groups are approximately coplanar, with a 20° twist to their planes. The closest edge to edge distance in subunit 1 is 9.7 Å, between N(5) of FMN and C-2A of the porphyrin haem edge. The intervening medium between the flavin and haem contains no peptide and is largely comprised of solvent molecules (Xia & Mathews, 1990).

1.4.4. Comparison of S. cerevisiae and H. anomala flavocytochromes b2

Attempts to crystallise H. anomala flavocytochrome b2 have been unsuccessful (Chapman et al., 1991), however, the gene encoding the enzyme has been cloned and the amino acid sequence deduced from the DNA sequence (Black et al., 1989a). Interestingly, flavocytochromes b2 from the two yeast species have different kinetic behaviour and the nature of the rate-limiting steps on the catalytic cycle also differ (Pompon et al., 1980; Capeillère-Blandin et al., 1986; Capeillère-Blandin, 1991). To understand the structural basis for these differences, the amino acid sequences of the mature proteins were compared in the context of the known
Figure 1.4.7. Amino Acid Sequence Comparison Between *H. anomala* and *S. cerevisiae* Flavocytochrome *b*-2

<table>
<thead>
<tr>
<th>Scb2</th>
<th>EPKLDNQKQ ISPAEVAHKN KPDDCVVIN GYVYDLRFL PNHPGGQVVI KFNAGKDVYA</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hab2</td>
<td>.DVPWHDIE LTDEIVSRQH KDDDLWNVLN QVQVLDLDFL PNHPGGQKII IYRAKDATK</td>
<td>59</td>
</tr>
<tr>
<td>SpGO</td>
<td>---------------------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>IFEPIDHADNY IDKYIADEKK LGPLQGSMPP ELVCPYPAP PMHEDIDARK BQLKSLPPLD</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>IFVPFHPPEY LGPLSRKRGQ KPYPY SDFPPHEL PHEEDIDRD RIER KFFPL</td>
<td>112</td>
</tr>
</tbody>
</table>

### *H. anomala* (Hab2)

- **Scb2**: NINILYDFEY LASQTLTKAQ WAYSQSGNAH VITVRRHHA YHRIFFPQKQ LDVRKDVIS
- **Hab2**: QMLNHDFET ARQIILPPPA LAYYCSAADD EVTLRENNA YHRIFNPQKQ LDVKKVIS
- **SpGO**: EITVMEYEA IAQJKLPKLV YDYYASGAED QWTLAENNIA PERILRFRPI LIYTVNDM

### *S. cerevisiae* (Scb2)

- **Scb2**: IFEPIDHADNY IDKYIADEKK LGPLQGSMPP ELVCPYPAP PMHEDIDARK BQLKSLPPLD
- **Hab2**: IFVPFHPPEY LGPLSRKRGQ KPYPY SDFPPHEL PHEEDIDRD RIER KFFPL
- **SpGO**: EITVMEYEA IAQJKLPKLV YDYYASGAED QWTLAENNIA PERILRFRPI LIYTVNDM

### Consensus (Con)

- **Hab2**: T-----E----A---- --F----------
- **SpGO**: E-----A----

### spinach glycolate oxidase (SpGO)

- **Scb2**: TDMLGSHDVPP FYVSYATLCLKGNPLEGK DVARGCQQQV TVKQGMISTLS ACSCPGBIII 240
- **Hab2**: TEEFPGKPTA PFBYSATLCLKGNPLEGK DVARGCQQQV TVKQGMISTLS ACSCPGBIAD 229
- **SpGO**: TEEFPGKPTA PFBYSATLCLKGNPLEGK DVARGCQQQV TVKQGMISTLS ACSCPGBIAD 229

### Con

- **Scb2**: QLYV--DR- ----- -V- -E- -G- -T- VPD-LG-RE --D-K-F-
- **Hab2**: EADS...DVQ G....DDEDIDR SQGASRALSL YVQGDPVLDK KDLKLVSGVE 410
- **SpGO**: TAPDASVRQQ...KQGRIPVFLDG

The amino acid sequences of the mature forms of flavocytochromes *b*-2 from *S. cerevisiae* (Scb2) and *H. anomala* (Hab2) are shown, along with a consensus (Con) wherein all the sequences are identical. The sequence alignment of spinach glycolate oxidase (SpGO) is also shown for comparison. The interdomain hinge region and the protease-sensitive loop of flavocytochrome *b*-2 are boxed (adapted from Chapman *et al.*, 1991).
crystal structure of the *S. cerevisiae* enzyme (Black *et al.*, 1989a). Overall, there is 60% identity, with key active site residues being conserved (Figure 1.4.7). However, two surface loops in particular are strikingly different in size and charge. One of these, the interdomain hinge region, covalently links the two domains (residues 89 to 103, with a net charge of -1 and residues 88 to 96, with a net charge of -6, in the *S. cerevisiae* and *H. anomala* enzymes, respectively). The other region is a proteolytically sensitive loop of the *S. cerevisiae* enzyme (Ghrir & Lederer, 1981), which is disordered in the crystal structure (Xia & Mathews, 1990). These structural differences are likely to have a significant effect upon the known catalytic differences between the two species.

1.4.5. The catalytic cycle of flavocytochrome *b*₂

Flavocytochrome *b*₂ can be described as a molecular transformer, as it mediates electron transfer from an organic substrate, lactate, which is a two electron donor, to a one electron acceptor, cytochrome *c*. It is able to do this by virtue of the fact that flavin can exist in three oxidation states; oxidised, semiquinone and reduced (Figure 1.4.8.), passing electrons one at a time to cytochrome *c* via *b*₂-haem (Suzuki & Ogura, 1966). There is no evidence that cytochrome *c* can accept electrons directly from the flavin in holo flavocytochrome *b*₂ (Forestier & Baudras, 1971).

The catalytic cycle is illustrated in Scheme 1.4.1. The first step is lactate binding at the active site to form the Michaelis complex and its subsequent dehydrogenation to pyruvate, generating fully reduced FMN. The catalytic mechanism by which this is thought to occur will be described in section 1.4.6. Steps 2 and 4 are interdomain electron-transfer events from fully reduced and semiquinone FMN to oxidised *b*₂-haem respectively. Steps 3 and 5 are interprotein electron-transfer events from reduced *b*₂-haem to oxidised cytochrome *c*. The very nature of the flavocytochrome *b*₂ catalytic cycle makes it an excellent model system for investigating protein mediated electron transfer; both intraprotein (FMN→haem) and interprotein (*b*₂-haem→cytochrome *c*) electron transfer can be studied. Also, the mechanism of L-lactate dehydrogenation has been thoroughly investigated (Lederer, 1991; Chapman *et al.*, 1991 and references therein).

1.4.6. Mechanism of L-lactate dehydrogenation

The chemistry of L-lactate dehydrogenation by *S. cerevisiae* flavocytochrome *b*₂ has been extensively studied by Lederer and co-workers (Lederer, 1991 and references therein) and more recently, the role of key active site residues in substrate binding, specificity and catalysis have been investigated by site-directed mutagenesis
Figure 1.4.8. Illustration of Three Oxidation States of Flavins

\[ R = \text{CH}_2(\text{CHOH})_2\text{CH}_2\text{OPO}_3^{2-} \]

Oxidised

\[ \text{H}^+ + \text{e}^- \]

Semiquinone

\[ \text{H}^+ + \text{e}^- \]

Reduced
Scheme 1.4.1. Catalytic Cycle of Flavocytochrome \( b_2 \)

The redox states of cytochrome \( c \) and the flavocytochrome \( b_2 \) flavin (F) and haem (H) are indicated by the subscripts 'ox' and 'red' for the oxidised and reduced forms, respectively. The flavin semiquinone is shown as Fsq.

1. Oxidation of lactate to pyruvate and reduction of FMN.
2. Electron transfer from fully reduced FMN to haem, resulting in the semiquinone form of FMN and reduced haem.
3. Reduction of the first cytochrome \( c \) molecule by electron transfer from the haem group of flavocytochrome \( b_2 \).
4. Electron transfer from the semiquinone form of FMN to haem, resulting in fully oxidised FMN and reduced haem.
5. Reduction of a second cytochrome \( c \) molecule by electron transfer from the haem group, which regenerates the fully oxidised enzyme. The enzyme is now ready to repeat the cycle.
Figure 1.4.9. The Initial Steps in L-lactate Dehydrogenation: αH-Abstraction from C-2 of L-lactate

Illustration of C-2 H-abstraction by (a) hydride transfer, or (b) by formation of a carbanion.
(Reid et al., 1988; Black et al., 1989a; Dubois et al., 1990; Chapman et al., 1991; Miles et al., 1992; Lederer et al., 1992; Rouvière-Fourmy et al., 1994; Daff et al., 1994).

The first step in L-lactate dehydrogenation, αH-abstraction from C-2 of L-lactate is the rate limiting one, with a deuterium kinetic isotope effect (KIE), measured by pre-steady-state kinetics, of 8 for FMN reduction (Lederer, 1974; Pompon et al., 1980 and Miles et al., 1992). There are two possible ways for catalysis to occur (Figure 1.4.9): either by hydride transfer (Figure 1.4.9.a), as in the nicotinamide pyridine linked dehydrogenases (Massey & Ghisla, 1983); or by formation of a carbanion (Figure 1.4.9.b), as has been proposed for the flavocytochrome b2 related flavoproteins, L-lactate monooxygenase (Ghisla & Massey, 1991), D-amino acid oxidase (Walsh et al., 1971) and glycolate oxidase (Lindqvist, 1991).

For flavocytochrome b2, a large amount of evidence has been accumulated, which indicates, but does not unambiguously prove, that the initial step in catalysis is the formation of a carbanion in the transition state (Lederer, 1991). The three main experiments whose results were taken as being indicative of a carbanion mechanism were: partition between elimination and oxidation of a halogenated substrate (Urban & Lederer, 1984); enzyme inactivation by the suicide substrate 2-hydroxy-3-butynoate (Lederer, 1974; Pompon & Lederer, 1985); and enzyme catalysed intermolecular tritium transfer (Urban & Lederer, 1985). However, there is some rationale for discriminating against a carbanion in favour of a hydride mechanism. The main factor is that, for a carbanion mechanism to operate, the active site base (His373) responsible for αH-abstraction from C-2 of L-lactate, would have to have a pKₐ of about 15 (in the reduced enzyme). Also, the pKₐ of flavin N(5) would have to be in the same region. These pKₐ values represent extreme shifts, even for a protein environment to impose. Further evidence which is not compatible with a carbanion mechanism, was derived from experiments with flavin analogues (Pompon & Lederer, 1979) and ethane nitronate. The latter acts as a carbanion transition state analogue in D-amino acid oxidase (Porter et al., 1973), but not in flavocytochrome b2, where it behaves as a competitive inhibitor (Genet and Lederer, 1990). Despite these ambiguities and for the sake of argument, the assumption will be made here, as is generally accepted in the literature (Lederer, 1991; Ghisla & Massey, 1991), that a carbanion is indeed formed.

The proposed mechanism of L-lactate dehydrogenation deduced from the crystal structure and kinetic studies of the enzyme (Xia & Mathews, 1990 and Lederer et al., 1988) is shown in Figure 1.4.10. A number of key active site residues
Figure 1.4.10. Proposed Mechanism of L-lactate Dehydrogenation by Flavocytochrome $b_2$

$$E_{ox} + S_{red} \rightleftharpoons \frac{1}{k_1} \frac{k_{-1}}{k_1}$$

**Michaelis Complex**

**Rate-Limiting Step**

**Carbanion Intermediate**

$k_2 \rightleftharpoons \frac{1}{k_2}$

$k_3 \rightleftharpoons \frac{1}{k_3}$
The rate determining step in catalysis is $\alpha$H-abstraction from C-2 of L-lactate to form a carbanion transition state ($k_{+2}/k_{-2}$). Electron transfer from substrate carbanion to FMN is shown here as proceeding via a covalent intermediate, although the existence of this intermediate has never been demonstrated and alternatives to this are shown in Figure 1.4.11 (adapted from Chapman et al., 1991).
Figure 1.4.11. Alternatives Routes of Electron Transfer From the Substrate Carbanion to the FMN of Flavocytochrome $b_2$

Oxidation of substrate can occur by a two electron transfer via a covalent intermediate ($A + E$); by formation of a radical pair followed by a second one electron transfer to give the covalent intermediate ($B+C+E$); and by formation of a radical pair, directly followed by a second one electron transfer to give reduced FMN ($B+D$) (Chapman et al., 1991).
are involved, these residues are totally conserved in the related flavoproteins L-lactate monooxygenase (Giegel et al., 1990), long chain \( \alpha \)-hydroxy acid oxidase 1988 (Urban et al., 1988; Lederer, 1991) and glycolate oxidase (Lindvist & Branden, 1989). The substrate is bound at the active site by interactions between its carboxylate group and the guanido N\( \delta \) of Arg376 which forms a hydrogen bond and an electrostatic interaction, and by the hydroxyl group of Tyr143, which hydrogen bonds to the other carboxylate oxygen. The active site base is His373, which abstracts the C-2 \( \alpha \)-hydrogen. Asp282 stabilises the resultant imidazolium ion. Tyr 254 is involved in transition state stabilisation. Electron transfer from the carbonium to FMN is facilitated by the positive charge on Lys349 stabilisation the anion at N(1) of the reduced flavin. As shown in Figure 1.4.10, the reduced flavin is thought to be in the N(1)N(5) dianion form, which is stabilised by the protein environment. The rate-limiting step in catalysis is at step \( k_2/k_{-2} \) in Figure 1.4.10. Whether the reaction proceeds via a radical species or by nucleophilic attack of the carbanion onto N(5) of the flavin, forming a covalent adduct intermediate (Figure 1.4.11.A), or by two, one electron transfers via a radical species (Figure 1.4.11.B), is speculation.

Unfortunately, it is very difficult to examine these possibilities, as the steps following the formation of the carbanion are faster than its rate of formation. As mentioned earlier, the catalytic roles ascribed to these active site residues have been extensively studied by site-directed mutagenesis, which by and large support the above described situation.

1.4.7. Interdomain (intraprotein) electron transfer

When considering interdomain electron transfer, one fundamental point which needs to be addressed, is whether electron transfer can occur between the prosthetic groups of the isolated domains. The flavin domain (Pallister et al, 1990) and the \( b_2 \)-core domain (Brunt et al., 1992) have been individually expressed in \( E. \) coli and the purified proteins have been extensively characterised. Both nuclear magnetic resonance (nmr) and circular dichroism spectroscopy have failed to detect any interaction between the separate domains (S. K. Chapman, G. A. Reid, C. E. Brunt, M. C. Cox & G. A. Moore, unpublished results). Also, no reduction of the \( b_2 \)-core domain was detected under pre-steady-state conditions, when excess reduced flavin domain was mixed with oxidised \( b_2 \)-core domain (S.K. Chapman & P. White, unpublished results). These data imply that the two domains need to be covalently linked by the hinge region for interdomain communication to occur.

Another key issue, is one of protein dynamics. Several lines of evidence indicate that the \( b_2 \)-core domain is mobile with respect to the flavin domain. Nmr
spectroscopy shows that the $b_2$-core domain has proton resonances whose linewidths are nowhere near as broad as would be anticipated for a protein as large as flavocytochrome $b_2$, this suggests that the $b_2$-core is fairly mobile (Labeyrie et al., 1988). As already mentioned, in the crystal structure of flavocytochrome $b_2$ there are two crystallographically distinct types of subunits, in which the $b_2$-core domains are either ordered or disordered (subunits 1 and 2, respectively). The fact that two of the $b_2$-core domains are disordered implies that they are mobile. Indeed, the rigidity of these domains in the other subunit may be due to crystal packing forces (Xia & Mathews, 1990).

Both of the above data indicate that the interdomain hinge region confers domain mobility to the $b_2$-core.

Intraprotein electron transfer in both $S. cerevisiae$ and $H. anomala$ flavocytochrome $b_2$ has been investigated by a wide variety of kinetic and spectroscopic techniques (Chapman et al., 1991; Lederer, 1991), the pertinent results and conclusions of which, will be described below.

1.4.7.1. Fully reduced FMN→haem electron transfer:

Many experiments, mainly involving pre-steady-state kinetics (Lederer, 1991; Chapman et al., 1991) have been performed upon both $S. cerevisiae$ and $H. anomala$ flavocytochromes $b_2$, in order to estimate the rate of interdomain electron transfer. For the $H. anomala$ enzyme, as the rate of flavin reduction is more rapid than that of haem reduction (fully reduced FMN→haem electron transfer), reliable estimates for the rate of this intraprotein electron transfer process have been obtained (Capeillère-Blandin et al., 1986; Capeillère-Blandin, 1991). However, for the $S. cerevisiae$ enzyme the situation is more complicated, as the converse is true; i.e. the rate of flavin reduction is slower than the rate of FMN→haem electron transfer (Pompon et al., 1980). In this case, simulation studies have been employed to describe the pre-steady-state experimental data (Capeillère-Blandin et al., 1975; Pompon, 1980), some with more success than others. Below, a 'new' scheme is described to estimate the rate constant for the first interdomain electron-transfer step, which correlates well with the stopped-flow data (Chapman et al., 1994).

As illustrated in steps 2 and 4 of Scheme 1.4.1, there are two interdomain electron-transfer steps in the flavocytochrome $b_2$ catalytic cycle. Under pre-steady-state conditions, in the absence of any external electron acceptors, the full reduction of the enzyme proceeds as illustrated in Scheme 1.4.2. The kinetic traces for flavin and haem reduction under most conditions, can be satisfactorily analysed as the sum of two exponential functions and a model has been proposed to explain the reduction
process (Capeillère-Blandin et al., 1975; Pompon et al., 1980). The rapid first phase (Scheme 1.4.2, steps 1 and 2) is a two-step process, in which two electrons enter a subunit at the flavin level and then redistribute between the flavin and the haem. The slower second phase is due to the entry of the third electron per subunit (corresponding to four electrons from two L-lactate molecules entering the tetramer), this is made possible by inter-subunit electron transfer. The slow phase is catalytically irrelevant during turnover of the enzyme, when it acts as a two-electron transferase under physiological conditions (Capeillère-Blandin et al., 1975; Pompon, 1980).

Scheme 1.4.2. The electron-transfer process occurring in one subunit of flavocytochrome $b_2$ during full reduction of the enzyme by L-lactate.

\[
\begin{align*}
F^+ H &\rightarrow F^+ H' \\
&\leftrightarrow F^+ H' \\
&\rightarrow F^+ H'
\end{align*}
\]

Step 1, reduction of flavin; Step 2, flavin to haem electron transfer. Step 3 is the entry of the third electron per subunit to generate the fully reduced enzyme. Abbreviations: F, flavin; H, haem and black dots denote 1 electrons.

The rate constant for flavin reduction by L-lactate is $604 \pm 60$ s$^{-1}$ (Scheme 1.4.2, $k_{+1}$) and the biphasic fit for the haem absorbance trace has a rapid rate constant of $445 \pm 50$ s$^{-1}$ (Miles et al., 1992). However, the fact that the haem reduction traces lag behind that of flavin reduction and that there are three steps in the reduction process, clearly indicates that fitting the data to a double exponential model is an approximation (Chapman et al., 1994). Thus, the rate constant for haem reduction by flavin will have contributions from all three steps in Scheme 1.4.2. However, as 'step 3' only contributes about 15 % of the total absorbance change and is much slower than the preceding steps, the contribution of 'step 3' over the initial 80 % of the haem absorbance change is small. The proportion of reduced haem in the initial 80 % absorbance change region can be described by the function:

\[
1 + \frac{(k_a e^{-k_b t} - k_b e^{-k_a t})}{k_b-k_a}
\]
This function describes the accumulation of C in an A→B→C consecutive process, with step B→C being in equilibrium (Chapman et al., 1994). If $k_a$ is the value of $k_{-1}$ in Scheme 1, $(604 \pm 60 \text{ s}^{-1})$ and the above function is fitted to the initial 80% of the haem absorbance change, a value for $k_b$ of $1600 \pm 300 \text{ s}^{-1}$ is obtained. This value of $k_b$ however, is slightly influenced by the rate of 'step 3', which will perturb the equilibrium of step 2. Thus, two limiting conditions are considered which take this into account: if 'step 3' is rapid and irreversible, $k_b$ will be equal to $k_{+2}$; but, if 'step 3' is slow $k_b$ will be equal to $k_{+2} + k_{-2}$. From the known mid-point potentials of the flavin and haem couples (Walker & Tollin, 1991), step 2 is calculated as lying 85% in favour of the reduced haem. Thus, the equilibrium constant for the ratio of $k_{+2}$ to $k_{-2}$ is 5.5:1, which gives limiting values of $k_{+2}$ and $k_{-2}$ of $1500 \pm 500 \text{ s}^{-1}$ and $270 \pm 90 \text{ s}^{-1}$, respectively. These values agree well with simulation studies, indicating that the approach is valid, especially as the nature of 'step 3' is not fully understood (Chapman et al., 1994). Hence, the rate of interdomain electron transfer from fully reduced flavin to haem is about $1500 \text{ s}^{-1}$.

1.4.7.2. Semiquinone FMN→haem electron-transfer:

The rate of semiquinone FMN→haem electron-transfer process (Scheme 1.4.1, step 4) is more difficult to estimate than that of the previous intraprotein electron-transfer step. Despite this, many investigators have attempted to measure this step, mainly on the H. anomala enzyme using a variety of techniques, including pre-steady-state kinetics (Janot et al., 1990), temperature-jump relaxation (Tegoni et al., 1984), pulse radiolysis (Capellère-Blandin et al., 1984), laser flash photolysis (Walker & Tollin, 1991, 1992), rapid freezing electron paramagnetic resonance spectroscopy (Capellère-Blandin et al., 1975, 1986; Tegoni et al., 1986; Capellère-Blandin, 1991) and redox potentiometry (Capellère-Blandin et al., 1986; Tegoni et al., 1986). The first three techniques mentioned, due to the nature of the experiments, only give a rough estimate of the semiquinone FMN→haem electron-transfer rate.

It is interesting to consider some contradictions in the literature concerning the effect of the reaction product pyruvate, on this intraprotein electron-transfer step. It is well documented that pyruvate can bind to and stabilise the flavosemiquinone form of l-lactate monooxygenase (Stankovich & Fox, 1983). This is also the case for flavocytochrome b2, where in the presence of 10 mM pyruvate, about 90% of the FMN is in the flavosemiquinone form and the potential of the flavosemiquinone/oxidised flavin couple is increased by about +100 mV in both the H. anomala (Tegoni et al., 1986) and S. cerevisiae enzymes (Walker & Tollin, 1991). Steady-state kinetics performed by Tegoni et al. (1990) on the H. anomala enzyme showed that pyruvate
exhibited mixed inhibition and that the global rate of catalysis was reduced 12-fold. However, laser flash photolysis experiments with flavocytochrome \( b_2 \) from both species gave results, which were interpreted by the authors as providing evidence for a conformational gating of intraprotein electron transfer, induced by pyruvate binding to the flavosemiquinone (Walker & Tollin, 1991, 1992). The laser flash generated substoichiometric amounts of reductant, so that only one electron transfer was observed between the redox centres. It was noted that intraprotein electron transfer only occurred in the presence of pyruvate, despite unfavourable thermodynamics due to pyruvate stabilization of the flavosemiquinone (the potential of the flavosemiquinone/flavin oxidised redox couple was more positive than that of the haem couple). In the absence of pyruvate, the thermodynamic driving force for electron transfer was about +100 mV, but in this situation no intraprotein electron transfer was detected (Walker & Tollin, 1991, 1992). However, there are many ambiguities in these experiments, which the authors themselves admit require further study, before any definitive conclusions can be drawn.

Despite many experimental approaches to date, a truly unambiguous value for the rate of haem reduction by the semiquinone form of FMN has yet to be determined (Chapman et al., 1994).

1.4.8. Interprotein electron transfer

The main investigations of interprotein electron transfer have been carried out between \( H. \text{ anomala flavocytochrome } b_2 \) and \( \text{ iso-1 cytochrome c } \) by Capeillère-Blandin et al. The interaction was strongly dependent on ionic strength, implying that it was governed by electrostatic interactions between the two proteins. The electron-transfer rate between a 1:1 stoichiometry of \( b_2 \)-haem and cytochrome c reached a saturating value of about 380 \( s^{-1} \) (extrapolated to zero ionic strength) at 5°C (Capeillère-Blandin, 1982). Also, experiments with the proteolytically derived, reduced cytochrome \( b_2 \)-core, showed that this transferred electrons to cytochrome c in an ionic strength dependent manner, with a second order rate constant of \( 10^7 \text{ M}^{-1}\text{s}^{-1} \) at 5°C and 0.2 M ionic strength (Capeillère-Blandin & Albani, 1987). Silvestrini et al., (1986) determined second order rate constants for the reduction of the non-physiological redox partners azurin and stellacyanin (blue copper proteins), by pre-reduced flavocytochrome \( b_2 \). The rate constants were of the order of \( 10^6 \text{ M}^{-1}\text{s}^{-1} \) (Silvestrini et al., 1986), implying that the reduction of redox proteins by flavocytochrome \( b_2 \) is relatively nonspecific, the major determinant being a favourable thermodynamic driving force for electron transfer (the difference in mid-
point potentials, $\Delta E^\circ$, of the $b_2$-haem and copper redox centres of azurin and stellacyanin are +340 and +204 mV respectively).

In contradiction to the previous finding, McLendon et al., (1987) have suggested a conformational gating (Hoffman & Ratner, 1987) of electron transfer between $S. cerevisiae$ flavocytochrome $b_2$ and horse heart cytochrome $c$. McLendon and co-workers investigated the rate of electron transfer in a pre-formed complex and found that this rate was independent of $\Delta G$, the thermodynamic driving force of the reaction ($\Delta G$ was varied by substituting the cytochrome $c$ haem with porphyrin and zinc porphyrin). However, the fact that the electron-transfer reaction was monitored in a preformed complex at very low ionic strength, could explain the observed 'conformational gating'. Cytochrome $c$ may have been associated with flavocytochrome $b_2$ in a non-productive conformation for electron transfer and the rate of cytochrome $c$ reduction limited by the time required to attain a favourable geometry (see chapter 3, section 1.3.3.1). Clearly these points require further study.

1.4.9. Determining the binding region of cytochrome $c$ on flavocytochrome $b_2$

Most of the binding studies performed between these two redox partners have been on the $H. anomala$ proteins, for which there are no available crystal structures. Chemical modification studies revealed that the major interacting surface on cytochrome $c$ for flavocytochrome $b_2$, was the area surrounding the haem crevice (Matsushima et al., 1986). Cytochrome $c$ has a cluster of conserved lysine residues in this region, which have been implicated in its binding to many other redox partners (Pettigrew and Moore, 1986). It is not surprising that cytochrome $c$ should bind to its redox partners in this fashion, as this would bring the haem group as close as possible to the redox partner, minimising the electron-transfer distance. Experiments to localise the binding area of cytochrome $c$ on flavocytochrome $b_2$ imply that cytochrome $c$ interacts with both the flavin and $b_2$-core domains, but the interaction with the flavin domain is about 10-fold stronger than that with $b_2$-core alone (Albani, 1985, 1990). The stoichiometry and degree of binding have also been investigated, with various results depending upon the experimental conditions and the source of the proteins (Tegoni et al., 1993 and references therein). However, from diffusing cytochrome $c$ into $S. cerevisiae$ flavocytochrome $b_2$ crystals, a 1:1 stoichiometry of cytochrome $c$ binding per subunit has been determined (Tegoni et al., 1983). Unfortunately, the cytochrome $c$ molecules cannot be visualised on electron density maps of these crystals, presumably because they are in equilibrium between the bound and unbound forms, or are positionally disordered.
Recently, Tegoni et al. (1993) have used the above information, along with the known crystal structures of *S. cerevisiae* flavocytochrome *b*₂ (Xia & Mathews, 1990) and *iso-l* cytochrome *c* (Louie & Brayer, 1990), to propose a hypothetical model for the interaction of cytochrome *c* with flavocytochrome *b*₂ in the crystalline state. The model was refined by energy minimisation techniques. The first attempt to describe the interaction on an atomic level (Figures 1.4.12.a and 1.4.12.b), show the proposed binding sites for four cytochrome *c* molecules per flavocytochrome *b*₂ tetramer. Figure 1.4.13 illustrates the surface complementarity of the interaction. The type of binding is such that each cytochrome *c* molecule interacts with different regions on three separate subunits (Figure 1.4.14); with the end of the C-terminal tail (Figure 1.4.14, subunit D), the interdomain hinge region (Figure 1.4.14, subunit B) and the flavin domain (Figure 1.4.14, subunit A), of separate subunits. The flavocytochrome *b*₂ and cytochrome *c* haems are coplanar, with an edge to edge distance of 14 Å (Figure 1.4.15). The contact surface area is about 800 Å² and comprises several electrostatic and hydrogen bond interactions (Tegoni et al., 1993). One important point to note about this model is that it is for cytochrome *c* interacting with crystalline flavocytochrome *b*₂. Therefore, a great deal of the flavocytochrome *b*₂ surface has been occluded by crystal contacts and so is prevented from being involved in binding. Thus, the applicability of this model to interactions in solution is questionable.

1.5. RESEARCH AIMS

The main aim of this work was to extend our knowledge of the role of the interdomain hinge region of flavocytochrome *b*₂ in protein mediated electron transfer; both intraprotein, FMN→haem (White et al., 1993; Sharp et al., 1994); and interprotein, flavocytochrome *b*₂-haem→cytochrome *c* (Sharp et al., 1994). To achieve this, three hinge-deletion mutant enzymes have been constructed, HA3, HA6 and HA9, with 3, 6 and 9 amino acids deleted from the hinge region respectively (Figure 1.5.1). These hinge-deletion enzymes have been extensively characterised by steady-state and pre-steady-state kinetics, in order to elucidate the effect of the deletions upon the above mentioned steps.
Figure 1.4.12.a. Molecular Model of the Flavocytochrome \( b_2 \):Cytochrome \( c \) Complex Viewed Down the Pseudo Four-Fold axis of symmetry.

The four subunits of the flavocytochrome \( b_2 \) tetramer are shown in blue, green, orange and pink, with the prosthetic groups in dark blue. The four cytochrome \( c \) molecules bound to the tetramer are shown in black.
Figure 1.4.12.b. Molecular Model of the Flavocytochrome $b_2$:Cytochrome $c$ Complex Viewed Perpendicular to the Symmetry Axis.

This is the equivalent complex to Figure 1.4.12.a, but rotated through 90°.
Figure 1.4.13. The Interacting Water Accessible Surfaces of Flavocytochrome $b_2$ (Blue) and Cytochrome $c$ (Pink).
Figure 1.4.14. Illustration of the Nature of Cytochrome c Binding to Flavocytochrome $b_2$

Stereo picture of the complex between a crystallographic dimer of flavocytochrome $b_2$ (subunits A and B) and a molecule of cytochrome c, the tail of subunit D is also shown. The order of increasing thickness of the lines depicting the α-carbon backbone is subunit A < B < cytochrome c < tail of D (from Tegoni et al., 1993).
Figure 1.4.15. Inter-Prosthetic Group Distances and Orientations in the Flavocytochrome $b_2$:Cytochrome $c$ Molecular Model

Depiction of the electron-transfer triad in the model complex: the FMN, $b_2$-haem and cytochrome $c$ haem. (A) Side view, with the haems perpendicular to the plane of the diagram. (B) Front view, with the inter-prosthetic group distances indicated (from Tegoni et al., 1993).
Figure 1.5.1. Construction of the Hinge-Deletion Flavocytochromes $b_2$

$^{89}PPELVCPPYAPGETK^{103}$

$\begin{array}{ll}
H\Delta3 & \text{APG} \\
H\Delta6 & \text{PPYAPG} \\
H\Delta9 & \text{LVCPPYAPG} \\
\end{array}$

The amino acid sequence of the interdomain hinge region from the $S.\ cerevisiae$ enzyme is shown, along with the deleted residues. Key: H, indicates the hinge region; $\Delta$ indicates a deletion; the number indicates the number of amino acid residues deleted.
Chapter 2

Materials and Methods
2.1. GROWTH AND MAINTENANCE OF STRAINS

2.1.1. Bacterial stocks

<table>
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<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
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<td><em>supE, hsdΔ5, thi, Δ(lac-proAB), F',</em></td>
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<td></td>
<td><em>trad36, proAB⁺, laqI, lacZΔM15</em></td>
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<td><em>E. coli</em> AR120</td>
<td><em>λN99 (F⁻, galK2, LAM⁺, rpsL200)</em></td>
<td>Gottesman &amp; Yarmolinsky, 1968</td>
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<td>derivative (ci, Δ-gal, nadA::Tn10)</td>
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<tr>
<td><em>E. coli</em> BW313</td>
<td><em>dut, ung, thi-1, relA, spoT1/F'lysA</em></td>
<td>Kunkel, 1985</td>
</tr>
</tbody>
</table>

2.1.2. Growth of bacterial cultures

Liquid cultures of bacteria were grown in the appropriate broth by inoculating a given volume of broth with a single colony using a sterile inoculating loop. Cultures of *E. coli* were grown at 37°C.

2.1.3. Storage of bacterial cultures

For long term storage 1 ml of fresh overnight culture of bacteria, grown in the appropriate medium, was mixed with 0.25 ml of 100% sterile glycerol and stored in a sterile vial at -80°C. Upon recovery, vials were thawed quickly and the culture streaked out on agar plates made with the appropriate medium, with antibiotics if required. TG1 was streaked out onto minimal agar plates to maintain the F' plasmid. After overnight incubation at the appropriate temperature, a single colony was picked to propagate a fresh bacterial culture. For short term storage (4 to 6 weeks), bacteria were stored on agar plates at 4°C.

2.1.4. Media for *Escherichia coli*

**Luria broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco Bacto tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Difco Bacto yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>6 g</td>
</tr>
</tbody>
</table>
Minimal medium

Per litre

20% glucose solution 10 ml
K₂HPO₄ 12 g
KH₂PO₄ 3 g
NH₄Cl 1 g
NaCl 0.5 g
dH₂O 800 ml

This was autoclaved and cooled to 40 °C, then the following were added:

1 M CaCl₂ 100 μl
1 M MgSO₄ 2 ml
2 mgml⁻¹ vitamin B₁ (thiamine.HCl) 200 μl

Plates of the above medium were made by adding 2% agar prior to autoclaving.

2.1.6. Antibiotics

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>[stock solution] (mgml⁻¹)</th>
<th>[working] (μgml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Carbenecillin</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

Stock solutions were sterilised by filtration through a 0.22 μm filter and stored at -20°C. Antibiotics were added after the media had been autoclaved and cooled.

2.2. SOLUTIONS

2.2.1. TE buffer

Tris/HCl 10 mM
EDTA 1 mM

2.2.2 10 x TBE

Per litre:

Tris base 108 g
Boric acid 55 g
0.5 M EDTA pH 8.0 40 ml
2.2.3. 10 x Loading buffer
0.25 % Bromophenol blue
15 % Ficoll 400

2.2.4. SDM buffer A
0.2 M Tris.HCl pH 7.5
0.1 M MgCl₂
10 mM DTT
Stored at -20°C.

2.2.5. SDM buffer B
SDM buffer A 1 µl
0.1 M DTT 1 µl
20 mM dATP, dCTP, dGTP and dTTP 0.5 µl of each
20 mM ATP 0.5 µl
dH₂O 4.5 µl
Stored at -20°C.

2.2.6. 0.1 M Protein purification buffer
Per 2 liters:
5 mM L-lactate 0.96 g
1 mM EDTA 0.74 g
0.2 M KH₂PO₄ solution 400 ml
0.2 M K₂HPO₄ solution 600 ml
adjusted to pH 7.0
dH₂O to 2 litres

2.2.7. 10 mM Tris/HCl buffer, pH 7.5 (I 0.10)
Per litre:
NaCl 5.265 g
1 M HCl 10 ml
dH₂O to 1 litre
adjusted to pH 7.5 with tris base
2.2.8. 10 mM Phosphate buffer, pH 7.0

Per 2 litres:

20 mM KH$_2$PO$_4$ solution 400 ml
20 mM K$_2$HPO$_4$ solution 600 ml
adjusted to pH 7.0
dH$_2$O to 2 litres

2.2.9. 20 mM Phosphate buffer, pH 7.0, in D$_2$O

20 mM KH$_2$PO$_4$ in D$_2$O 20 ml
20 mM K$_2$HPO$_4$ in D$_2$O until pH is 7.0

2.2.10. 10 mM CAPS buffer, pH 7.0 (I 0.10)

Per litre:

NaCl 5.265 g
1 M NaOH 10 ml
dH$_2$O to 1 litre
CAPS until pH is 11.0

2.2.11. DNA sequencing gel (6 % acrylamide)

Per 80 ml:

Urea 33.6 g
Protogel (30 % acrylamide; 0.8 % bis-acrylamide) 16 ml
10 x TBE 8 ml
dH$_2$O to 80 ml
10 % APS 180 µl
TEMED 180 µl

All reagents except APS and TEMED were mixed thoroughly. APS and TEMED were added immediately before pouring the gel.

2.2.12. 4 x Resolving buffer for SDS-PAGE

Per litre:

Tris base 181.6 g
SDS 4.0 g
dH$_2$O 600 ml
conc.HCl 10 ml
adjust to pH 8.8
### 2.2.13. 4 x Stacking buffer for SDS-PAGE

Per 500 ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>30.28 g</td>
</tr>
<tr>
<td>SDS</td>
<td>2.0 g</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>450.0 ml</td>
</tr>
<tr>
<td>conc. HCl</td>
<td>15.0 ml</td>
</tr>
</tbody>
</table>

Adjust to pH 6.8

### 2.2.14. 2 x SDS-PAGE loading buffer

Per 100 ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris.HCl pH 6.8</td>
<td>3.31 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>9 ml</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5 ml</td>
</tr>
<tr>
<td>1 % bromophenol blue</td>
<td>1 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>80 ml</td>
</tr>
</tbody>
</table>

### 2.2.15. 10 % Resolving gel for SDS-PAGE

Per 30 ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protogel (30 % acrylamide; 0.8 % bis-acrylamide)</td>
<td>10 ml</td>
</tr>
<tr>
<td>4 x Resolving buffer</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>12.3 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>190 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

### 2.2.16. 5 % Stacking gel for SDS-PAGE

Per 10 ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protogel (30 % acrylamide; 0.8 % bis-acrylamide)</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>4 x Stacking buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>5.86 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>30 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>
2.2.17. 5 x Tris-glycine electrophoresis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>15.1 g</td>
</tr>
<tr>
<td>glycine</td>
<td>94 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

2.2.18. 10 x Transfer buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris.HCl pH 8.3</td>
<td>250 ml</td>
</tr>
<tr>
<td>Glycine</td>
<td>112.6 g</td>
</tr>
</tbody>
</table>

2.2.19. Tris-buffered saline (TBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris.HCl pH 7.5</td>
<td>10 ml</td>
</tr>
<tr>
<td>4 M NaCl</td>
<td>37.5 ml</td>
</tr>
</tbody>
</table>

2.3. SUPPLIERS

2.3.1. Enzymes

T<sub>4</sub> DNA ligase, restriction endonucleases, DNA kinase and the Klenow fragment of DNA polymerase were obtained from Gibco-BRL, Paisley, UK. Taq DNA polymerase was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Lysosome and Pancreatic ribonuclease A were obtained from Sigma, the ribonuclease A was boiled for 10 minutes to remove any DNase activity before use. Sequenase® was obtained from United States Biochemical Corporation, Cleveland, Ohio.

2.3.2. Antisera

HRP-conjugated goat anti-rabbit IgG was obtained from the Scottish Antibody Production Unit (SAPU).

2.3.3. Isotopes - Amersham International.

2.3.4. General laboratory chemicals - Sigma chemical company, Poole, Dorset or, BDH, Poole, Dorset.
2.4. PHAGE

M13K07 helper phage was used to obtain single-stranded DNA (Vieira & Messing, 1987).

2.5. PLASMIDS

Description and use:  
Reference:

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGR401</td>
<td>phagemid/yeast shuttle vector</td>
<td>Reid et al. (1988)</td>
</tr>
<tr>
<td>pDS6</td>
<td>transcriptional vector</td>
<td>Black et al. (1989b)</td>
</tr>
</tbody>
</table>

2.6. OLIGONUCLEOTIDE PRIMERS

Sequence 5'-3':

<table>
<thead>
<tr>
<th>Name</th>
<th>Priming position relative to start of flavocytochrome $b_2$ presequence coding region:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGCACAATATTTCAAGC</td>
<td>primes in ADH promoter 102A 233 to 248</td>
</tr>
<tr>
<td>TAGACACAACAAGCCGAA</td>
<td>E1K 358 to 378</td>
</tr>
<tr>
<td>CTACCAATATGCCAGGTGGG</td>
<td>H43M 448 to 467</td>
</tr>
<tr>
<td>GTCATCGACAAGATATATAGC</td>
<td>XCl 450 522 to 538</td>
</tr>
<tr>
<td>TCCTCCTTGTCTCCTTG</td>
<td>Y97F 660 to 676</td>
</tr>
<tr>
<td>GTGGGCCTTCTATTTCT</td>
<td>Y143F 715 to 729</td>
</tr>
<tr>
<td>AATGCCTATCATAGG</td>
<td>$b_2$ 730 993 to 1009</td>
</tr>
<tr>
<td>CCACTATTTGTAACT</td>
<td>Y254F 1075 to 1094</td>
</tr>
<tr>
<td>GTCACGTGGAATGCTCCAAG</td>
<td>D282N 1147 to 1164</td>
</tr>
<tr>
<td>GGTCACAATACATGAAGAG</td>
<td>Cla 1155 1279 to 1296</td>
</tr>
<tr>
<td>GTTATACAGGTTTCAA</td>
<td>K349R 1360 to 1377</td>
</tr>
<tr>
<td>GGTGGTAAACATTACAT</td>
<td>R376K 1494 to 1511</td>
</tr>
<tr>
<td>CTTGAAAGCTTTATGTCT</td>
<td>Hind 1500 521 to 551</td>
</tr>
<tr>
<td>GTCCCTCTATTAGAATAAGGA</td>
<td>HΔ3 512 to 551</td>
</tr>
<tr>
<td>AACTTGTCTGTGAATACCTAGGA</td>
<td>HΔ6 503 to 551</td>
</tr>
<tr>
<td>TGCCCTCTGAAGAAACTAAGGA</td>
<td>HΔ9 503 to 551</td>
</tr>
</tbody>
</table>
2.7. TRANSFORMATION AND SELECTION PROCEDURES

2.7.1. Preparation of competent cells

1 litre of LB broth was inoculated with 10 ml of an overnight culture and shaken at 37°C until an OD_{600nm} of 0.3 was reached. Cells were placed on ice for 10 minutes to arrest growth, then pelleted by centrifugation in 4 x 250 ml sterile Sorvall pots at 5000g (Sorvall SS-34 rotor) for 15 minutes at 4°C. The pellets were resuspended in 125 ml of fresh 100 mM calcium chloride. After 30 min on ice, the cells were pelleted at 9000g (Sorvall SS-34 rotor) for 10 minutes at 4°C. Each pellet was resuspended in 25 ml of 100 mM calcium chloride, and combined in one of the pots. Glycerol (20 ml) was added (final concentration of 17%) then the cells were divided into 1 ml aliquots, frozen in liquid nitrogen and stored at -80°C.

2.7.2. Transformation of *E. coli*

Supercoiled plasmid DNA (5 to 10 ng), or half of a ligation reaction, were added to 100 µl of competent *E. coli* cells in a 1.5 ml microcentrifuge tube and left on ice for 30 minutes. The cells were then heat-shocked at 42°C for 3 min. Luria broth (1 ml), was added to the cells, which were incubated at 37°C for 1 hour. The cells were then pelleted in a microfuge and resuspended in 100 µl of Luria broth. Aliquots (25 µl and 75 µl) were spread onto Luria agar petri dishes containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C.

2.8. ISOLATION OF DNA

2.8.1. Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using the alkaline lysis method essentially as described by Birnboim and Doly (1979).

2.8.1.1. Small scale isolation

Luria broth (5 ml), supplemented with ampicillin to a final concentration of 100 µg/ml, was inoculated with a single colony of the plasmid-bearing strain and incubated at 37°C overnight with shaking. Half of the culture (1.5 ml) was pelleted by centrifugation in a microfuge and resuspended in 100 µl of 25 mM Tris/ HCl (pH 8.0), 10 mM EDTA, 50 mM glucose (TEG). Lysis buffer (200 µl of 0.2 M NaOH, 1% SDS) was added and after gentle mixing, placed on ice for 5 min. Following the lysis 150 µl of 3 M sodium acetate (pH 5.0) was added to precipitate chromosomal
DNA, SDS and proteins. After a further incubation on ice for 5 minutes the precipitate was removed by centrifugation in a microfuge for 10 minutes. Absolute ethanol (1 ml) was added to the supernatant, mixed, incubated at -20°C for 15 minutes to precipitate the DNA and then centrifuged in a microfuge to pellet the plasmid DNA. The DNA was washed with 70 % ethanol, dried under vacuum, and resuspended in 50 µl of TE buffer. Plasmid 'miniprep' DNA was stored at 4°C.

2.8.1.1. Large scale isolation

A 250 ml culture of bacteria carrying the desired plasmid was incubated overnight at 37°C with vigorous shaking in Luria broth supplemented with 100 µg/ml-1 of ampicillin. The cells were pelleted by centrifugation at 9000g (Sorvall GSA rotor) and resuspended in 4 ml of TEG. Cells were lysed on ice by adding 8 ml of 0.2 M NaOH, 1 % SDS and left on ice for 20 min. Addition of 6 ml of 3 M sodium acetate (pH 5.0) precipitated chromosomal DNA, SDS and proteins which were spun down at 17000g (Sorvall SS-34 rotor) for 15 minutes at 4°C. Isopropanol (11 ml) was added to the supernatant and left at room temperature for 30 min to precipitate plasmid DNA. The DNA was pelleted by centrifugation at 20000g (Sorvall SS-34 rotor) for 20 minutes at 4°C. The pellet was washed in 70 % ethanol, dried under vacuum, resuspended in 10 ml TE buffer containing 10 µg/ml-1 RNase and incubated at 37°C for 30 minutes. Plasmid DNA (9.4 ml) was transferred to a fresh tube to which 100 µl of 10 mg/ml-1 ethidium bromide and 9.02 g of CsCl were added, giving a density of 1.55 g/ml-1. The DNA was banded by centrifugation at 40000g (Beckman TL-100; TLA-100.3 rotor) for 12 hours at 20°C. DNA was visualized by side illumination with a UV light. The lower band containing supercoiled plasmid DNA was removed by puncturing the tube with a 19 gauge needle and syringe. A second 19 gauge needle was inserted at the top of the tube to allow pressure release. The ethidium bromide was removed by extraction several times with butanol, and the CsCl was removed by dialysis against 2 litres of TE buffer for 8 to 15 hours at room temperature. The DNA was then stored at 4°C for up to 12 months or under ethanol at -80°C for longer term storage.

2.8.2. Isolation of single stranded DNA from E. coli

Single stranded DNA from plasmids with the F1 origin of replication (phagemids) were prepared from E. coli using M13KO7 helper phage. This method yielded approximately 1 µg of single stranded DNA which was suitable for use in sequencing reactions and a 5-fold scaled up preparation yielded approximately 5 µg
of single stranded DNA which, when resuspended in 50 µl of TE, was sufficiently concentrated for use as the template DNA in site-directed mutagenesis.

A single colony of *E. coli* host containing the phagemid was grown in 2 ml of selective medium with shaking, at 37°C to mid-log phase (OD₆₀₀nm = 0.5). M13KO7 helper phage was added to a multiplicity of infection of 10 and the culture was shaken vigorously. After 1 hour, 400 µl of infected cells were mixed with 10 ml of selective medium, to which kanamycin was added at a final concentration of 70 µg/ml⁻¹, to select for the phage. The culture was grown overnight at 37°C, with vigorous shaking to give good aeration.

Cells were removed from the culture supernatant by centrifuging 1.5 ml of the overnight culture at 12000g for 5 minutes. To precipitate the phage, 1.2 ml of the supernatant was added to 0.3 ml of NaCl/PEG solution (2.5 M NaCl, 20 % polyethylene glycol 6000), and left at room temperature for 15 minutes. The phage were pelleted at 12000g for 5 minutes. After removing all the NaCl/PEG solution, the phage were resuspended in 100 µl TE. Phenol (50 µl) was added to the phage. The suspension was vortexed and centrifuged for 1 minute. DNA, which was located in the aqueous layer, was removed and cleaned by Geneclean™, finally being resuspended in 50 µl of TE.

### 2.9. GEL ELECTROPHORESIS OF DNA

#### 2.9.1. Agarose gel electrophoresis of DNA

DNA was separated in 0.8 - 2 % (w/v) agarose BRL electrophoresis grade with 0.5 µg/ml⁻¹ ethidium bromide in 1 x TBE. Prior to loading, DNA samples were mixed with 0.1 x volume of loading buffer (20 % glycerol; 20 mM EDTA; 0.1 % bromophenol blue). Electrophoresis was carried out horizontally across a potential difference of 1-10 Vcm⁻¹. Bacteriophage λc1857 DNA restricted with HindIII was used as size markers. DNA was visualised by UV illumination and photographed.

#### 2.9.2. Recovery of DNA from agarose gels

DNA was electrophoresed through 1 % agarose BRL in 1 x TBE, containing 0.5 µg/ml⁻¹ ethidium bromide. The desired fragment was visualised by UV illumination, cut out, and extracted from the agarose using Geneclean™. The agarose was weighed and 0.5 volume of TBE modifier and 4.5 volumes of 6 M NaI solution were added. The agarose was dissolved by heating to 55°C for 5 minutes with occasional mixing, and then cooled on ice for 5 minutes. The molten agarose was
treated with 5 µl of 'glass milk' (a silica matrix suspended in water), and left for 5 minutes on ice with occasional mixing to allow the DNA to bind to the silica matrix. The 'glass milk' was pelleted by centrifugation at 15000g (microfuge) for 1 minute. The supernatant was discarded and the pellet washed three times with 500 µl of 'NEW wash' (NaCl/ethanol/water mix). After a final spin all the NEW wash was discarded and the DNA was eluted from the 'glass milk' in 5-20 µl of TE buffer or water at 55°C. The mixture was spun in a microfuge, and the supernatant containing the DNA, transferred to a fresh microfuge tube and stored at 4°C. Recovery of DNA fragments of ≥500 base pairs was usually around 80%.

2.10. DNA MANIPULATION TECHNIQUES

2.10.1. Cleavage of DNA with restriction enzymes

All restriction enzyme digests were performed using BRL enzymes and buffers. DNA (0.1 to 20 µg) was cut in 10 - 200 µl of 1 x appropriate 'React' buffer for 2 to 8 hours at the appropriate temperature. For double digests involving enzymes with different recommended buffers, the buffers were checked individually in double digests to determine which gave the most efficient restriction.

2.10.2. Ligation of DNA ends

50 to 100 ng of vector (cut with the appropriate restriction enzyme) was incubated with an excess of fragment in 1 x ligation buffer (10 mM Tris/HCl pH 7.2; 1 mM EDTA; 10 mM MgCl₂; 10 mM DTT; 1 mM ATP) with 10 EU of T₄ ligase. The reaction was made up to a final volume of 10 µl and incubated overnight at 16°C.

2.11. SITE-DIRECTED MUTAGENESIS

This was performed by the method of non-phenotypic selection, essentially as described by Kunkel (1985). The 3, 6 and 9 amino acid hinge-deletion flavocytochromes b₂ were constructed using the oligonucleotides H₃, H₆ and H₉, respectively. Section 2.6 gives the sequences of, and the priming positions of these mutagenic oligonucleotides on the flavocytochrome b₂ gene sequence.
2.11.1 Phosphorylation of oligonucleotide

Reaction mix:
- 200 pmoles oligonucleotide in a volume of 23 μl TE
- 1 M Tris/HCl, pH 8.0: 3 μl
- 0.3 M MgCl₂: 1 μl
- 0.1 M DTT: 1.5 μl
- 20 mM ATP: 1.5 μl
- 5 EU Polynucleotide kinase: 0.5 μl

The reaction mix was incubated at 37°C for 45 minutes and then incubated at 68°C for 10 minutes to inactivate the enzyme and stop the reaction. The phosphorylated oligonucleotide was used immediately.

2.11.2. Mutagenesis

**Annealing**
- 1 μg single stranded DNA template in 5 μl TE
- 4 pmoles phosphorylated mutagenic oligonucleotide in 2.5 μl SDM buffer A
- dH₂O: 6 μl

The annealing mix was incubated at 70°C for 3 minutes and then allowed to cool slowly to 4°C over about 30 minutes.

**Extension/ligation.**

to annealing mix add:
- SDM buffer B: 10 μl
- dH₂O: 17 μl
- 3 EU of Klenow fragment of DNA polymerase: 0.5 μl
- 3 EU T₄ DNA ligase: 1 μl

This was incubated at 16°C for 15 hours.

2.11.3. Screening of potential mutants

In order to test the efficiency of the extension/ligation reactions, a 5 μl volume of the SDM reaction mix was electrophoresed in a 1.5 % agarose gel alongside single stranded and double stranded pGR401 DNA as controls. If the reaction product DNA co-migrated with the double stranded DNA and not the single stranded DNA control, the reaction was deemed successful and 25 μl of the reaction mix was used to transform *E. coli* TG1 cells.
Potential mutants were screened directly by DNA sequencing of single stranded DNA. As the efficiency of the mutagenesis was at its lowest about 5%, only a maximum of 20 colonies needed to be screened to obtain the required site-directed mutant.

2.12. SEQUENCING OF SINGLE STRANDED DNA

Sequencing of DNA was carried out using the Sequenase™ Version 2.0 kit (United States Biochemicals), which uses the dideoxy chain termination method. The flavocytochrome \( b_2 \) gene was sequenced using a combination of the oligonucleotide primers listed in section 2.6.

Appropriate sequencing primer (1 \( \mu \)l of 3 ng\( \mu \)l\(^{-1} \)) was annealed to 7 \( \mu \)l of template DNA (approximately 1 \( \mu \)g DNA) in 2 \( \mu \)l of 5 x reaction buffer (200 mM Tris/HCl, pH 7.5; 100 mM MgCl\(_2\); 250 mM NaCl) by heating to 65°C for 2 minutes then cooled slowly to below 37°C. Extension from the annealed primer was done by adding 1 \( \mu \)l 0.1 M DTT, 2 \( \mu \)l dGTP label mix (a 1 in 4 dilution of 7.5 \( \mu \)M dGTP, dCTP, dATP and dTTP), 0.5 \( \mu \)l \( \alpha \)-[\( ^{35} \)S]-dCTP (400 Ci/mmole) and 2 \( \mu \)l diluted sequenase™ (a 1 in 8 dilution of sequenase at 1 EU\( \mu \)l\(^{-1} \) in 10 mM Tris.HCl, pH 7.5; 5 mM DTT; 0.5 mg\( \mu \)ml\(^{-1} \) BSA). The extension mixture was left at room temperature for 2 to 5 min. Further extension and termination was performed by dispensing 3.5 \( \mu \)l of extension mix into four tubes preheated to 37°C containing 2.5 \( \mu \)l of one of the four termination mixes:

- ddGTP mix - 80 \( \mu \)M dNTPs; 8 \( \mu \)M ddGTP; 50 mM NaCl
- ddATP mix - 80 \( \mu \)M dNTPs; 8 \( \mu \)M ddATP; 50 mM NaCl
- ddCTP mix - 80 \( \mu \)M dNTPs; 8 \( \mu \)M ddCTP; 50 mM NaCl
- ddTTP mix - 80 \( \mu \)M dNTPs; 8 \( \mu \)M ddTTP; 50 mM NaCl

The termination reaction was allowed to proceed at 37°C for 5 minutes and the reaction was stopped by the addition of 4 \( \mu \)l of stop solution (95 % formamide; 20 mM EDTA; 0.05 % bromophenol blue). In some cases where extensive secondary structure occurred along the flavocytochrome \( b_2 \) DNA sequence causing premature termination in all four reaction mixes, the termination reactions were performed at 75°C instead of 37°C. Taq DNA polymerase (which is stable at this temperature) was added to the termination mixes at a final concentration of 1 EU. This ensured that the
DNA was fully denatured and eliminated any sequencing artefacts due to secondary structure.

Extension products were separated by electrophoresis through a 6 % denaturing polyacrylamide gel made up by mixing 25.2 g of urea, 6 ml 10 x TBE, 12.5 ml Protogel (30 % acrylamide; 0.8 % bis-acrylamide) made up to a final volume of 60 ml with water. Polymerisation of the acrylamide was achieved by adding 140 μl of 10 % ammonium persulphate and 140 μl of TEMED immediately prior to pouring the gel. Sequencing reactions were heat denatured at 75°C for 3 minutes, loaded onto the gel and electrophoresed in 1 x TBE buffer at 65 Watts for 1 hour 45 minutes to 6 hours. The gel was fixed in 10 % acetic acid (v/v), washed with water and then dried under vacuum at 80°C for 1 to 2 hours. The gel was then autoradiographed, overnight, at room temperature.

2.13. ONE DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

This technique was used to separate proteins according to their molecular weight, (Laemmli, 1970). The polyacrylamide gel was prepared in two phases, a resolving gel for the separation of the protein samples and a stacking gel for the concentration of the protein samples before separation. The resolving gel was mixed well, poured between two glass plates and overlaid with water saturated butanol. It was then left to polymerise for 1 hour. Once the resolving gel had polymerised the water saturated butanol was rinsed off with distilled water and the stacking gel was prepared. The stacking gel was mixed well and then poured on top of the resolving gel. A comb was then inserted into the top of the stacking gel and the gel was allowed to polymerise for 30 minutes. Once polymerised the gel was clamped into a vertical electrophoresis tank filled with 1 x running buffer. The comb was then carefully removed and the protein samples were loaded into the wells. The gel was then run at 10 vcm⁻¹ for 4 to 5 hours. The gels were stained with 1 % PAGE Blue Electran in 20 % (v/v) methanol, 5 % (v/v) acetic acid, to visualise the electrophoresed proteins.

2.13.1. Western transfer

An SDS-PAGE gel was run and then soaked in 1 x transfer buffer for two minutes. It was then assembled into a "sandwich" with the gel adjacent to a piece of nylon membrane (Hybond-N) placed between 2 by 2 layers of 3 MM filter paper and
foam sponge, (all pre-soaked in 1 x transfer buffer). The proteins were then transferred onto the membrane, by immersing the sandwich in a tank containing 1 x transfer buffer and passing a current of 1 Amp through it for 2 hours. The membrane was on the positive electrode side of the gel, since proteins migrate towards the positive electrode. The membrane was then dried or used immediately for immunodetection.

2.13.2. Filters blocked with skimmed milk proteins

The filters were blocked for 6 hours in 5 % (w/v) skimmed milk powder in TBS, at 25°C overnight. The solution was then replaced with 20 ml of 2 % (w/v) skimmed milk powder in TBS. The primary antibody, anti-flavocytochrome b2 polyclonal sera from rabbit (30 μl) was added and the membranes/filters incubated at 25°C for 2 hours. After incubation with the antibody the membrane was washed in 100 ml of TBS, four times for 5 minutes. The membrane was then placed in fresh skimmed milk powder in TBS (at the same relative concentration) and second antibody was added. After incubation for 2 hours the membrane was washed as before and then developed.

2.13.3. Primary antibody detection with HRP-conjugated secondary antibody

Horse radish peroxidase-conjugated goat anti-rabbit IgG (Scottish Antibody Production Unit) was used as the secondary antibody. Antibody (10-20 μl) was added to a small volume of the diluted blocking agent and incubated with the washed membranes at 25°C for 2 hours. The membranes were then washed thoroughly and developed by shaking the membrane in 10 ml of developing solution (0.5 ml of 5 mgml⁻¹ dianisidine, 1 ml of 0.1 M imidazole (pH 7.4), 0.1 ml of 30 % H₂O₂ and 8.4 ml of water) until an orange band appeared. Where larger membranes were used the volumes were scaled up accordingly. The reaction was stopped by rinsing the membrane in distilled water and left to dry. The developed membrane was photographed, as the colour tended to fade with time.

2.14. PURIFICATION OF FLAVOCYTOCHROME b₂

2.14.1. Growth and harvesting of E. coli cells over-expressing flavocytochrome b₂

Initially, about 50 ml of LB media containing 25 μgmL⁻¹ of the antibiotic carbenecillin as a selectable marker (LBcarb), was inoculated with a single bacterial
colony and incubated overnight at 37°C, with shaking, and the cells allowed to grow
to stationary phase. Carbenecillin is used in preference to ampicillin as it has longer
half-life and thus gives stonger selection for bacteria containing pDSb₂, resulting in
greater over-expression of flavocytochrome b₂.

A small volume (1.5 ml) of the stationary phase cells was centrifuged and the
colour of the pellet noted, pink (over-expressing) pellets were washed and
resuspended in 1 ml TE. The suspended cells were lysed by sonication for 30
seconds and 0.2 ml of assay buffer (10 mM L-lactate and 1 mM ferricyanide in TE)
was added to the sonicate to check for activity; rapid loss (< 30 seconds) of the
yellow ferricyanide colour as ferrocyanide was formed indicated that the expressed
protein was active.

One ml aliquots of the stationary phase cells were then added to 500 ml of
LBcarb and incubated overnight at 37°C, with shaking. Typically, 6 litres of culture
were grown. The stationary phase cells were harvested in a Sorvall RC-5B centrifuge
with a GS3 rotor run at 5000g for 20 minutes. The resulting 'wet pellet' of cells was
stored at -20°C until required, the yield was typically 5 g of wet cells per litre of
medium.

2.14.2. Cell lysis

The frozen pellet of cells containing over-expressed protein was snap-cooled
in liquid nitrogen to facilitate cell lysis and then resuspended in about 50 ml of
purification buffer per 5 g cells, the buffer is 0.1 M phosphate pH 7.0, with 1 mM
EDTA and 5 mM L-lactate. The EDTA chelates calcium ions released upon cell lysis
and the lactate maintains the flavocytochrome b₂ in its more stable reduced form.
Lysozyme was added to a concentration of approximately 0.2 mgml⁻¹ and the
mixture stirred for one hour at 4°C (the protein solution was maintained at 4°C
throughout the entire purification). After this time, the cell debris and any unlysed
cells were removed from suspension by centrifugation at 39,000g for 10 minutes. A
red/pink colouration of the supernatant indicated the presence of flavocytochrome b₂.
If the pelleted material was still fairly red in colour, then it was subjected to another
round of snap-freezing in liquid nitrogen and lysis. All the resulting supernatants
containing flavocytochrome b₂ were pooled.

2.14.3 Ammonium sulphate fractionation

This is the first stage of the purification and involves the principle of
selectively precipitating macromolecules out of solution by increasing the salt
concentration. Ammonium sulphate is generally used for this purpose as it has a high solubility and it does not significantly alter the pH of the solution.

After lysis, the supernatant was adjusted to 40 % ammonium sulphate saturation and the precipitated proteins removed by centrifugation at 39,000g for 10 minutes. This supernatant was then further adjusted to 70 % saturation, which is sufficient to precipitate flavocytochrome \( b_2 \) out of solution, and the protein was removed by repeating the centrifugation. The enzyme was sufficiently pure at this stage to be used for steady-state kinetic experiments. All the hinge-deletion flavocytochromes \( b_2 \) were preliminarily analysed at this stage, to ascertain their maximal activities while the samples were still fresh.

2.14.4. Dialysis

Prior to further purification by column chromatography, the protein has to be dialysed in order to remove the ammonium sulphate and any other low molecular weight contaminants. Seamless dialysis tubing with a molecular weight exclusion limit of 12 kDa was used. The 70 % ammonium sulphate precipitate of protein was redissolved in a minimum amount of purification buffer and sealed in dialysis tubing, which had been thoroughly rinsed in the buffer. The protein solution was dialysed overnight against a large volume (typically 10-fold greater than dialysis tubing volume) of half-strength buffer under nitrogen.

2.14.5. Column chromatography

Following dialysis, the protein solution was centrifuged at 39,000g for 5 minutes, to remove any insoluble material. Two columns were used to purify flavocytochrome \( b_2 \) to homogeneity; firstly, a DEAE ion-exchange column and then a hydroxyapatite column.

**Ion-exchange column**: the column material consists of diethyl-aminoethyl groups covalently cross-linked to a cellulose matrix. The material used was Whatman DE-52 which was prepared by pre-swelling in the purification buffer and adjusted the pH to 7.0 by addition of HCl. Typically, a 15 x 2.5 cm column was poured and equilibrated at 4°C. The protein solution was loaded onto the column and as flavocytochrome \( b_2 \) does not bind to DEAE, but passes straight through the column, protein fractions were collected immediately that the red/pink colour of flavocytochrome \( b_2 \) eluted.

The eluted fractions of flavocytochrome \( b_2 \) had a UV/Vis ratio \( (A_{269 \text{ nm}} / A_{423 \text{ nm}}) \) between 1.5 and 2.0, compared to a ratio of >10 after dialysis. This ratio is a measure of purity; the UV wavelength quantitates total protein and the Vis
wavelength quantitates flavocytochrome \(b_2\), a pure flavocytochrome \(b_2\) solution has a ratio of 0.5. The column material was discarded after use.

**Hydroxyapatite column:** Hydroxyapatite is the crystalline form of Ca\(_{10}(PO_4)_6\)OH\(_2\). Binding is believed to occur via the phosphate groups and bound proteins are eluted by increasing the ionic strength of the eluting buffer. As the column material consists of a majority of negatively charged groups, neutral and positively-charged molecules bind well, which explains why *S. cerevisiae* flavocytochrome \(b_2\) binds well.

The eluted protein fractions were loaded onto a 10 x 2.5 cm hydroxyapatite column, which had previously been equilibrated with two column volumes of purification buffer. Flavocytochrome \(b_2\) bound in a tight band at the top of the column and was washed with several column volumes of purification buffer. Flavocytochrome \(b_2\) was eluted using a 0 to 10 % ammonium sulphate gradient, the fractions being collected manually. Each fraction was assayed for degree of purity as described previously and generally fractions with a ratio <1 were pooled, adjusted to 70 % ammonium sulphate saturation and centrifuged at 39,000g for 10 minutes. The resulting pellets were then stored at 4°C under a nitrogen atmosphere. Wild-type flavocytochrome \(b_2\) could be kept for several weeks under these storage conditions, without suffering any significant loss of activity. However, the stability of the hinge-deletion flavocytochromes \(b_2\) was less than that of the wild-type enzyme (generally, the more severe the mutation, the lower the stability) and so experiments involving these enzymes were performed within days of purification.

Typical yields of purified wild-type flavocytochrome \(b_2\) were about 10 mg of enzyme per litre of culture media. However, the yield was generally lower for the hinge-deletion mutants, especially the HA9 enzyme (chapter 3, section 3.2).

The hydroxyapatite column was regenerated by washing with 200 ml of purification buffer containing 1 M NaCl and then re-equilibrated by washing with two column volumes of buffer. The column material was generally reused three times.

### 2.14.6. Modifications to standard purification procedure for hinge-deletion flavocytochromes \(b_2\)

The described purification procedure is ideal for wild-type flavocytochrome \(b_2\), however with the hinge-deletion enzymes, especially HA6 and HA9, significant activity loss occurred during purification. When the pure enzymes were assayed for the molar ratios of incorporated FMN to haem in the holoenzymes, they were found to be significantly less than the desired 1:1 ratio, typically, about 50 % FMN loss had...
occurred. Presumably, one effect of truncating the hinge-region of flavocytochrome $b_2$ is to increase the dissociation constant, $K_d$ for FMN (over many weeks, FMN loss is the major cause of activity loss for the wild-type enzyme).

To counteract this FMN loss, purification of the hinge-deletion enzymes was performed in buffer containing 10 $\mu$M FMN. As FMN is negatively charged, it binds to the DEAE column and is thus removed from the buffer, however the eluted flavocytochrome $b_2$ was immediately loaded onto a hydroxy-apatite column, where it binds tightly and is stable to FMN loss. The eluted, pure hinge-deletion enzymes are stored as ammonium sulphate pellets, as previously described.

**2.15. PREPARATION OF SUBSTRATES**

**2.15.1. L-[2-$^{1}$H] lactate**

All the solutions were made as standards by the addition of the required buffer (10 mM Tris/HCl, pH 7.5, I 0.10 for kinetic experiments) to the calculated amount of the lithium salt of L-lactic acid.

**2.15.2. Synthesis of L-[2-$^{2}$H] lactate**

A coupled enzymatic procedure was used, essentially by the method of Shapiro and Dennis (1965). The following starting solution was prepared:

50 ml volume:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM phosphate buffer, pH 7.0</td>
<td>to 49 ml</td>
</tr>
<tr>
<td>1.8 mM NAD</td>
<td></td>
</tr>
<tr>
<td>70 mM pyruvate</td>
<td></td>
</tr>
<tr>
<td>90 mM hexadeuterated ethanol</td>
<td>1 ml stock</td>
</tr>
<tr>
<td>2 mg yeast alcohol dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>12 mg beef heart lactate dehydrogenase</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 24 hours, with shaking, after which time the reaction was quenched by heating at 72°C for 3 minutes. The solution was then filtered and allowed to cool.

The deuterolactate produced was purified by anion exchange chromatography using Dowex 1 x 8-200 resin, which is strongly basic in nature. A 15 x 2.5 cm Dowex column, equilibrated in deionised water was used. The filtered solution was added to the column and fractions were collected immediately. The column was
washed with one volume of deionised water and then the deuterolactate was eluted using a linear, shallow gradient of 0 to 0.36 M formic acid in deionised water. The fractions were adjusted to pH 7.5 and crudely assayed for the presence of deuterolactate by taking a small sample of eluate (0.1 ml) and adding ferricyanide to 1 mM, along with a catalytic amount of wild-type flavocytochrome \( b_2 \) (approximately 1 nM), shaken and left for several minutes. The presence of deuterolactate is confirmed by the loss of the yellow ferricyanide colour, as ferrocyanide is formed.

As the pK\(_a\) of lactate is 3.73 and that of pyruvate is 2.39, lactate will elute ahead of the pyruvate as the pH of the column is progressively lowered by the formic acid concentration gradient, however the last fractions of lactate tend to be contaminated by pyruvate. The degree of contamination was assessed by use of a pyruvate diagnostic kit. All the pyruvate-free fractions were pooled and concentrated to about a 2 ml volume using a high vacuum trigol/carcide rotary evaporator. Isotopic purity was confirmed by nmr spectroscopy. The concentrated stock solution was diluted in 10 mM Tris.HCl, pH 7.5 (\( I \) 0.10) and the concentration of deuterolactate determined by adding a known volume to an assay solution containing 1 mM ferricyanide and a catalytic amount of wild-type flavocytochrome \( b_2 \) (1 nM). The total absorbance change, monitored at 420 nm using UV/Vis spectroscopy was used to calculate the stock concentration of \( L-[2-\text{2H}] \) lactate.

**2.16. DETERMINATION OF DEGREE OF PROSTHETIC GROUP INCORPORATION**

Flavocytochrome \( b_2 \), stored as a 70 % ammonium sulphate precipitate was dissolved in a minimum amount of 10 mM CAPS buffer, pH 11.0 (\( I \) 0.10). At this high pH the FMN prosthetic group fully dissociates from the protein. The dissociated FMN and protein-bound haem were separated by gel-filtration on a 15 x 1 cm Sephadex G-25 column, equilibrated and eluted with CAPS buffer at 4°C in darkness (to prevent photo-dissociation of the FMN). The protein-bound haem and the FMN fractions were well resolved and collected separately. The molar amounts of FMN and protein-bound haem were determined by their known visible absorption maxima of 12,500 M\(^{-1}\)cm\(^{-1}\) at 450 nm and 183,000 M\(^{-1}\)cm\(^{-1}\) at 423 nm respectively.
2.17. STEADY-STATE KINETIC ANALYSIS

2.17.1. General background

In enzyme kinetics, steady-state behaviour refers to dynamic situations, where the rate of formation of product is balanced by the rate of removal of substrate. This is an approximation, as the substrate is being depleted with time, but as activity measurements are recorded over short time periods, any concentration changes are negligible and the steady-state is a good approximation.

The initial rate, \( V \), of an enzyme catalysed reaction tends towards a maximal, limiting value, \( V_{\text{max}} \), with increasing substrate saturation. This kinetic behaviour can be described by the basic equation governing enzyme kinetics, the Michaelis-Menten equation:

\[
V = \frac{V_{\text{max}} [S]}{[S] + K_m}
\]

The kinetic parameters which can be determined experimentally are; \( k_{\text{cat}} \), the catalytic turnover number, which represents the maximum number of substrate molecules converted to product per active site per unit time (s\(^{-1}\)), and \( K_m \), the Michaelis constant which in some cases is effectively \( K_s \), the dissociation constant for the enzyme-substrate complex (M). \([S]\) is the substrate concentration (M).

In some cases, substrate inhibition was observed at higher substrate concentrations (chapter 3, sections 3.3.2 and 3.4.2). In these situation, plots of rate constants against substrate concentration were fit to the equation described below:

\[
V = \frac{(V_{\text{max}} [S])}{[S] \{1 + (\frac{[S]}{K_i})\} + K_m}
\]

The kinetic parameters are the same as the Michaelis-Menten equation, except an additional parameter, \( K_i \) has been introduced, which is the substrate inhibition constant.

2.17.2. Kinetic parameter determination

Flavocytochrome \( b_2 \) catalyses a two electron oxidation and is capable of transferring electrons two a wide variety of external electron acceptors. The turnover of the enzyme under steady-state conditions can thus be monitored by observing the reduction of an external electron acceptor under saturating conditions. The physiological electron acceptor is cytochrome \( c \), however, artificial electron...
acceptors such as ferricyanide can also be used to provide useful kinetic information (see chapter 3, section 3.3.1 and chapter 4, section 4.3.4 for details).

Experiments were performed using either a Beckman DU62 or a Shimadzu 2101PC spectrophotometer. All kinetic experiments were carried out at 25 ± 0.1°C in 10 mM Tris/HCl at pH 7.5 (I 0.10) and the assays were performed in high quality glass or quartz cuvettes with either 1 or 0.2 cm path-lengths.

The kinetic parameters, $k_{cat}$ and $K_m$ (in some cases, $K_i$ and $[S]_{opt}$ as well) for substrate were determined by carrying out assays at varying substrate concentration over a suitable range. This is generally 0 to 15 mM substrate, under saturating conditions of electron acceptor and vice-versa to elucidate the $K_m$ for the electron acceptor.

2.17.3. Ferricyanide as the acceptor

Assays were performed at saturating concentrations for ferricyanide. However, due to the high $K_m$ values of the hinge-deletion flavocytochromes $b_2$ for this electron acceptor, compared to the value for the wild-type enzyme, assays were performed at 2 and 8 mM ferricyanide concentration for the HA3 and HA6/HA9 enzymes respectively (see chapter 3, section 3.3.1 for full details). This necessitated all the assays being performed in 0.2 cm path-length cuvettes, for the hinge deletion enzymes. Activities were calculated from the absorbance decrease at a wavelength of 420 nm, using an extinction coefficient of $\varepsilon_{\text{ox-red}} = 1010 \text{ M}^{-1}\text{cm}^{-1}$.

2.17.4. Cytochrome c as the acceptor

Horse heart cytochrome c (type VI - Sigma) was used in the assays and was always freshly made up in the required buffer prior to use. Concentrations were determined by measuring the absorbance at 550 nm of dithionite reduced cytochrome c, using an extinction coefficient $\varepsilon_{\text{red}} = 30,900 \text{ M}^{-1}\text{cm}^{-1}$. Assays were performed in both 0.2 and 1 cm path-length cuvettes. The cytochrome c assay concentration used was 35 μM for all three hinge-deletion flavocytochromes $b_2$ (see chapter 3, section 3.3.2 for full details). Activities were calculated from the absorbance increase at a wavelength of 550 nm, using the extinction coefficient $\varepsilon_{\text{ox-red}} = 22,640 \text{ M}^{-1}\text{cm}^{-1}$.

2.17.5. Protein concentration determination

All flavocytochrome $b_2$ and cytochrome c concentrations were determined spectrophotometrically, using previously published extinction coefficients. The majority of the flavocytochrome $b_2$ concentrations were measured using the Soret
peak at 423 nm in the reduced spectrum, which has an extinction coefficient $\varepsilon_{\text{red}} = 183,000 \text{ M}^{-1}\text{cm}^{-1}$.

2.18. STOPPED-FLOW KINETIC ANALYSIS

2.18.1. General background

The stopped-flow technique allows the analysis of a single enzymatic turnover in a pre-steady-state situation. The principle of the technique is that small, equal volumes of enzyme and substrate are rapidly mixed and the time lag between the mixing and reaction monitoring is known as the dead time of the apparatus, which is typically $\leq 1 \text{ ms}$. After this, the solution ages normally with time and the reaction can be monitored by suitable spectroscopic methods. In the case of flavocytochrome $b_2$, stopped-flow analysis can give useful information regarding a number of electron transfer steps; from substrate to FMN (monitoring FMN reduction by lactate), interdomain electron transfer from FMN to haem (monitoring haem reduction by lactate, this will be explained in chapter 3, section 3.4.2) and interprotein electron transfer between flavocytochrome $b_2$ haem and cytochrome $c$ (monitoring cytochrome $c$ haem at a flavocytochrome $b_2$ isosbestic).

2.18.2. Practical details

All presteady-state kinetic experiments were performed on an Applied Photophysics SF.17 Micro Volume stopped-flow spectrofluorimeter. Data were collected, displayed in the absorbance mode and were processed using the SF.17MV spectrofluorimeter software package.

2.18.3. Preparation of enzyme

Flavocytochrome $b_2$ was prepared for stopped-flow kinetics by dissolving the 70 % ammonium sulphate protein precipitate in a minimal volume of 10 mM Tris.HCl buffer, pH 7.5 ($I$ 0.10). The concentrated enzyme solution was then loaded onto a 1.5 x 10 cm Sephadex G25 column, equilibrated and eluted with Tris buffer, to remove salts and lactate. The eluted enzyme was fully oxidised.

2.18.4. Presteady-state oxidation of L-lactate

Experiments were performed in Tris buffer (section 2.19.3) at a temperature of $25 \pm 0.1^\circ\text{C}$. Reduction of the FMN prosthetic group was monitored at 438.3 nm, which is a haem isosbestic and haem reduction was monitored at either 423 or 557
nm (the results were identical at both wavelengths). The software package facilitated analysis of the data by non-linear regression analysis, including multiexponential processes. The data could then be used to calculate the kinetic parameters as described in section 2.17.2.

2.18.5. Pre-steady-state reduction of cytochrome c

The pre-steady-state reduction of cytochrome c (both horse heart and yeast) by fully pre-reduced flavocytochrome \( b_2 \) was monitored with the stopped flow apparatus. The reaction was followed at 416.5 nm, which is an isosbestic for flavocytochrome \( b_2 \). Flavocytochrome \( b_2 \) was prepared for such experiments as described above, except that the enzyme, which eluted from the G25 column in the oxidised state, was then fully reduced by the addition of L-lactate (>1.0 mM). Experiments were performed under aerobic conditions as auto-oxidation of flavocytochrome \( b_2 \) did not occur to any significant extent over the time scale of the experiment (2 to 3 hours). Cytochrome \( c \) was fully oxidised by addition of a few crystals of ferricyanide and the excess oxidant was removed by passing the mixture through a G25 column, equilibrated and eluted with Tris buffer.

To ensure that the reduction of cytochrome \( c \) occurred under pseudo-first-order conditions, flavocytochrome \( b_2 \) was always present in excess. Reduction was carried out over a wide range of flavocytochrome \( b_2 \) concentrations (1.5 to 15 \( \mu \)M). Cytochrome \( c \) concentration was 0.75 \( \mu \)M. The data was processed as described above.

2.19. REDOX POTENTIOMETRY

2.19.1. General background

The redox mid-point potential of a redox couple is a measure of its tendency to donate or accept electrons. The value gives an indication of the relative stability of the oxidised and reduced states. Any factor which results in the stabilisation of the reduced form will make the couple a better electron acceptor and give rise to a more positive redox potential. It is important to consider the redox potentials of the prosthetic groups in flavocytochrome \( b_2 \) mutants, to be sure that any kinetic differences observed between mutant and wild-type enzymes are 'real' and not due to an altered redox potential.

The method described by Dutton (1978) coupled with specrophotometry was used to measure the haem mid-point potentials of all three hinge-deletion
flavocytochromes $b_2$. Measurements were carried out with a platinum working electrode used in conjunction with an Ag/AgCl reference electrode, under anaerobic conditions.

2.19.2. Preparation of redox standard solutions

Fe (III)/EDTA solution: EDTA was dissolved in 50 ml of dH$_2$O to 40 mM concentration and the pH adjusted to 7.0. Iron (III) ammonium sulphate was dissolved in this solution to 2 mM concentration by heating gently.

Fe (II) solution: Iron (II) ammonium sulphate was dissolved in 10 ml of degassed dH$_2$O to 100 mM concentration and maintained under strict anaerobic conditions.

Dithionite solution: 30 mg of sodium dithionite was dissolved in 5 ml of degassed 0.1 M phosphate buffer, pH 7.0 and maintained under strict anaerobic conditions.

Ferricyanide solution: 30 mg of potassium ferricyanide was dissolved in 5 ml of 10 mM Tris/HCl buffer, pH 7.5 (I 0.10M).

2.19.3. Redox mediators

Redox centres of biological molecules are often shielded from solvent by the protein framework and this can impede effective contact with the electrode surface. Redox mediators are small organic or inorganic reagents which act as fluxes between the redox couple and the measuring electrode. The mediators must be stable, react reversibly and not chemically modify the redox couple. The mediators used have $E_m$ values cover the range of the redox couple(s) $E_m(s)$.

The mediators used for the redox potential determination of the haem group of flavocytochrome $b_2$ are listed below. 5 mM stock solutions were prepared in dH$_2$O (with 20 % ethanol for DAD and HNQ) and were foil wrapped to prevent light induced decomposition.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>$E_m$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>flavin mononucleotide (FMN)</td>
<td>-200</td>
</tr>
<tr>
<td>2-hydroxy-1, 4-napthaquinone (HNQ)</td>
<td>-140</td>
</tr>
<tr>
<td>phenazine methosulphate (PMS)</td>
<td>+60</td>
</tr>
<tr>
<td>phenazine ethosulphate (PES)</td>
<td>+80</td>
</tr>
<tr>
<td>2, 3, 5, 6-tetramethyl-p-phenylenediamine (DAD)</td>
<td>+220</td>
</tr>
</tbody>
</table>
2.19.4. Calibrating the electrode

The calibration solution used was 5 ml of 0.3 mM Fe (III), 8 mM EDTA and 0.25 M acetate buffer at pH 5.0. This solution was thoroughly degassed with water saturated nitrogen, prior to (for 10 minutes) and during calibration. To calibrate, 10 µl of Fe (II) solution was injected and the potential was recorded. A further 15 µl was injected, bringing the solution to a concentration of 0.5 mM Fe (III), and the potential was recorded. These data were plotted on a Nernst plot, from which the values of the slope and intercept could be calculated, which should ideally be +59 and +108 mV respectively, for a well calibrated electrode.

2.19.5. Measurement of the haem redox potential

Oxidised flavocytochrome b₂ was prepared exactly as described in section 2.17.3. After calibration of the electrode had been achieved, the following solution was prepared:

stock flavocytochrome b₂ solution 10 to 20 µM
35 µl 2 mM Fe (III)/40 mM EDTA solution 20 µM
10 µl of each mediator 14 µM
Tris buffer to 3.5 ml

This solution was thoroughly degassed with water saturated nitrogen for 1 hour prior to and during the entire experiment. The solution was stirred by means of a magnetic 'flea'.

Flavocytochrome b₂ was reduced by titrating with the anaerobic dithionite solution. Visible absorption spectra were sequentially recorded over the range 530 to 570 nm, at a scan speed of 2 nm s⁻¹ and the potentials were noted each time for the haem α-peak at 557 nm. Flavocytochrome b₂ was oxidised by titrating with the ferricyanide solution and the potentials recorded as before. All the potentials were corrected for the Ag/AgCl reference electrode so that they were relative to the standard hydrogen electrode (SHE), $E_h = E + 196$ mV.

The ratios of [ox]/[red] flavocytochrome b₂ were determined from the absorption spectra and the data presented as a Nernst plot of $\log_{10} [\text{ox}]/[\text{red}]$ against $E_h$, for both the reductive and oxidative experiments (Nernst equation is shown overleaf). If the system is in equilibrium, then both of these plots should overlay with a slope equal to $+59 \pm 10$ mV. The haem mid-point potential is calculated from the intercept of these plots with the $E_h$-axis.
\[ E = E_h + \frac{RT}{nF} \ln\left(\frac{[\text{ox}]}{[\text{red}]}\right) \] for the redox couple: \( \text{ox} + n\text{e}^- = \text{red} \)

Where, \( E \) is the measured potential (V), \( E_h \) is the mid-point potential (V), \( R \) is the molar gas constant (J mol\(^{-1}\)K\(^{-1}\)), \( T \) is the absolute temperature (K), \( F \) is the Faraday constant (C mol\(^{-1}\)), \( n \), is the number of electrons, \( [\text{ox}] \) is the concentration of the oxidised species, \( [\text{red}] \) is the concentration of the reduced species.

2.20. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

2.20.1. General background

Nuclear magnetic resonance (nmr) spectroscopy serves a continually expanding role in biology, especially with regard to the structure determination of macromolecules in solution (Wüthrich 1986). Such studies are becoming increasingly sophisticated with regard to the methodology of structure determination and the amount of useful information that can be extracted from the spectra (MacArthur et al., 1994). Nmr spectroscopy has a wide range of other applications and it can be used to give useful information concerning interprotein interactions; it is to this end that the technique has been exploited to obtain fundamental information about the flavocytochrome \( b_2 \)-cytochrome \( c \) redox complex. More specifically, the stoichiometry and affinity of binding of cytochrome \( c \) to flavocytochrome \( b_2 \) (see chapter 3, section 3.7.1 for details).

2.20.2. Practical details

All nmr spectra were recorded on a Varian VXR 600S spectrometer operating at 600 MHz, at a temperature of 25°C and in \( D_2O \) as solvent. All chemical shifts are quoted relative to the standard 4, 4-dimethyl-4-silapentane sodium sulphonate (DSS). The samples were contained in high quality 7” nmr tubes (Wilmad). All one-dimensional (1D) spectra were recorded using a standard one dimensional pulse sequence (1.5 s saturation pulse, followed by a 5 μs excitation pulse at 90° to the saturation pulse. The data were then acquired for 1.8 s after the excitation pulse). In a typical experiment, either 256, 512 or 1024 separate acquisitions of data were performed, depending on the signal to noise ratio (see later). The acquired data was averaged, Fourier transformed (FT), usually with 2 to 10 Hz linewidth broadening to smooth the signal, and analysed using the Varian VXR 600S software.
2.20.3. Sample preparation

Flavocytochrome $b_2$: Pure protein, stored as a 70 % ammonium sulphate precipitate was dissolved in a minimum amount of 20 mM phosphate buffer, pH 7.0 in D$_2$O. The solvent was exchanged three times using a centricon-10 microconcentrator (Amicon) by centrifuging at 4,500g, at 4°C, for about 1 hour after each buffer addition. Protein recovery from the membrane was achieved by inversion of the centricon and centrifugation at 300g for 2 minutes. The flavocytochrome $b_2$ sample was fully oxidised.

Cytochrome $c$: Freeze-dried horse heart cytochrome $c$ was prepared in the same way as flavocytochrome $b_2$ and 0.8 ml of 0.1 mM oxidised cytochrome $c$ solution was placed in the nmr tube.

A stock solution was prepared, containing 1.0 mM flavocytochrome $b_2$ subunit and 0.1 mM cytochrome $c$.

2.20.4. Experimental details

The cytochrome $c$ solution was placed in the nmr spectrometer, the sample manually shimmed in the x, y and z planes, to ensure that the applied magnetic field was uniform in nature and the water peak removed by pulse saturation. Initially, 256 data acquisitions were collected for the oxidised cytochrome $c$ solution over the chemical shift range -10 to 40 ppm, in one dimension. These data were averaged, Fourier transformed with 2 Hz linewidth broadening and analysed.

It is important that the sample remain oxidised at all times, as the cytochrome $c$ haem resonances of interest are paramagnetically shifted away from the bulk of the protein backbone and side chain resonances, by the ferric iron of the haem. If the sample were to be reduced, then these resonances would no longer occur.

The chemical shifts of haem methyl resonances 3 and 8 were measured accurately (under the experimental conditions, these were typically 32.5 and 35.5 ppm, respectively). It is the change in chemical shift of these resonances ($\Delta\delta$), when flavocytochrome $b_2$ is titrated into the cytochrome $c$ sample that is monitored throughout the experiment. Flavocytochrome $b_2$ has no paramagnetically shifted resonances and so does not interfere with the cytochrome $c$ spectrum.

The nmr tube was removed and a small aliquot of the stock solution (section 2.19.3) added, such that the total concentration of flavocytochrome $b_2$ in the nmr tube is accurately known (the concentration of total cytochrome $c$ will always be 0.1 mM). The sample was well mixed, placed back into the nmr spectrometer, reshimmed automatically in the z-plane (the shims in the x and y planes did not need to be adjusted, as only the depth of the sample had changed) and a further 256 acquisitions.
collected. The data was analysed as before and any change in the haem methyl $\Delta\delta$ values noted. In a typical experiment, about 20 aliquots were added and the haem methyl $\Delta\delta$ values recorded in each case. For flavocytochrome $b_2$ subunit:cytochrome $c$ stoichiometries less than 1:3, 256 data acquisitions were performed. However, above this stoichiometry, the signal to noise ratio increased so, 512 acquisitions were collected to improve the signal strength. At even higher stoichiometries of 2:1 flavocytochrome $b_2$ subunits:cytochrome $c$, 1024 acquisitions needed to be collected (the signal to noise ratio improves by a factor proportional to the square root of the number of data acquisitions performed).

2.20.5. Data analysis

The stoichiometry of cytochrome $c$ binding to flavocytochrome $b_2$ was analysed by plotting the change in chemical shift of the cytochrome $c$ haem methyl resonances ($\Delta\delta$) against the ratio of flavocytochrome $b_2$ tetramer to cytochrome $c$ concentration ([fcb$_2$ tet]/[cyt $c$]) and the curve fitted manually as described in chapter 3, section 3.7.3. The affinity of cytochrome $c$ binding to flavocytochrome $b_2$ was evaluated either by plotting the cytochrome $c$ haem $\Delta\delta$ values against flavocytochrome $b_2$ subunit concentration, or by a double reciprocal plot of the above parameters. The data were fitted to the equation shown below and analysed as described in chapter 3, section 3.7.4.

$$\Delta\delta = \frac{\Delta\delta_{\text{max}} \text{[fcb}_2\text{ subunit]}}{(K + \text{[fcb}_2\text{ subunit]})}$$

Where, $\Delta\delta$ is the change in chemical shift, $\Delta\delta_{\text{max}}$ is the maximal change in chemical shift upon complexation, $K$ is the apparent dissociation constant (chapter 3, section 3.7.4) and [fcb$_2$ subunit] is the concentration of flavocytochrome $b_2$ subunit.
Chapter 3

Results
3.1. INTRODUCTION.

This chapter describes the construction, over-expression, purification and subsequent characterisation of the three hinge-deletion flavocytochromes $b_2$, to determine the consequences of sequentially truncating the interdomain hinge region.

Sections 3.3 to 3.5 describe the results of the steady- and pre-steady-state kinetic experiments used to investigate the effect of these mutations on intra-protein (interdomain: FMN→haem) electron transfer, the formation of a catalytically competent electron-transferring complex between flavocytochrome $b_2$ and cytochrome $c$, and the subsequent inter-protein electron transfer. Section 3.6 gives the results of potentiometric experiments used to measure the effect of the imposed structural changes (the hinge deletions) upon the mid-point potentials of the flavocytochrome $b_2$ prosthetic groups.

Additionally, section 3.7 describes the results of preliminary experiments involving the use of nmr spectroscopy to investigate the stoichiometry and affinity of cytochrome $c$ binding to wild-type and HΔ6 flavocytochromes $b_2$ (the equivalent experiments on HΔ3 and HΔ9 have yet to be done), to test the proposed theories for the nature of the electron-transferring complex between flavocytochrome $b_2$ and cytochrome $c$ (Tegoni et al., 1993).

3.2. CONSTRUCTION AND EXPRESSION OF HINGE-DELETION FLAVOCYTOCHROMES $b_2$.

The three hinge-deletion mutants of flavocytochrome $b_2$ are termed, HΔ3, HΔ6 and HΔ9, with 3, 6 and 9 amino acids deleted from the interdomain hinge region respectively. These mutants were constructed in the M13 phagemid/yeast shuttle vector pGR401 (Reid et al., 1988) by the Kunkel method of non-phenotypic selection (Kunkel, 1985), as described in chapter 2, section 2.11.

The entire 1.8 kb mature flavocytochrome $b_2$ coding region was sequenced for all three hinge-deletion mutants, as described in chapter 2, section 2.12 to ensure that during the site-directed mutagenesis procedure, no secondary mutations had been introduced elsewhere in the gene.

The mutant constructs were excised from pGR401 and subcloned into the bacterial transcription vector pDS6, which directs the over-expression of flavocytochrome $b_2$ constructs (Black et al., 1989b). All three constructs were successfully over-expressed in strain TG1 of the bacterium Escherichia coli, as
Figure 3.1. Western Blot of Whole Cell Extracts of *E. coli* strains TG1 and AR120 Over-Expressing the Three Hinge-Deletion Flavocytochromes b₂.

Conditions were as described in chapter 2, section 2.13. The numbers 3, 6 and 9, denote HA3, HA6 and HA9 flavocytochromes b₂ respectively. The larger bands at about 57 kDa correspond to intact flavocytochrome b₂ subunits and the smaller bands correspond to proteolysed b₂ peptide.
indicated by the colour of the colonies when grown on the appropriate selective media (Black et al., 1989b). The degree of over-expression varied between the three mutant enzymes, with HΔ3 and HΔ6 expressing to a greater extent than HΔ9. This was inferred from the colour of the colonies and by the yield of pure flavocytochrome $b_2$ from a known mass of $E. coli$ cells.

Western blotting of whole cell extracts and SDS/PAGE of the purified proteins was used, as described in chapter 2, section 2.13, to check the degree of proteolysis of the flavocytochrome $b_2$ constructs in the $E. coli$ cytosol. It is well established for the wild-type enzyme that proteolysis causes alterations in the kinetic behaviour (Jacq & Lederer, 1972). Significant proteolysis of the HΔ6 and HΔ9 mutants occurred in the TG1 strain, as opposed to HΔ3 flavocytochrome $b_2$, which did not appear to be proteolysed to the same extent. To minimise this problem, the protease deficient $E. coli$ strain AR120 was used for the subsequent expression of the HΔ6 and HΔ9 mutants. Figure 3.1 shows a Western blot of the total cell extract for the three constructs expressed in the two different $E. coli$ strains. Initial characterisation of the HΔ3 mutant was performed on the enzyme over-expressed and purified from the TG1 $E. coli$ strain. However, there was no difference in the activity of HΔ3 flavocytochrome $b_2$, whether it was over-expressed in TG1 or AR120.

3.3. STEADY-STATE KINETIC PROPERTIES OF THE HINGE-DELETION FLAVOCYTOCHROMES $b_2$.

Table 3.1 presents the results of the steady-state kinetic measurements on HΔ3, HΔ6 and HΔ9 flavocytochromes $b_2$ using $^1$H-lactate and $^2$H-lactate (L-[2-$^1$H] lactate and L-[2-$^2$H] lactate respectively) as substrates and with ferricyanide (Table 3.1.1) and cytochrome $c$ (Table 3.1.2) as electron acceptors. These results are compared to previously reported values for the wild-type enzyme.

3.3.1. Ferricyanide as the electron acceptor

From the data in Table 3.1.1, it is clear that all three hinge-deletion mutants are good L-lactate dehydrogenases as judged by the $k_{cat}$ with ferricyanide as electron acceptor; only a 40% decrease for HΔ3 and HΔ6, whereas, in the case of HΔ9 the $k_{cat}$ is identical to that of wild-type flavocytochrome $b_2$ (see Figure 3.2.1 for an example of a Michaelis-Menten fit of the kinetic data for HΔ3 flavocytochrome $b_2$). The $K_m$ values for $^1$H-lactate fall in value from HΔ3 through to HΔ9, with HΔ6 having the same value as wild-type flavocytochrome $b_2$,
although these $K_m$ values for the hinge-deletion mutants are not strikingly different to that observed for the wild-type enzyme. The ratio of $k_{cat}/K_m$ gives a measure the catalytic efficiency of an enzyme and comparing these values for $\text{H}\Delta 3$ and $\text{H}\Delta 6$ to that of wild-type flavocytochrome $b_2$ show that they are 40 % and 70 % of the value for the wild-type enzyme respectively. The $k_{cat}/K_m$ for $\text{H}\Delta 9$ however, is the same within experimental error as the value reported for wild-type flavocytochrome $b_2$. In the wild-type enzyme, $\alpha$H-abstraction from C-2 of L-lactate is the major rate-limiting step in the catalytic cycle (Pompon et al., 1980) and from the data presented in Table 3.1.1, it can be seen that $\text{H}\Delta 6$ has the same deuterium kinetic isotope effect (KIE), within experimental error, as wild-type flavocytochrome $b_2$. The KIE values for $\text{H}\Delta 3$ and $\text{H}\Delta 9$ are the same within experimental error and are slightly lower than the value for the wild-type enzyme, implying that other factors caused by the mutations, aside from $\alpha$H-abstraction at C-2 of L-lactate also contribute to rate-limitation.

### 3.3.2. Cytochrome $c$ as the electron acceptor

The ferricyanide reduction data illustrate that there are no major kinetic differences between wild-type and hinge-deletion flavocytochromes $b_2$ when this inorganic anion is used as an electron acceptor. However, when the physiological electron acceptor, cytochrome $c$ is used, the values of $k_{cat}$ for $\text{H}\Delta 3$, $\text{H}\Delta 6$ and $\text{H}\Delta 9$ flavocytochromes $b_2$ are significantly decreased by factors of 5-, 6- and 26-fold, with respect to the value for the wild-type enzyme (Table 3.1.2 and see Figure 3.2.2 for an example of a Michaelis-Menten fit of the kinetic data for $\text{H}\Delta 3$ flavocytochrome $b_2$). The fact that $k_{cat}$ values differ depending upon whether ferricyanide or cytochrome $c$ is used as electron acceptor implies that electron flow to these acceptors has been affected in different ways by the hinge deletions, with electron flow to cytochrome $c$ being impaired in all three hinge-deletion enzymes. Thus, the three hinge-deletion flavocytochromes $b_2$ are all good L-lactate dehydrogenases, but are poor cytochrome $c$ reductases.

Values of $K_m$ for $1^H$-lactate show a steep fall from $\text{H}\Delta 3$ through to $\text{H}\Delta 9$ when compared to the wild-type value. $\text{H}\Delta 3$ has the same $K_m$ within experimental error as wild-type flavocytochrome $b_2$, however, the values for $\text{H}\Delta 6$ and $\text{H}\Delta 9$ are significantly decreased (3- and 12-fold lower respectively). The catalytic efficiencies ($k_{cat}/K_m$) for the hinge-deletion enzymes are lower than wild-type flavocytochrome $b_2$ by factors of 5- and 2.5-fold for $\text{H}\Delta 3$ and $\text{H}\Delta 6/\text{H}\Delta 9$ respectively. The $k_{cat}/K_m$ value for $\text{H}\Delta 3$ is lower than that observed for both $\text{H}\Delta 6$ and $\text{H}\Delta 9$ as the decrease
Figure 3.2.1. Michaelis-Menten Plot of Steady-State Reduction of Ferricyanide by HΔ3 Flavocytochrome $b_2$

![Michaelis-Menten Plot of Steady-State Reduction of Ferricyanide by HΔ3 Flavocytochrome $b_2$](image)

Figure 3.2.2. Michaelis-Menten Plot of Steady-State Reduction of Cytochrome $c$ by HΔ3 Flavocytochrome $b_2$

![Michaelis-Menten Plot of Steady-State Reduction of Cytochrome $c$ by HΔ3 Flavocytochrome $b_2$](image)
Figure 3.3. Substrate Inhibition Plot of Steady-State Cytochrome c Reduction by HΔ6 Flavocytochrome $b_2$
in the $k_{cat}$ for cytochrome $c$ reduction is not compensated by a corresponding decrease in the $K_m$.

The deuterium KIE values for the hinge-deletion mutants when cytochrome $c$ is used as electron acceptor, are lower than the value reported for wild-type flavocytochrome $b_2$, with the exception of HA6, which is the same within experimental error. When the KIE values for both electron acceptors are considered, it is clear that HA3 and HA9 have significantly lower values compared to the wild-type enzyme. This implies that electron-transfer reactions following $\alpha$H-abstraction from C-2 of L-lactate contribute to overall rate limitation in these enzymes to a greater extent than in the wild-type enzyme. Interestingly, the HA6 enzyme has the same KIE values within experimental error as wild-type flavocytochrome $b_2$ for both electron acceptors. This implies that there is no significant change in the nature of the rate-determining step that can be detected by macroscopic (steady state) kinetics. Clearly, electron flow to cytochrome $c$ has been affected in HA6, but this is due to a decrease in the microscopic rate constant for haem reduction (see section 3.2.2), compared to that for wild-type flavocytochrome $b_2$.

An interesting observation regarding the kinetic behaviour of HA6 and HA9 flavocytochromes $b_2$ when cytochrome $c$ is used as the electron acceptor, is the marked substrate inhibition exhibited by these mutant enzymes over the L-lactate concentration range investigated (Table 3.1.2 and Figure 3.3). The degree of inhibition was much smaller over an equivalent concentration range for both HA3 and wild-type flavocytochrome $b_2$ (Table 3.1.2). Also no significant inhibition was observed for any mutant or wild-type enzyme when ferricyanide was used as the electron acceptor. This kinetic phenomenon, which becomes more apparent as the length of the hinge region is progressively truncated (the inhibition constant, $K_i$ falls from $92 \pm 5$ mM to $21.1 \pm 2.2$ mM and $14.0 \pm 1.5$ mM for HA3, HA6 and HA9 respectively) may be due to greater accessibility of lactate to the active site for these hinge-deletion enzymes, compared to wild-type flavocytochrome $b_2$. This point will be further discussed in chapter 4, section 4.3.3.

Another significant difference in the steady-state kinetic behaviour between the hinge-deletion and wild-type flavocytochromes $b_2$ is the dependence of the reaction rates on electron acceptor concentration (Table 3.1.3). The most obvious difference is found with ferricyanide, under the experimental conditions used in this study (10 mM Tris/HCl, pH 7.5, $I = 0.10$ M) no concentration dependence was observed above 100 $\mu$M ferricyanide. The hinge-deletion enzymes however, had
Tables 3.1. Steady-State Kinetic Parameters and Deuterium Kinetic Isotope Effects for Wild-Type and Hinge-Deleted Flavocytochromes $b_2$.

All experiments were performed at 25°C in 10 mM Tris/HCl buffer, pH 7.5, ionic strength ($I$) adjusted to 0.10 M by the addition of NaCl. Concentrations of electron acceptors used with the hinge deletion and wild-type flavocytochromes $b_2$ were as follows: [ferricyanide], wild-type = 1 mM, HΔ3 = 2 mM, HΔ6 and HΔ9 = 8 mM (these concentrations were greater than 90% saturating in all cases); [cytochrome $c$], wild-type = 30 μM (>75% saturating), HΔ3, HΔ6 and HΔ9 = 35 μM (>80% and >90% saturating for HΔ3 and HΔ6/HΔ9, respectively). The $k_{cat}$ values are expressed as moles of electrons transferred per second per mole of enzyme (as L-lactate is a two-electron donor, these values can be halved to express them in terms of moles of substrate reduced per second).

Table 3.1.1. Ferricyanide as Electron Acceptor

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (x10$^5$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^1$H-lac</td>
<td>$^2$H-lac</td>
<td>$^1$H-lac</td>
</tr>
<tr>
<td>wild-type</td>
<td>400 ± 10</td>
<td>86 ± 5</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td>HΔ3</td>
<td>257 ± 10</td>
<td>70 ± 2</td>
<td>0.72 ± 0.07</td>
</tr>
<tr>
<td>HΔ6</td>
<td>276 ± 10</td>
<td>61 ± 6</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>HΔ9</td>
<td>400 ± 10</td>
<td>120 ± 10</td>
<td>0.37 ± 0.04</td>
</tr>
</tbody>
</table>

### Table 3.1.2. Cytochrome c as Electron Acceptor

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ ($\times 10^5$ M$^{-1}$s$^{-1}$)</th>
<th>[L-lactate] (mM)</th>
<th>$k_{\text{cat}}/K_m$ ($\times 10^5$ M$^{-1}$s$^{-1}$)</th>
<th>$K_i$ (mM)</th>
<th>KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type$^a$</td>
<td>207 ± 10</td>
<td>70 ± 10</td>
<td>0.24 ± 0.04</td>
<td>8.6 ± 2.3</td>
<td>1.5 ± 0.6</td>
<td>6.2 ± 0.6</td>
<td>173 ± 13</td>
</tr>
<tr>
<td>HA3$^b$</td>
<td>39 ± 1</td>
<td>20 ± 1</td>
<td>0.25 ± 0.03</td>
<td>1.6 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>4.5 ± 0.3</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>HA6</td>
<td>33.5 ± 1.4</td>
<td>14.0 ± 0.4</td>
<td>0.09 ± 0.01</td>
<td>3.7 ± 0.7</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>21.1 ± 2.2</td>
</tr>
<tr>
<td>HA9</td>
<td>7.9 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td>0.02 ± 0.01</td>
<td>3.4 ± 2.1</td>
<td>N.D.</td>
<td>0.6 ± 0.2</td>
<td>14.0 ± 1.5</td>
</tr>
</tbody>
</table>

Table 3.1.3. Values of $K_m$ for the electron acceptors ferricyanide and cytochrome c

The L-lactate concentration was 10 mM throughout, except for experiments with HA6 and HA9 flavocytochromes $b_2$, using cytochrome c as the electron acceptor. In these cases, [L-lactate] was 1 mM, which is close to the optimum substrate concentration.
Km values for ferricyanide which increased as the length of the hinge region was progressively truncated (Table 3.1.3). This necessitated kinetic assays being performed at higher ferricyanide concentrations for the hinge-deletion enzymes than is routine for wild-type flavocytochrome b2. A possible explanation for these increases in Km will be discussed in chapter 4, section 4.3.4.

3.4. PRESTEADY-STATE KINETIC PARAMETERS FOR L-LACTATE OXIDATION BY HINGE-DELETION FLAVOCYTOCHROMES b2.

Microscopic rates for the reduction of the FMN and haem prosthetic groups of hinge deleted and wild-type flavocytochromes b2 by 1H-lactate and 2H-lactate were determined directly using stopped-flow spectrophotometry, as described in chapter 2, section 2.18. The kinetic parameters are summarised in Table 3.2.

3.4.1. FMN Reduction

A typical FMN reduction trace for HA3 flavocytochrome b2 is illustrated in Figure 3.4. In the case of the HA3 and HA6 enzymes, the effect of the hinge deletions upon the rate of FMN reduction by L-lactate is insignificant, kcat is only decreased by 15 %, compared to the value for the wild-type enzyme (Table 3.2.1 and see Figure 3.5 for a Michaelis-Menten plot of the kinetic data for HA3 flavocytochrome b2). Conversely, HA9 shows the opposite trend to HA3 and HE6. It has a rate constant which is 15 % higher than that for the wild-type enzyme, but again, this is not a significant difference. These results clearly support the steady-state ferricyanide kinetic data that all three hinge-deletion mutants are good L-lactate dehydrogenases, compared to wild-type flavocytochrome b2.

Values of Km for L-lactate show a steady decrease from wild-type through successive truncations of the hinge region to HA9, the effect of this on the catalytic efficiency of L-lactate dehydrogenation is that the HA3 and HA6 enzymes have the same kcat/Km value within experimental error as the wild-type enzyme. However, the value for HA9 is 3-fold higher than that for wild-type. This is the first mutant flavocytochrome b2 enzyme to be characterised that is a more efficient L-lactate dehydrogenase than the wild-type enzyme, with regards to FMN reduction.

The measured deuterium kinetic isotope effects are the same, within experimental error, as the value for the wild-type enzyme, for all the hinge-deletion flavocytochromes b2. The HA9 enzyme, however, has a higher KIE than both HA3 and HA6 flavocytochromes b2, implying that the nine amino acid deletion has a more
Figures 3.4. FMN Reduction Trace for HΔ3 Flavocytochrome $b_2$

Figure 3.4.1. FMN reduction trace

Experiment was performed at 25°C in 10 mM Tris/HCl buffer, pH 7.5 ($I = 0.10$ M), as described in chapter 2, section 2.18. [HΔ3 flavocytochrome $b_2$] = 10 μM, [L-lactate] = 10 mM.

Figure 3.4.2. FMN reduction trace fitted to a double exponential equation

Fitting function: $y = Ae^{-k_1t} + Be^{-k_2t}$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude phase 1 (A)</td>
<td>$(5.62 \pm 0.12) \times 10^{-2}$</td>
</tr>
<tr>
<td>Rate phase 1 ($k_1$)</td>
<td>$514 \pm 16.9 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>Amplitude phase 2 (B)</td>
<td>$(5.45 \pm 1.46) \times 10^{-3}$</td>
</tr>
<tr>
<td>Rate phase 2 ($k_2$)</td>
<td>$129 \pm 40.3 \text{ s}^{-1}$</td>
</tr>
</tbody>
</table>
Figure 3.5. Michaelis-Menten Plot of HΔ3 Flavocytochrome b$_2$ FMN Pre-Steady-State Reduction
pronounced effect upon αH-abstraction from C-2 of L-lactate, than the smaller three and six amino acid deletions (Table 3.2.1).

### 3.4.2. Haem Reduction

A typical haem reduction trace for the HA3 enzyme is given in Figures 3.6. The effect of the hinge-deletion mutations on haem reduction is more striking than that for FMN reduction. The rate constant for haem reduction by L-lactate decreases in value from that of the wild-type enzyme as the length of the hinge region is progressively truncated (Table 3.2.2 and see Figure 3.7 for a Michaelis-Menten plot of the kinetic data for HA3 flavocytochrome $b_2$). The values of $k_{cat}$ are 5-, 16- and 50-fold lower for HA3, HA6 and HA9 respectively, than the corresponding value for the wild-type enzyme. However, as described in chapter 1, section 1.4.7, the true rate of interdomain electron transfer from FMN to haem in wild-type flavocytochrome $b_2$ is $1500 \pm 500\; s^{-1}$. The values of $k_{cat}$ for haem reduction of the hinge-deletion enzymes presented here truly reflect the rate of FMN to haem interdomain electron transfer, even though the entire reductive traces are fitted to double (HA3 enzyme), or treble (HA6 and HA9 enzymes) exponential equations. For all three hinge-deletion enzymes, FMN reduction is very fast compared to the subsequent haem reduction rate, and no appreciable time lag occurs before sufficient reduced FMN is formed prior to haem reduction. Thus, the rate of FMN $\rightarrow$ haem electron transfer has actually fallen 17-, 50- and 170-fold for HA3, HA6 and HA9 respectively, compared to the value for wild-type flavocytochrome $b_2$. This shows that as the hinge region is progressively truncated, the degree of impairment of haem reduction increases. Thus, maintaining the structural integrity of the hinge region is crucial for effective interdomain electron transfer between the FMN and haem prosthetic groups of flavocytochrome $b_2$.

The $K_m$ of the HA3 enzyme for L-lactate is slightly lower than the value for the wild-type enzyme. However, the HA6 and HA9 enzymes have $K_m$ values which are about 10-fold lower than that for wild-type flavocytochrome $b_2$ (Table 3.2.2). It is difficult to interpret these $K_m$ values quantitatively (even though haem reduction is a microscopic event), as several kinetic steps are involved in the scheme for haem reduction.

The deuterium kinetic isotope effects for all three hinge-deletion enzymes are significantly lower than the value for wild-type flavocytochrome $b_2$, implying that for these mutants, αH-abstraction from C-2 of L-lactate contributes much less to rate limitation than for the wild-type enzyme and in the case of the HA9 enzyme, not at all (a KIE < 2 is considered insignificant).
Figures 3.6. Haem Reduction Trace for HΔ3 Flavocytochrome $b_2$

Figure 3.6.1. Haem reduction trace alone

![Graph](image)

Experiment was performed at 25°C in 10 mM Tris/HCl buffer, pH 7.5 ($I = 0.10$ M), as described in chapter 2, section 2.18. $[HΔ3$ flavocytochrome $b_2] = 10 \mu$M, $[L$-lactate] = 10 mM.

Figure 3.6.2. Haem reduction trace fitted to a double exponential equation

![Graph](image)

Fitting function: $y = Ae^{-ka t} + Be^{-kb t}$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude phase 1 (A)</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td>Rate phase 1 ($k_a$)</td>
<td>85.8 ± 1.6 s$^{-1}$</td>
</tr>
<tr>
<td>Amplitude phase 2 (B)</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Rate phase 2 ($k_b$)</td>
<td>14.9 ± 1.3 s$^{-1}$</td>
</tr>
</tbody>
</table>
Figure 3.7. Michaelis-Menten Plot of HΔ3 Flavocytochrome b\textsubscript{2} Haem Pre-Steady-State Reduction
Interestingly, the HΔ6 and HΔ9 enzymes, which exhibit strong substrate inhibition in the steady-state reduction of cytochrome c, suffer no inhibition under the pre-steady-state conditions of haem reduction. A possible explanation for this will be presented in chapter 4, section 4.3.3. Unlike the haem reduction behaviour of some of the hinge deleted enzymes, no substrate inhibition occurred over the equivalent concentration ranges for FMN reduction (data not shown), which is a similar situation to that observed for the steady-state reduction of ferricyanide.

The haem reduction traces of HΔ6 and HΔ9 flavocytochromes b2 fit more accurately to a triphasic, rather than a biphasic exponential equation, as illustrated in Figures 3.8.3 and 3.8.4, for the HΔ9 enzyme. There is a lag phase for the HΔ9 enzyme prior to haem reduction (much longer in time scale than the dead-time of the stopped-flow apparatus) of about 15 ms, despite the FMN being fully reduced after about 2 ms, consequently all traces were fitted after this lag (Figure 3.8.2). The rate of the first phase is taken to be the kinetically relevant rate of haem reduction for both enzymes, despite the fact that it only comprises about 20% of the total amplitude of the trace over most of the L-lactate concentrations investigated (Figure 3.8.4). This is a reasonable assumption, because the kcat values obtained are the same, within experimental error as the kcat values for the steady-state reduction of cytochrome c. The other two phases are too slow to be kinetically relevant in the catalytic cycle and could represent a number of non-physiological intraprotein electron-transfer events. In the case of both enzymes, increasing the flavocytochrome b2 concentration has no effect upon the rate constants for haem reduction (data not shown), implying that none of the phases observed are due to interprotein electron transfer. In fact, a triphasic exponential is probably the minimum fit that can adequately describe the haem reduction trace, which may well consist of many more than three components.

3.4.3. Prosthetic Group Reoxidation

An interesting phenomenon that was observed while performing pre-steady-state reduction of HΔ9 flavocytochrome b2 haem, was the subsequent haem reoxidation. At the very low L-lactate concentrations (≤ 50 μM) required to accurately determine the low Km value (Table 3.2.2), over the time scale of the experiment (6 s) the absorbance change reached a plateau and then started to fall in amplitude. This suggested that the haem was initially reduced by the lactate and then once the substrate had been consumed, the enzyme was reoxidised by an external electron acceptor. When the time scale of the experiment was increased to 50 s, the haem trace eventually returned to its original amplitude (at time zero), that is, it was
Figures 3.8. Haem Reduction Trace for H∆9 Flavocytochrome $b_2$

Figure 3.8.1. Haem reduction trace

Experimental conditions were as for Figure 3.6, except [H∆9 flavocytochrome $b_2$] = 2.5 µM.

Figure 3.8.2. Haem reduction trace.

As for Figure 3.8.1, but over a shorter time scale to illustrate the lag phase.
Figure 3.8.3. Haem reduction trace fitted to a double exponential equation

Fitting function: $y = Ae^{-k_at} + Be^{-k_bt}$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude phase 1 (A)</td>
<td>$(9.13 \pm 0.04) \times 10^{-2}$</td>
</tr>
<tr>
<td>Rate phase 1 ($k_a$)</td>
<td>$5.38 \pm 0.05 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>Amplitude phase 2 (B)</td>
<td>$(1.47 \pm 0.05) \times 10^{-2}$</td>
</tr>
<tr>
<td>Rate phase 2 ($k_b$)</td>
<td>$0.81 \pm 0.01 \text{ s}^{-1}$</td>
</tr>
</tbody>
</table>
Figure 3.8.4. Haem reduction trace fitted to a treble exponential equation

\[ y = A e^{-k_a t} + B e^{-k_b t} + C e^{-k_c t} \]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude phase 1 (A)</td>
<td>((5.13 \pm 0.05) \times 10^{-2})</td>
</tr>
<tr>
<td>Rate phase 1 ((k_a))</td>
<td>(9.45 \pm 0.09 \text{ s}^{-1})</td>
</tr>
<tr>
<td>Amplitude phase 2 (B)</td>
<td>((9.46 \pm 0.06) \times 10^{-2})</td>
</tr>
<tr>
<td>Rate phase 2 ((k_b))</td>
<td>(2.11 \pm 0.02)</td>
</tr>
<tr>
<td>Amplitude phase 3 (C)</td>
<td>((9.79 \pm 0.09) \times 10^{-2})</td>
</tr>
<tr>
<td>Rate phase 3 ((k_c))</td>
<td>(0.63 \pm 0.01 \text{ s}^{-1})</td>
</tr>
</tbody>
</table>
Table 3.2. Pre-Steady-state Kinetic Parameters and Deuterium Kinetic Isotope Effects for Reduction of FMN and Haem in Wild-Type and Hinge-Deletion Flavocytochromes $b_2$.

All experiments were performed at 25°C in 10 mM Tris/HCl buffer, pH 7.5, ionic strength ($I$) adjusted to 0.10 M by the addition of NaCl. Stopped-flow data was analysed as described in chapter 2, section 2.18.4. Values of $k_{cat}$ are expressed as number of prosthetic groups reduced per second. The $k_{cat}$ values reported correspond to the rapid phase of biphasic traces, unless otherwise indicated.

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (x10⁵ M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^1$H-lac</td>
<td>$^2$H-lac</td>
<td>$^1$H-lac</td>
</tr>
<tr>
<td>wild-type</td>
<td>604 ± 60</td>
<td>75 ± 5</td>
<td>0.84 ± 0.20</td>
</tr>
<tr>
<td>H$\Delta$3</td>
<td>518 ± 17</td>
<td>71 ± 3</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>H$\Delta$6</td>
<td>514 ± 21</td>
<td>83 ± 3</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>H$\Delta$9</td>
<td>690 ± 16</td>
<td>64 ± 3</td>
<td>0.30 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$Miles et al. (1992), $^b$Sharp et al. (1994), $^c$Optimal substrate for the fastest observed rate, $^d$this work, $^e$the actual rate constant for haem reduction by electron transfer from fully reduced FMN is 1500 ± 500 s⁻¹, the value quoted in the table is a consequence of the inaccurate biphasic fit (Chapman et al., 1994), $^l$rapid phase of triphasic trace.
<table>
<thead>
<tr>
<th>ENZYME</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (x10$^5$ M$^{-1}$s$^{-1}$)</th>
<th>$^1$H-lac Inhibition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type$^a$</td>
<td>$^1$H-lac 445 ± 50$^e$</td>
<td>$^2$H-lac 71 ± 5</td>
<td>$^1$H-lac 0.53 ± 0.05</td>
<td>$^2$H-lac 0.68 ± 0.05</td>
</tr>
<tr>
<td>HA3$^b$</td>
<td>91 ± 3</td>
<td>32 ± 1</td>
<td>0.38 ± 0.07</td>
<td>0.80 ± 0.13</td>
</tr>
<tr>
<td>HA6$^i$</td>
<td>29 ± 1</td>
<td>15 ± 1</td>
<td>0.04 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>HA9$^i$</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
<td>0.07 ± 0.02</td>
<td>0.21 ± 0.06</td>
</tr>
</tbody>
</table>
completely reoxidised. The experiment was repeated at various low L-lactate concentrations and as the substrate concentration decreased, so did the duration and the maximal amplitude of the plateau phase of absorbance (Figure 3.9).

The stopped-flow experiment was repeated monitoring FMN reduction of HA9 flavocytochrome $b_2$ and a similar situation occurred, where at equivalent substrate concentrations to those used in the haem reoxidation experiments, FMN was initially reduced and then the absorbance returned to its original amplitude upon reoxidation (Figure 3.14). To estimate the rates of reoxidation, the reoxidation regions of both the haem and FMN traces were fitted to a monophasic exponential equation (Figures 3.11 and 3.12). The rates were independent of L-lactate concentration and were about 0.2 s$^{-1}$ and 1.5 s$^{-1}$, for haem and FMN reoxidation respectively.

The only plausible electron acceptor under the experimental conditions used, is oxygen in solution. It has been previously reported that wild-type flavocytochrome $b_2$ can reduce oxygen (Boeri & Rippa, 1960) and to check that this reoxidation process was not an intrinsic property of the HA9 enzyme, caused by the hinge-deletion mutation, the experiments were repeated with wild-type flavocytochrome $b_2$ (Figures 3.13 and 3.14). The same situation was observed for the wild-type enzyme as for the HA9 mutant, with the rate constants for FMN and haem reoxidation being the same for both enzymes, within experimental error.

To unambiguously prove that this reoxidation was caused by oxygen in solution, the experiments were repeated with wild-type flavocytochrome $b_2$ in which all the solutions used were degassed with nitrogen for some time, to remove as much oxygen as possible. Figure 3.15 shows a comparison of the traces obtained for haem reduction/oxidation, between non-degassed and degassed samples. It is clear that for the degassed sample, the rate of reoxidation is much slower (rate constant is about 0.03 s$^{-1}$, fit not shown) than that of the non-degassed sample. It should be noted that the stopped-flow apparatus used is not ideally suited for performing anaerobic experiments and as such, the solutions used were probably semi- as opposed to fully-anaerobic. Despite this, one can be confident that the reoxidation is caused by oxygen and presumably if fully anaerobic conditions could be attained, the absorbance trace would reach a steady maximum upon reduction. The mechanism by which reoxidation probably occurs is described in chapter 4, section 4.4.
Figure 3.9. Haem Reduction/Oxidation Traces for HΔ9 Flavocytochrome b₂ at Varying L-Lactate Concentrations

Conditions were as for Figure 3.4. [HΔ9 flavocytochrome b₂] = 2.5 μM. [L-Lactate] were, (1.) = 30 μM, (2.) = 20 μM and (3.) = 10 μM.

Figure 3.10. FMN Reduction/Oxidation Traces for HΔ9 Flavocytochrome b₂ at Varying L-Lactate Concentrations

Conditions were as for Figure 3.4. [HΔ9 flavocytochrome b₂] = 10 μM. [L-Lactate] were, (1.) = 20 μM and (2.) = 10 μM.
Figure 3.11. Fit of a Monophasic Exponential Equation to HA9 Flavocytochrome $b_2$ Haem Reoxidation Trace

Fitting function: $y = Ae^{-kt}$

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>$0.58 \pm 0.01$</td>
</tr>
<tr>
<td>rate constant</td>
<td>$0.17 \pm 0.01$ s$^{-1}$</td>
</tr>
</tbody>
</table>

Figure 3.12. Fit of a Monophasic Exponential Equation to HA9 Flavocytochrome $b_2$ FMN Reoxidation Trace

Fitting function: $y = Ae^{-kt}$

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>$0.19 \pm 0.02$</td>
</tr>
<tr>
<td>rate constant</td>
<td>$1.29 \pm 0.01$ s$^{-1}$</td>
</tr>
</tbody>
</table>
Figure 3.13. Haem Reduction/Oxidation Traces for Wild-Type Flavocytochrome \( b_2 \) at varying L-lactate concentrations

Conditions were as for Figure 3.4. \([\text{H} \Delta 9 \text{ flavocytochrome } b_2] = 2.5 \ \mu\text{M}. \) [L-Lactate] were, (1.) = 30 \ \mu\text{M}, (2.) = 20 \ \mu\text{M} and (3.) = 30 \ \mu\text{M}.

Figure 3.14. FMN Reduction/Oxidation Traces for Wild-Type Flavocytochrome \( b_2 \) at varying L-lactate concentrations.

Conditions were as for Figure 3.4. \([\text{wild-type flavocytochrome } b_2] = 10 \ \mu\text{M}. \) [L-Lactate] were, (1.) = 30 \ \mu\text{M}, (2.) = 20 \ \mu\text{M} and (3.) = 10 \ \mu\text{M}.
Figure 3.15. A Comparison of the Haem Reduction/Oxidation Traces Under Semi-Anaerobic and Aerobic Conditions for Wild-Type Flavocytochrome $b_2$

Conditions were as for Figure 3.4. [wild-type flavocytochrome $b_2$] = 2.5 μM, [L-lactate] = 20 μM. (1) semi-anaerobic conditions and (2) aerobic conditions.
3.5. PRE-STEADY-STATE KINETIC PARAMETERS FOR CYTOCHROME c REDUCTION BY PRE-REDUCED WILD-TYPE AND HINGE-DELETION FLAVOCYTOCHROMES \( b_2 \).

Reduction of cytochrome \( c \) by wild-type and hinge-deletion flavocytochromes \( b_2 \) was monitored using stopped flow spectroscopy as described in chapter 2, section 2.18.5. The bimolecular rate constants for cytochrome \( c \) reduction are shown in Table 3.3. Figures 3.16 shows typical reductive traces for horse heart and yeast cytochrome \( c \). Figure 3.17 shows plots of \( k_{\text{obs}} \) for cytochrome \( c \) reduction against flavocytochrome \( b_2 \) concentration fitted to a linear regression analysis. These data measure the rate of formation of a catalytically competent electron-transferring complex between flavocytochrome \( b_2 \) and cytochrome \( c \) and do not reflect the rate of electron transfer from \( b_2 \)-haem to cytochrome \( c \) haem in the pre-formed complex, which would have to be measured by techniques other than rapid mixing.

Considering the data for horse heart cytochrome \( c \); the largest decrease in the second order rate constant is for the HA3 enzyme, which is only an order of magnitude less than the corresponding value for wild-type flavocytochrome \( b_2 \). The values for the HA6 and HA9 enzymes are only 5- and 2-fold lower respectively, than that for the wild-type enzyme. This trend is somewhat surprising in view of the fact that as the hinge region is progressively truncated, the second order rate constant for cytochrome \( c \) reduction increases in magnitude, to a value half that of the wild-type enzyme. As opposed to decreasing in value, as might have been anticipated by the increasing severity of the successive mutations.

The data for the reduction of yeast cytochrome \( c \) shows that for all the hinge-deletion mutants and wild-type flavocytochromes \( b_2 \), the second order rate constant is approaching that for a diffusion controlled reaction (Fersht, 1988). Interestingly, all three hinge-deletion enzymes have the same second order rate constant within experimental error, which are only 1.5-fold lower than that for wild-type flavocytochrome \( b_2 \). This implies that yeast cytochrome \( c \) shows less selectivity toward the hinge deletions than that exhibited by horse heart cytochrome \( c \).

These data from two different cytochrome \( c \) species indicate that contrary to what had previously been supposed (Sharp et al., 1994), the structural integrity of the hinge region of flavocytochrome \( b_2 \) does not appear to be strongly implicated in effective cytochrome \( c \) binding. Possible reasons for this will be discussed in chapter 4, sections 4.4.1 and 4.4.2.

The horse heart cytochrome \( c \) reductive traces fitted well to monophasic exponential equations for wild-type and hinge-deletion flavocytochromes \( b_2 \) (Figure
Figures 3.16. Traces of Presteady-State Reduction of Cytochrome c by Wild-Type Flavocytochrome b₂

Figure 3.16.1. Horse heart cytochrome c

Trace alone. Experiment was performed at 25°C, in 10 mM Tris/HCl buffer, pH 7.5 (I = 0.10 M). Protein concentrations were; 0.75 μM oxidised cytochrome c and 4.1 μM reduced wild-type flavocytochrome b₂. L-lactate was present at approximately 1 mM.

Figure 3.16.2. Trace 3.18.1 fitted to a single exponential equation

Fitting function: \( y = Ae^{-ka_t} \)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>((3.97 \pm 0.07) \times 10^{-2})</td>
</tr>
<tr>
<td>rate constant</td>
<td>(175 \pm 4 \text{ s}^{-1})</td>
</tr>
</tbody>
</table>
Figure 3.16.3. Yeast cytochrome c

Trace alone (average of 3 traces, as single traces were noisy), experimental conditions as described in Figure 3.18.1. Protein concentrations were; 0.75 μM oxidised cytochrome c and 3.3 μM wild-type flavocytochrome b₂. L-lactate was present at approximately 1mM.

Figure 3.16.4. Trace 3.18.3 fitted to a single exponential equation

fitting function: \( y = Ae^{-kt} \)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>((2.62 \pm 0.04) \times 10^{-2})</td>
</tr>
<tr>
<td>rate constant</td>
<td>(331 \pm 5 \text{ s}^{-1})</td>
</tr>
</tbody>
</table>
Figure 3.16.5. Trace 3.18.3 fitted to a double exponential equation

![Graph showing fitted trace]

fitting function: \( y = Ae^{-k_a t} + Be^{-k_b t} \)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude phase 1 (A)</td>
<td>((2.86 \pm 0.03) \times 10^{-2})</td>
</tr>
<tr>
<td>Rate phase 1 (k_a)</td>
<td>(445 \pm 7 \text{ s}^{-1})</td>
</tr>
<tr>
<td>Amplitude phase 2 (B)</td>
<td>((2.9 \pm 0.1) \times 10^{-3})</td>
</tr>
<tr>
<td>Rate phase 2 (k_b)</td>
<td>(47 \pm 5 \text{ s}^{-1})</td>
</tr>
</tbody>
</table>

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Figures 3.17. Linear Regression Analysis of Second Order Rate Constants for Cytochrome c Reduction by Prereduced Hinge-Deletion and Wild-Type Flavocytochromes $b_2$

Figure 3.17.1. Horse Heart Cytochrome c Reduction

Figure 3.17.2. Yeast Cytochrome c Reduction
Table 3.3. Bimolecular Rate Constants for the Reduction of Cytochrome c by Pre-Reduced Hinge-Deletion and Wild-Type Flavocytochromes $b_2$

All experiments were performed at 25°C in 10 mM Tris/HCl buffer, pH 7.5, ionic strength ($I$) adjusted to 0.10 M by the addition of NaCl. The second order rate constants were determined as described in chapter 2, section 2.18.5.


<table>
<thead>
<tr>
<th>ENZYME</th>
<th>Second order rate constant (x 10^7 M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>horse heart cyt c</td>
</tr>
<tr>
<td>wild-type</td>
<td>4.7 ± 0.2$^a$</td>
</tr>
<tr>
<td>HA3</td>
<td>0.43 ± 0.04$^a$</td>
</tr>
<tr>
<td>HA6</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>HA9</td>
<td>2.2 ± 0.1</td>
</tr>
</tbody>
</table>
3.16.3), as expected for a one electron-transfer reaction from $b_2$-haem to cytochrome c haem under pseudo first-order conditions. In the case of yeast cytochrome c which is a mixture of two isomers, *iso*-1 and *iso*-2, present in the approximate proportions *iso*-1, 95% and *iso*-2, 5%, most of the traces fitted better to a biphasic exponential equation at lower flavocytochrome $b_2$ to cytochrome c ratios (Figures 3.16.4 and 3.16.5). However, flavocytochrome $b_2$ was still in excess over cytochrome c, so the pseudo first order relationship does not break down. At higher ratios the traces fitted equally well to both monophasic and biphasic exponential equations, but these traces were noisy and thus there exists a wide margin of error for fitting the data. For biphasic traces, the rate of the large amplitude, fast phase was taken to be $k_{obs}$ for cytochrome c reduction. It is unlikely that the smaller second phase is solely due to reduction of the *iso*-2 component as this only constitutes at the most, 5% of the total cytochrome c present. One possible explanation for the second phase is electron transfer from one cytochrome c haem to another, within a disulphide-linked cytochrome c dimer. It is known that yeast *iso*-1 has a propensity to dimerise, due to a redox sensitive thiol on Cys102 at the 'back' face of the molecule relative to the haem crevice (Mauk & Mauk, 1988). This conclusion is supported by the observation that the amplitude and rate constant for this second phase is invariant and independent of flavocytochrome $b_2$ concentration (data not shown), indicating an 'intramolecular' (between two disulphide linked cytochromes c) rather than an intermolecular electron transfer. The conclusion could be tested by repeating the experiment using a site-directed mutant of yeast *iso*-1 cytochrome c, Cys102→Thr, which can no longer dimerise, as the redox sensitive thiol group of amino acid 102 has been replaced by a redox insensitive one (Cutler *et al.*, 1987). If only a single phase were to be observed for cytochrome c haem reduction in the C102T mutant, this would give unequivocal support for the conclusion drawn here.

### 3.6. HAEM REDOX MID-POINT POTENTIALS OF HINGE-DELETION FLAVOCYTOCHROMES $b_2$

There was a possibility that the construction of deletion mutations in the hinge region of flavocytochrome $b_2$ might affect the redox potentials of the prosthetic groups. Clearly, there can have been very little or no effect on the FMN mid-point potential for all three hinge-deletion enzymes, as the $k_{cat}$ values for FMN reduction are not significantly different between these enzymes and wild-type flavocytochrome $b_2$. However, it is possible that a large change in the haem mid-point potential could
Table 3.4. Haem Mid-Point Redox Potentials of Wild-Type, \(b_2\)-Core and Hinge-Deletion Flavocytochromes \(b_2\)

All experiments were performed in 10 mM Tris/HCl buffer, pH 7.5, ionic strength adjusted to 0.10 M by the addition of NaCl, at room temperature. The mid-point potentials were determined as described in chapter 2, section 2.19.

1 independently expressed and purified cytochrome \(b_2\) domain of flavocytochrome \(b_2\), \(^a\) Miles et al (1992), \(^b\) Brunt et al (1992), \(^c\) Sharp et al (1994).

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>Haem redox potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type (^a)</td>
<td>-17 ± 3</td>
</tr>
<tr>
<td>(b_2)-core (^b,1)</td>
<td>-31 ± 2</td>
</tr>
<tr>
<td>(\Delta 3) (^c)</td>
<td>-14 ± 5</td>
</tr>
<tr>
<td>(\Delta 6)</td>
<td>-23 ± 5</td>
</tr>
<tr>
<td>(\Delta 9)</td>
<td>-33 ± 5</td>
</tr>
</tbody>
</table>
have contributed to the decrease in the values of $k_{cat}$ for haem reduction, in some or all of the hinge-deletion enzymes. To check this, the haem mid-point potentials for the three hinge-deletion flavocytochromes $b_2$ were measured (Table 3.4. and Figure 3.18). Both the $H\Delta 3$ and $H\Delta 6$ enzymes have the same haem potential within experimental error as the wild-type enzyme. However, $H\Delta 9$ has a slightly lower haem potential by about 15 mV than the value for wild-type flavocytochrome $b_2$. In fact, the haem potential of the $H\Delta 9$ enzyme is the same within experimental error as the value determined for the independently expressed and purified cytochrome $b_2$ domain of flavocytochrome $b_2$, $b_2$-core (Brunt et al., 1992). This indicates that the haem environment in the cytochrome $b_2$ domain of the $H\Delta 9$ enzyme resembles that of $b_2$-core to a greater extent than that of wild-type flavocytochrome $b_2$, implying that perhaps the haem edge is more exposed to solvent than in the wild-type enzyme. This will be discussed in more detail in chapter 4, section 4.3.5.

3.7. INVESTIGATION OF CYTOCHROME C BINDING TO HINGE-DELETION AND WILD-TYPE FLAVOCYTOCHROMES $b_2$ BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

As described in chapter 1, section 1.3.3, nuclear magnetic resonance (nmr) spectroscopy has been widely used to investigate complex formation between physiological and non-physiological redox partners. In most cases, the two redox partners are small proteins (< 15 kDa) and are thus ideally suited to investigation by nmr spectroscopic techniques. The information that can be obtained from such studies are the stoichiometry of binding, the strength of binding - association or dissociation constants (Eley & Moore, 1983; Burch et al., 1990; Whitford et al., 1990; Moench et al., 1992), and in some cases where extensive nmr information is available for the individual proteins (such as complete assignment of resonances in the one and two dimensional nmr spectra and/or a knowledge of the backbone amide exchange dynamics), the nature of the interacting surfaces can be deduced (Burch et al., 1990; Jeng et al., 1994).

In the present study, the issues addressed are the stoichiometry and affinity of binding of cytochrome c to wild-type and hinge-deletion flavocytochromes $b_2$. Unlike many of the other systems mentioned (chapter 1, section 1.3.3), flavocytochrome $b_2$ is a very large protein to study by nmr spectroscopy, it has a molecular weight of 230 kDa (Pajot & Groudinsky, 1970). However, in the nmr experiments, it is the cytochrome c paramagnetically shifted haem resonances that
are monitored and this protein has a molecular weight of about 12 kDa (Pettigrew and Moore, 1986).

The experiment was performed as described in chapter 2, section 2.20 and the results are illustrated in Figure 3.19 which shows a stacked plot of the cytochrome c nmr spectra in the chemical shift range -10 to 50 ppm. Each spectrum is of cytochrome c with an increasing ratio of wild-type flavocytochrome b2 present (from the bottom spectrum upwards). As the molar ratio of flavocytochrome b2:cytochrome c increases, the resonances of the substituent methyl groups on the cytochrome c haem become shifted, due to a change in their chemical environment upon complex formation between the two redox proteins. The linewidths of these shifted resonances also broaden, due to the fact that the small cytochrome c molecules are now effectively bound in equilibrium to the much larger flavocytochrome b2. The degree of linewidth broadening is directly proportional to the rate of molecular tumbling, which in turn is related to the shape and size of the molecule or complex investigated. Figure 3.20 gives a graphical illustration of this phenomenon.

3.7.1. Determination of the stoichiometry of cytochrome c binding to wild-type flavocytochrome b2

Figure 3.21. shows the data plotted as the change in cytochrome c haem methyl chemical shift ($\Delta$ ppm) against the ratio of flavocytochrome b2 tetramer to cytochrome c ([b2 tet]/[cyt c]). For a stoichiometry of one cytochrome c molecule per flavocytochrome b2 subunit, $\Delta$ should reach a maximum at a [b2 tet]/[cyt c] ratio of 0.25, which the initial portion (region 1) of the haem methyl 8 $\Delta$ plot does. At higher ratios though, the HM8$\Delta$ continues to increase (region 2) after reaching an initial maximum (actually best described as an inflexion in the curve) and this further increase could be due to the formation of higher order non-physiological complexes. This phenomenon has been observed between smaller redox partners such as cytochrome c and cytochrome b5, even though the available surface area for binding is much smaller (Whitford et al., 1990). In the present study however, flavocytochrome b2 has a large surface area, where it is possible to accommodate several cytochrome c molecules around one subunit. This may account for the further increase in HM8$\Delta$, due to binding of more cytochrome c molecules.

The haem methyl 3 $\Delta$ (HM3$\Delta$) could be described as increasing in a linear manner with increasing [b2 tet]/[cyt c] over the entire range of ratios investigated, which is in marked contrast to the behaviour of the haem methyl 8 resonance, implying that the two methyl groups experience different chemical environments.
Figure 3.19. Stack Plots of Cytochrome c Resonances from -10 to 45 ppm Illustrating the Change in Nature of the Peaks With Increasing Stoichiometry of Flavocytochrome $b_2$

The 600 MHz spectra were recorded at 25°C in 20 mM phosphate/D$_2$O buffer, pH 7.0. Spectrum (a) is of 0.4 mM wild-type-$b_2$ subunit (0.1 mM tetramer); (b) an 8:1 mixture of wild-type-$b_2$ and cytochrome c respectively; (c) 4:1, (d) 8:3, (e) 4:2; (f) 4:3; (g) 4:4; (h) 4:6 and (i) is an electronic addition spectrum of free wild-type flavocytochrome $b_2$ and cytochrome c. '8' and '3' are the cytochrome c haem methyl resonances at 35.5 and 32.5 ppm respectively.
Figure 3.20. Graphical Illustration of Linewidth Broadening and Chemical Shift Change Upon Complexation

(A) shows free cytochrome c in solution (cyt c), the molecule tumbles rapidly and so the haem methyl resonances appear at a certain chemical shift (X ppm), with a narrow linewidth (LwX Hz) at the half-maximal peak height. (B) shows the changes in the haem methyl resonance upon complexation of cytochrome c with flavocytochrome b₂ (cyt c:fcb₂). The linewidth of the cytochrome c haem methyl resonance has increased (LwY Hz), due to the slower tumbling of the complex compared to free cytochrome c. Also, the peak position has shifted due to a change in the chemical environment of the haem methyl resonance upon complexation (Y ppm).
Figure 3.21. Plot of Cytochrome c Haem Methyl Chemical Shift Changes During Titration Against Wild-Type Flavocytochrome $b_2$: Stoichiometry Determination

Experimental conditions are as described in Figure 3.19.
These data when considered together are difficult to rationalise. The HM8Δδ values indicate the initial formation of a complex with a 1:1 stoichiometry of cytochrome c per flavocytochrome b2 subunit, which is in agreement with the proposed molecular model and other experimental data (Tegoni et al., 1993), but at higher ratios, this stoichiometry can increase. The haem methyl 3 Δδ values however, imply that a fixed stoichiometry does not exist. Clearly, only one of these situations is applicable and ideally, the experiments should be repeated before any strong conclusions can be drawn.

3.7.2. Determination of dissociation constants for cytochrome c binding to wild-type and HA6 flavocytochromes b2

3.7.2.1. Wild-type flavocytochrome b2

Figure 3.22 shows the plot of the data obtained for the change in chemical shift of cytochrome c haem methyl resonances (Δδ) during the titration of wild-type flavocytochrome b2 against cytochrome c. For the haem methyl 8 Δδ values, good fits were obtained when the data were fitted to an equation describing a single binding site for cytochrome c per flavocytochrome b2 subunit (Figure 3.22.1) and to a double reciprocal linear regression analysis (Figure 3.22.2). In both cases the same value within experimental error, 15 ± 1 μM (Table 3.5) was found for the cytochrome c concentration at the half maximal Δδ, K1/2. As for the stoichiometry plots, the haem methyl 8 Δδ values continued to increase after reaching an initial maximum, but these data points were omitted from the fit in Figure 3.22.1 and 3.22.2.

In the simplest case of one equivalent cytochrome c binding site per flavocytochrome b2 subunit, the K1/2 determined here should represent the dissociation constant, Kd for cytochrome c binding to flavocytochrome b2. If however, the binding sites for cytochrome c are non-equivalent, in that the binding of one cytochrome c molecule to one subunit affects the binding of another cytochrome c molecule to a different subunit (negative cooperativity), then fitting the data as in Figure 3.22 is an oversimplification of the binding equilibrium. Despite this, a Kd of 15 ± 1 μM is in reasonable agreement with previously determined values of Kd by different experimental techniques (Tegoni et al., 1993 and references therein).

In the case of HM3Δδ values (Figure 3.22, plots 3 and 4), reasonable fits were not obtained for either plots. The calculated K1/2 was about 500 μM (Table 3.5), which is 35-fold higher than that calculated from the HM8Δδ data. This value cannot
represent a true $K_d$ and again, as for the stoichiometry data, it is difficult to rationalise the difference between the two haem methyl $\Delta\delta$ data.

3.7.2.2. HΔ6 flavocytochrome $b_2$

Figure 3.23 shows the equivalent data for HΔ6 flavocytochrome $b_2$ as Figure 3.24 for the wild-type enzyme. Good fits were obtained for the cytochrome $c$ HM8$\Delta\delta$ values (Figure 3.23.1 and 3.23.2) when it was titrated against HΔ6 flavocytochrome $b_2$, the $K_{1/2}$ value being $30 \pm 5 \mu M$. For the HM3$\Delta\delta$ values, reasonable fits were obtained, with $K_{1/2}$ being about $45 \pm 11 \mu M$. These two values for $K_{1/2}$ are the same within experimental error (Table 3.5). This value of about $35 \mu M$ is only twice that of the $K_{1/2}$ for the wild-type enzyme, as determined from the cytochrome $c$ haem methyl $8 \Delta\delta$ values and if this is the 'true' $K_d$, then introducing a six amino acid truncation into the hinge region has not disrupted cytochrome $c$ binding to flavocytochrome $b_2$ by a significant extent.

The haem methyl $3 \Delta\delta$ cannot represent the binding equilibrium between cytochrome $c$ and flavocytochrome $b_2$, as the $K_{1/2}$ determined for cytochrome $c$ binding to the wild-type enzyme is about 20-fold higher than that for HΔ6 flavocytochrome $b_2$. 
Figures 3.22. Plot of Cytochrome c Haem Methyl Chemical Shift Changes Upon Titration of Wild-Type Flavocytochrome $b_2$: Affinity Determination

Figure 3.22.1.(i) Hyperbolic fit of Chemical Shift Change of Haem Methyl 8 Against Titrated Flavocytochrome $b_2$ Concentration

Figure 3.22.1.(ii) Double Reciprocal Fit of Haem Methyl 8 Chemical Shift Change Against Titrated Flavocytochrome $b_2$ Concentration
Figure 3.22.2.(i) Hyperbolic Fit of Haem Methyl 3 Chemical Shift Change Against Titrated Flavocytochrome $b_2$ Concentration

![Hyperbolic Fit Graph]

Figure 3.22.2.(ii) Double Reciprocal Plot of Chemical Shift Change of Haem Methyl 3 Against Titrated Flavocytochrome $b_2$ Concentration

![Double Reciprocal Plot Graph]
Figures 3.23. Plot of Cytochrome c Haem Methyl Chemical Shift Changes Upon Titration of HΔ6 Flavocytochrome b$_2$: Affinity Determination

Figure 3.23.1.(i) Hyperbolic Fit of Haem Methyl 8 Chemical Shift Change Against Titrated Flavocytochrome b$_2$ Concentration

Figure 3.23.1.(ii) Double Reciprocal Plot of Chemical Shift Change of Haem Methyl 8 Against Titrated Flavocytochrome b$_2$ Concentration.
Figure 3.23.2.(i) Hyperbolic Fit of Haem Methyl 3 Chemical Shift Change Against Titrated Flavocytochrome $b_2$ Concentration

Figure 3.23.2.(ii) Double Reciprocal Plot of Chemical Shift Change of Haem Methyl 3 Against Titrated Flavocytochrome $b_2$ Concentration
Table 3.5. Apparent Dissociation Constants for Cytochrome c Binding to Wild-Type and HΔ6 Flavocytochromes b₂

NMR spectroscopy was performed on a Varian 600 MHz NMR spectrometer at 25°C, the buffer was 20 mM phosphate, pH 7.0 (uncorrected for the small isotope effect), [cytochrome c] = 0.1 mM.

a [flavocytochrome b₂] at which the cytochrome c haem methyl chemical shift change is half maximal, the apparent dissociation constant. b,c K₁/₂ determined from data derived from haem methyls 8 and 3 respectively.

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>methyl 8 b</th>
<th>methyl 3 c</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>15 ± 1</td>
<td>500 ± 200</td>
</tr>
<tr>
<td>HΔ6</td>
<td>30 ± 5</td>
<td>45 ± 11</td>
</tr>
</tbody>
</table>

K₁/₂ \(\text{a} (\mu\text{M})\)
Chapter 4

Discussion
The two domains of *S. cerevisiae* flavocytochrome *b*<sub>2</sub> are connected by a typical hinge sequence that contains proline, glycine and various charged residues (Chapter 1, Figure 1.4.7). It has been proposed that the most likely role of this hinge region is to confer domain mobility, allowing movement of the cytochrome domain with respect to the flavin domain. This proposal is supported by a number of observations, involving crystallography and nmr spectroscopy. In the three-dimensional structure of *S. cerevisiae* flavocytochrome *b*<sub>2</sub>, two crystallographically distinct types of subunit are visible in the asymmetric unit. In one subunit, neither product nor substrate is bound at the active site and the cytochrome domain is resolved. In the other, pyruvate is bound at the active site and no electron density is observed for the cytochrome domain, implying that it is positionally disordered (Xia & Mathews, 1990). However, the fact that two of the cytochrome domains in the crystallised tetramer are positionally ordered may be due to restricted mobility imposed by crystal packing forces. In solution, nmr spectroscopy shows that the cytochrome domain is substantially more mobile than would be expected for a protein as large as the flavocytochrome *b*<sub>2</sub> tetramer; the observed linewidths for the haem group resonances in the cytochrome domain are not broadened to the degree anticipated for a large protein, implying considerable flexibility of this domain (Labeyrie et al., 1988; S. K. Chapman, G. A. Reid, G. A. Moore, M. C. Cox & C. E. Brunt, unpublished results). Thus, a large body of structural information exists highlighting the importance of the hinge region in interdomain movement.

Recently, data have been published reporting the kinetic properties of a mutant flavocytochrome *b*<sub>2</sub>, designed to further investigate the role of the hinge in interdomain communication (White et al., 1993). This enzyme was termed "hinge-swap" as it comprised the bulk of the *S. cerevisiae* enzyme, but with the native hinge region replaced by the equivalent region of the *H. anomala* enzyme. The most striking difference between the wild-type and hinge-swap enzymes, was the 1000-fold decrease in the rate of interdomain electron transfer, implying that this mutation had severely impaired interdomain communication. These data support the conclusions from structural studies and stress the importance of maintaining the overall structural integrity of the hinge region for efficient interdomain interactions.

To further our understanding of the role of the hinge region in intra- and inter-protein communication, three hinge-deletion mutant flavocytochromes *b*<sub>2</sub> have been constructed; HΔ3 (Sharp et al., 1994), HΔ6 and HΔ9, with 3, 6 and 9 amino acids deleted from the hinge region respectively. These enzymes exhibit some
interesting differences in their electron-transfer properties when compared with wild-type and mutant flavocytochromes $b_2$.

4.2. EFFECT OF THE HINGE-DELETIONS ON CATALYSIS OF L-LACTATE DEHYDROGENATION BY FLAVOCYTOCHROME $b_2$

The catalytic cycle for flavocytochrome $b_2$ is shown diagrammatically in Scheme 4.1. The first step, flavin reduction by lactate is virtually unaffected in all three hinge-deletion flavocytochromes $b_2$; the $k_{\text{cat}}$ values for the pre-steady-state reduction of FMN by L-lactate in HΔ3 and HΔ6 are only 15% lower than the value for the wild-type enzyme. However, in the case of HΔ9, $k_{\text{cat}}$ for FMN reduction is 15% higher than the wild-type value and intriguingly, as reported in the results chapter (section 3.4), HΔ9 is the first mutant flavocytochrome $b_2$ enzyme to be characterised which has a higher catalytic efficiency for L-lactate dehydrogenation than the wild-type enzyme.

In the case of the hinge-swap enzyme (White et al., 1993) a similar situation was observed, where $k_{\text{cat}}$ for FMN reduction was only 2-fold lower than that for the wild-type enzyme. Also, the $k_{\text{cat}}$ for FMN reduction by Y143F flavocytochrome $b_2$ (Miles et al., 1992; Rouvière-Fourmy et al., 1994) is 15% higher than the value for the wild-type enzyme and the same within experimental error as the $k_{\text{cat}}$ reported here for the HΔ9 enzyme (Y143F is a mutant enzyme which will be discussed in section 4.3). However, the $K_m$ for FMN reduction by L-lactate is much higher for the Y143F enzyme (Miles et al., 1992 and Rouvière-Fourmy et al., 1994) than both wild-type and HΔ9 flavocytochromes $b_2$. Also, the measured deuterium kinetic isotope effects (KIE) with L-2-[2H]-lactate are the same for the hinge-deletion enzymes compared to that for wild-type flavocytochrome $b_2$. So, the nature of the rate-limiting step for L-lactate dehydrogenation, $\alpha$H-abstraction from C-2 of L-lactate, has not been altered by the hinge-deletion mutations. These data imply that structural integrity of the interdomain hinge region is not of great importance for efficient FMN reduction by L-lactate. Thus, all the hinge-deletion flavocytochromes $b_2$ remain good L-lactate dehydrogenases compared to the wild-type enzyme. This conclusion is supported by the steady-state kinetic measurements using ferricyanide as the electron acceptor (section 3.3.1).
Scheme 4.1. Catalytic Cycle of Flavocytochrome $b_2$.

The redox states of cytochrome $c$ and the flavocytochrome $b_2$ flavin (F) and haem (H) are indicated by the subscripts 'ox' and 'red' for the oxidised and reduced forms respectively. The flavin semiquinone is shown as Fsq.

1. Oxidation of lactate to pyruvate and reduction of FMN; this is the major rate-limiting step in the case of the wild-type enzyme.

2. Electron transfer from fully reduced FMN to haem, resulting in the semiquinone form of FMN and reduced haem (this is the rate-limiting step in the case of the HΔ6 and HΔ9 enzymes).

3. Reduction of the first cytochrome $c$ molecule by electron-transfer from the haem group of flavocytochrome $b_2$.

4. Electron-transfer from the semiquinone form of FMN to haem, resulting in fully oxidised FMN and reduced haem (this is the rate-limiting step in the HΔ3 enzyme).

5. Reduction of a second cytochrome $c$ molecule by electron-transfer from the haem group which results in the regeneration of the fully oxidised enzyme. The enzyme is now ready to repeat the cycle.
4.3. INTERDOMAIN (INTRAPROTEIN) ELECTRON TRANSFER

4.3.1. Effect of the hinge-deletion mutations on the rate of interdomain electron transfer

The second step in the catalytic cycle of flavocytochrome \( b_2 \) is interdomain electron-transfer from fully reduced flavin to oxidised haem (Scheme 4.1, step 2). The nature of this step is altered in the case of the hinge deletion, hinge swap and Y143F mutant enzymes. As described in chapter 1, section 1.4.7, the true rate of interdomain electron transfer in the wild-type enzyme is 1500 ± 500 s\(^{-1}\). For the hinge-deletion enzymes, however, the rate of interdomain electron-transfer is 16-, 50- and 170-fold lower for HA3, HA6 and HA9 respectively, than the value for the wild-type enzyme. These results show that as the interdomain hinge region is sequentially truncated, the rate constant for haem reduction decreases in magnitude, implying that maintaining the structural integrity of the hinge region is crucial for effective interdomain communication. This conclusion is supported by the results reported for the rate of interdomain electron transfer in hinge-swap flavocytochrome \( b_2 \) (White et al., 1993), where the rate constant for haem reduction was about 1.6 s\(^{-1}\), 1000-fold lower than the value for the wild-type enzyme. Hinge swap is an interspecies hybrid enzyme comprising the bulk of the \( S.\ crerevisiae \) enzyme, but with the hinge region from the \( H.\ anomala \) enzyme; it has 29 amino acid residues from the \( S.\ crerevisiae \) flavocytochrome \( b_2 \) replaced by 23 residues from the shorter and more acidic interdomain hinge region of the \( H.\ anomala \) enzyme (White et al., 1993). Thus, the hinge swap is a more drastic mutation than any of the hinge deletions (although, accurate assessment of the degree of any gross structural change can only be achieved by obtaining, and comparing, the crystal structures of these mutant flavocytochromes \( b_2 \) to that of the wild-type enzyme). It is not surprising that the effect of the hinge swap on the rate of interdomain electron transfer is more severe than that caused by the hinge deletions.

A similar situation occurs for Y143F flavocytochrome \( b_2 \), where a single point mutation, incurring the loss of one hydrogen bond causes the rate of interdomain electron transfer to fall 70-fold, from 1500 s\(^{-1}\) in the wild-type enzyme to 21 s\(^{-1}\) in the Y143F mutant (Miles et al., 1992). As described in section 4.1, the crystal structure of flavocytochrome \( b_2 \) shows two crystallographically distinct subunits in the asymmetric unit. In subunit one, both domains are clearly visible in the electron-density map and Y143 forms an interdomain hydrogen bond with a haem propionate group. In subunit two, however, there is a molecule of pyruvate located in the active site with its carboxylate group hydrogen bonded to Y143 and in
In this case, electron density for the cytochrome domain is not apparent due to positional disorder. Thus, it appears that Y143 is uniquely positioned to play a important role in controlling electron transfer from substrate to flavin and from flavin to haem. The pre-steady-state experiments of Miles et al., (1992) mentioned above clearly support this later proposal. The crystal structure of the Y143F flavocytochrome b2-sulphite complex has been solved to 3 Å resolution (Tegoni et al., 1994) and is isostructural with both the native pyruvate-ligated enzyme (Mathews and Xia 1990) and the recombinant flavocytochrome b2-sulphite complex (Tegoni et al., 1994). A difference map of the wild-type and mutant structures shows a peak of negative electron density at the position of side chain 143, attributed to the loss of a hydroxide group. There are no other significant conformational changes in the Y143F mutant compared to wild-type flavocytochrome b2. Clearly, the effect of the Y143F mutation on the rate of interdomain electron transfer cannot be due to structural changes, but must be attributed solely to the loss of a single interdomain hydrogen bond.

In the flavocytochrome b2 crystal structure, the edge to edge distance between the prosthetic groups in subunit one, from N(5) of FMN to C-2A of the haem, is 9.7 Å, the prosthetic groups are approximately coplanar and the intervening medium comprises Y143 and several water molecules (Mathews & Xia, 1990; Figure 4.1.1). The potential difference between fully reduced flavin and oxidised haem is of the order of +100 mV (Tollin & Walker, 1991). If one considers these parameters, then the theoretical rate of interdomain electron transfer between these prosthetic groups should be >>1500 s⁻¹ (Marcus and Suttin, 1985). Clearly, in the case of flavocytochrome b2, some other factor must be rate limiting this electron-transfer step, as the observed rate is 1500 s⁻¹. In the light of the kinetic and crystallographic experiments on Y143F flavocytochrome b2, it is not surprising that deletion mutations within the hinge region of flavocytochrome b2 decrease the rate of interdomain electron transfer. A likely explanation which supports the conclusions drawn from the hinge-deletion, hinge-swap and Y143F mutations, is that conformational gating is rate limiting the interdomain electron-transfer reaction and not the electron-transfer event itself. In the hinge-deletion enzymes, correct juxtapositioning of the cytochrome domain with respect to the flavin domain may be impaired, hindering the formation of an optimal conformation for efficient electron transfer. This could be due to truncation of the hinge resulting in decreased flexibility of this region, impairing optimal conformational exploration.

Recently, the crystal structures of two other flavocytochromes have been elucidated, although the structures are still undergoing refinement (Mathews et al.,...
Figures 4.1. Schematic Representation of the Flavin:Haem Geometries in Two Flavocytochromes

Figure 4.1.1. Flavocytochrome $b_2$ (Mathews & Xia, 1990)

The closest edge to edge distance between the flavin and haem groups in subunit 2 is 9.7 Å, from N(5) of FMN to C2-A of the haem. N(5) of FMN faces towards the propionate edge of the haem and the prosthetic groups are almost coplanar. (a), (b) and (c) show three orthogonal views of the haem and the flavin groups of flavocytochrome $b_2$ in isolation.
The methyl groups of FAD face towards the haem edge and the closest edge to edge distance is 9 Å. The haem propionates face away from the flavin by an angle of 90° and the prosthetic groups are tilted at an angle of 65° to one another.
1991; F. S. Mathews, personal communication). These are p-cresol methylhydroxylase (a flavocytochrome c) and flavocytochrome c sulphide dehydrogenase. Both of these proteins are composed of two distinct subunits, a small cytochrome domain (di-haem in sulphide dehydrogenase) and a larger subunit with flavin bound at the active site. It is interesting to compare the haem-flavin interactions between these proteins and flavocytochrome $b_2$. In all three cases, the distance between the prosthetic groups is about 9 Å, however, the orientation of the prosthetic groups differs. In flavocytochrome $b_2$, N(5) of the FMN faces towards the propionate edge of the haem and the prosthetic groups are almost coplanar (Figure 4.1.1). For p-cresol methylhydroxylase, the methyl groups of the FAD face towards the haem edge, but as is the case with sulphide dehydrogenase, the haem propionates face away from the flavin by an angle of 90°, and the prosthetic groups are tilted at an angle of 65° to one another (Figure 4.1.2). In sulphide dehydrogenase, the propionate groups of the nearest haem face are at 90° relative to the pyridine ring of FMN, which faces towards the haem, and the prosthetic groups are tilted at an angle of 30° to one another (not shown - data not published). Thus, the crystal structures of these three enzymes show that whilst the inter-prosthetic group distance is similar, the orientation of the prosthetic groups differs. Laser flash photolysis experiments measuring the rate of intersubunit electron-transfer in sulphide dehydrogenase (FAD → haem) showed that it was >10,000 s$^{-1}$ (G. Tollin, 1994, unpublished work, communicated by F. S. Mathews). It is revealing to compare this value to that obtained by stopped-flow studies with flavocytochrome $b_2$ (1500 s$^{-1}$). In sulphide dehydrogenase, there are extensive intersubunit contacts and as the distance between the prosthetic groups is fixed, the intersubunit electron-transfer rate is not controlled by conformational gating, but by some other factor, such as the reorganisation energies of the redox centres upon electron transfer. Flavocytochrome $b_2$ as described earlier, has the possibility of movement of the two domains with respect to one another by virtue of the hinge region. This may limit the rate of interdomain electron transfer to the number of productive encounters between the two domains: i.e. conformational gating.

4.3.2. Nature of the rate-limiting steps in the catalytic cycle of hinge-deletion flavocytochromes $b_2$

The catalytic cycle of flavocytochrome $b_2$ can be represented in a linear manner, as shown in Figure 4.2. Step 2 of this figure reports the rate constants, determined by stopped-flow spectroscopy, for interdomain electron transfer from FMN→haem in wild-type and hinge-deletion flavocytochromes $b_2$. Step 3 reports the
Figure 4.2. Linear Representation of the Catalytic Cycle of Lactate Oxidation and Cytochrome c Reduction by Flavocytochrome b₂

Rate constants at 25°C are reported for the wild-type (WT) and hinge deletion (Δ3, Δ6, Δ9) enzymes. (1) L-lactate→FMN electron transfer (stopped flow); (2) fully reduced FMN→haem electron transfer (stopped flow); (3) L-lactate→cytochrome c electron transfer (steady state). Abbreviations: Fₚₑₓₜ, reduced FMN; Fₒₓ, oxidised FMN; Fₛₚₜ, semiquinone form of FMN; Hₒₓ, oxidised haem; Hₚₑₓₜ, reduced haem; Cytₚₒₓ, oxidised cytochrome c; Cytₚₑₓₜ, reduced cytochrome c; LAC, L-lactate and PYR, pyruvate.
LAC  PYR

\[ F_{ox} H_{ox} \rightarrow F_{red} H_{ox} \rightarrow F_{sq} H_{red} \rightarrow F_{sq} H_{ox} \rightarrow F_{ox} H_{red} \rightarrow F_{ox} H_{ox} \]

--- (1) ---

WT = 604 ± 60 s⁻¹
ΔH₃ = 518 ± 17 s⁻¹
ΔH₆ = 514 ± 21 s⁻¹
ΔH₉ = 690 ± 16 s⁻¹

--- (2) ---

WT = 1500 ± 500 s⁻¹
ΔH₃ = 91 ± 3 s⁻¹
ΔH₆ = 29 ± 1 s⁻¹
ΔH₉ = 9 ± 1 s⁻¹

--- (3) ---

WT = 207 ± 7 s⁻¹
ΔH₃ = 39 ± 1 s⁻¹
ΔH₆ = 33.5 ± 1.4 s⁻¹
ΔH₉ = 7.9 ± 0.4 s⁻¹
rate constants for cytochrome c reduction by wild-type and the hinge-deletion enzymes under steady-state conditions. For the hinge-deletion enzymes, HA6 and HA9, the $k_{cat}$ values for haem and cytochrome c reduction are the same within experimental error (30 s$^{-1}$ and 8 s$^{-1}$, for the HA6 and HA9 enzymes respectively), implying that interdomain electron transfer from FMN→haem is the rate-limiting step in these enzymes.

In the case of the HA3 enzyme, the rate of interdomain electron-transfer is 91 s$^{-1}$, but the steady-state rate constant for cytochrome c reduction is 39 s$^{-1}$. Thus, interdomain electron transfer is not totally rate limiting in the HA3 enzyme, some other electron-transfer step must contribute to rate limitation. Pseudo first order reduction of cytochrome c (horse heart) by pre-reduced HA3 flavocytochrome $b_2$ under pre-steady-state conditions (chapter 3, section 3.5) yields a second order rate constant of $4.3 \times 10^6$ M$^{-1}$s$^{-1}$. This value was determined by fitting a plot of $k_{obs}$ against HA3 concentration to a linear regression analysis. Over the concentration range investigated, the plot did not deviate from a straight line fit, indicating that the system was not saturating and $k_{obs}$ values up to 80 s$^{-1}$ were measured (Chapter 3, Figure 3.19.1). This implies that electron transfer from $b_2$-haem to cytochrome c cannot be rate limiting in the steady-state reduction of cytochrome c. Step 4 in Scheme 4.1 illustrates interdomain electron transfer from the semiquinone form of FMN to oxidised haem (unfortunately this step cannot be monitored directly by stopped-flow kinetics, due to the nature of the flavocytochrome $b_2$ catalytic cycle). In the light of the above evidence, it is reasonable to propose that in the HA3 enzyme, FMN semiquinone→haem electron transfer has a large contribution to rate limitation and one could tentatively suggest that the rate of this step is about 20 s$^{-1}$, as two cytochrome c molecules are reduced per flavocytochrome $b_2$ catalytic cycle (Scheme 4.1).

The above conclusions are supported by the deuterium kinetic isotope effect (KIE) values reported in Figure 4.3. The wild-type enzyme has the same KIE value for L-lactate→haem electron transfer, within experimental error as that for FMN reduction by L-lactate, implying that $\alpha$H-abstraction from C-2 of L-lactate is still rate-limiting (this is not surprising in view of the fact that the rate of FMN→haem electron transfer is faster than the rate constant for FMN reduction by L-lactate). However, the KIE values for haem reduction by L-lactate in the hinge-deletion enzymes are significantly lower than their corresponding KIE values for FMN reduction by L-lactate (Figure 4.3, steps 1 and 2 respectively). The haem reduction KIE values for the hinge-deletion enzymes appear to follow a trend: as the length of the hinge region is progressively truncated, the magnitude of the KIE values decrease
Figure 4.3. Comparison of the Deuterium Kinetic Isotope Effects for Wild-Type and Hinge-Deletion Flavocytochromes $b_2$

Kinetic isotope effects (25°C) are reported for wild-type (WT) and hinge-deletion (HΔ3, HΔ6 and HΔ9) flavocytochromes $b_2$. (1) L-lactate →FMN electron transfer (stopped-flow); (2) L-lactate→haem electron transfer (stopped flow) and (3) L-lactate → cytochrome $c$ electron transfer (steady state).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>WT</th>
<th>HΔ3</th>
<th>HΔ6</th>
<th>HΔ9</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) L-lactate → FMN</td>
<td>8.1 ± 1.4</td>
<td>7.3 ± 0.6</td>
<td>6.2 ± 0.5</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>(2) L-lactate → haem</td>
<td>6.3 ± 1.2</td>
<td>2.8 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>(3) L-lactate → cytochrome $c$</td>
<td>3.0 ± 0.6</td>
<td>2.0 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>
dramatically. In the case of the HΔ6 and HΔ9 enzymes, where the KIE values are insignificant (<2), αH-abstraction from C-2 of L-lactate no longer contributes to rate limitation of haem reduction. This implies that deleting six or more residues from the hinge region causes FMN→haem electron transfer to become totally rate limiting. This conclusion is supported by the fact that for the HΔ6 enzyme, the KIE value for steady state cytochrome c reduction by L-lactate (Figure 4.3, step 3) is the same within experimental error as that for pre-steady-state haem reduction. For the HΔ9 enzyme the KIE for haem reduction is lower than that for cytochrome c reduction (1.1 versus 1.7 respectively), however, this may be an artefact of the manner in which the latter KIE was determined (ratio of the rate constant at 1 mM L-lactate for 1H- and 2H-lactate, rather than the ratio of the kcat values obtained from Michaelis-Menten fits of the kinetic data) and the true KIE may be lower than the value reported here. For the HΔ3 enzyme, the KIE value for haem reduction by L-lactate is 2.8 and although this is much lower than the corresponding KIE value of 7.3 for FMN reduction, it is still significant, implying that αH-abstraction from C-2 of L-lactate still contributes slightly to rate limitation of haem reduction in the HΔ3 enzyme, but much less so than in wild-type flavocytochrome b₂. The KIE value for steady-state cytochrome c reduction is lower than that for haem reduction in the HΔ3 enzyme, implying that an electron-transfer step subsequent to fully reduced FMN→haem electron transfer, contributes to rate limitation.

In summary, for the hinge-deletion enzymes, the KIE values are consistent with the kcat data for FMN, haem and cytochrome c reduction, since both the kcat and the KIE values erode in a similar fashion along the electron-transfer pathway.

4.3.3. Conclusions

All the kinetic data discussed thus far, concerning interdomain electron transfer are consistent with the proposal that maintaining the structural integrity of the hinge region is crucial for efficient interdomain communication: large deletions, >6 amino acids, render interdomain FMN→haem electron transfer totally rate limiting; a smaller deletion, of three amino acids has more complicated repercussions, with both intraprotein electron-transfer steps being affected (Scheme 4.1, steps 2 and 4).

Interestingly, for hinge-swap (White et al., 1993) and Y143F (Miles et al., 1992) flavocytochromes b₂, the rate-limiting step in the catalytic cycle is interdomain electron transfer from fully reduced FMN→haem. As is the case for the HΔ6 and HΔ9 enzymes, the pre-steady-state rate of haem reduction is the same within experimental error as the steady-state rate of cytochrome c reduction for both hinge-
swap and the Y143F enzymes (1.6 s\(^{-1}\) and 21 s\(^{-1}\) respectively). Also, the KIE values are the same within experimental error for both processes, and are < 2, indicating that FMN→haem electron transfer is indeed totally rate limiting. These results reinforce the conclusions drawn about the effect of the hinge-deletion mutations on interdomain communication, in that structural intergrity of the domain linker region (the hinge) is essential. Furthermore, in the case of the Y143F mutant enzyme, conservation of a key interacting residue at the domain interface is crucial.

4.3.4. Substrate-inhibition by hinge-deletion flavocytochromes \(b_2\)

As reported in Chapter 3, section 3.3.2, the hinge-deletion enzymes exhibit marked substrate inhibition for steady-state cytochrome \(c\) reduction but not for ferricyanide reduction. The \(K_i\) values are 2-, 9- and 12-fold lower for the HA3, HA6 and HA9 enzymes respectively, than the corresponding \(K_i\) value for the wild-type enzyme (Chapter 3, table 3.1.2). In the case of the HA6 and HA9 enzymes, the inhibition is severe. There are two possible explanations for this: either excess lactate binds at a secondary site, once the active site is occupied and this interferes with subsequent electron transfer to cytochrome \(c\); or, excess lactate interacts with and stabilises the hydroquinone or semiquinone form of FMN, inhibiting subsequent interdomain electron transfer. The latter explanation better explains the substrate inhibition pattern observed in the hinge-deletion enzymes. It is interesting to note that neither the HA6 or HA9 enzyme appear to exhibit substrate inhibition at the level of haem reduction by L-lactate (although this cannot be established unambiguously, as fitting the traces to a treble exponential equation gives a large scatter of rates at lactate concentrations greater than 15 mM). The HA3 enzyme, however, does exhibit substrate inhibition at the level of haem reduction, with a \(K_i\) which is a little higher in value than that obtained for steady-state cytochrome \(c\) reduction.

Wild-type flavocytochrome \(b_2\) does exhibit very weak substrate inhibition with regard to ferricyanide, cytochrome \(c\) and haem reduction (chapter 3, tables 3.1.2 and 3.2.2; Miles, 1993). The \(K_i\) is the same (175 mM) within experimental error for all three processes, implying that the inhibition is caused by the same factor. Making large deletions (6 and 9 amino acids) in the hinge region increases the inhibition observed for cytochrome \(c\) reduction but does not give rise to any significant substrate inhibition of ferricyanide or haem reduction over an equivalent concentration range of L-lactate. This suggests that in the HA6 and HA9 enzymes, the substrate inhibition might arise by lactate binding to and stabilising flavosemiquinone, inhibiting the second interdomain electron-transfer step (Scheme 4.1, step 4). However, it is also possible that lactate can bind to and stabilise the
flavohydroquinone. The absence of observed haem reduction inhibition under pre-steady-state conditions, could be due to the fact that the first interdomain electron-transfer step is so slow (Scheme 4.1, step 2), that it is totally rate limited anyway and the additional stabilisation of the flavohydroquinone by lactate has no effect. For the HΔ3 enzyme, the slight inhibition observed may be due to lactate binding to and stabilising both the hydro- and semiquinone forms of the flavin, inhibiting interdomain electron transfer from both forms of FMN, since the $K_1$ value for cytochrome $c$ reduction is slightly lower than that for haem reduction.

The increase in the degree of inhibition observed as the length of the hinge region is progressively truncated maybe due to greater accessibility of lactate to the active site. This could be due to impaired contact between the core and flavin domains, resulting in greater exposure of the interdomain interface to solvent.

4.3. Effect of the hinge deletions upon ferricyanide reduction

The fact that the rate of fully reduced FMN→haem electron transfer is drastically decreased in the hinge-deletion enzymes, compared to that for wild-type flavocytochrome $b_2$ may help to explain the higher $K_m$ values observed in steady-state experiments using ferricyanide as the electron acceptor (Chapter 3, Table 3.1.2). The $k_{cat}$ values for the steady-state reduction of ferricyanide are 3-, 9- and 50-fold higher than the $b_2$-haem reduction rate, for HΔ3, HΔ6 and HΔ9 flavocytochromes $b_2$ respectively. It follows that, in the hinge-deletion enzymes, ferricyanide can accept electrons from the flavohydroquinone and flavosemiquinone, effectively bypassing the haem group, as illustrated in Scheme 4.2.

The degree of haem bypassing increases as the length of the hinge region is progressively truncated. For the HΔ3 enzyme, there is only a 3-fold difference in the rate of ferricyanide and haem reduction and the haem group can still compete with ferricyanide for electrons, resulting in a slightly elevated $K_m$ for ferricyanide compared to the value for the wild-type enzyme ($0.15$ mM compared to $<<0.1$ mM respectively). In the case of the HΔ6 and HΔ9 enzymes, there is a larger difference in the rates of ferricyanide and haem reduction and it follows that ferricyanide must be removing electrons mainly from the flavin, bypassing the haem. This results in an increased $K_m$ for ferricyanide in the HΔ6 and HΔ9 enzymes compared to that for wild-type flavocytochrome $b_2$ (at least 4- and 6-fold higher respectively).

It is worth considering experiments performed on the haem-free (dehaemo-) form of flavocytochrome $b_2$ (Iwatsubo et al., 1977) and comparing that situation to the present one. Dehaemo-flavocytochrome $b_2$ shows a high $K_m$ for ferricyanide. However, $K_m$ values for electron acceptors measured under steady-state conditions in
Scheme 4.2. Electron Transfer Pathway From L-lactate to Ferricyanide as Catalysed by the Hinge-Deletion Flavocytochromes $b_2$

The scheme describes electron transfer in mutant flavocytochromes $b_2$ with disrupted interdomain communication (HΔ3, HΔ6, HΔ9, Y143F and hinge-swap enzymes). In the wild-type enzyme, route A is fast. However, in the above mentioned enzymes, route A is slowed down to varying degrees and route B is thought to become important. A significant contribution to the overall electron-transfer rate from route B would explain the marked dependence of reaction rate on ferricyanide concentration. This is because electron transfer to ferricyanide is fast from haem and flavosemiquinone, but is slow from flavohydroquinone.
systems where there are multiple electron-transfer steps, such as flavocytochrome \( b_2 \),
are complex and do not represent true dissociation constants. Iwatsubo et al. (1977)
have proposed a scheme in which ferricyanide accepts electrons from the
flavohydroquinone and flavosemiquinone in the dehaemo enzyme. An equation for
the dependence of electron-transfer rate on acceptor concentration at saturating
lactate was derived, expressing the \( K_m \) for ferricyanide in terms of a combination of
rate constants for electron transfer: from substrate to flavin; from flavohydroquinone
to ferricyanide and from flavosemiquinone to ferricyanide. With the ferricyanide
being reduced about 20-fold faster by flavosemiquinone than flavohydroquinone.
The rationale given by Iwatsubo et al. (1977) to explain the difference in \( K_m \) values
for ferricyanide between holo- and dehaemo-flavocytochrome \( b_2 \) may also be used to
explain the difference observed between the wild-type and hinge-deletion enzymes,
particularly \( H\Delta 6 \) and \( H\Delta 9 \) flavocytochromes \( b_2 \).

Hinge-swap (White et al., 1993) and Y143F (Miles et al., 1992; Rouvière-
Fourmy et al., 1994) flavocytochromes \( b_2 \) also have high \( K_m \) values of 1.1 mM and
3.3 mM respectively, for steady state ferricyanide reduction under saturating
substrate conditions. Interestingly, these \( K_m \) values are higher than those reported
here for the hinge-deletion enzymes, but the same rationale proposed by Iwatsubo et
al. (1977) can be used to explain them.

4.3.6. Effect of the hinge-deletion mutations on the haem group mid-point
potentials

As already discussed, the most probable cause of the decreased rate of
interdomain electron transfer in hinge-deletion flavocytochromes \( b_2 \), is that the
truncated hinge region has reduced flexibility, resulting in impaired recognition
between the flavin and cytochrome domains. The possibility that these decreased
rates could have been caused by a change in the thermodynamic driving force for
electron transfer, due to a large shift in the haem group mid-point potential, has been
eliminated. The haem potentials of the \( H\Delta 3 \) and \( H\Delta 6 \) enzymes are the same within
experimental error as the wild-type enzyme (Chapter 3, Table 3.3). The \( H\Delta 9 \) enzyme
has a haem potential which is about 15 mV lower than that of the wild-type enzyme,
but is the same, within experimental error as that for the independently expressed and
purified cytochrome \( b_2 \) domain of flavocytochrome \( b_2 \), \( b_2 \)-core (Brunt et al., 1992).
However, this change in the haem potential of the \( H\Delta 9 \) enzyme, is too small to alter
the driving force of the FMN→haem electron-transfer reaction, to such an extent that
it would account for the large decrease observed in the interdomain electron-transfer
rate. It is interesting that both \( b_2 \)-core and the \( H\Delta 9 \) enzyme have the same haem
potential. The pKₐ values of the haem propionates in b₂-core have been determined (Brunt et al., 1992) and are close in value to that of free haem propionic acid groups (Moore and Pettigrew, 1990), implying that the propionate groups of b₂-core must be exposed to solvent. The exposure of the haem edge to solvent in b₂-core probably accounts for the change in redox potential of the haem group, compared to the wild-type enzyme. Thus, it is plausible to suggest that the haem edge is more exposed to solvent in the HA9 enzyme than in wild-type flavocytochrome b₂. This idea is consistent with the proposal that the large nine amino acid deletion in the hinge region of the HA9 enzyme has impaired interdomain communication by 'pulling' the cytochrome domain away from the flavin domain, hindering effective contact.

4.4. PROSTHETIC GROUP REOXIDATION

As described in Chapter 3, section 3.4.3 an interesting phenomenon that was observed with the HA9 enzyme and subsequently confirmed to occur with wild-type flavocytochrome b₂ was prosthetic group reoxidation by molecular oxygen. The steady-state auto-oxidation of wild-type flavocytochrome b₂ by oxygen has been previously reported (Boeri & Rippa, 1960). The nature of the reoxidation processes are exactly the same in the two enzymes, indicating that this property is intrinsic to wild-type flavocytochrome b₂ and not an artefact of the hinge-deletion mutation (prosthetic group reoxidation was not investigated in the HA3 and HA6 enzymes). The fact that the rate constants for FMN and haem reoxidation are different, about with values of about 1.5 s⁻¹ and 0.2 s⁻¹ respectively, implies that reoxidation by molecular oxygen is occurring independently at both prosthetic groups, and the nature of these processes differs between the two sites.

Flavin reoxidation: A probable mechanism for FMN reoxidation by oxygen is shown in scheme 4.3. The physiological role of flavin in flavocytochrome b₂ is not as an oxidase, but as an electron transferase. In the light of this, it is informative to compare the oxidative half reactions of flavocytochrome b₂ with that of a related flavoprotein, whose physiological function is as an oxidase, glycolate oxidase. The reaction it catalyses is: glycolate + O₂ → glyoxylate + H₂O₂. The crystal structure of glycolate oxidase from spinach has been solved to 2 Å resolution (Lindqvist, 1989), however, comparatively little kinetic studies have been performed. Despite this, comparisons of the active site structures of these enzymes give an insight into the molecular basis for their different reactivities.
Figure 4.4. Comparison of the Active Site Geometry of Glycolate Oxidase and Flavocytochrome \( b_2 \)

Stereo diagrams of FMN and loop 1 in (a) glycolate oxidase and (b) flavocytochrome \( b_2 \). There is a difference in the main chain conformation resulting in a hydrogen bond between Ala NH-198 and FMN (N)5 in flavocytochrome \( b_2 \), which is absent in glycolate oxidase (Lindqvist et al., 1991).
Figure 4.5. Schematic Diagram of Flavocytochrome $b_2$ and Glycolate Oxidase, Illustrating the Hydrogen Bond Network

(a) Glycolate oxidase and (b) flavocytochrome $b_2$ (subunit with visible cytochrome domain) (Lindqvist et al., 1991).
The proposed oxidative half reaction catalysed by glycolate oxidase is similar in mechanism to Scheme 4.3 proposed for flavocytochrome $b_2$ flavin reoxidation (Lindqvist, 1991). The physiological half reaction of the two enzymes are clearly very different, what accounts for these differences? The active site structures of flavocytochrome $b_2$ and glycolate oxidase are very similar (Figure 4.4), with the positions of key catalytic residues virtually superimposable. However, in the latter, a buried water molecule occupies a cavity on the re-side of the FMN ring close to the C(4)O position and it is in this pocket that oxygen is postulated to bind for catalysis (Figure 4.4; Lindqvist 1991). In flavocytochrome $b_2$, however, the re-side of the flavin is inaccessible to solvent and hence oxygen, as the hydrogen bonds from the side chains of Gln-252 and Ser-228 prevent access to FMN (N)3 and O(4) respectively. Also, the FMN isoalloxazine ring system lies closer to β-strand 1 and the amide of Ala-198 forms a hydrogen bond to the flavin N(5), further inhibiting access (Figures 4.4 and 4.5; Mathews and Xia, 1990; Lindqvist, 1991).

It seems that the emergence of the flavooxidase and flavodehydrogenase functions of these two enzymes have proceeded by divergent evolution from a common precursor, and the subtle differences in the FMN orientation have a strong influence on the ultimate function of these enzymes. It is known that free reduced flavin in solution can be effectively oxidised by oxygen (Gibson & Hastings, 1962; Ballou et al., 1969 Elberlein & Bruice, 1982; Ghisla & Massey, 1989 and references therein) and so oxygen reactivity in these flavoproteins is likely to be governed by control of binding of oxygen to the active site in a suitable position for catalysis and not by influences of the protein on the chemistry of reduced flavin. Flavocytochrome $b_2$ still possesses some oxidase activity, which is only apparent at very low lactate concentrations. A possible explanation for this, is that in the presence of a limiting concentration of lactate, total conversion to product occurs, which then dissociates. Following this, oxygen enters and binds at the active site on the accessible si-face of the FMN ring, where it oxidises the reduced flavin. Thus, a partitioning exists.

![Scheme 4.3.](attachment:Scheme_4.3.png)

Abbreviations: E, flavocytochrome $b_2$; $F_{ox}$, oxidised FMN; $F_{red}$, reduced FMN; OOH; FMN-4a-hydroperoxy intermediate; LAC, lactate and PYR, pyruvate.
between flavin reoxidation by $b_2$-haem and oxygen, with the former being far more efficient.

**Haem reoxidation**: this phenomenon is altogether more difficult to explain than the observed flavin reoxidation. The $b$-type haem in flavocytochrome $b_2$ is low spin and is axially coordinated by two histidines on either side of the haem plane (Mathews & Xia, 1990). It is difficult to imagine how oxygen could bind to the haem iron in a suitable conformation for catalysis without displacing one of the axially coordinating histidines. Reoxidation of the $b_2$-haem would result in the formation of superoxide anion, unless additional electron transfer from flavin to haem allowed the further reduction of superoxide to hydrogen peroxide. It is interesting to note that cytochrome $b_5$, which is structurally very similar to cytochrome $b_2$-core, is also capable of being reoxidised by oxygen (Berman et al., 1976).

### 4.5. INTERPROTEIN RECOGNITION AND ELECTRON TRANSFER

#### 4.4.1. A kinetic investigation of cytochrome c:flavocytochrome $b_2$ association

The steady-state rate constants for cytochrome $c$ reduction by hinge-deletion flavocytochromes $b_2$ indicate that electron transfer from L-lactate to cytochrome $c$, as catalysed by flavocytochrome $b_2$ has been disrupted by the deletion mutations. However, steady-state experiments when considered alone, can only give information regarding the global electron-transfer rate from L-lactate through flavocytochrome $b_2$ to cytochrome $c$. Thus, to address the above question more fully, a single microscopic step, flavocytochrome $b_2$:cytochrome $c$ association was investigated.

Steps 3 and 5 in Scheme 4.1 illustrate flavocytochrome $b_2$ haem→cytochrome $c$ electron transfer (the final steps in the physiological catalytic cycle of flavocytochrome $b_2$). Recently, molecular modelling studies of the flavocytochrome $b_2$:cytochrome $c$ complex have been reported (Tegoni et al., 1993) and the nature of this complex is discussed in chapter 1, section 1.4.9. This model postulates that the interdomain hinge region of flavocytochrome $b_2$ forms a substantial part of the binding region for cytochrome $c$. To test this proposal, the effect of the hinge-deletion mutations upon flavocytochrome $b_2$:cytochrome $c$ association was investigated by pre-steady-state reduction of cytochrome $c$, by pre-reduced flavocytochrome $b_2$, as reported in chapter 3, section 3.5.

Scheme 4.4, shows the association step, subsequent electron transfer and dissociation step of the flavocytochrome $b_2$:cytochrome $c$ interprotein electron-transfer event.
Scheme 4.4.  
\[
\begin{align*}
\text{k}_{\text{on}} & \quad \text{k}_{\text{et}} & \quad \text{k}_{\text{diss}} \\
\text{fcb}_2\text{red} + \text{cyt c}_\text{ox} & \rightleftharpoons [\text{fcb}_2\text{red cyt c}_\text{ox}] & \rightarrow [\text{fcb}_2\text{ox cyt c}_\text{red}] & \rightarrow \text{fcb}_2\text{ox} + \text{cyt c}_\text{red} \\
\text{k}_{\text{off}} & & & \\
\end{align*}
\]

Abbreviations: \text{fcb}_2\text{red}, fully reduced flavocytochrome \text{b}_2; \text{fcb}_2\text{ox}, oxidised \text{b}_2-haem; \text{cyt c}_\text{ox}, oxidised cytochrome \text{c}; \text{cyt c}_\text{red}, reduced cytochrome \text{c}; \text{k}_{\text{on}}, bimolecular association rate constant (M$^{-1}$s$^{-1}$) - the step measured in the stopped-flow experiments; \text{k}_{\text{off}}, dissociation rate constant (s$^{-1}$) of the reactant complex; \text{k}_{\text{et}}, rate of interprotein electron transfer (s$^{-1}$) and \text{k}_{\text{diss}}, the dissociation rate constant of the product complex (s$^{-1}$).

As described in the results chapter, \text{k}_{\text{on}} was investigated using both horse heart and yeast cytochrome \text{c} for wild-type and hinge-deletion flavocytochromes \text{b}_2. Considering the results with horse heart cytochrome \text{c}, the largest change in \text{k}_{\text{on}} was for the HA3 enzyme, which was an order of magnitude lower than the corresponding value for the wild-type enzyme. This implies that deletion of three amino acids from the hinge region of flavocytochrome \text{b}_2 has impaired interprotein association between the HA3 enzyme and cytochrome \text{c}. In the case of the HA6 and HA9 enzymes, however, the \text{k}_{\text{on}} values are 4- and 2-fold lower than that for the wild-type enzyme and are larger in magnitude than that for the HA3 enzyme. This is surprising, in view of the fact that one would expect the 6 and 9 amino acid deletions in the hinge region to have a greater effect upon cytochrome \text{c} binding than the smaller 3 amino acid deletion, if only because of the larger structural change imposed.

When yeast cytochrome \text{c} was used (the true physiological cytochrome), \text{k}_{\text{on}} for the wild-type enzyme was 3-fold higher than the equivalent value obtained with horse heart cytochrome \text{c} and had a value approaching that for a diffusion controlled reaction (Fersht, 1988). Intriguingly, the \text{k}_{\text{on}} values for all three hinge deletion enzymes were the same within experimental error, and only 1.5-fold lower in magnitude than \text{k}_{\text{on}} for wild-type flavocytochrome \text{b}_2. A decrease in \text{k}_{\text{on}} by a factor of 1.5-fold is insignificant and implies that in the case of yeast cytochrome \text{c}, large changes in the length of the hinge region of flavocytochrome \text{b}_2 have very little, if any effect upon cytochrome \text{c} association.

It is interesting to compare the \text{k}_{\text{on}} values obtained for horse heart and yeast cytochrome \text{c}. The former exhibits greater specificity for the type of hinge deletion, but not in a manner that is readily interpretable in terms of the structural changes.
imposed. The fact that the true physiological cytochrome exhibits very little specificity to the degree of hinge truncation, and that the $k_{on}$ values for the hinge-deletion and wild-type enzymes are so similar, strongly implies that the hinge region cannot be an integral part of the proposed cytochrome $c$ binding site on the surface of flavocytochrome $b_2$ as proposed by Tegoni et al. (1993). Also, as the $k_{on}$ values are affected differently in the hinge-deletion enzymes, depending upon whether horse heart or yeast cytochrome $c$ is used, implies that these two cytochromes $c$ may associate with different surface regions of flavocytochrome $b_2$.

Clearly, in the light of these results, the nature of the proposed molecular model between the flavocytochrome $b_2$:cytochrome $c$ complex requires further investigation.

4.4.2. Use of nmr spectroscopy to investigate cytochrome $c$:flavocytochrome $b_2$ association

Chapter 3, section 3.5 reports the results of preliminary nmr experiments performed on wild-type and HΔ6 flavocytochromes $b_2$, in order to characterise the nature of the cytochrome $c$ interaction with wild-type flavocytochrome $b_2$ and how this was affected in a hinge-deletion enzyme. Two parameters were investigated; the stoichiometry of cytochrome $c$ binding to flavocytochrome $b_2$ and the affinity of the cytochrome $c$:flavocytochrome $b_2$ interaction.

As described in Chapter 3, section 3.7.1, the wild-type enzyme interacts with cytochrome $c$ with a stoichiometry of at least one cytochrome $c$ molecule per flavocytochrome $b_2$ subunit, at lower molar ratios of cytochrome $c$ to $b_2$-subunit (chapter 3, figure 3.23). At higher ratios of cytochrome $c$, it appears that further binding can occur, and the stoichiometry increases.

Wild-type and HΔ6 flavocytochromes $b_2$ have $K_{1/2}$ values of about 15 μM and 60 μM respectively (Chapter 3, section 3.7.2.1 and 3.7.2.2). $K_{1/2}$ is the concentration of flavocytochrome $b_2$ which causes a half maximal change in the chemical shift of the cytochrome $c$ haem methyl resonances. As described in section 3.7.1.1, $K_{1/2}$ is not a true dissociation constant, but rather, a combination of all the individual dissociation constants for all the cytochrome $c$:flavocytochrome $b_2$ associated complexes. Scheme 4.5, shows a situation which would arise for multiple binding of cytochrome $c$ to a single flavocytochrome $b_2$ subunit. If the stoichiometry of binding is indeed one cytochrome $c$ per $b_2$-subunit, then $K_{1/2} = K_1$. However, if the stoichiometry is $> 1$, then $K_{1/2}$ will be a function of a number of equilibrium constants.
Scheme 4.5.

\[
K_1 \quad K_2 \quad K_n \\
\text{fcb}_2 + \text{cyt} c \rightarrow [\text{fcb}_2: \text{cyt} c] + \text{cyt} c \rightarrow [\text{fcb}_2: (\text{cyt} c)_2] + n \ \text{cyt} c \rightarrow [\text{fcb}_2: (\text{cyt} c)n]
\]

Abbreviations: fcb$_2$, flavocytochrome $b_2$ (oxidised); cyt c, cytochrome $c$ (oxidised); K, equilibrium dissociation constant.

If the $K_{1/2}$ value for cytochrome $c$ binding to wild-type flavocytochrome $b_2$ is regarded as a dissociation constant, then a value of 15 $\mu$M, under the conditions of the experiment (20 mM phosphate buffer, pD 7.0), is indicative of tight binding. It is clear that the binding of cytochrome $c$ to flavocytochrome $b_2$ has not been greatly disrupted by the six amino acid deletion in the hinge region, as the $K_{1/2}$ for the HA6 enzyme is only 2-fold higher than that for the wild-type enzyme. This observation supports the data discussed in section 4.4.1, concerning the $k_0$ rate constants for the hinge-deletion enzymes compared to wild-type flavocytochrome $b_2$, where truncation of the hinge-region does not have a large effect upon cytochrome $c$ association.

Thomas et al. (1987) investigated cytochrome $c$ binding to flavocytochrome $b_2$ and the cytochrome $b_2$-core domain, with the proteins all isolated from the yeast $H. anomala$. The experiments were similar to those described here, where the cytochrome $c$ haem methyl 8 chemical shift change upon binding was monitored as a measure of the association between flavocytochrome $b_2$ and cytochrome $c$. Additionally, the induced shifts of a $b_2$-haem resonance at 19.4 ppm were also monitored for $b_2$-core and cytochrome $c$ association. The results indicated that $b_2$-core binds to cytochrome $c$ with a 1:1 stoichiometry. However, for the flavocytochrome $b_2$:cytochrome $c$ titration, the stoichiometry attained was only 4:10, for $b_2$ subunit:cytochrome $c$ (spectra above this stoichiometry being unresolved), so no reliable determination of binding stoichiometry could be achieved (Thomas et al., 1987). An important point to note is that the authors do not state whether the quoted flavocytochrome $b_2$:cytochrome $c$ ratios are with regard to the flavocytochrome $b_2$ tetramer or subunit. In the interpretation of their results, I have assumed the latter.

4.4.3. Conclusions concerning the cytochrome $c$:flavocytochrome $b_2$ interaction

The pre-steady-state and nmr data when considered together, indicate that the affinity of the interaction between flavocytochrome $b_2$ and cytochrome $c$ is quite strong. Also, maintaining the structural integrity of the hinge region is not important for effective cytochrome $c$ binding and subsequent electron transfer.
4.5. OVERALL CONCLUSIONS

From these studies on the hinge-deletion mutants of flavocytochrome $b_2$, the following general conclusions can be drawn:

(i) The interdomain hinge has little influence on the lactate dehydrogenase function of the enzyme.

(ii) Maintaining the structural integrity of the hinge region is essential for efficient flavin to haem electron transfer (intraprotein electron-transfer): Fully reduced FMN $\rightarrow$ haem electron transfer is the rate-limiting step in the HA6 and HA9 enzymes; semiquinone FMN$\rightarrow$haem electron transfer is the rate-limiting step in the HA3 enzyme.

(iii) Contrary to the proposed molecular model of the flavocytochrome $b_2$:cytochrome $c$ complex, the hinge region does not appear to be an important structural determinant for cytochrome $c$ binding. Indeed, making large deletions (up to 9 amino acid residues) in the hinge region does not greatly alter the association rate constant for cytochrome $c$ binding to flavocytochrome $b_2$. This suggests that there must be a certain degree of 'plasticity' in the interaction between these redox partners (chapter 1, section 1.3.3.1).
Chapter 5

Appendices
5.1. FUTURE WORK

In view of the results presented in this thesis, there are some obvious experiments which could shed more light onto the importance of maintaining the structural integrity of the hinge region, for efficient interdomain electron-transfer.

One idea which will be investigated, is to lengthen, as opposed to shortening the hinge region. Two oligonucleotide primers have been constructed which will insert 3 and 6 amino acid residues into the hinge region, as illustrated in Figure 5.1. The hinge lengthened flavocytochromes $b_2$ will be termed H13 and H16, for the 3 and 6 amino acid residues inserted respectively. These hinge lengthened enzymes will be characterised in a similar manner to the hinge-deletion flavocytochromes $b_2$. The main goal is to examine whether increasing the length and presumably the flexibility of the hinge region, impairs interdomain communication and whether the degree of impairment is related to the increase in length. In the unlikely event that these mutations do not have a significant effect upon interdomain electron transfer efficiency, than much larger insertions will be made.

Clearly, in order to interpret the kinetic data regarding the effect of the hinge deletions at the molecular level, the crystal structures of the three mutants will have to be determined. This goal is already underway, in collaboration with M. Tegoni and C. Cambillau at the University of Marseille, France. To date, microcrystals of the HA3 enzyme have been prepared, but as yet, no diffraction studies have been performed.
Figure 5.1. Construction of the Hinge Lengthened Flavocytochromes \( b_2 \)

The amino acid sequence of the interdomain hinge region from the \( S. \) \textit{cerevisiae} enzyme is shown, along with the lengthened amino acid sequence of the hinge insertion mutants (the inserted residues are shown in italics)

\[
\downarrow
\]

\[
89 \text{ PPELVCPYAPG}^{103}
\]

H/3 \[
89 \text{ PPELVCPYAPGAPG}^{(103+3)}
\]

H/6 \[
89 \text{ PPELVCPYAPGAPGAPG}^{(103+6)}
\]
5.2. REFERENCES


Oritz de Montellano P. R., editor (1986) *Cytochromes P450: Structure, Mechanism and Biochemistry (B)*


Wuthrich K. (1986) *Nmr of Proteins and Nucleic Acids*


5.3. PUBLICATIONS

Stephen K. Chapman, Patricia White, Simon Daff, Graeme A. Reid, R. Eryl Sharp and Forbes D. C. Manson
Probing the Structure and Function of Flavocytochrome b$_2$ Using Protein Engineering Methods.

R. Eryl Sharp, Patricia White, Stephen K. Chapman and Graeme A. Reid
Role of the Interdomain Hinge of Flavocytochrome b$_2$ in Intra- and Inter-Protein Electron Transfer.

Stephen K. Chapman, Graeme A. Reid, Simon Daff, R. Eryl Sharp, Patricia White, Forbes D. C. Manson and Florence Lederer
Flavin to Haem Electron Transfer in Flavocytochrome b$_2$.
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Role of the Interdomain Hinge of Flavocytochrome \( b_2 \) in Intra- and Inter-Protein Electron Transfer†

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ABSTRACT: The two distinct domains of flavocytochrome \( b_2 \) (L-lactate:cytochrome \( c \) oxidoreductase, EC 1.1.2.3) are connected by a hinge peptide. Kinetics experiments [White, P., Manson, F. D. C., Brunt, C. E., Chapman, S. K., & Reid, G. A. (1993) Biochem. J. 291, 89–94] have illustrated the importance for efficient interdomain electron transfer of maintaining the structural integrity of the hinge. To probe the role of the hinge in a more subtle manner, we have constructed a mutant enzyme, HΔ3, which has a three amino acid deletion in the hinge region. Intra- and inter-protein electron transfer within HΔ3 flavocytochrome \( b_2 \) and the HΔ3:cytochrome \( c \) redox complex was investigated by steady-state and stopped-flow kinetics analysis. The HΔ3 mutant enzyme remains a good L-lactate dehydrogenase, as is evident from steady-state experiments with ferricyanide as electron acceptor (40% less active than wild-type enzyme) and stopped-flow experiments monitoring flavin reduction (15% less active than wild-type enzyme). The global effect of the deletion is to lower the enzyme’s effectiveness as a cytochrome \( c \) reductase. This property of the HΔ3 enzyme is manifested at two electron-transfer steps on the catalytic cycle of flavocytochrome \( b_2 \). First, the rate of heme reduction has fallen 5-fold in HΔ3 compared with the wild-type enzyme (from 445 to 91 s\(^{-1}\)), due to poor interdomain electron transfer from flavin to heme. Second, the rate of cytochrome \( c \) reduction in the steady-state has fallen 5-fold (from 207 to 39 s\(^{-1}\)), indicating that \( b_2 \) heme to cytochrome \( c \) electron transfer has also been disrupted. These data, along with the measured kinetic isotope effects, indicate that cytochrome \( c \) reduction has become the rate-limiting step in the catalytic cycle for the HΔ3 enzyme. Further evidence for the importance of the hinge in inter-protein electron transfer is obtained from second-order rate constants for cytochrome \( c \) reduction by prereduced flavocytochrome \( b_2 \); the rate constant for HΔ3 is an order of magnitude less than the corresponding value for the wild-type enzyme, with values of \( 4 \times 10^6 \) and \( 4.7 \times 10^7 \) M\(^{-1}\) s\(^{-1}\), respectively. From our data, we conclude that the hinge plays an important role in facilitating both intra- and inter-protein electron transfer.

Flavocytochrome \( b_2 \) (L-lactate:cytochrome \( c \) oxidoreductase, EC 1.1.2.3) from baker’s yeast (Saccharomyces cerevisiae) is a homotetramer of subunit molecular weight 57 500 (Jacq & Lederer, 1974). The enzyme is a soluble component of the mitochondrial intermembrane space (Daum et al., 1982), where it catalyzes the oxidation of L-lactate to pyruvate with subsequent electron transfer to cytochrome \( c \) (Appleby & Morton, 1954). The crystal structure of flavocytochrome \( b_2 \) has been solved to 0.24-nm resolution (Xia & Mathews, 1990) and shows that each subunit consists of two distinct domains: an N-terminal domain containing protoheme IX and a C-terminal domain containing flavin mononucleotide. The two domains are connected by a single segment of polypeptide chain which constitutes the interdomain hinge (Figure 1). Crystallographic (Xia & Mathews, 1990) and NMR data (Labeyrie et al., 1988) support the idea that this segment of polypeptide functions as a hinge, by indicating that the cytochrome domain is mobile relative to the flavin domain.

The primary structure of flavocytochrome \( b_2 \) from another yeast species, Hansenula anomala, has been determined (Black et al., 1989a), and although there is an overall 60% identity between the amino acid sequences of the Saccharomyces and Hansenula enzymes, there are striking differences in the primary structure and net charge of the hinge region. The idea that these marked differences may account, at least in part, for the known kinetic differences between the two flavocytochromes \( b_2 \) has been tested by White et al. (1993). They investigated the role of the hinge in intramolecular electron transfer between the two domains of flavocytochrome \( b_2 \) by the construction of an interspecies hybrid enzyme comprising the bulk of the Saccharomyces enzyme but with the hinge region from the Hansenula enzyme. This "hinge-swap" enzyme, in which 29 residues from the Saccharomyces flavocytochrome \( b_2 \) had been replaced by 23 residues from the shorter and more acidic interdomain region from the Hansenula enzyme, was still a good \( b_2 \) dehydrogenase, but it was a poor cytochrome \( c \) reductase compared to the wild-type \( S. \) cerevisiae flavocytochrome \( b_2 \). Data showed that the rate of \( b_2 \) heme reduction was at least 300-fold lower in the hinge-swap enzyme compared to that of the wild-type enzyme, implying that the structural integrity of the hinge region is crucial in mediating electron transfer between the flavin- and heme-containing domains of flavocytochrome \( b_2 \) (White et al., 1993).

To more subtly probe the role of the hinge region in intra-protein and possibly inter-protein electron transfer within the flavocytochrome \( b_2 \) cytochrome \( c \) redox complex, we have constructed a mutant flavocytochrome \( b_2 \), HΔ3, which has a three amino acid deletion of residues 98–100 (APG) in the sequence (Figure 2). These residues were selected for deletion

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Figure 1: Graphic representation of a single wild-type flavocytochrome b subunit, generated using the MOLSCRIPT molecular graphics program (Kraulis, 1991). α helices and β sheets are shown as ribbons, the remaining carbon backbone is shown as a wire, and ball and stick atoms represent the prosthetic groups. The arrow indicates the position of the hinge region, and the position of the three residues deleted in the HΔ3 mutant is highlighted in black.

**FIGURE 1**

89 PPELVCPPYAPGETK → PPELVCPPYETK

**TABLE 1**

| FIGURE 2: Construction of HΔ3 flavocytochrome b2. The amino acid sequence of the interdomain hinge is shown. Amino acid residues APG (residues 98-100) are deleted in the mutant. |

Materials and Methods

**DNA Manipulation, Strains, and Growth.** Site-directed mutagenesis was performed by the Kunkel method of non-phenotypic selection (Kunkel, 1985) using the oligonucleotide 326N (GTCCTCCTTTAGAAAATAAGGA) (Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K.). Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation, and transformation were performed as described in Sambrook *et al.* (1991). *E. coli* strain TG1 was used for expression of wild-type and mutant flavocytochromes b2.

**Enzymes.** Wild-type and HΔ3 flavocytochromes b2 expressed in *E. coli* were isolated from cells which had been stored at -20 °C, using a previously reported purification procedure (Black *et al.*, 1989b). Purified enzyme preparations were stored under a nitrogen atmosphere at 4 °C as precipitates in 70% (NH4)2SO4. Enzyme concentrations were calculated by using previously published extinction coefficients (Pajot & Groudinsky, 1970).

**Kinetics Analysis.** All kinetics experiments were carried out at 25 ± 0.1 °C in Tris/HCl at pH 7.5 and 0.10 M. The buffer concentration was 10 mM in HCl with I adjusted to 0.10 M by addition of NaCl.

Steady-state kinetics measurements involving the enzymatic oxidation of L-lactate were performed using a Beckman DU62 spectrophotometer with either horse heart cytochrome c (type VI, Sigma) or ferriyanide (potassium salt, BDH Chemicals) as electron acceptor, as previously described (Miles *et al.*, 1992).

Stopped-flow measurements involving single turnover of L-lactate were carried out with an Applied Photophysics SF.17 MV stopped-flow spectrophorimeter. Flavocytochrome b2 was prepared for stopped-flow kinetics by dissolving the 70% (NH4)2SO4 protein precipitate in a minimal amount of Tris buffer; this was passed through a G25 Sephadex column equilibrated and eluted with Tris buffer, to remove salts and lactate. The eluted enzyme was fully oxidized. Flavin reduction was monitored at 438.3 nm (a heme isosbestic point), and heme reduction was monitored at either 423 or 557 nm (the results were identical at both wavelengths). Collection and analysis of data were as previously described (Miles *et al.*, 1992). *Km* and *kcat* parameters were determined using nonlinear regression analysis.

Kinetic isotope effect analysis was performed with L-[2-2H]lactate. Purification (Pompon *et al.*, 1980) and measurements of kinetic isotope effects (KIEs), using this substrate, were as previously described (Miles *et al.*, 1992).

**Abbreviations:** FMN, flavin mononucleotide; KIE, kinetic isotope effect; [H]Lac, L-[2-2H]lactate; [H]Lac, L-[2-2H]lactate; ferri, ferri cyanide; cyt c, cytochrome c.
The pre-steady-state reduction of cytochrome c (type VI, Sigma) by fully prereduced flavocytochrome b2 was monitored with the stopped-flow apparatus. The reaction was followed at 416.5 nm, which is an isobestic point for flavocytochrome b2. Flavocytochrome b2 was prepared for such experiments as described above except that the enzyme, which eluted from the G25 column in the fully oxidized state, was then fully reduced by addition of excess L-lactate (20 mM). Experiments were performed under aerobic conditions, as autooxidation of flavocytochrome b2 did not occur to any significant extent over the time scale of the experiment (2-3 h). Cytochrome c was fully oxidized by addition of a few crystals of ferricyanide, and the excess oxidant was removed by passing the mixture through a Sephadex G-25 column, equilibrated and eluted with Tris buffer.

To ensure that the reduction of cytochrome c occurred under pseudo-first-order conditions, flavocytochrome b2 was always present in excess. Reduction was carried out over a range of flavocytochrome b2 concentrations (2-15 μM). Cytochrome c concentration was 0.75 μM. The traces were fitted to single-exponential nonlinear regression analysis. At least six runs were performed at each flavocytochrome b2 concentration. Second-order rate constants were determined by plotting the \( k_{obs} \) for electron transfer (rate of cytochrome c reduction) against flavocytochrome b2 concentration and fitting the data to a linear regression analysis.

**Measurement of Redox Potential.** The midpoint potential of the heme group in HΔ3 was determined spectrophotometrically by using a previously published redox potentiometry method (Dutton, 1978). The mediators, N-ethylphenazonium ethyl sulfate, N-methylphenazonium methyl sulfate, 2,3,5,6-tetramethylphenylenediamine, 2-hydroxy-1,4-naphthoquinone, and flavin mononucleotide were used as previously described (Dutton, 1978). The enzyme concentration was 10 μM.

Reduction was achieved by titrating with sodium dithionite under anaerobic conditions, and oxidation, by titrating with potassium ferricyanide (Brunt et al., 1992). Changes in heme absorbance at 557 nm were measured with changing electrode potential. The system was buffered with the same Tris buffer as for kinetics experiments. The Nernst plots for both reductive and oxidative sequences showed no hysteresis, implying that the system was at equilibrium.

**RESULTS**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>electron acceptor</th>
<th>( k_{cat} (s^{-1}) )</th>
<th>( K_m ) (mM)</th>
<th>( k_{cat}/K_m ) (X10^3 M^{-1} s^{-1})</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>ferri</td>
<td>400 ± 10</td>
<td>86 ± 5</td>
<td>0.49 ± 0.05</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>HΔ3</td>
<td>ferri</td>
<td>257 ± 10</td>
<td>70 ± 2</td>
<td>0.72 ± 0.07</td>
<td>0.94 ± 0.11</td>
</tr>
<tr>
<td>wild type</td>
<td>cytc</td>
<td>207 ± 10</td>
<td>70 ± 10</td>
<td>0.24 ± 0.04</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>HΔ3</td>
<td>cytc</td>
<td>39 ± 1</td>
<td>20 ± 1</td>
<td>0.25 ± 0.03</td>
<td>0.61 ± 0.07</td>
</tr>
</tbody>
</table>

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (f.0.10). Concentrations of acceptors used with the HΔ3 enzyme were as follows: [cytochrome c], 35 μM (84% saturating); [ferricyanide], 2 mM (93% saturating). The \( k_{cat} \) values are expressed as moles of electrons transferred per second per mole of enzyme (as L-lactate is a two-electron donor, these values can be halved to express them in terms of moles of substrates reduced per second).

The value of the \( K_m \) for L-lactate observed with HΔ3 when ferricyanide is used as the electron acceptor implies (Miles et al., 1992) that electron flow to these acceptors has been affected in different ways by the hinge deletion, with electron flow to cytochrome c being impaired to a greater extent. Thus, the HΔ3 enzyme remains a good L-lactate dehydrogenase, but is a poor cytochrome c reductase.

In the wild-type enzyme, proton abstraction at C-2 of lactate is the rate-limiting step. From the data presented in Table 1, it can be seen that the deuterium kinetic isotope effect values for the HΔ3 enzyme are lower than those for the wild-type enzyme with both electron acceptors. This indicates that electron-transfer reactions following C-2 proton abstraction contribute to overall rate limitation in the HΔ3 enzyme to a greater extent than in wild-type flavocytochrome b2.

The value of the \( K_m \) for L-lactate observed with HΔ3 when ferricyanide is used as the electron acceptor is slightly higher than that for the wild-type enzyme. However, with cytochrome c as the electron acceptor, the apparent \( K_m \) for L-lactate is identical for HΔ3 and wild-type enzymes. The net effect of these changes in the kinetic parameters on the catalytic efficiency \( (k_{cat}/K_m) \) between HΔ3 and wild-type enzymes is a 2-fold decrease when ferricyanide is used as electron acceptor and a more significant 5-fold decrease when cytochrome c is used as electron acceptor.

**Stopped-Flow Kinetic Parameters for L-Lactate Oxidation.** Reduction of the FMN and heme prosthetic groups of HΔ3 flavocytochrome b2 by L-[2-1H]-lactate and L-[2-1H]-lactate was monitored directly using stopped-flow spectrophotometry. The kinetic parameters are summarized in Table 2. The effect of the hinge deletion on the rate of FMN reduction is insignificant \( (k_{cat} \) is lowered by 15% in HΔ3 flavocytochrome b2 compared to that of the wild-type enzyme). The rate of heme reduction, however, is 5-fold lower in HΔ3 compared to that for the wild-type enzyme (Table 2). These results imply that introducing the deletion mutation in the hinge region of flavocytochrome b2 has only a very slight effect on FMN reduction by lactate, but must have a greater effect on electron transfer from FMN to heme. This conclusion is supported by the KIE values reported in Table 2.

**Redox Potential.** We considered the possibility that the deletion mutation in the hinge region might have affected the redox potentials of the prosthetic groups. Clearly, there can have been little or no effect on the flavin potential, as the \( k_{cat} \) values for flavin reduction are not significantly different between HΔ3 and the wild-type enzyme. However, it seemed possible that a large change in the heme potential could have contributed to the decrease in the \( k_{cat} \) value for heme reduction...
Table 2: Stopped-Flow Kinetic Parameters and Deuterium Kinetic Isotope Effects for Reduction of FMN and Heme in Wild-Type and HΔ3 Flavocytochromes b₂

<table>
<thead>
<tr>
<th>enzyme</th>
<th>prosthetic group reduction</th>
<th>kₘ(s⁻¹) [H]Lac</th>
<th>kₘ(s⁻¹) [D]Lac</th>
<th>KIE</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>FMN</td>
<td>604 ± 60</td>
<td>75 ± 5</td>
<td></td>
<td>Miles et al. 1992</td>
</tr>
<tr>
<td>HΔ3</td>
<td>FMN</td>
<td>518 ± 17</td>
<td>71 ± 3</td>
<td></td>
<td>this work</td>
</tr>
<tr>
<td>wild type</td>
<td>heme</td>
<td>445 ± 50</td>
<td>71 ± 5</td>
<td></td>
<td>Miles et al. 1992</td>
</tr>
<tr>
<td>HΔ3</td>
<td>heme</td>
<td>91 ± 3</td>
<td>32 ± 1</td>
<td></td>
<td>this work</td>
</tr>
</tbody>
</table>

* All experiments were carried out at 25 °C in Tris/HCl buffer, I = 0.1. Stopped-flow data were analyzed as described in the Materials and Methods section. Values of kₘ are expressed as number of prosthetic groups reduced per second. Where biphasic kinetics were observed, the values reported correspond to values of the rapid phase as previously described (Miles et al., 1992).

in the HΔ3 enzyme. To check this, we measured the heme redox potential (at pH 7.5) for the HΔ3 enzyme and found that the value was the same within experimental error as that previously determined for wild-type flavocytochrome b₂: wild type = -17 ± 3 mV (Miles, 1992; White et al., 1993); HΔ3 = -14 ± 3 mV.

**Stopped-Flow Kinetic Parameters for Cytochrome c Reduction.** Reduction of cytochrome c by wild-type and HΔ3 flavocytochromes b₂ was monitored directly using stopped-flow spectrophotometry as described in the Materials and Methods section. The second-order rate constant for cytochrome c reduction by HΔ3 was an order of magnitude lower than the corresponding value for the wild-type enzyme. These data reflect the rate of electron transfer from flavocytochrome b₂ heme to cytochrome c within the flavocytochrome b₂ cytochrome c complex [(4.0 ± 0.3) × 10⁶ and (4.7 ± 1.0) × 10⁶ M⁻¹ s⁻¹ for wild-type and HΔ3 flavocytochromes b₂, respectively]. Clearly, b₂ heme to cytochrome c heme electron transfer has been disrupted in the HΔ3 enzyme, implying that the hinge region has an important role in maintaining the integrity of the complex.

**DISCUSSION**

The two domains of *S. cerevisiae* flavocytochrome b₂ are connected by a typical hinge sequence that contains proline, glycine, and various charged residues (Figure 2). It has been proposed that the most likely role of this hinge region is to confer domain mobility, allowing movement of the cytochrome domain with respect to the flavin domain. This proposal is supported by a number of observations, involving crystallography and NMR spectroscopy. In the three-dimensional structure of *S. cerevisiae* flavocytochrome b₂, two crystallographically distinct types of subunit are seen in the asymmetric unit. In one subunit, neither product nor substrate is bound at the active site and the cytochrome domain is resolved. In the other, pyruvate is bound at the active site and no electron density is observed for the cytochrome domain, implying that it is positionally disordered (Xia & Mathews, 1990). However, the fact that two of the cytochrome domains in the crystallized tetramer are positionally ordered may be due to restricted mobility imposed by crystal packing forces. In solution, NMR spectroscopy shows that the cytochrome domain is substantially more mobile than would be expected for a protein as large as the flavocytochrome b₂ tetramer; the observed line widths for heme group resonances in the cytochrome domain are not broadened to the degree anticipated for a large protein, implying considerable flexibility of this domain (Labeyrie et al., 1988; S. K. Chapman, C. E. Brunt, M. Cox, and G. Moore, unpublished results). Thus, a large body of structural information exists highlighting the importance of the hinge in interdomain interactions. Recently, we have published data concerning the kinetic properties of a mutant flavocytochrome b₂, designed to further investigate the role of the hinge in interdomain communication (White et al., 1993). This enzyme was termed hinge-swap, as it comprised the bulk of the *S. cerevisiae* enzyme, but with the native hinge region replaced by the equivalent region of the *H. anomala* enzyme. The most striking difference between the wild-type and hinge-swap enzymes was the 300-fold decrease in the kₘ value for heme reduction by lactate, implying that this mutation had severely impaired interdomain communication. These data support the conclusions from structural studies and stress the importance of maintaining the structural integrity of the hinge region for efficient interdomain communication.

To further our understanding of the role of the hinge region in intra- and inter-protein communication, we have constructed a mutant flavocytochrome b₂ with a three amino acid deletion in the hinge region. This enzyme, HΔ3, has some interesting differences in its electron-transfer properties when compared with wild-type and hinge-swap flavocytochromes b₂.

**FIGURE 3:** Catalytic cycle of flavocytochrome b₂. The redox states of cytochrome c and the flavocytochrome b₂ flavin (F) and heme (H) are indicated by the subscripts ox and red for the oxidized and reduced forms, respectively. The flavin semiquinone is shown as F₄ox. (1) Oxidation of lactate to pyruvate and reduction of FMN; this is the rate-limiting step in the case of the wild-type enzyme. (2) Electron transfer from fully reduced FMN to heme resulting in the semiquinone form of FMN and reduced heme (this is the slowest step in the case of the hinge-swap enzyme). (3) Reduction of the first cytochrome c molecule by electron transfer from the heme group of flavocytochrome b₂ (this is the slowest step in the HΔ3 enzyme). (4) Electron transfer from the semiquinone form of FMN to heme resulting in fully oxidized FMN and reduced heme. (5) Reduction of a second cytochrome c molecule by electron transfer from the heme group, which regenerates the fully oxidized enzyme. The enzyme is now ready to repeat the cycle.
where $k_{cat}$ for FMN reduction was only 2-fold less than that for the wild-type enzyme. These data imply that the hinge is not of great importance in FMN reduction by lactate, and thus $\Delta H_3$ remains a good $\Delta$-lactate dehydrogenase. This conclusion is supported by the steady-state measurements with ferricyanide as electron acceptor (Table 1).

The steps following FMN reduction are very different in wild-type, hinge-swap (White et al., 1993), and $\Delta H_3$ enzymes. The second step in the catalytic cycle (Figure 3) is intraprotein electron transfer from fully reduced flavin to oxidized heme. The $k_{cat}$ values for heme reduction by 1-lactate in the $\Delta H_3$ and hinge-swap enzymes are 5-fold and 300-fold lower, respectively, than in the wild-type enzyme. These results show that, in the case of the $\Delta H_3$ enzyme, there has been a significant impairment of electron transfer from FMN to heme. Thus, the three amino acid deletion in the hinge region has disrupted interdomain communication, but to a much lesser extent than the hinge-swap mutation. A likely explanation for this is that the truncation of the hinge region in $\Delta H_3$ and the hinge-swap enzymes (3 and 6 amino acids shorter, respectively, than the wild-type enzyme) has led to restriction of hinge flexibility, thereby preventing efficient recognition between the two domains. The much more pronounced effect observed for the hinge-swap enzyme compared to the $\Delta H_3$ enzyme is probably due to the more drastic structural change caused by the hinge-swap compared to the deletion mutation.

The final steps in the catalytic cycle involve reduction of cytochrome $c$, the physiological electron acceptor (Figure 3). In the case of the hinge-swap enzyme, $k_{cat}$ for the steady-state reduction of cytochrome $c$ was the same within experimental error as the $k_{cat}$ for $b_2$ heme reduction (1.6 s$^{-1}$), implying that cytochrome $c$ reduction does not contribute to rate limitation in the catalytic cycle and that the slowest step in the catalytic cycle must be FMN to heme electron transfer (White et al., 1993). In contrast, the $\Delta H_3$ enzyme has a $k_{cat}$ for pre-steady-state $b_2$ heme reduction of 91 s$^{-1}$, but the $k_{cat}$ for steady-state cytochrome $c$ reduction is slower at 39 s$^{-1}$. Direct evidence for impaired electron transfer between the hemes of the $\Delta H_3$ enzyme:cytochrome $c$ complex was obtained from the measurement of the second-order rate constants for cytochrome $c$ reduction under stopped-flow conditions. The rate constant for the $\Delta H_3$ enzyme was an order of magnitude less than the corresponding value for wild-type flavocytochrome $b_2$. Thus, we can conclude that the three amino acid deletion in the hinge region has resulted in impaired electron transfer from mutant flavocytochrome $b_2$ to cytochrome $c$, as well as disruption of interdomain communication.

The above conclusions are supported by the deuterium KIE values reported in Tables 1 and 2. For FMN reduction the KIE is the same within experimental error for $\Delta H_3$ and wild-type enzymes. This confirms that proton abstraction at C-2 of lactate is still rate-limiting for FMN reduction. However, for heme reduction there is a more significant lowering of the KIE value from 6.3 in the wild type to 2.8 in $\Delta H_3$. This indicates that proton abstraction at C-2 of lactate still contributes to rate-limitation of heme reduction in the $\Delta H_3$ enzyme, but much less so than in wild type. For cytochrome $c$ reduction in the steady-state, the KIE is 2.0 for $\Delta H_3$ compared to 3.0 for the wild-type enzyme, implying that, for the mutant, proton abstraction at C-2 of lactate is less rate-limiting for cytochrome $c$ reduction than for wild type. In summary, this is consistent with the $k_{cat}$ data for FMN, $b_2$ heme, and cytochrome $c$ reduction, since both the $k_{cat}$ and the KIE values erode in a similar fashion along this electron-transfer pathway for $\Delta H_3$.

As already mentioned, the most probable reason for the effect of the hinge deletion mutation on intra- and inter-protein electron transfer is that for the former, the shorter hinge region has reduced flexibility and impaired recognition between the cytochrome and flavin domains and for the latter, the cytochrome $c$ binding site has been disrupted. We have excluded the possibility that the heme redox potential was altered by the $\Delta H_3$ mutation, as the measured values for the wild-type and $\Delta H_3$ enzymes are identical within experimental error.

Previously published experiments investigating cytochrome $c$ binding to both $S$. cerevisiae and $H$. anomala flavocytochrome $b_2$ have concentrated on determining the stoichiometry and the dissociation constant, $K_4$, for cytochrome $c$ binding (Baudras & Spyridakis, 1971; Baudras et al., 1971; Baudras, 1971; Prats, 1977; Tegoni et al., 1983, 1990) and localizing the binding area to either the flavin or the cytochrome domains (Thomas et al., 1983; Albani, 1985) but not to any specific region on flavocytochrome $b_2$. In this paper, we have shown that maintaining the structural integrity of the hinge region is important for efficient cytochrome $c$ reduction, and thus it seems reasonable to propose that the hinge may form part of a recognition site for cytochrome $c$. This idea is supported by recent molecular modeling studies of the flavocytochrome $b_2$:cytochrome $c$ complex, which was based on the known crystal structures of the two proteins and energy minimization techniques (Tegoni et al., 1993). The model predicts that, within the complex, a significant number of inter-protein interactions occur between the hinge region and cytochrome $c$ which could be important determinants for cytochrome $c$ binding.

CONCLUSIONS

From our studies on $\Delta H_3$ flavocytochrome $b_2$, we draw the following conclusions: (i) The interdomain hinge has little influence on the lactate dehydrogenase function of the enzyme. (ii) The hinge is crucial in mediating electron transfer between the flavin- and heme-containing domains of the enzyme (intraprotein electron transfer). (iii) The three amino acid deletion results in $b_2$ heme to cytochrome $c$ heme electron transfer becoming the slowest step in the catalytic cycle (inter-protein electron transfer). (iv) The hinge region forms at least part of the binding site for cytochrome $c$ on flavocytochrome $b_2$. (v) The three amino acid deletion has little or no effect on the redox potential of the heme group.

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REFERENCES

electron transfer and haem oxygen binding are very rapid. Therefore the initial optical spectrum observed will be that of the haem oxy-complex and flavin radical. Flavin radical is then oxidized by oxygen at a rate of 0.15 s⁻¹, re-generating FAD. However, in the presence of oxygen bound at the haem site, flavin reduction by NAD(P)H is inhibited, and the flavin remains oxidized in the steady state. While attractive, such a model requires more direct evidence of haem–flavin interactions and oxygen consumption by FAD*, before it is confirmed.

Hmp binds oxygen and reduces it and oxidizes NAD(P)H. This intriguing diaphorase might, therefore, be able to 'sense' NAD(P)H/NAD(P) ratios and/or cellular oxygen concentrations. Experiments to investigate both possibilities are in progress in these laboratories.

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**Flavin to haem electron transfer in flavocytochrome b₂**

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**Introduction**

Flavocytochrome b₂ is a homotetrameric enzyme from yeast mitochondria which catalyses the oxidation of L-lactate to pyruvate with subsequent electron transfer to cytochrome c [1,2]. The enzyme from *Saccharomyces cerevisiae* has been successfully expressed at a high level in *Escherichia coli* [3] and it has been shown that the kinetic properties of flavo-

Abbreviation used: RDS, rate-determining step.

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flavocytochrome \( b_2 \) exhibits two crystallographically distinguishable subunits in the asymmetric unit [4]. In subunit 1, the electron-density map clearly shows the presence of both cytochrome and flavodehydrogenase domains, with Tyr-143 hydrogen bonding to a haem propionate (Figure 1). However, in subunit 2, the cytochrome domain is not visible, due to positional disorder (Gly-100 is the first visible residue), and Tyr-143 is seen to be hydrogen bonded to the carboxylate of a pyruvate molecule (the reaction product) which is located in the active site of the flavodehydrogenase domain.

Analysis of this three-dimensional information has allowed the identification of particular structural elements and amino acid residues which might be important in controlling electron transfer from one prosthetic group to another. For example, the two domains are linked by a typical hinge peptide and it is clear that this region of the protein has a significant influence on flavin to haem electron transfer [6,7]. In addition, it has been clearly demonstrated that one particular interface residue, Tyr-143 (Figure 1), plays a pivotal role in the modulation of interdomain electron transfer [8]. In the present paper we shall draw together previous work and new results to give an overall view of flavin to haem electron transfer in flavocytochrome \( b_2 \).

Materials and methods

DNA manipulation

Standard methods for growth of \( E. \ coli \), DNA manipulation and transformation were performed as described by Sambrook et al. [9]. Site-directed mutagenesis was performed as described elsewhere [10].

Enzymes

Wild-type and mutant flavocytochromes \( b_2 \) were isolated from \( E. \ coli \) cells and purified as previously reported [3].

Kinetic analysis

All kinetic parameters reported here were determined at 25 ± 0.1°C in 0.01 M Tris/HCl, pH 7.5, with \( I \) adjusted to 0.10 by addition of NaCl. Steady-state results were obtained as previously described [8]. Pre-steady-state kinetic measurements were made using an Applied Photophysics SF.17MV stopped-flow spectrophotometer as previously described [8]. Analysis of data was performed using the SF.17MV software and simulations were carried out using the Applied Photophysics Global Analysis package.

Results and discussion

How fast is flavin to haem electron transfer in flavocytochrome \( b_2 \)?

The electron flow through flavocytochrome \( b_2 \) proceeds from \( L \)-lactate to flavin, from flavin to \( b_2 \)-haem and finally from \( b_2 \)-haem to cytochrome \( c \). This process includes two intramolecular electron-transfer steps [11,12]: electron transfer from fully reduced flavin to \( b_2 \)-haem, and electron transfer from flavin semiquinone to \( b_2 \)-haem. Under pre-steady-state conditions, in the absence of any electron acceptor such as cytochrome \( c \), the full reduction of flavocytochrome \( b_2 \) proceeds as illustrated in Scheme 1. The various electron-transfer

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steps in this process have been studied by stopped-flow spectrophotometry [8] in which the reduction of the flavin and haem can be monitored at 438.3 nm (an isosbestic point in the visible spectrum of the haem) and 557 nm respectively [11,13]. As in previous studies, under most conditions the kinetic traces could be satisfactorily analysed as the sum of two exponentials.

Two models have been proposed to explain the reduction process [14,15]. In these models, the rapid first phase is a two-step process in which two electrons from L-lactate enter a subunit at the flavin level and are redistributed between flavin and haem in each subunit. The slower second phase is dominated by the entry of a third electron per subunit (corresponding to four electrons from two L-lactate molecules entering the full tetramer), which is made possible by inter-subunit electron transfer. This slow phase (equivalent to step 3 in Scheme 1) is kinetically irrelevant during catalytic turnover of the enzyme when it acts as a two-electron transferase [11,13].

In this paper we are focusing on flavin to haem electron transfer, which is step 2 in Scheme 1. This step is reversible, with the position of the equilibrium lying 85% in favour of reduced haem (as calculated from redox potentials [16]). The preceding step, flavin reduction by L-lactate, has a measured rate constant of 604 ± 60 s⁻¹ [8], which corresponds to \( k_{+1} \) in Scheme 1. The absorbance versus time trace for haem reduction yields a rate constant of 445 ± 50 s⁻¹ if fitted to a biphasic model [8]. However, the fact that these traces show an appreciable lag behind flavin reduction, coupled with the fact that there are three steps in Scheme 1, clearly indicates that fitting such data to a biphasic model is an approximation. Consequently, the value of the rate constant for haem reduction will have contributions from all three steps in Scheme 1. Step 3, however, contributes only ~15% of the total absorbance change and is much slower than steps 1 and 2. Therefore, during the initial 80% of the haem absorbance change the contribution of step 3 is comparatively small. Hence, in this region of the trace, the proportion of reduced haem can be described by the function

\[
\frac{1 + (k_a e^{-k_h t} - k_c e^{-k_h t})}{k_b - k_a}
\]

This function describes the accumulation of C in an A \( \rightarrow \) B \( \rightarrow \) C consecutive process, the second step of which may be an equilibrium. By fixing \( k_a \) to be the value of \( k_{+1} \) in Scheme 1, i.e. 604 ± 60 s⁻¹, and fitting the above function to the initial 80% of the haem absorbance change, a value for \( k_b \) of 1600 ± 300 s⁻¹ can be deduced. The value of \( k_b \) is, however, still slightly influenced by the rate of step 3, which perturbs the equilibrium of step 2. In order to take all the possible contributions into account, the limiting conditions must be considered. If step 3 is rapid and irreversible, the equilibrium in step 2 would be displaced and \( k_b \) would be equal to \( k_{+2} \). If, on the other hand, step 3 is detectably slow, then \( k_b \) would be equal to \( k_{+2} + k_{-2} \). The ratio of \( k_2 \) to \( k_{-2} \) can be calculated, from the equilibrium constant, to be 5.5:1 and this allows the limiting values for \( k_{+2} \) and \( k_{-2} \) to be calculated. From the limiting values one can estimate values for the rate constants \( k_{+2} \) (1500 ± 500 s⁻¹) and \( k_{-2} \) (270 ± 90 s⁻¹). Simulations indicate that this is a valid approach, especially in the absence of a more detailed understanding of step 3. These rate constants have been evaluated previously under very different experimental conditions [14,15]. Thus, at 25°C and at pH 7.5, the rate constant for electron transfer from fully reduced flavin to \( b_2 \)-haem is ~1500 s⁻¹.

The rate of electron transfer from flavin semiquinone to \( b_2 \)-haem is more difficult to estimate however. This step has been more extensively studied using the enzyme from Hansenula anomala with experimental approaches involving T-jump relaxation [17], stopped-flow spectrophotometry [18], stopped-flow coupled with e.p.r. [19,20] and laser flash photolysis [16,21]. The major conclusion from these experiments is that the stability of the flavin semiquinone is greatly enhanced in the presence of pyruvate, which causes an increase in the oxidized-semiquinone redox couple of ~100 mV. There is at present conflicting evidence about how this affects electron transfer from flavin semiquinone to \( b_2 \)-haem [16,18,19,21].

Is the hinge important for interdomain electron transfer?

As mentioned in the introduction, the cytochrome and flavodehydrogenase domains of flavocytochrome \( b_2 \) are linked by a segment of polypeptide chain which constitutes the interdomain hinge. This allows the cytochrome domain to be mobile with respect to the flavodehydrogenase domain, as demonstrated by crystallographic work [4] and supported by n.m.r. data [22]. The primary structure of the hinge region is strikingly different between flavocytochromes \( b_2 \) from two yeast species, S. cerevisiae and Hansenula anomala, even though there is 60% identity between the amino acid sequences of the two enzymes as a whole [23].
In order to probe the role of the hinge, an interspecies hybrid enzyme was constructed which consisted of the bulk of the *Saccharomyces* enzyme but had the hinge region replaced with that from the *Hansenula* enzyme [6]. This ‘hinge-swap’ enzyme retained the ability to be a good L-lactate dehydrogenase but was a very poor cytochrome c reductase. It was clear that the major effect of the hinge-swap was on flavin to haem electron transfer, which was at least 300-fold slower in the hybrid enzyme compared with wild-type [6] (Table 1). The implication was that structural integrity around the hinge was crucial in mediating electron transfer between the domains.

To probe more subtly this effect, two hinge mutations of flavocytochrome b$_2$ (HΔ3 and HΔ6) were constructed. The HΔ3 enzyme has a three-amino-acid deletion of residues 98–100 (APG) and the HΔ6 enzyme a six-amino-acid deletion of residues 95–100 (PYPAPG). These residues were chosen for deletion since they lie within the hinge region at the interdomain boundary. Both HΔ3 and HΔ6 enzymes remain good L-lactate dehydrogenases; flavin reduction is only 15% slower than for the wild-type enzyme (Table 1), implying that the hinge is of little importance in flavin reduction by L-lactate. The most striking effect of these deletions is, not surprisingly, on the rate of haem reduction, indicating that flavin to haem electron transfer has been impaired. The $k_{\text{cat}}$ values for haem reduction in the HΔ3 and HΔ6 enzymes are some 5-fold and 17-fold lower respectively than in the wild-type enzyme (Table 1). These results show that as the hinge segment is sequentially truncated the rate of flavin to haem electron transfer decreases. One possible explanation for this is that the truncation restricts the flexibility of the hinge, thereby impairing productive recognition between the two domains. In any case, it is now clear that the nature of the hinge region is critical for efficient electron transfer from flavin to haem.

### Table 1

Values of $k_{\text{cat}}$ for the reduction of flavin and haem by L-lactate for wild-type and mutant flavocytochromes b$_2$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Flavin reduction</th>
<th>Haem reduction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>604 ± 60</td>
<td>445 ± 50</td>
<td>[8]</td>
</tr>
<tr>
<td>Y143F</td>
<td>735 ± 80</td>
<td>21 ± 2</td>
<td>[8]</td>
</tr>
<tr>
<td>HΔ3</td>
<td>518 ± 17</td>
<td>91 ± 3</td>
<td>[7]</td>
</tr>
<tr>
<td>HΔ6</td>
<td>520 ± 12</td>
<td>26 ± 1</td>
<td>R. E. Sharp, unpublished work</td>
</tr>
<tr>
<td>Hinge-swap</td>
<td>240 ± 12</td>
<td>1.6 ± 0.4</td>
<td>[6]</td>
</tr>
</tbody>
</table>

All values were determined by the stopped-flow method at 25°C in Tris/HCl buffer, pH 7.5, $I=0.10$. Values, obtained from non-linear least-squares fit of Michaelis–Menten curves, are expressed as number of prosthetic groups reduced per second (mean ± S.D.). Data were collected and analysed as described previously [8].

*Does Tyr-143 control flavin to haem electron transfer?*

From Figure 1, Tyr-143 appears to be uniquely placed to play a central role in mediating electron transfer both from lactate to flavin and from flavin to haem. The role of this residue has been examined by the construction and extensive characterization of a mutant flavocytochrome b$_2$ (Y143F-b$_2$) in which Tyr-143 has been replaced by phenylalanine [8,24]. The most significant effect of the mutation was found to be a change in the rate-determining step (RDS) for the enzyme. In the wild-type enzyme the main RDS is proton abstraction at C-2 of L-lactate, as shown by the primary $^2$H-kinetic isotope effect [8,13]. However, in Y143F-b$_2$ the RDS is intramolecular electron transfer from flavin to haem. The rate of flavin reduction is identical (within error; Table 1) for the mutant and wild-type enzymes under the conditions employed in [8], whereas the $k_{\text{cat}}$ for haem reduction in Y143F-b$_2$, as determined from stopped-flow experiments, is some 20-fold lower than that measured for wild-type enzyme (Table 1) [8]. Therefore we can conclude immediately that Tyr-143 plays a key role in facilitating electron transfer from flavin to haem.
Why does the Y143F mutation have such an effect on flavin to haem electron transfer? It is known that the effect is not due to gross structural changes since the X-ray crystal structure of Y143F-\(b_2\) is essentially the same, overall, as that of the wild-type enzyme [25]. The most likely explanation is that the mutation removes a critical inter-domain hydrogen bond between the phenolic OH of Y143 and a haem propionate (Figure 1) and this removal significantly impairs interaction and electron transfer between the domains.

**Does pH influence flavin to haem electron transfer?**
The catalytic activity of flavocytochrome \(b_2\) is pH-dependent and pre-steady-state kinetics indicate that the rate of flavin to haem electron transfer is substantially decreased at low pH. This suggests that protonation of an ionizable group close to the haem or flavin has a significant influence on this electron-transfer step. The \(pK_a\) of this ionizable group is estimated, from kinetic experiments, to be a little above 5.0. One can suggest, from Figure 1, that a suitable candidate for this group would be haem-propionate 7 (HP7) which hydrogen-bonds to Tyr-143. N.m.r. experiments on the isolated cytochrome domain indicate that HP7 has a \(pK_a\) of \(4.8\) [26], and a similar n.m.r. study on a monomeric form of flavocytochrome \(b_2\) gives a value of \(\sim 5.0\) (P. White, unpublished work). These results are consistent with HP7 being the ionizable group which affects flavin to haem electron transfer. It is possible to visualize the protonation of HP7 causing a disruption of the network of hydrogen-bonding between the flavin and haem (Figure 1) with the resulting effect on electron transfer between the two redox centres.

**Conclusions**
There are several factors which affect the rate of electron transfer in proteins, such as the driving force, the distance between redox centres and the intervening medium between these centres. In this paper we have focused on electron transfer from flavin to haem in flavocytochrome \(b_2\). It has been shown that this intramolecular electron-transfer step is strongly influenced by a number of structural features of the enzyme, including the inter-domain hinge, Tyr-143 and haem-propionate 7. In addition, it is now evident that properties such as domain mobility are important in modulating inter-domain electron transfer.

We are indebted to Professor F. S. Mathews, Dr. M. Tegoni and Dr. C. Cambillau for helpful discussions. This work was supported by the Science and Engineering Research Council (SERC) through research grants and by the Royal Society through an equipment grant. We are grateful to SERC for postdoctoral support for P.W. and F.D.C.M. and for postgraduate support for S.D. and R.E.S. We thank the European Community FLAPS network for travel funds.

Metal-redox centre interactions in photosynthetic reaction centres
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Introduction
Photosynthetic reaction centres are supramolecular structures which convert light energy into electrochemical energy [1]. The general design of these structures and the mechanism of energy conversion is the same in all chlorophyll-containing photosynthetic organisms. The photochemically driven electron-transfer process depends on a highly organized electron-transfer chain containing a mixture of metal centres and organic redox centres. The latter are held in a protein matrix which provides them with very precise positions and orientations. The efficiency of the photochemical system depends on the positioning of the redox components, which controls the rate of electron transfer. The precise positioning and close relationship of the organic and metal redox centres results in magnetic interactions between the centres which can be detected by magnetic resonance techniques, providing information about the distances between the centres and a sensitive measure of changes in the local environment of the centres. These interactions can provide comparative information to allow modelling of reaction centre structures and mechanisms and also provide novel information about the mechanism of electron transfer.

The essential reaction centre structure contains a primary photochemically activated electron donor and an acceptor. The basic photochemical mechanism appears to be essentially the same in all reaction centres:

\[ \text{Photochemical donor} \rightarrow \text{first acceptor} \rightarrow \text{secondary acceptors} \]

However, there are two types of reaction centres, in which the protein structure and the nature of the redox centres of the electron acceptor complex are very different. One type (type A) is found in purple anoxygenic photosynthetic bacteria, some green bacteria and photosystem 2 of oxygenic organisms. It is well characterized, with X-ray crystal structures available for two purple bacterial reaction centres and extensive spectroscopic characterization of reaction centres available for both oxygenic and anoxygenic organisms. Type A has two major polypeptides which bind the redox components and show significant sequence similarities among the different groups of organism. In these reaction centres the first electron acceptor is a pheophytin and the secondary acceptors are two quinone molecules coupled to a ferrous iron atom. In bacteria, the electron donors to this type of centre are cytochromes, and in photosystem 2 the water-oxidizing complex is the electron donor.

The second type of reaction centre (type B), found in green sulphur bacteria, Heliobacteria and photosystem 1 of oxygenic photosynthetic organisms, is less well characterized structurally. Crystals of cyanobacterial photosystem 1 are available, but the structure has not yet been completed, although data at 6 Å has been published [2]. Again two main polypeptides bind the initial redox components; however, these are much larger than in type A, share no sequence similarity with type A polypeptides and bind a large number of light-harvesting chlorophyll molecules as well as the redox-active components. There are a number of smaller polypeptides associated with type B reaction centres, one of which binds two iron-sulphur centres of the electron acceptor complex. In these reaction centres the first electron acceptor is a chlorophyll and the secondary acceptors include both quinone and iron-sulphur centres. While the purple bacterial reaction centre is the best characterized type A centre, with much of our knowledge of photosystem 2 coming from application of the bacterial model, photosystem 1 is the best known type B centre, with the green bacteria and Helio-bacterial centres rather poorly understood because...