Cytochrome $c_3$ modules as electron transfer nanowires

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For my wife and parents
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Abstract

The tetraheme cytochrome c<sub>3</sub> from *Desulfovibrio vulgaris* Miyazaki F is involved in sulphate reduction. It contains four c-type hemes covalently bound to a single polypeptide of only 107 amino acids. The protein surface is highly positively charged at physiological pH with a prevalence of lysine residues. All four hemes are bis-histidine ligated and their reduction potentials are very low. With a cyclic heme arrangement and their partial exposure to solvent, cytochrome c<sub>3</sub> can transfer electrons in all directions. The purpose of this work was to develop novel methods of controlling the crosslinking selectivity of proteins constructing biological "nanowires" with cytochrome c<sub>3</sub> modules. The crosslinking functional groups are bismaleimide derivatives and the cysteine thiol. Construction strategies involve electrostatic selection by controlling the surface charge distribution to regulate protein crosslinking, and the use of protein-peptide recognition (i.e. Calmodulin and its binding peptide) to block/protect the crosslinking sites. Another aim of this project is to exploit the electrochemical properties of cytochrome c<sub>3</sub> by covalently attaching it to cytochrome P450 BM3 heme domain and electrodes to form unique bioelectronic components, which could facilitate electron transfer between enzymes and electrodes.

In order to utilize the cytochrome c<sub>3</sub> for protein conjugation, three cysteine mutants (K40C, S107C, and K40C:S107C) were constructed, successfully expressed in *Shewanella oneidensis* MR-1, purified and characterized by fluorescent labelling and thiol counting. Protein crosslinking was performed with a bismaleimide crosslinker, 1,8-bis-Maleimidodiethyleneglycol (BM(PEO)<sub>2</sub>) on the cysteine mutants to produce cytochrome c<sub>3</sub> dimers and polymers. The homodimer formation showed that S107C was more efficient in forming homodimers than K40C probably because it is at the flexible and exposed C terminus of the protein. The K40C and S107C heterodimer (yield: ~ 25 %) was generated upon the production of a bismaleimide-modified S107C intermediate. The two-step reaction shows that protein ligation can be performed in a controllable multi-step way. Two methods were used for polymer production: disulfide crosslinking and bismaleimide crosslinking. The polymers generated through disulfide crosslinking were cleavable whereas bismaleimide crosslinked polymers are stable in a reducing environment. Both gave a high degree of polymerization yielding up to the 12mer at least for disulfide crosslinking and up to
the 8mer at least for bismaleimide crosslinking. The chemical nature of heme groups in the cytochrome $c_3$ modules remained unchanged before and after polymerization. OTTLE potential titration shows that the polymerisation of the cytochrome $c_3$ modules leads to a reduction potential shift towards the negative from $-304$ mV (wild type) to $-336$ mV (disulfide-linked cytochrome $c_3$ polymers) and $-388$ mV (bismaleimide-linked cytochrome $c_3$ polymers). A possible reason is that the interaction among hemes in the cytochrome $c_3$ modules makes the polymer more difficult to reduce.

The affinity tags: Calmodulin Binding Peptide (CBP) and Strep Tag II, were are exploited to control the protein ligation. The cytochrome $c_3$ mutants tagged with CBP did not bind to the Calmodulin (CaM) affinity resin during purification. Possible reasons might be that the linker between protein and CBP was not long enough or that the CBP was partly proteolysed during expression. A K40C cytochrome $c_3$ with a “New Strep Tag” containing a cysteine (nstK40C) was constructed, expressed and purified. Strep-Tactin was used to block the cysteine in New Strep Tag of nstK40C cytochromes $c_3$ allowing the cysteine 40 to react, in order to control the ligation process. A controlled protein ligation strategy was successfully developed and a short wire was generated using three different cytochrome $c_3$ modules in spite of low yield. Cytochrome $c_3$ was crosslinked to P450 close to its active site heme. The heme of P450 had a similar environment to the heme of wild type P450. P450 heme domain could not be reduced directly by the electrode due to its buried active site and it was slowly reduced by cytochrome $c_3$ ($\sim 10$ hours). However, there was no electron transfer observed under the same condition for the conjugate of cytochrome $c_3$ and P450 BM3 heme domain. Probably the cytochrome $c_3$ was not crosslinked to the right position of P450 BM3 close enough to the P450 BM3 heme.
### Abbreviations and units

#### A. Amino Acids

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C. Abbreviations

ATP: Adenosine triphosphate
BM(PEO)$_2$: 1,8-bis-Maleimidodiethyleneglycol
BV: Benzyl viologen
CaM: Calmodulin
CBB: Coomassie Brilliant Blue
CBP: Calmodulin binding peptide
CDI: Carbonyldiimidazole
CO: Carbon monoxide
CPR: Cytochrome P450 reductase
CYP: Cytochrome P450
DMSO: Dimethyl sulfoxide
DTNB: 5,5'‐Dithiobis(2-nitrobenzoic acid)
DTT: Dithiothreitol
EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA: Ethylene diamine tetraacetic acid
EGTA: Ethylene glycol tetraacetic acid
F5M: Fluorescin-5-maleimide
FAD: Flavin adenine dinucleotide
FMN: Flavin mononucleotide
FNR: FAD-dependent ferredoxin reductase
FPLC: Fast protein liquid chromatography
Frd: Fumarate reductase
GST: Glutathione S-transferase
HMC: High-molecular-weight cytochrome c
MBP: Maltose binding peptide
MOPS: 3-(N-morpholino)-propanesulfonic acid
MV: Methyl viologen
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NHE: Normal hydrogen electrode
NHS: N-Hydroxysuccinimide
OTTLE: Optically transparent thin layer electrode
PAGE: Polyacrylamide gel electrophoresis
PEG: Polyethylene glycol
PCR: Polymerase chain reaction
PDB: Protein data bank
2-PDS: 2,2'-Dipyridyl disulfide
PMSF: Phenylmethylsulphonyl fluoride
SBP: Streptavidin binding peptide
SDS: Sodium dodecyl sulphate
SHE: Standard hydrogen electrode
TCEP: Tris(2-carboxyethyl) phosphine hydrochloride
TMBZ: 3,3',5,5'-tetramethylbenzidine
TNB: Thio-2-nitrobenzoic acid
Tris: 2-Amino-2-hydroxymethyl-propane-1,3-diol
TSS: Transformation and storage solution
UV-Vis: Ultraviolet-visible
WT: Wild-type
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Introduction
1.1 Electron Transfer in Biology

Electron transfers without chemical bond formation or breakage are fundamentally important in biological systems, such as the systems of respiration, and photosynthesis (Fig. 1.1). Both systems involve redox centres, strategically placed between initial donor and final acceptor, transporting electrons to the required site forming ‘biological currents’. Most redox centres contain transition metals, although flavins, quinones and other organic species also facilitate electron transfers.

Figure 1.1: photoreaction center of the purple bacterium Rhodopseudomonas viridis, (PDB entry: 1PRC). Panel a: the system has four components: three subunits, H, M, and L (cyan, yellow, and purple, respectively), and a fourth protein, cytochrome c (green) associated with complex at the membrane surface. Panel b: shown as stick-and-ball models are the prosthetic groups that participate in the photochemical events. Shown at the top of the figure are four heme groups (red) of c-type cytochrome associated with the reaction center. Bound to the L and M chains are two pairs of bacteriochlorophyll molecules (green); one of the pairs (the “special pair,” (Chl)$_2$) is the site of the first photochemical changes after light absorption. Also incorporated in the system is a pair of bacteriochrome molecules (blue); two quinones, menaquinone ($Q_A$) and ubiquinone ($Q_B$) (yellow and orange), and a single nonheme Fe (red) located in the center between the quinones. The excited special pair passes an electron to bacteriochrome, from which the electron moves rapidly to the tightly bound menaquinone, $Q_A$. This quinone passes electrons much more slowly to
the diffusible ubiquinone, \( Q_B \), through the nonheme Fe. Meanwhile, the "electron hole" in the special pair is filled by an electron from a heme of cytochrome c.

The rate of electron transfer decreases exponentially with the distance between redox centres. However, it has been shown that biological electron transfer is productive within 14 Å, which is still fast enough to enable substrate binding and chemical bond formation or breakage without being the rate-limiting step in a catalytic cycle [1]. Naturally, most of the distances between redox centers are limited to less than about 14 Å and chains of redox centers are exploited to relay electrons over a long distance.

1.2 Electron Tunnelling

In the quantum mechanics, the behavior of an electron is described as a wave function. All wave equations exhibit evanescent wave coupling effects in the right conditions regardless of the existence of the energy barrier. It means that, if the wave of an electron on the donor can reach the acceptor penetrating far enough through the energy barrier, the electron will transfer from the donor to the acceptor. Electrons penetrating the narrow potential energy well between the donor and the acceptor, is defined as "electron tunnelling" [2]. In contrast with the classical transition state theory, thermal energy (generated by the collision of the reactants) is required to overcome the energetic barrier.

The coupling of donor and acceptor in an electron transfer reaction is only dependent upon the distance between them:

\[
H_{AB}^2 \sim e^{-r}
\]  

(Eq. 1.1)

where \( H_{AB} \) represents the electronic coupling factor between the reactants (A and B) and \( r \) the distance between them.

The electron is potentially able to oscillate in the donor and acceptor coupling system. The resonance vibration depends on the distance (\( r \)) and on the relative energy of the wells of the donor/acceptor couple. In a biological system, donor and acceptor will have different energy states, enough to break the resonance state and allow the electron transfer to progress to completion. The whole system must satisfy both the Franck-Condon and energy conservation principles [3,4]. The Franck-Condon
principle is the approximation that an electronic transition is most likely to occur without changes in the positions of the nuclei in the molecular entity and its environment.

1.3 Marcus Theory and Electron Transfer Rate

Marcus and co-workers, taking the Franck-Condon and energy conservation principles into consideration, developed a convenient model to describe the electron transfer reactions both in classical and quantum mechanistic terms [5,6].

Considering a simple example of an electron transfer reaction,

$$\text{Reactant (R)} \xrightarrow{e^-} \text{Product (P)}$$

the energy configurations (E) of both reactant and product can be represented by a parabola plotted against the reaction coordinate (r) (Fig. 1.2). Both the profiles of reactant and product indicate ground states with minimum energy configuration. To transfer the electron, the reactant needs to be activated to the transition state, the intersection of the two parabolic curves, where the nuclear configurations are isoenergetic ($E_R = E_P$). Then, the energy of the reaction system goes along the curve of product to the lowest point, forming the stable product. The difference between the ground states of reactant and product is defined as the total Gibbs free energy change ($\Delta G$) of the electron-transfer reaction, representing the driving force for the reaction. The activation energy ($\Delta G^+$) describes the energy required to push the reactant to the energy level at the intersection of the two potential energy surfaces. The reorganization energy ($\lambda$) is also introduced as the energy required for all geometrical adjustments (nuclear configuration change) from reactant to product without transferring an electron.
Cytochrome c₃ modules as electron transfer nanowires

Figure 1.2: plot of the potential energy (E) against nuclear coordinates (r). The blue parabola curve shows the energy configuration of the reactant while the black curve shows the energy configuration of the product. \( \lambda \), the reorganization energy; \( \Delta G^\dagger \), the activation energy; \( \Delta G \), the free energy

Marcus theory provides a relationship between the activation energy (\( \Delta G^\dagger \)), the driving force (\( \Delta G \)) of a chemical reaction and the reorganization energy (\( \lambda \)) by an equation:

\[
\Delta G^\dagger = \frac{(\Delta G + \lambda)^2}{4 \lambda} \tag{Eq. 1.2}
\]

In quantum theory interpretation of electron transfer, the energy potential curves are simple harmonic oscillators, where only some energy levels are permitted: these levels correspond to the frequency of the oscillator (\( E = \hbar \omega \)). In this case the transfer depends upon the overlap of the harmonic oscillator wave functions of the two reactants.

In the adiabatic case, the rate of electron transfer reaction is related to the activation energy (\( \Delta G^\dagger \)) according to the equation:
\[ k_{ET} = \kappa \exp \left(-\frac{\Delta G^+}{k_B T}\right) \]  
(Eq. 1.3)

where \( k_{ET} \) represents the observed electron-transfer rate, \( \kappa \) is the electron-transfer rate when \( \Delta G^0 \) is zero (maximum rate of electron transfer), \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature.

Combining Eqs. 1.2 & 1.3, it would give

\[ k_{ET} = \kappa \exp \left(-\frac{(\Delta G + \lambda)^2}{4\lambda k_B T}\right) \]  
(Eq. 1.4)

**Figure 1.3:** plots of the nuclear potential energy vs. nuclear coordinates. The blue parabola curve shows the energy state of the reactant while the black curve shows energy state of the product. Three groups of parabola curves represent: (a) normal case of electron transfer; (b) activationless case of electron transfer; (c) inverted case of electron transfer. \( \lambda \), the reorganization energy; \( \Delta G \), the free energy

According to the Eq. 1.4, three possible cases can be deduced:
(a) **Normal region** (Fig 1.3a): when $\Delta G + \lambda > 0$, which means $\lambda > -\Delta G$;

(b) **Activationless region** (Fig 1.3b): when $\Delta G + \lambda = 0$, which means $\lambda = -\Delta G$, and $k_{ET} = \kappa$. The reaction reaches the maximum rate without activation energy;

(c) **Inverted region** (Fig 1.3c): when $\Delta G + \lambda < 0$, which means $\lambda < -\Delta G$

The driving force ($\Delta G$) varies in reverse direction of the change of electron transfer rate.

The relationship of the logarithm of electron-transfer rate ($\ln k_{ET}$) and reaction driving force ($\Delta G$) is illustrated in Fig. 1.4. As the driving force ($\Delta G$) increases, $k_{ET}$ keeps going up until it reaches a summit ($-\Delta G = \lambda$), and then comes down ($-\Delta G > \lambda$).

*Figure 1.4: plot of $\ln k_{ET}$ against reaction's driving force ($\Delta G$).*
In the quantized view the dependence upon temperature (T) of the electron-transfer rate in Eq. 1.4 is not allowed and T is replaced by the quantum energy of the oscillator, \( E = h\omega \).

Other factors that affect electron transfer are not only the distance between the donor-acceptor pair, but also the intervening medium, that contributes to propagating the electron transfer. The electron-transfer rate is dependent on the intervening medium and the distance between the donor and acceptor according to the equation below.

\[
K \propto e^{-1/2\beta r} \quad \text{(Eq. 1.5)}
\]

where \( \beta \) is the coefficient that describes the decay through the medium and \( r \) is the travelling distance of the electron.

To summarize the above equations, a new equation is given below.

\[
k_{ET} \propto e^{-1/2\beta r} \exp\left(-\left(\Delta G + \lambda \right)^2 / \left(4 \lambda k_B T\right)\right) \quad \text{(Eq. 1.6)}
\]

The Eq. 1.6 indicates that electron-transfer rates are determined by two major factors: the thermodynamic oscillation that bring the reactant and the product to their isoenergetic states and the probability of electron tunnelling between isoenergetic states. The thermodynamic oscillation depends on the contribution of the macroscopic environment to the reaction system. The electron tunnelling probability involves the chemical and structural properties of the donor-acceptor pair [7-10].

1.4 Biological Electron Transfer Models

Two major theory models have been developed to explain biological electron transfer through redox proteins:

1) The through-space theory model describes the one step electron transfer in terms of direct orbital overlap between the donor and acceptor sites regardless the network of chemical bonds. As the result of this mechanism, there is only
one single $\beta$ value, averaged over the entire pathway.

2) **The through-bond theory model** considers that the electrons are tunnelled along the routes composed of covalent bonds or hydrogen bonds between donor and acceptor sites. The $\beta$ values vary with different specific routes.

In biological systems the value of the decay coefficient ($\beta$) is about $1.4 \pm 0.2 \, \text{Å}^{-1}$ for protein, which is much lower than for a complete vacuum space ($\beta = 2.8 - 3.5 \, \text{Å}^{-1}$) [11-12], indicating that the polypeptide chain clearly facilitates electron transfer among the redox centres. This is the common ground of the two theory models, although there are many arguments with experimental evidence between them [9, 13-14].

Dutton and co-workers [1,11] proposed average values of reorganization energy ($\lambda = 0.7 \, \text{eV}$, intra-protein electron transfer; $1.0 \, \text{eV}$, inter-protein electron transfer) and decay coefficient ($\beta = 1.4 \, \text{Å}^{-1}$) and calculate the electron transfer rates of various redox proteins with the edge-to-edge distances ($r$) obtained from their crystal structures. They find that electron transfer reactions are productive in biological systems when the distances between redox centres are within 14 Å.

Gray and co-workers exploited metal-labelling techniques [7] to study electron transfer within biological systems. Upon attaching the ruthenium to proteins, such as cytochrome $c$, cytochrome $b_{562}$, myoglobins and iron proteins [15], the modified proteins were exposed to laser light and the electron transfer between ruthenium and redox centers investigated in detail. The light-driven electron transfers at differing distances between ruthenium and the redox center indicate that the rate and coupling correlate with the length of the tunneling pathways that are comprised of covalent bonds, hydrogen-bonds and through-space jumps.

**1.5 Electrical Wiring of Redox Enzymes**

The electrical wiring of redox enzymes is of great interest in bioelectronics and it leads to develop biosensors and bioreactores like biofuel cells. However, the direct electron tunnelling from redox proteins to electrodes is usually difficult, as the insulative polypeptide shells of the enzymes separate their internal redox center from
the conductive surfaces [16]. Many redox mediators like quinones and its derivatives [17-24], viologens [25-29], hexacyano-metal complexes [30-34], ferrocene-devatives [35], are utilised to facilitate the electron transfer between redox proteins and electrodes. These electron transfers are controlled by the free diffusion of mediators. The immobilizations of redox proteins on the electrodoses are developed to enhance the electronic communication of them. Small organic molecules can be used to anchor and electronically connect redox proteins to electrodes [16, 36-38]. Willner and his co-workers reconstitute redox enzymes on the electron relay units' monolayers on the electrodes. The electron relay units include pyrroloquinoline-quinone [39], nanoparticles [40] and nanotubes [41]. The electron relay units and well-defined alignment and orientation of redox proteins towards electrodes hugely improve their electronic interactions. Self-assembled films [42-43] and electron-conducting redox hydrogels [44] are also involved in the entrapment or attachment of biomolecules onto the electrodes’ surfaces. They electrically connect enzymes’ reaction centers to electrodes regardless of the spatial orientation of enzymes at the electrode surface and also connect multiple enzyme layers. In addition, metallized peptides [45] and small electron transportation proteins like cytochrome c [46] and flavodoxin [47-49] can be employed to wire redox enzymes, which enable the electrons to hop through the small peptides or proteins.

1.6 Biological Redox Centres, Electron Relay Units

Since the distance of productive biological electron transfer is within 14 Å, nature has evolved a large number of redox centers strategically placed over long distances in the biological systems to relay electrons from initial donor to final acceptor. Commonly, there are two groups of redox active species used as electron transfer cofactors in a protein matrix. Inorganic metal complexes like heme groups and iron-sulfur clusters contain metal centers with multiple redox states, and they can shorten the electron transfer distances (r_{edge-to-edge} < r_{center-to-center}). Organic molecules such as flavins and quinones have delocalized π-bonding systems.
1.6.1 Heme

Hemes consist of a porphyrin macrocycle with an iron atom coordinating four ring nitrogen atoms in the centre. They have several common representations (Fig. 1.5).

![Hemes](image)

*Figure 1.5: Hemes commonly found in biological systems*

Heme-\(a\) is found in cytochrome \(c\) oxidase. Compared with that of Heme-\(b\), the structure of heme-\(a\) shows that a methyl side chain of the porphyrin ring is changed into a formyl group, and one of the vinyl side chains is replaced by an isoprenoid chain.

Heme-\(b\), also termed protoheme IX, is the most abundant heme, found in hemoglobin, myoglobin, \(b\)-type cytochromes and the cytochromes P-450. Heme-\(b\) is not covalently bound to the protein in which it is contained.

Heme-\(c\) is the prosthetic group of \(c\)-type cytochromes. These are usually electron transfer proteins. Heme-\(c\) is covalently linked to the protein via two thioether linkages between cysteine residues on the protein and vinyl groups at positions 2 and 4 of the porphyrin ring, which therefore differs from heme-\(b\).

According to the coordination chemistry of iron, it is able to form two additional coordinating bonds, on either side of the heme plane. These two coordinating sites are called the fifth and sixth coordination positions. The iron centres in the electron transfer hemoproteins, which require minimal structural change, are commonly hexa-
coordinate and low spin. In contrast, hemoproteins that are involved in catalytic reactions or oxygen transportation require an available coordination site at the heme iron. Therefore the iron centres are usually penta-coordinate and high spin.

Three oxidation states of iron can be found in biological systems. Those are the ferrous (+2) oxidation state, the ferric (+3) oxidation state, and ferryl (+4) as postulated in the cytochromes P450. The coordination and oxidation state of the iron play essential roles in the hemoproteins.

1.6.2 Iron-Sulfur Centres
Besides heme groups, iron-sulfur (Fe-S) clusters are another category of iron-containing cofactors found in the redox active proteins. The coordination chemistry of iron atoms in the Fe-S clusters is mainly in tetrahedral arrangements, different from that in the hemes. Three common kinds of Fe-S clusters are discussed below.

The simplest Fe-S cluster is the rubredoxin center, where the sulfur atoms provided by four cysteine residues are tetrahedrally coordinated to only a single iron atom (Fig. 1.6a). [2Fe-2S] clusters, are constituted by two iron ions bridged by two sulfur atoms and coordinated by the sulphydryl groups of four cysteines (in Fe₂S₂ ferredoxins, Fig. 1.6b) or by the sulphydryl groups of two cysteines and the imidazole groups of two histidines (in Rieske proteins). The oxidized state of an [2Fe-2S] cluster is Fe(III)-Fe(III), whereas the reduced state is Fe(III)-Fe(II). The “saturated” Fe-S cluster is the [4Fe-4S] motif found in Fe₄S₄ ferredoxins. Four iron and sulfur atoms spatially occupy the corners of a cube and four cysteine residues are tetrahedrally coordinated to the [4Fe-4S] cube (Fig. 1.6c). The oxidation states range from 3Fe(III)-Fe(II), 2Fe(III)-2Fe(II) to Fe(III)-3Fe(II).
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Figure 1.6: Common examples of iron sulphur clusters in biological systems.

1.6.3 Flavin

Flavins are a group of organic compounds containing an isoalloxazine ring. In biological systems, flavin-containing species are always found as riboflavin, Flavin Adenine Dinucleotide (FAD) or Flavin MonoNucleotide (FMN) (Fig. 1.7).
Figure 1.7: the structures of the oxidized forms of FAD (entire molecule) and FMN (orange portion of molecule).

The isoalloxazine ring is the redox active motif and it utilises two oxidation states during electron transportation. The oxidation states are commonly known as flavoquinone (fully oxidized), flavosemiquinone (half-reduced) and flavohydroquinone (fully reduced) (Fig. 1.8).

Figure 1.8: three different redox states of isoalloxazine motif: fully oxidized (quinone), one-electron reduced (flavosemiquinone) and two-electron reduced (hydroquinone).
1.6.4 Ubiquinone and Menaquinone

Ubiquinone and menaquinone are both hydrophobic benzoquinone derivatives with large isoprenoid tails. Ubiquinone (Fig. 1.9A), also termed coenzyme Q, is an oil-soluble vitamin-like substance primarily present in the mitochondria of most eukaryotic cells. It is an essential component of the aerobic respiratory chain that generates energy in the form of Adenosine TriPhosphate (ATP). Menaquinone (Fig. 1.9C), commonly known as vitamin K$_2$, is produced by bacteria in the intestines and plays an important role in blood coagulation. Upon accepting a single electron, quinone, the core of ubiquinone/menaquinone, is reduced to semiquinone. Semiquinone is able to convert to quinol by one electron's further reduction.

![Figure 1.9: structures of the quinone/quinol groups. Panel A: Ubiquinone; Panel B: Ubiquinol; Panel C: Menaquinone; Panel D: Menaquinol. Usually $n = 10$ for ubiquinone/ubiquinol whereas $n = 6$ for menaquinone/menaquinol.](image-url)
1.7 Cytochromes

1.7.1 Cytochromes c

Cytochromes c are proteins that link one or more heme c moieties via two thioether linkages between cysteine residues on the protein and vinyl groups on the porphyrin ring. The characteristic heme-attachment sequence motif is either -Cys-Xaa-Xaa-Cys-His- or -Cys-Xaa-Xaa-Xaa-Cys-His- (where Xaa is any amino acid).

Cytochromes c are crudely divided into six classes, depending on the number of hemes, the properties of the axial iron ligands, and the redox potential [50-51].

Class I includes the classical soluble cytochromes c of mitochondria, the main role of which is to transfer electrons between coenzyme Q-cytochrome c oxidoreductase and cytochrome c oxidase. The heme-attachment site is towards the N-terminus. Usually the fifth ligand of a c-type heme is a histidine residue and the sixth ligand is a methionine residue about forty residues further on towards the C-terminus. Most class I cytochromes c are similar in their three-dimensional structures.

Class II includes the high spin cytochromes c' and various low-spin cytochromes with histidine residues as the fifth ligands of c-type hemes. The heme-attachment site is close to the C-terminus. In the low-spin proteins the sixth ligand is likely to be a methionine residue close to the N-terminus, and in the high-spin proteins there is no ligand in the sixth coordination position. The three-dimensional structures of class II cytochrome c is completely different from the others with the proteins folding as a cluster of four α-helices.

Class III comprises the low redox potential multiheme cytochromes: cytochrome c₃, cytochrome c₇ and high-molecular-weight cytochrome c (HMC), with only around thirty amino acids per heme. All the c-type hemes are bis-histidine ligated.

Class IV consists of complex proteins that have other prosthetic groups as well as heme c, such as flavocytochromes c.

Class V includes several recently characterized cytochromes c sharing similarities in the heme arrangement. Such an arrangement is thought to be relevant for the type of electron-transfer processes, in which all of the proteins belonging to this group are involved. These cytochromes c includes cytochrome c₁ and cytochrome f, which function as electron carriers in photosynthetic process, hydroxylamine oxidoreductase ([HAO) which contains eight covalently bound hemes [52]), and the tetraheme c₅₅₄ from Nitrosomonas europaea, pentaheme cytochrome c₅₅₂ from Sulfurospirillum
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deleyianum; and the flavocytochrome c₃ fumarate reductase from Shewanella species [53].

Several cytochromes c cannot be assigned to any of the above groups. They are the decaheme cytochromes c from Shewanella oneidensis [54], and the diheme cytochromes c from Pseudomonas aeruginosa [55], Rhodobacter sphaeroides [56] and Rhodobacter adriaticum [57].

1.7.1.1 Tetraheme cytochrome c₃ from Desulfovibrio vulgaris Miyazaki F

The tetraheme cytochrome c₃ (cyt c₃), which is found in the bacterium Desulfovibrio vulgaris Miyazaki F, is involved in sulfate-reduction [58]. This cyt c₃ contains four hemes covalently bound to a single polypeptide of only 107 amino acids (Fig. 1.10), with a size of ~14 kDa. In the fifth and sixth coordination positions, the ligands of the four hemes are histidines.

Figure 1.10: three dimensional crystal structure of globular cytochrome c₃ from Desulfovibrio vulgaris Miyazaki F (hemes in red, ligating histidines in magenta and protein backbone in green, alpha helix in orange, and beta sheets in blue, PDB entry: 1J0O)
The hemes of cyt $c_3$ have a much greater exposure to solvent than does the single heme group of the mitochondrial cytochromes $c$ [59]. The macroscopic reduction potentials range from -240 to -357 mV versus the normal hydrogen electrode (NHE) [60]. Compared with the reduction potentials of $\frac{1}{2}O_2/H_2O$ (+0.815 V) and bacterial ferredoxins (-0.432 V) [61], the reduction potentials of the four cyt $c_3$ heme groups are very low. The surface of this cyt $c_3$ is highly positively charged. Cytochrome $c_3$ can interact with an electrode without the presence of any electron transfer mediators [62]. The oxidation/reduction of cyt $c_3$ solution by the electrode is reversible and diffusion controlled [63]. Even in solid state, cyt $c_3$ demonstrates good electrical conductivity [64].

An overexpression system for Desulfovibrio vulgaris Miyazaki F cyt $c_3$ was generated using Shewanella oneidensis MR-1 [65], which is a facultative anaerobe that can be grown easily and rapidly, and produce plentiful multiheme cytochromes. This system was the basis for much of the work presented in this thesis.

### 1.7.2 Cytochrome P450

The cytochromes P450 (CYP or P450) are a huge and diverse superfamily of heme-containing proteins present in bacteria, fungi, insects, plants, animals, and humans. These colored P450s show very high affinity for carbon monoxide (CO) in their reduced states, forming characteristic Soret peaks at 450 nm. It is also the reason the family of proteins are named P450 (Pigment at 450 nm). Usually, P450s all contain a $b$-type heme axially coordinated by the side chain of a conserved cysteine residue, leaving another axial coordination position free for catalysis.

Commonly the typical catalysis reaction of P450s is monooxygenation, which can be illustrated simply below:

$$\text{RH} + O_2 + 2H^+ + 2e^- \xrightarrow{\text{P450}} \text{ROH} + H_2O$$

The monooxygenation catalysed by P450s is the insertion of an oxygen atom derived from molecular oxygen into an organic substrate (RH), while consuming two electrons from an electron transport chain and two protons from the environment. The substrates of P450s have a broad range, from small molecules (nitric oxide) [66], long
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chain fatty acids (arachidonate) [67], to macro rings (epithilone) [68]. The oxygenation of organic substrates increases their hydrophilicity, making them much more easily excreted by the biological systems.

P450s activate molecular oxygen (triplet state) in order for it to react with an organic substrate (singlet state). Molecular oxygen binding to iron produces highly reactive species like the peroxo-ferric (Fe(III)-O₂⁻), hydroperoxy-ferric (Fe(III)-OOH⁻) and ferryl (Fe(IV)=O) complexes [69]. A schematic showing the P450 catalytic cycle is shown in Fig. 1.11.

Figure 1.11: P450 catalytic cycle depicting 1: substrate binding, 2: first electron transfer, 3: Oxygen binding, 4: second-electron transfer, 5: formation of oxy-ferryl species, 6/7: rebound
Cvtochrome C3 modules as electron transfer nanowires

mechanism for oxygen insertion and 8: product dissociation. The 1, 2 and 4 electron uncoupling pathways to superoxide, peroxide and water are shown as dotted lines.

The catalytic centers (hemes) of P450s are not stand-alone and they need the support of electron transfer cofactors to achieve the goal of catalysis. Hence the heme domains of P450s and their supporting parts form multi-component electron transfer chains, generally called P450 containing systems. The systems mainly can be classified into two groups:

1) Class I P450s (Fig. 1.12A), commonly found in bacteria [70], are composed of three proteins: FAD-dependent ferredoxin reductase (FNR), ferredoxin (containing an 2Fe-2S cluster), and a P450. The FNR acquires two electrons from NADH and transfers them sequentially to the P450 via a 2Fe-2S cluster (ferredoxin).

2) Class II P450s (Fig. 1.12B), are associated with the endoplasmic reticulum in eukaryotes, and consist of two enzymes: an FAD and FMN-containing reductase and a P450 [71-72]. Compared with class I P450s, the 2Fe-2S cluster is replaced by an FMN cofactor and the FAD and FMN are organised together by a single polypeptide. The cytochrome P450 reductase (CPR) facilitates electron transfer from NADPH to the P450 heme.
Fig 1.12: representation of the diverse classes of cytochromes P450. P450s or its heme domains are represented in red and other proteins or components in yellow (FAD-containing proteins/domains), green (FMN-containing proteins/domains) and blue (iron-sulfur-containing proteins/domains). (A): class I system comprised of FNR, ferredoxin and P450. (B): class II system comprised of FAD/FMN reductase and P450. (C): class III system formed by fused FAD/FMN reductase and P450. (D) and (E): other unique combinations of redox cofactors such as in P450 Rhf and P450 cin.
This classification system is suitable for most of the P450s. However, there are some examples excluded from the above system. The most famous one is flavocytochrome P450 BM3 from *Bacillus megaterium* (Fig. 1.12C, Fig. 1.13) [73]. It is a large soluble protein with only a single polypeptide involving FAD, FMN, and heme together. From a structural point of view, P450 BM3 can be considered the fusion of an FAD/FMN domain and a heme domain from a class II P450 model. Flavocytochrome P450 BM3 has been studied intensively because of its easy expression in *E. coli* and as an ideal model for P450 systems without the recognition(binding of other components complicating the system.

**Figure 1.13:** three dimensional crystal structure of cytochrome P450 BM3 from *Bacillus megaterium* (hemes in red, ligated cysteine in yellow and protein backbone in wheat, alpha helix in cyan, and beta sheets in magenta, PDB entry: 2IJ2)
Other examples with unusual redox cofactor arrangements include P450 Rhf (fusion of FMN-2Fe2S-heme, Fig. 1.12D) [74-75], P450 cin (separated FAD, FMN and heme domains, Fig. 1.12E) [76-77] and P450 nor [78] gaining electrons directly from the environment without the any support of cofactors.

In some cases, cytochromes P450 also receive electrons from cytochrome $b_5$ [79] (Fig. 1.14), a 16.7-kDa electron transfer protein found in the endoplasmic reticulum of liver cells and in erythrocytes [80].

Figure 1.14: electron transfer between cytochrome P450, cytochrome $b_5$ and their reductases

The presence of cytochrome $b_5$ either stimulates, inhibits or has no effect on P450 catalysis. The interaction between P450 and cytochrome $b_5$ depends on the the P450 isoform, the specific substrate and experimental conditions [81]. Some experimental evidences showed that binding of cytochrome $b_5$ to P450 causes structural influences [82-84]. In the presence of cytochrome $b_5$, cytochrome P450 17 can catalyse hydroxylation of pregnenolone to dehydroepiandrosterone; without cytochrome $b_5$, the hydroxylation product of pregnenolone by P450 17 is cortisol. The cytochrome $b_5$ regulation of hydroxylation of pregnenolone by cytochrome P450 17 does not require electron transfer [83]. It is believed that cytochrome $b_5$ induces a conformational change at the active site of P450 17 [85]
1.8 Bioconjugation Techniques

Bioconjugation involves the covalent linking of two or more biomolecules to form a novel complex having the combined properties of its individual components [86]. Hence either natural proteins or synthetic polypeptides with unusual amino acids can be chemically crosslinked together to derive new conjugates that not only retain the original individual activities but also add newly designed properties.

According to the targets of bioconjugation, the crosslinking methods can be crudely classified into three types: protein crosslinking, carbohydrate and glycan conjugation, and polynucleotide crosslinking [86]. The crosslinking of two proteins can be simply achieved by adding a homobifunctional reagent. The reagent will react with one of the proteins first forming a coupling-active intermediate, and then the activated protein will produce conjugates with another protein (ideal products) or create homodimers (side products). If there is more than one conjugation site on the protein, there is large probability of producing undesired polymeric conjugates that sometimes precipitate from the reaction solution. The progress of conjugation is necessary to control and crosslinking sites should be selected with high specificity. The carbohydrate and glycan conjugation strategies always involve a multi-step reaction sequence. The first step is to process the adjacent hydroxyl groups in the sugar residues of polysaccharides through mild sodium periodate oxidation to produce aldehyde groups. The aldehyde group is highly active towards amine or hydrazide containing compounds, forming a stable covalent linkage. If the second molecule has an amine or hydrazide group, the direct mixing of the oxidized intermediate and the second molecule in a reducing environment can produce the conjugate. Otherwise, it is necessary to introduce an amine or hydrazide group onto the second molecule before conjugation. The weakness of the carbohydrate conjugation is that the periodate oxidation always destroys the three-dimensional structures of polysaccharides. The polynucleotide crosslinking method is quite different from that with any other biomolecules. Without any interference from base pairing, conjugates can be formed at the 3'-end or 5'-end upon modification.
1.8.1 Bioconjugate-active groups

Generally the reactive groups for bioconjugation involve natural amino acid residues like primary amines, sulfhydryl, carboxyl, hydroxyl, together with synthetic aldehyde/ketone, photochemically active groups, etc [86]. In relation to the aim of the project, the introduction of reactive groups will focus on the natural amino acid residues.

1. Primary amine (-NH₂)

The amine group is up to now the most common reactive group for bioconjugation. Amine-involved conjugation could happen with most proteins due to the abundance of lysines and arginines within polypeptides. The reaction types for amine coupling are mainly acylation and alkylation. The coupling process is very fast and efficient at a suitable pH, where amine shows high nucleophilic activity, and produces a stable product like amide or secondary amine. Several amine-coupling reactions occur with commercially available reagents such as N-hydroxysuccinimide (NHS) ester-amine reaction, imidoester-amine reaction and carbodiimide-amine reaction that are utilised in large number of bioconjugations.

(1) N-hydroxysuccinimide (NHS) ester [87-88]

An NHS (N-hydroxysuccinimide) ester can be created by the addition of NHS to the carboxylate in presence of a carbodiimide. The NHS ester can react with a primary amine at physiological pH yielding an amide bond in the product. Hydroxyl group, sulfhydryl group, and imidazolyl ring of histidine will competitively react with the NHS ester. Their conjugates are unstable and easily hydrolyzed.

![Scheme 1.1: the conjugation of primary amine and N-hydroxysuccinimide (NHS) ester](image-url)
(2) **Imidoester** [89-92]

Imidoester crosslinks to a primary amine to form an amidine bond. The resulting amidine is protonated at physiological pH and bears a positive charge. Like NHS esters, imidoesters have high specificity for amine.

![Scheme 1.2: the conjugation of primary amine and imidoester](image)

(3) **Carbodiimide** [93-95]

The carbodiimide is considered to be a zero-length crosslinker between carboxylate and primary amine. Zero length means that there are not any additional interconnecting spacers between the components in the conjugate.
Scheme 1.3: the conjugation of nucleophilic regents (primary amine, water, sulfo-
NHS ester) and carbodiimide activated carboxylate.

In the progress of coupling, EDC reacts with a carboxyl group on biomolecule A
forming a short-lived O-acylisourea intermediate. This intermediate has a very high
reactivity towards nucleophilic reagents. It can react with another biomolecule B
containing a primary amine yielding a heterodimer, or is hydrolysed back to the
biomolecule A, or coupled to a soluble sulfo-NHS to become a stable amine-reactive NHS ester that may crosslink to biomolecule B.

2. **Sulfhydryl (-SH)**

As the functional group of the amino acid cysteine, the sulfhydryl or thiol group, plays an important role in biological systems. In the presence of oxidants, two cysteines' thiol groups, which are near each other, can form a disulfide bond that helps proteins maintain their tertiary structure or connect the heavy chain and light chain together in antibodies. In this project, thiol groups link the heme groups to the polypeptide chain in cytochromes c or coordinate to the b-type hemes in P450s.

The sulfhydryl group is the second crosslinking target widely used for bioconjugation. At pH > 7, the thiol group deprotonates ($pK_a \approx 8.3$) and forms thiolate (-$S^-$), which behaves as a strong nucleophilic reagent. Although the coupling activity of the sulfhydryl group is comparable to primary amine, thiol groups are less common in a large number of biomolecules. Therefore, thiol groups must be introduced to biomolecules before conjugation, either by chemical conversion or mutagenesis.

The reaction types involving thiol coupling are alkylation and disulfide interchange. Typical coupling reactions include the haloacetyl derivative-thiol reaction, maleimide-thiol reaction, vinylsulfone-thiol reaction, and thiol-disulfide interchange.

(1) **Haloacetyl derivative [96]**

The representative reaction is the conjugation between iodoacetyl derivatives and thiol containing biomolecules, since the other haloacetyl derivatives are not so reactive towards thiol group at physiological pH.

However, selectivity of iodoacetyl derivatives during conjugation is poor. In addition to the thiol group of cysteine, they will react with the imidazole group of histidine, the thioether of methionine, the primary amine of lysine and the N-termini of proteins. It limits the application of iodoacetyl derivatives in many areas.
Maleimides are the products of the reaction between maleic anhydride and ammonia or primary amine. The functional site for coupling is the double bond, which is easily attacked by strong nucleophilic reagents like thioates or amines. At pH 6.5 ~ 7.5, the maleimide group can react specifically with thiol groups, forming a stable thioether linkage [97-100]. At pH 7.0, maleimides react with thiols 1,000-fold faster than with amines [101]. This is the most suitable condition for specific thiol-maleimide coupling. However, at pH >8.5, the primary amine-maleimide coupling becomes the main reaction [102]. Maleimides do not react with weak nucleophilic reagents contributed by the residues of tyrosines, histidines or methionines.

Unfortunately, the maleimide group always faces the problem of hydrolysis, especially at pH > 8.0 [103]. The hydrolysis breaks the five-member ring and makes the maleimide group unreactive to the coupling reagent.
Vinylsulfone [104]

A vinylsulfone group can crosslink to nucleophilic groups like thiol, amine, and even hydroxyl groups. At pH 7.2–9.2, the vinylsulfone group mainly reacts with the thiol group to give a stable thioester. Unlike the maleimide group, the vinylsulfone group does not give stereoisomers upon addition of a thiol.

![Scheme 1.6: the conjugation of thiol and vinylsulfone](image)

At pH > 9.5, the primary amine reacts with the vinylsulfone group competitively, although the thiol-vinylsulfone coupling is still the fastest reaction in the same system. Compared with the maleimide group, the conjugation of the vinylsulfone group is less efficient and slow, but the vinylsulfone group is resistant to hydrolysis.

Thiol-disulfide interchange

Thiol-disulfide interchange is the attack of thiolate on a sulfur atom in a disulfide bond resulting in the breakage of the old disulfide bond and the formation of a new one. The common disulfides used are DTNB (5,5'-DiThiobis(2-NitroBenzoic acid)) [105-106] and 2-PDS (2,2'-diPyridyl DiSulfide) [107]. They are both classical thiol-counting reagents due to the formation of chromophores after the breakage of their disulfide bond. Either the original or newly formed disulfide bond is easily cleaved by thiol-reducing reagents, e.g. TCEP (Tris(2-CarboxyEthyl)Phosphine), DTT (DiThioThreitol), 2-mercaptopoethanol or 2-mercaptopoethylamine.
Scheme 1.7: thiol-disulfide interchange. The yellow chromophore is 5-Thio-2-NitroBenzoic acid (TNB) or 2-pyridinethione

3. Carboxylate (-COO⁻)

Carboxylate groups show low nucleophilicity, even when the pH is much higher than 7 in aqueous solutions. This is due to the delocalization of electrons within the carboxylate group conjugation system. Carboxylates are therefore not very reactive for the conjugation. In order to crosslink at the carboxylate site, this functional group must be activated first. The common activation regents are carbonyldiimidazoles and carbodiimides (see the coupling reactions of primary amine).

(1) Carbonyldiimidazole [108]

Carbonyldiimidazole, abbreviated as CDI, can react with carboxylate groups forming intermediate N-acylimidazoles. These intermediates are very reactive towards amines or hydroxyls, yielding stable amides or esters. The intermediate can also react with another carboxylate group in alkaline solution.
4. **Hydroxyl (-OH)**

The hydroxyl group is more reactive than the carboxylate group. However, in many conjugation applications, they are modified to increase their reactivity and specificity.

1. **Carbonyldiimidazole**

Similarly, carbonyldiimidazole can react with the hydroxyl group. The intermediate is an imidazole carbamate. This intermediate is active towards nucleophilic regents as well, like amines. And the coupling product is a stable $N$-alkyl carbamate.

---

**Scheme 1.8: the conjugation of CDI activated carboxylate and primary amine**

**Scheme 1.9: the conjugation of CDI activated hydroxyl group and primary amine**
(2) \( N,N'-\text{Disuccinimidyl carbonate}/N\text{-hydroxysuccinimidyl chloroformate} \)

In terms of hydroxyl group, the goal of \( N,N'-\text{disuccinimidyl carbonate} \) or \( N\text{-hydroxysuccinimidyl chloroformate} \) is to produce a succinimidyl carbonate. The succinimidyl carbonate is highly active toward nucleophilic reagents and usually crosslinks to primary amines creating a carbamate with good stability.

\( \text{Scheme 1.10: the conjugation of } N,N'-\text{disuccinimidyl carbonate or } N\text{-hydroxysuccinimidyl chloroformate activated hydroxyl group and primary amine} \)

1.8.2 Crosslinkers for bioconjugation

The selection of crosslinkers is based on their coupling reactivities to particular functional groups and the best crosslinker for a specific bioconjugation must be determined empirically. Generally, several important points should be under consideration while choosing the most suitable crosslinker.

1. Chemical reactivity and specificity

Crosslinking normally involves two or more functional groups and they commonly include primary amines, sulfhydryls, carboxylates and hydroxyls. The reactivity and
specificity towards these functional groups are very important for the conjugation process.

2. The length of spacer arm

The selection of the length of spacer arm depends on the specific applications. Some applications require the components in conjugates to be separated by a certain distance without affecting their respective tertiary structures. Some applications need short spacer arms to keep the components closer. Different lengths of spacer may act as molecular rulers in some systems.

3. Water solubility and cell membrane permeability

The classification of crosslinkers is on the basis of the solubilities of the reactants in aqueous solution or organic solvents. In bioconjugation the solubility of a crosslinker must be considered carefully because if too much organic solvent is used for dissolving the crosslinker, the enzymes in aqueous solution could precipitate or denature.

4. Homobifunctional / heterobifunctional crosslinkers

Homobifunctional crosslinkers connect reactants at the same functional groups and they are usually used in one-step conjugation. Heterobifunctional crosslinkers are utilised in controlled multi-step conjugation, and react with different functional groups.

5. Cleavability

In certain applications, the crosslinked conjugates are designed to allow cleavage after use. Normally the cleavable domains of crosslinkers locate in the middle of the spacer arm and they are disulfide groups (cleaved by thiol), diol groups (cleaved by periodate), ester groups (cleaved by hydroxylamine), or sulfone groups (cleaved by base).

1.9 Protein Tag

To produce highly pure and well-characterized recombinant proteins is a major task for the protein chemists working in scientific laboratories. Many different peptides, domains and proteins (called affinity tags) have been developed for purification of over-expressed recombinant proteins. Generally these tag systems have the following
common features: (a) one-step purification for target proteins; (b) a minimal effect on
target proteins' tertiary structures and biological activities; (c) easy and specific
removal from the recombinant proteins; (d) simple and accurate assay of the
recombinant protein during purification; (e) applicability to a number of different
proteins [109]. Nevertheless, there is no a single tag suitable for all proteins and it is
difficult to decide on the best affinity tag for a specific protein of interest. This
depends on the target protein itself (e.g. stability, hydrophobicity), the expression
system, and the application of the purified protein. Table 1.1 below lists most
frequently used and interesting tag-protein fusion systems.
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</tbody>
</table>
Small peptide tags such as Arginine Tag [110-111], Histidine Tag [112-115], FLAG Tag [116-122], c-myc Tag [123-125], S Tag [126-128], and Strep Tag II [129-131], are very commonly used. These tags have nearly no effects on the expression, structure and biological activity of most proteins. And usually there is no need to remove the tag after purification. Large tag systems like Glutathione S-transferase (GST) [132] and Maltose binding protein (MBP) [133-135], can increase the yield or enhance the solubility of recombinant proteins besides introducing one-step purification in some cases. The disadvantage is that the tag must be removed for several applications e.g. crystallization or antibody production.

1.9.1 Calmodulin and Calmodulin Binding Peptide (CBP)

Calmodulin is a small, acidic, Ca$^{2+}$-binding protein constituted of 148 amino acids with a mass of 16706 Daltons. The apo-Calmodulin is dumb-bell-shaped. Two globular domains, also called lobes, are connected by a flexible α-helical linker. There are two helix-loop-helix Ca$^{2+}$ binding sites which are commonly called EF hands in each globular domain. In the presence of Ca$^{2+}$, Calmodulin undergoes a major conformational change and “wraps-around” its target peptide or protein (Figure 1.15) [136-139].

![Calmodulin with 4 Ca$^{2+}$](image1.png)

![Complex of Calmodulin and CBP](image2.png)

*Figure 1.15: Calmodulin and Calmodulin Binding Peptide (CBP). Calmodulin is in green; Calcium ions are in red; CBP is in yellow*
1.9.2 Streptavidin and Streptavidin Binding Peptide (SBP)

Streptavidin is a tetrameric protein, obtained from the bacterium *Streptomyces avidinii*, which binds extraordinarily tightly to the small molecule, biotin ($K_d \sim 10^{-15}$ M). Compared with most biomolecules, streptavidin is extremely stable and survives tough environments, such as 8 M urea or guanidine, 0.5 M NaOH, or 50 % formamide. Common proteases, including pepsin, papain, subtilisin, thermolysin, and elastase, do not cleave streptavidin. Even in the presence of SDS, streptavidin keeps its integrity and disassociates into monomers only at high temperature (> 60°C). *Strep*-Tactin is a streptavidin derivative, which is mainly used as the column material for affinity purification of proteins with the *Strep*-Tactin association peptide.

Strep tag II is a small peptide with only 8 amino acids (WSHPQFEK) and it is found that it displays a very high affinity towards *Strep*-Tactin (Fig 1.16) [129-131]. Strep tag II is widely used for affinity purification as a tag placed either at the C-terminus or N-terminus. Generally, it does not interfere with protein folding or bioactivity, does not react with heavy metal ions, has no ion exchange properties and does not induce protein aggregation. There is no need to remove it after purification in most case.

![Streptavidin derivative and Strep Tag II](image)

*Figure 1.16: Streptavidin derivative and Strep Tag II. Streptavidin derivative is in cyan and Strep Tag II is in magenta stick representation.*
1.10 Aims of this Thesis

The aims of project include:

1. Constructing cytochrome c₃ mutants for use in conjugation studies;
2. Characterizing the constructed cytochrome c₃ mutants by gel electrophoresis, UV-Vis spectroscopy, fluorescent labelling, and thiol counting;
3. Constructing simple dimers of cytochrome c₃ mutants;
4. Constructing polymers of cytochrome c₃ mutants ("biological nanowires") and characterizing them with gel electrophoresis, UV-Vis spectroscopy, and OTTLE (Optically Transparent Thin Layer Electrode) potentiometry;
5. Developing a controllable conjugation strategy through constructing a well-defined conjugate of cytochrome c₃ mutants;
6. Constructing P450 BM3 mutants for use in conjugation studies;
7. Characterizing the constructed cytochrome c₃ mutants by gel electrophoresis and UV-Vis spectroscopy, and substrate binding;
8. Constructing a conjugate of P450 BM3 mutant and cytochrome c₃ mutant, characterizing them with gel electrophoresis, UV-Vis spectroscopy, and size exclusion chromatography, and investigating the electron transfer between P450 BM3 mutant and cytochrome c₃ mutant by an OTTLE.

How can multiple redox proteins be coupled together in a controlled way to generate a "nanowire"? The keys are to have very selective chemical ligating reactions and effective protecting/deprotecting strategies.

For native proteins the most selective chemical ligation site is a cysteine thiol group. These can be placed anywhere on the protein surface by mutagenesis. It’s easy to link two proteins together by using either a disulfide bond directly or a synthetic linker. The best synthetic linkers are bis-maleimide cross-linking reagents (Fig. 1.17), which can react with cysteine thiol groups specifically, and the cross-linking products are stable at physiological pH. Moreover, the lengths of these linkers can be changed due to different spacers between two maleimides.

Introduction
In making a hetero-dimer (A-B), homo-dimers (A-A and B-B) will be a problem. So A should be treated with a linking agent first, prior to reaction with B.

**Scheme 1.11**: two-step conjugation to produce A-B heterodimer, hereby A, B represents two different cysteine mutants of cytochrome c\textsubscript{3}, respectively

Linking 3 proteins together in a controlled way is much more difficult. To make an A-B-C module, B requires two exposed cysteine thiols to form the bridge. In order to connect A to B without forming A-B-A, one of the cysteine sites must be protected. Strategies for this are detailed below:

1) Electrostatic control

Electrostatics could be used to control reactivity. By generating a dipole (Fig. 1.18) across a c\textsubscript{3} molecule near the cysteine site through mutagenesis, connection will be favoured in one orientation.

**Figure 1.18**: a cyt c\textsubscript{3} dipole controls the ligation
(2) Affinity tags
The choices for affinity tag strategies include calmodulin (CaM) / calmodulin binding peptide (CBP) and Strep-Tactin / Strep Tag II.
As an example, calmodulin binding peptide (CBP) is tagged at the N- or C-terminus of a protein near the cysteine site (Fig. 1.19). While binding to the CBP, CaM can physically obstruct the cysteine thiol group to achieve the aim of protection (Fig 1.20). It is easy to remove CaM by decreasing the calcium concentration using EGTA.

![Figure 1.19: the CBP is tagged at the C-terminus of cytochrome c₃ double cysteine mutants. The big circle with 4 diamonds represents the cytochrome c₃ and 4 hemes; the small circles in magenta and cyan represent cysteine thiol groups at different sites on the surface of cytochrome c₃; the green triangle represents the CBP.](image)

![Figure 1.20: CaM binds to the CBP and the thiol group in (or near) the tag is involved by CaM so that it becomes unreactive towards conjugation.](image)

A postulated procedure to create a well-organized cytochrome c₃ heterotrimer is illustrated in Fig 1.21.
Figure 1.21: a postulated multi-step procedure to produce a cytochrome c₃ heterotrimer. A, B represent different cysteine mutants of cytochrome c₃; the magenta star represent the bismaleimide crosslinker.

The b-type heme in the P450 BM3 is deeply buried in the protein (Fig 1.22).
Physiologically, it needs electron transfer cofactors (FAD and FMN) to gain electrons to achieve catalysis. This aim is to connect cytochrome $c_3$ near to the active site of the P450 BM3 and utilize the electrode to provide the electrons to the P450 heme domain via the cytochrome $c_3$ module (Fig 1.23).
Figure 1.23: postulated electrode-driven catalysis of P450 BM3 heme domain-cytochrome c₃ conjugate.
Chapter 2

Materials and methods
## 2.1 Bacterial plasmids and strains

### Table 2.1: bacterial plasmids

<table>
<thead>
<tr>
<th>Bacterial plasmids</th>
<th>Main purpose of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKF 19k</td>
<td>Cloning vector for <em>Desulfovibrio vulgaris</em> Miyazaki F cytochrome <em>c</em>&lt;sub&gt;3&lt;/sub&gt; and its mutants; Compatible with the expression system of <em>Shewanella oneidensis</em> MR-1 [65]</td>
</tr>
<tr>
<td>pUC 118</td>
<td>Cloning vector for the heme domain of cytochrome P450 BM3 [140]</td>
</tr>
<tr>
<td>pET-11d</td>
<td>Cloning vector for Calmodulin Binding Peptide (CBP) [141]</td>
</tr>
</tbody>
</table>

### Table 2.2: bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Main purpose of use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>Cloning strain for wild type <em>Desulfovibrio vulgaris</em> Miyazaki F cytochrome <em>c</em>&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Lab stock</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>Cloning strain for mutants of <em>Desulfovibrio vulgaris</em> Miyazaki F cytochrome <em>c</em>&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Lab stock</td>
</tr>
<tr>
<td><em>Escherichia coli</em> TG1</td>
<td>Cloning and Expression strain for the heme domain of cytochrome P450 BM3</td>
<td>Lab stock</td>
</tr>
<tr>
<td><em>Shewanella oneidensis</em> MR-1</td>
<td>Expression strain for <em>Desulfovibrio vulgaris</em> Miyazaki F cytochrome <em>c</em>&lt;sub&gt;3&lt;/sub&gt; and its mutants; Rifampicin resistance</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>
2.2 Growth media and antibiotics

*Table 2.3: LB Broth (per litre)*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre.

*Table 2.4.1: SOC Broth (per litre)*

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Tryptone</td>
<td>20 g</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre and the solution was sterilized by autoclaving at 121 °C for 20 minutes before use.

The following filter-sterilized supplements were added prior to use:

*Table 2.4.2: additives of SOC broth (per litre)*

<table>
<thead>
<tr>
<th>Chemicals (aqueous concentration)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (1 M)</td>
<td>10 ml</td>
</tr>
<tr>
<td>KCl (0.25 M)</td>
<td>10 ml</td>
</tr>
<tr>
<td>MgCl₂ (1 M)</td>
<td>10 ml</td>
</tr>
<tr>
<td>MgSO₄ (1 M)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Glucose (2 M)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
Table 2.5: TB Broth (per litre)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Tryptone</td>
<td>12 g</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>24 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4 ml</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>23.12 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>125.41 g</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre and the solution was sterilized by autoclaving at 121 °C for 20 minutes before use.

Table 2.6: LB Agar (per litre)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>14 g</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre and autoclaved for 20 minutes at 121 °C. It was allowed to cool before addition of antibiotics.
Table 2.7: antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock concentration (mg / ml)</th>
<th>Final concentration (µg / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>50 (molecular cloning)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 (protein expression)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

The stock solution of kanamycin, ampicillin, or carbenicillin was prepared in deionised water (millipore), passed through a 0.22 µM syringe-driven filter and stored at -20 °C. The stock solution of rifampicin was prepared in methanol without being filtered and stored at -20 °C.

2.3 Genetic manipulation

2.3.1 Primers and plasmids

Table 2.8: Plasmids and cloning primers (see separated page)
### Table 2.8: summarization of plasmids and cloning primers in the project

<table>
<thead>
<tr>
<th>Name of plasmid</th>
<th>Forward and reverse primes for PCR (5' -&gt; 3')</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKFC3k</td>
<td>N/A</td>
<td>encoding wild type <em>Desulfovibrio vulgaris</em> Miyazaki F cytochrome $c_3$, obtained from Japan</td>
</tr>
<tr>
<td>pSL1</td>
<td>CACCCGGTCAACGGCTGTGAAGACTACCAGAAG</td>
<td>encoding K40C cytochrome $c_3$</td>
</tr>
<tr>
<td>pSL2</td>
<td>CACCCGGTCAACGGCTGTGAAGACTACCAGAAG</td>
<td>encoding S107C cytochrome $c_3$</td>
</tr>
<tr>
<td>pSL3</td>
<td>GCCAGTGAATTCTTAGCAATGGCACTTGGAGGCC</td>
<td>encoding K40C:S107C cytochrome $c_3$</td>
</tr>
<tr>
<td>pSL4</td>
<td>ACGCCAAAGCTTGCATGC</td>
<td>encoding K40C:S107C cytochrome $c_3$ and a calmodulin binding peptide (CBP) with a short linker, glycine and threonine</td>
</tr>
<tr>
<td>pSL5</td>
<td>GTGCCATTGCACCAACGGCACTTGGAGG</td>
<td>encoding K40C:S107C cytochrome $c_3$ and a calmodulin binding peptide (CBP) with a short linker, threonine</td>
</tr>
<tr>
<td>pSL6</td>
<td>ACGCCAAAGCTTGCATGC</td>
<td>encoding K40C:S107C cytochrome $c_3$ and a calmodulin binding peptide (CBP) with a thrombin proteolysis site (Met-Tyr-Pro-Arg*-Gly-.Asn)</td>
</tr>
<tr>
<td>pSL7</td>
<td>ACGCCAAAGCTTGCATGC</td>
<td>encoding K40C:S107C cytochrome $c_3$ and a Strep Tag II with a short linker, serine and alanine</td>
</tr>
<tr>
<td>pSL8</td>
<td>ACGCAAGCTGCTGCAATGGCAGCCATTTGGAAC</td>
<td>encoding S107C cytochrome c3 and a Strep Tag II with a short linker, serine and alanine</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pSL9</td>
<td>CCAAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding K40C cytochrome c3 and a New Strep Tag with a short linker, serine and alanine</td>
</tr>
<tr>
<td>pSL10</td>
<td>CCAAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding wild type cytochrome c3 and a New Strep Tag with a short linker, serine and alanine</td>
</tr>
<tr>
<td>pSL11</td>
<td>GCCAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding K40C cytochrome c3 and a His6 Tag with a short linker, glycine and serine</td>
</tr>
<tr>
<td>pSL12</td>
<td>GCCAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding wild type cytochrome c3 and a His6 Tag with a short linker, glycine and serine</td>
</tr>
<tr>
<td>pSL13</td>
<td>GCCAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding K40C cytochrome c3 and a Strep Tag II with a short linker, serine and alanine</td>
</tr>
<tr>
<td>pBM20</td>
<td>N/A</td>
<td>encoding heme domain of wild type P450 BM3, obtained from lab stock</td>
</tr>
<tr>
<td>pSLa</td>
<td>CAGTCTGCTAACCCATACACCTACATACACATAGTGTGTTGTTGTTGTTGTTGTTGTTG</td>
<td>encoding C156S heme domain of P450 BM3</td>
</tr>
<tr>
<td>pSLb</td>
<td>TCCAAGTGCCATAGCGCGTTGTTGCACCACCGCAG</td>
<td>encoding C156S heme domain of P450 BM3 and a His6 Tag (C62P450)</td>
</tr>
<tr>
<td>pSLc</td>
<td>CCGTTTAAAGAAGCAAGCGATGAATCACGC</td>
<td>encoding C62S:C156S:Q387C heme domain of P450 BM3 and a His6 Tag (C387P450)</td>
</tr>
<tr>
<td>pCAL-c</td>
<td>N/A</td>
<td>encoding Calmodulin Binding Peptide (CBP), purchased from Stratagene</td>
</tr>
</tbody>
</table>
2.3.2 Point mutagenesis for production of K40C, S107C, K40C:S107C cytochromes $c_3$ (pSL1, pSL2, and pSL3, in collaboration with Dr. Caroline Miles)

(1) Small scale plasmid DNA extraction with QIAprep spin miniprep kit (Qiagen)

5 ml of LB broth supplemented with 50 µg / ml of kanamycin, was inoculated from the stock of pKFC3k|DH5α in the -80 °C freezer using a sterile inoculating loop and grown at 37 °C in an incubator at 225 rpm overnight. The cells were harvested by centrifugation at 5,000 rpm for 3 minutes. The pellet was resuspended in 250 µl of Buffer P1 and the mixture was transferred to a sterilized 1.5-ml eppendorf. 250 µl of Buffer P2 was added to the mixture and mixed thoroughly by inverting the eppendorf 4-6 times. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the eppendorf 4-6 times again. The cloudy mixture was spun at 13,000 rpm for 10 minutes and the supernatant was transferred carefully to the QIAprep spin column. The column was spun at 13,000 rpm for 1 minute and the flow-through was discarded. At this moment, plasmid DNA was bound to the resin of the column. 500 µl of Buffer PB and 750 of Buffer PE, respectively, were applied to wash the spin column and the residual buffers were discarded by centrifugation at 13,000 rpm for 1 minute. DNA was eluted into a clean sterilized eppendorf upon addition of 50 µl of Buffer EB and centrifugation at 13,000 rpm for 1 minute. The extracted DNA was used immediately or stored in -20 °C freezer.

(2) Detection of DNA by Agarose gel electrophoresis
Table 2.9: TAE buffer (50× stock solution) per litre

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH8.0)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre.

Table 2.10: gel sample loading buffer (6× stock solution)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll</td>
<td>15 %</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.25%</td>
</tr>
<tr>
<td>Xylene Cyanoll</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

Table 2.11: electrophoresis sample preparation

<table>
<thead>
<tr>
<th></th>
<th>1 Kb DNA Marker* / µl</th>
<th>Plasmid DNA / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA stock solution</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Sample loading buffer (6×)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Sterilized deionised water</td>
<td>11.5</td>
<td>7.5</td>
</tr>
<tr>
<td>total</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

* New England Biolabs

Table 2.12: 0.8 % Agarose gel composition for each gel

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose (multi-purpose)</td>
<td>0.24 g</td>
</tr>
<tr>
<td>TAE buffer (1×)</td>
<td>30 ml</td>
</tr>
<tr>
<td>Ethidium Bromide (10 mg / ml)</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

The agarose gel was run in 1× TAE buffer at 15 ~ 20 V/cm for 30 ~ 60 minutes according to the migration of colored frontier. DNA bands on the gel could be monitored and photographed under a UV lamp.
(3) Site-directed mutagenesis with QuikChange mutagenesis kit (Stratagene)

Table 2.13: PCR (Polymer Chain Reaction) sample preparation for K40C (pSL1) or S107C (pSL2) mutants of cytochrome c₃

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKFC3k</td>
<td>1 (~ 25 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>QuikSol</td>
<td>3</td>
</tr>
<tr>
<td>Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38</td>
</tr>
<tr>
<td>Pfu Turbo DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
</tr>
</tbody>
</table>

* pKFC3k was the extraction of 5-ml overnight LB broth; Primers were ordered from Sigma and made 100 ng/µl stock solution; other reagents were provided by QuikChange mutagenesis kit.

Table 2.14: PCR sample preparation for K40C:S107C (pSL3) mutant of cytochrome c₃

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKFC3k</td>
<td>1</td>
</tr>
<tr>
<td>forward primers for K40C and S107C</td>
<td>1.25 + 1.25</td>
</tr>
<tr>
<td>reverse primers for K40C and S107C</td>
<td>1.25 + 1.25</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>QuikSol</td>
<td>3</td>
</tr>
<tr>
<td>Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>35.5</td>
</tr>
<tr>
<td>Pfu Turbo DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>total</td>
<td>51</td>
</tr>
</tbody>
</table>
Table 2.15: PCR program for K40C (pSL1), S107C (pSL2), or K40C:S107C (pSL3) mutant of cytochrome c₃

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>68</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

*DpnI*, restriction enzyme provided in the kit, was added to the PCR product directly. The reaction mixture was incubated at 37 °C for 80 minutes to digest the parental double strand DNA.

A 100-µl aliquot of XL 10-Gold Ultracompetent Cells (Stratagene) was thawed on ice. 4 µl of the β-ME mix provided with the kit was added to the cells and mixed very gently. The cells were incubated on ice for 10 minutes and mixed gently every 2 minutes. Then, 2 µl (single mutant) or 3 µl (double mutant) of PCR product was added to the cells. The cells were mixed very gently and incubated on ice for another 30 minutes. The mixture was incubated at 42 °C for 30 seconds (The duration of the heat pulse is critical) and then, chilled on the ice for 2 minutes. 900 µl of NZY⁺ broth, preheated at 42°C, was added the mixture and the entire mini-culture was shaken at 225 rpm at 37 °C for 1 hour. 250 µl of the mini-culture was spreaded on the LB Agar plate supplemented with 50 µg / ml kanamycin and incubated at 37 °C overnight.
Table 2.16.1: NZY broth per liter

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ amine</td>
<td>10 g</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Deionised water (millipore) was added to 1 litre. The solution was adjusted to pH 7.5 using NaOH and sterilized by autoclaving at 121 °C for 20 minutes before use.

The following filter-sterilized supplements were added prior to use:

Table 2.16.2: additives of SOC broth (per litre)

<table>
<thead>
<tr>
<th>Chemicals (aqueous concentration)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂ (1 M)</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>MgSO₄ (1 M)</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>Glucose (2 M)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

(4) General method for DNA sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

A single colony was picked up using a sterile inoculating loop from LB Agar plate supplemented with 50 µg / ml kanamycin. Then the colony was transferred into 5 ml LB Broth with 50 µg / ml kanamycin, and grown at 37 °C overnight in an incubator at 225 rpm. 930 µl of overnight culture was pipetted into a clean sterile 1.5-ml eppendorf and 70 µl of sterilized DMSO was added to the eppendorf. The mixture was swirled very gently and put into a -80 °C freezer for long-term storage. Plasmid DNA was extracted from the rest of the overnight culture with QIAprep spin miniprep kit (Qiagen) and checked by agarose gel electrophoresis.
Table 2.17: general sample preparation for DNA sequencing

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL1/pSL2/pSL3</td>
<td>10 (~250 ng)</td>
</tr>
<tr>
<td>primer</td>
<td>2 (~200 ng)</td>
</tr>
<tr>
<td>Terminator Ready Reaction Mix (kit reagent)</td>
<td>4</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>4</td>
</tr>
<tr>
<td>total</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.18: general PCR program for DNA sequencing

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

The forward and reverse primers for sequencing pSL1, pSL2, and pSL3 are 5'-ATGACCATGATTACGCCAAGCTTGC and 5'-GTCACGACGTTTGTTAAAACGACGGGTC, respectively. And there is only one primer, not a pair of primers, in a sequencing sample. 10 µl of PCR product was submitted to School of Biological Science Sequencing Service (SBSSS) at the University of Edinburgh.

(5) DNA sequence analysis

The sequencing results, returned from SBSSS, were aligned with nucleotide sequence in the NCBI (National Center for Biotechnology Information) database (access number: D31702), using an online sequence alignment tool ‘EMBOSS Pairwise Alignment Algorithms’ from EBI (European Bioinformatics Institute). The reverse sequence was converted to its reverse-complement part with an online tool ‘Reverse Complement’ from Bioinformatics Organization, Inc.
2.3.3 Overlap PCR for tagging calmodulin binding peptide (CBP) to K40C:S107C cytochromes c₃ (pSL4, pSL5, pSL6)

(1) Overview of CBP tagging strategy

Figure 2.1: CBP tagging strategy. The Plasmids are in linear representation. Nucleotide segment encoding cytochrome c₃ is in red; nucleotide segment encoding CBP is in green.
Table 2.19: primers for CBP tagging*

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1 (forward)</td>
<td>ACGCCAAAGCTTGCATGC</td>
</tr>
<tr>
<td>Primer 2(pSL4r)</td>
<td>GCTTGGTAGCCGCAATGGGCACCTGGGAGC</td>
</tr>
<tr>
<td>Primer 3(pSL4f)</td>
<td>GTGCCATTGGCGTAGAACAGGACGATG</td>
</tr>
<tr>
<td>Primer 4 (reverse)</td>
<td>CATCGAATTCAAAGTGCACCCGGAGG</td>
</tr>
<tr>
<td>Primer 5(pSL5f)</td>
<td>GTGCCATTGGGACCAAGCGACGATGGGA</td>
</tr>
<tr>
<td>Primer 6(pSL5r)</td>
<td>GTCGCTTGCGCAATGGGCACCTGGGAGC</td>
</tr>
<tr>
<td>Primer 7(pSL6f)</td>
<td>GTGCCATTGGCAGTAGATCCACGATGGAGA</td>
</tr>
<tr>
<td>Primer 8(pSL6r)</td>
<td>GTGGATACATGCAATGGGCACCTGGGAGC</td>
</tr>
</tbody>
</table>

*Sequences in pink are restriction-cutting sites of HindIII and EcoRI; sequences in red are the fragments annealing to cytochrome c3 encoding sequences; sequences in green are the fragments annealing to CBP encoding sequences. pSL4: K40C:S107C cyt c3-Gly-Thr-CBP, pSL5: K40C:S107C cyt c3-Thr-CBP, pSL6: K40C:S107C cyt c3-thrombin-CBP

The cytochrome c3 encoding sequence and CBP encoding sequence were amplified by PCR, respectively (Fig 2.1). The amplified product of PCR a1 contained an intact cytochrome c3 encoding sequence with a HindIII restriction cutting site on the upstream and a short sequence encoding N-terminus of CBP after cytochrome c3 encoding sequence (~ 500 bp). Similarly, the product of PCR a2 contained an intact CBP encoding sequence with an EcoRI restriction cutting site on the downstream and a short sequence encoding C-terminus of cytochrome c3 immediately before the CBP encoding sequence (~ 100 bp). The products of PCR a1 and a2, purified by a commercial kit, were combined together and amplified by another PCR b yielding a product encoding CBP tagged cytochrome c3 (~ 600 bp). This product was purified, digested by HindIII and EcoRI, ligated to the vector of pKF19k, and transformed into E. coli JM109.

(2) PCR for CBP tagging
### Table 2.20: general PCR a1/a2 sample preparation for pSL4/pSL5/pSL6

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL3 / pCAL-c</td>
<td>1 (~25 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>2 (~200 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>2 (~200 ng)</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Reaction Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38.5</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>total</td>
<td>50</td>
</tr>
</tbody>
</table>

* pCAL-c (Stratagene); Pfu DNA polymerase and 10× Reaction Buffer (Promega); dNTPs (Roche)

### Table 2.21: general PCR b sample preparation for pSL4/pSL5/pSL6

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>purified PCR a1 product</td>
<td>1</td>
</tr>
<tr>
<td>purified PCR a2 product</td>
<td>1</td>
</tr>
<tr>
<td>forward primer</td>
<td>2 (~200 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>2 (~200 ng)</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Reaction Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>37.5</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>total</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 2.22: general PCR program for pSL4/pSL5/pSL6

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>360 (120 for PCR a2)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>420 (300 for PCR a2)</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

The product of PCR a1 or a2 was purified though QIAquick PCR Purification Kit (Qiagen). 250 μl of Buffer PBI was added to the PCR solution (50 μl) and mixed throughly. The mixture was transferred to the QIAquick spin column and the column was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and PCR product was bound to the resin of the column. 750 μl of Buffer PE was applied to wash the column and the residual buffers were discarded by centrifugation at 13,000 rpm for 1 minute. DNA was eluted into a clean sterilized eppendorf upon addition of 50 μl of Buffer EB and centrifuged at 13,000 rpm for 1 minute and checked by agarose gel electrophoresis. The purified PCR a1/a2 product was used immediately or stored in a -20 °C freezer. (The kit includes buffers PBI, PE, EB, and QIAquick spin columns.)

After the amplification of PCR b, the product was also purified with the same kit and checked by agarose gel electrophoresis.

(3) Double restriction digestion with HindIII and EcoRI
Cytochrome c₃ modules as electron transfer nanowires

Table 2.23: general double restriction digestion sample preparation

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>HindIII</td>
<td>5</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5</td>
</tr>
<tr>
<td>Buffer E (10×)</td>
<td>5</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>0.5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>4.5</td>
</tr>
<tr>
<td>total</td>
<td>50</td>
</tr>
</tbody>
</table>

The sample was mixed throughly and incubated at 37 °C in a water bath for 3 hours.

Enzymes and Buffer E were purchased from Promega.

The purified product of PCR b was digested by both HindIII and EcoRI and purified though QIAquick PCR Purification Kit (Qiagen). The purified digested product (insert) was used immediately or stored in a -20 °C freezer.

(3) pKF19k vector preparation by gel extraction

The plasmid pSL3 was digested by HindIII and EcoRI. The big fragment of the digestion products (~ 2.2 kbp) could be separated from the reaction mixture by agarose gel electrophoresis and purified though QIAquick Gel Extraction Kit (Qiagen).

The double digestion mixture (50 μl) together with 10 μl of gel sample loading buffer (Table 2.10) was loaded into a 0.8 % agarose gel (Table 2.12). The gel was run at 15 ~ 20 V/cm for 40 minutes. The gel fragment containing higher molecular weight (~ 2.2 kbp) was cut off by a scalpel under a UV lamp. The operation was as quick as possible because the DNA could be cleaved upon UV exposure. The gel slice was weighed out in a clean sterile eppendorf and 3 volumes of Buffer QG were added to 1 volume of gel slice (1 mg ~ 1 μl). The eppendorf was incubated at 50 °C in a water bath until the gel slice was totally dissolved. 1 gel volume of
isopropanol was added to the solution and mixed thoroughly. The mixture was transferred to the QIAquick spin column and the column was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and DNA was bound to the resin of the column. 500 µl of Buffer QG and 750 µl of Buffer PE, respectively, was applied to wash the column and the residual buffers were discarded by centrifugation at 13,000 rpm for 1 minute. DNA was eluted into a clean sterilized eppendorf upon addition of 50 µl of Buffer EB and centrifugation at 13,000 rpm for 1 minute. The purified double digestion product (vector) was used immediately or stored in a -20 °C freezer. (The kit includes buffers QG, PE, EB, and QIAquick spin columns.)

(4) Ligation
The purified digested PCR b product (insert, ~ 600 bp) and big fragment of digested pSL3 (pKF19k vector, ~ 2.2 kbp) were ligated by T4 ligase (Promega)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>insert</td>
<td>7</td>
</tr>
<tr>
<td>Vector</td>
<td>1</td>
</tr>
<tr>
<td>Ligation Buffer (10×)</td>
<td>1</td>
</tr>
<tr>
<td>T4 Ligase</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

The sample was mixed throughly and incubated at 10 °C overnight. T4 ligase and Ligation Buffer were purchased from Promega.

(5) One-step chemical transformation (4) of pSL4, pSL5, or pSL6 into *E. coli* JM109
5 ml of LB broth was inoculated from the stock of *E. coli* JM109 in the -80 °C freezer using a sterile inoculating loop and grown at 37 °C in an
incubator at 225 rpm overnight. A new 5-ml of LB was inoculated with 200 μl of overnight culture and grown until the optical density at 600 nm reached approximate 0.3 ~ 0.4. The cells were harvested by centrifugation at 5,000 rpm for 5 min and resuspended in 500 μl of ice-cold transformation and storage solution (TSS). 200-μl aliquots of cells were dispensed into pre-chilled eppendorfs.

Table 2.25: transformation and Storage Solution (TSS)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td>-</td>
</tr>
<tr>
<td>PEG 8000</td>
<td>10% (wt/vol)</td>
</tr>
<tr>
<td>DMSO</td>
<td>5% (vol/vol)</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

The solution was adjusted to pH 6.5 using NaOH and sterilized by passing through a 0.22 μM syringe-driven filter. TSS could be used immediately or put in a 4 °C fridge for short-term storage.

10 μl of ligation mixture was added to an aliquot of cells and incubated for 30 min at 4 °C. 800 μl of SOC broth was added to the mixture and the entire mini-culture was shaken at 225 rpm at 37 °C for 1 hour. The cells were pelleted at 6,000 rpm for 4 minutes and resuspended in 200 μl of SOC broth. Then, the cells were spread on an LB Agar plate supplemented with 50 μg / ml kanamycin and incubated at 37 °C overnight.

(6) DNA sequencing analysis for pSL4, pSL5, and pSL6

DMSO stocks were made for picked-up colonies. A forward primer, 5’-ACGCCAAGCTTTGATGC, was found to sequence all cytochrome c₃ mutants. All the sequencing procedures and data analysis followed above-mentioned protocols.
2.3.4 Overlap PCR for tagging Strep Tag II to K40C:S107C cytochromes $c_3$
(pSL7 and psL8)

(1) Overview of Strep tagging strategy

Figure 2.2: Strep tagging strategy. The Plasmids are in linear representation. Nucleotide segment encoding cytochrome $c_3$ is in red; nucleotide segment encoding Strep Tag II is in yellow.
The cytochrome $c_3$ encoding sequence was amplified by PCR (Fig 2.2). The amplified product of PCR a contained an intact cytochrome $c_3$ encoding sequence with a *HindIII* restriction cutting site on the upstream and a short sequence encoding the N-terminus of Strep Tag II after the cytochrome $c_3$ encoding sequence (∼ 500 bp). The purified product of PCR a and a short sequence C3ST (51 bp), which encoded the C-terminus of K40C:S107C cytochrome $c_3$, Strep Tag II, and an *EcoRI* cutting site, were combined together and amplified by another PCR b yielding a product encoding Strep tagged cytochrome $c_3$ (∼ 550 bp). This product was purified, digested by *HindIII* and *EcoRI*, ligated to the pKF19k vector, and transformed into *E. coli* JM109. The strategy worked in a similar way to CBP tagging.

(2) PCR for Strep tagging
### Table 2.27: general PCR a sample preparation for pSL7/pSL8

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL3 / pSL2</td>
<td>1 (~25 ng)</td>
</tr>
<tr>
<td>primer 1</td>
<td>2 (~ 200 ng)</td>
</tr>
<tr>
<td>primer 9</td>
<td>2 (~ 200 ng)</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Reaction Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38.5</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>total</td>
<td>50</td>
</tr>
</tbody>
</table>

### Table 2.28: general PCR b sample preparation for pSL7/pSL8

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>purified PCR a product</td>
<td>1</td>
</tr>
<tr>
<td>C3ST</td>
<td>1 (~ 10 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>2 (~ 200 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>2 (~ 200 ng)</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Reaction Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>37.5</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>total</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 2.29: general PCR program for pSL7/pSL8

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>65</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

The product of PCR a was purified though QIAquick PCR Purification Kit and used immediately or stored in a -20 °C freezer. After the amplification of PCR b, the product was again purified with the same kit and checked by agarose gel electrophoresis.

All the following steps in the Strep tagging procedure shared the same protocols and regents with corresponding steps in CBP tagging procedure.

(3) Double restriction digestion with *HindIII* and *EcoRI*

(4) Ligation

(5) One-step chemical transformation of pSL7 or pSL8 into *E. coli* JM109

(6) DNA sequencing analysis for pSL7 and pSL8

2.3.5 Point mutagenesis for production of K40C and wild type cytochromes c₃ with New Strep Tags (pSL9 and pSL10)

(1) Small scale plasmid DNA extraction with QIAprep spin miniprep kit (Qiagen)

pSL9 and pSL10 were produced on the basis of pSL7 and pSL8. Plasmid DNA solutions of pSL7 and pSL8 were extracted from 5-ml overnight mini-cultures with 50 μg / ml of kanamycin of pSL7JM109 and pSL8JM109, respectively.

(2) Detection of DNA by Agarose gel electrophoresis
The extracted DNA solutions of pSL7 and pSL8 were checked by 0.8 % agarose gel electrophoresis.

(3) Site-directed mutagenesis with QuikChange mutagenesis kit (Stratagene) and one-step transformation into *E. coli* JM109

**Table 2.30: PCR (Polymer Chain Reaction) sample preparation for pSL9 and pSL10**

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Usage / μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL7 / pSL8</td>
<td>1 (~ 25 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>QuikSol</td>
<td>3</td>
</tr>
<tr>
<td>Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38</td>
</tr>
<tr>
<td>Pfu Turbo DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
</tr>
</tbody>
</table>

**Table 2.31: PCR program for pSL9 and pSL10**

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>55</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>68</td>
<td>210</td>
<td>1</td>
</tr>
<tr>
<td>68</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

The forward primer (5'-CCAAGTGCCATAGCAGCGCTTGGTGCCACCCGCAG), and reverse primer (5'-CTGCGGGTTGCCACCAAGCGCTGCTATGGGCACTTGG) were used to convert the cysteines 107 of strep-tagged cytochrome c3
mutants (pSL7 and pSL8) to serines and the serines in the Strep Tag II to cysteines.

After restriction digestion of the parental template by DpnI, 5 μl of reaction solution was transferred into 200 μl of the freshly made E. coli JM109 cells in TSS. After 30-minute incubation on ice, 800 μl of SOC broth was added and the mini-culture was grown up at 225 rpm at 37 °C for 1 hour. Then, the cells were pelleted, resuspended in 200 μl of SOC broth, spread onto an LB agar plate with 50 μg / ml of kanamycin, and incubated overnight at 37 °C.

(4) DNA sequencing and data analysis for pSL9 and pSL10

This procedure was identical to the previous procedure with the general cytochrome c₃ sequencing primer, 5'-ACGCCAAGCTTGATGC.

2.3.6 Long oligonucleotide insertion for the attachment of His₆ tags to K40C and wild type cytochromes c₃ (pSL11 and pSL12)

In this project, it was found that the QIAprep spin miniprep kit (Qiagen) not only worked well for point mutagenesis, but also had good performance in the long oligo nucleotide insertion to the plasmid DNA, which made the tagging a small peptide to a protein easier during genetic manipulation. Here, the template plasmid DNAs were pSL1 and pKFC3k.

(1) Small scale plasmid DNA extraction with QIAprep spin miniprep kit (Qiagen)

Plasmid DNA solutions of pSL1 and pKFC3k were extracted from 5-ml overnight mini-cultures with 50 μg / ml of kanamycin of pSL1JXL10 and pKFC3kDH5α, respectively.

(2) Detection of DNA by Agarose gel electrophoresis

The extracted DNA solutions of pSL1 and pKFC3k were checked by 0.8 % agarose gel electrophoresis.

(3) Site-directed mutagenesis with QuikChange mutagenesis kit (Stratagene) and one-step transformation into E. coli JM109
Table 2.32: PCR (Polymer Chain Reaction) sample preparation for pSL11 and pSL12

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL1 / pKFC3k</td>
<td>1 (~ 25 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>QuikSol</td>
<td>3</td>
</tr>
<tr>
<td>Buffer (10x)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38</td>
</tr>
<tr>
<td>Pfu Turbo DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
</tr>
</tbody>
</table>

Table 2.33: PCR program for pSL11 and pSL12

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>68</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

The forward long primer containing a His6 tag was 5'-TCCAAGTGCCATAGCGGATCGCATCACCATCACCATCACTAAG AATTCACTGGC, and the corresponding reverse long primer was 5'-GCCAGTGAATTCTTAGTGATGGTGATGGTGATGGTGATGCGATCCGCTA TGGCACTTGGGA.

After restriction digestion of the parental template by DpnI, 5 µl of reaction solution was transferred into 200 µl of the freshly made E. coli JM109 cells in TSS. After 30-minute incubation on ice, 800 µl of SOC
broth was added and the mini-culture was grown up at 225 rpm at 37 °C for 1 hour. Then, the cells were pelleted, resuspended in 200 μl of SOC broth, spread onto an LB agar plate with 50 μg/ml of kanamycin, and incubated overnight at 37 °C.

(4) DNA sequencing and data analysis for pSL11 and pSL12

This procedure was identical to the previous procedure with the general cytochrome c₃ sequencing primer, 5'-ACGCAAAGCTTGCATGC.

2.3.7 Point mutagenesis for production of K40C cytochromes c₃ with a Strep Tag II (pSL13)

In order to produce a K40C cytochrome c₃ with a Strep Tag II at the C-terminus, point mutagenesis could be exploited though a template plasmid DNA, pSL9. All reagents and programs were identical to section 2.3.5, except the forward primer, 5'-AGCGCTTGGAGCCACCG, and the reverse primer, 5'-CGGGTGGCTCCAAGCGCT.

2.3.8 Site-directed mutagenesis for production of the C156S mutant of P450 BM3 heme domain (pSLa), the C156SHis6 mutant (pSLb), and the C62S:C156S:Q387CHis6 mutant (pSLc)

According to previous experience, pSLa and pSLc could be produced though point mutagenesis while pSLb was created though long nucleotide insertion. All the genetic operations were achieved using the QuickChange mutagenesis kit (Stratagene). The template DNA was pBM20, which encoded the wild type heme domain of P450 BM3 [140].

(1) Small scale plasmid DNA extraction with QIAprep spin miniprep kit (Qiagen)

Plasmid DNA solutions of pBM20 was extracted from 5-ml overnight mini-cultures with 100 μg/ml of ampicillin of pBM20|TG1.

(2) Detection of DNA by Agarose gel electrophoresis
The extracted DNA solution of pBM20 was checked by 0.8 % agarose gel electrophoresis.

(3) Site-directed mutagenesis with QuikChange mutagenesis kit (Stratagene) and one-step transformation into *E. coli* JM109

*Table 2.34: PCR (Polymer Chain Reaction) sample preparation for pSLa, pSLb, or pSLc*

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Usage / µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBM20 / pSLa / pSLb</td>
<td>1 (~ 25 ng)</td>
</tr>
<tr>
<td>forward primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>reverse primer</td>
<td>1.25 (~ 125 ng)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5</td>
</tr>
<tr>
<td>QuikSol</td>
<td>3</td>
</tr>
<tr>
<td>Buffer (10×)</td>
<td>5</td>
</tr>
<tr>
<td>sterilized deionised water</td>
<td>38 (35.5 *)</td>
</tr>
<tr>
<td>Pfu Turbo DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
</tr>
</tbody>
</table>

*Four primers were used in the production of pSLc. Therefore, the water volume was adjusted to 35.5 µl.*
Table 2.35: PCR program for pSLa, pSLb, or pSLc

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Incubation time / s</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30</td>
<td>18 (16 in pSLa production)</td>
</tr>
<tr>
<td>55</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>68</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>68</td>
<td>420</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Table 2.36: plasmids and primers for the site-directed mutagenesis of heme domain of P450 BM3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Forward and reverse primes for PCR (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM20</td>
<td>N/A</td>
</tr>
<tr>
<td>pSLa</td>
<td>CAATTGGTCTTTAGCGGCTTTAAC</td>
</tr>
<tr>
<td></td>
<td>GTAAAGCCGCTAAAGCAATTTG</td>
</tr>
<tr>
<td>pSLb</td>
<td>CAGTCTGCTAAAGAAGTACGCCACCACTACATTGACTCTCTAGGAGTGG</td>
</tr>
<tr>
<td></td>
<td>CGACTCTAGGATCTATTAGTGATGTTTAGTGATGCGCTACTTTTTTTAGCAGACTG</td>
</tr>
<tr>
<td>pSLc</td>
<td>CTAATTAAGAGCAAGCGATGAATCAGC</td>
</tr>
<tr>
<td></td>
<td>GCCATTCATCGCTCTTCTTTAAATTAG</td>
</tr>
<tr>
<td></td>
<td>CCAGTTGCGATTTCCGTGCCGCGTTATTAAACC</td>
</tr>
<tr>
<td></td>
<td>CGGTCTAAACGCAATTCGCACTGTGACTGG</td>
</tr>
</tbody>
</table>

After restriction digestion of the parental template by DpnI, 5 μl of reaction solution was transferred into 200 μl of the freshly made E. coli TG1 cells in TSS. After 30-minute incubation on ice, 800 μl of SOC broth was added and the mini-culture was grown up at 225 rpm at 37 °C for 1 hour. Then, the cells were pelleted, resuspended in 200 μl of SOC broth, spreaded onto LB agar plate with 100 μg / ml of ampicillin, and incubated overnight at 37 °C.

(4) DNA sequencing and data analysis for pSLa, pSLb, or pSLc

This procedure was identical to the previous procedure with the general sequencing primer pair for the mutants of heme domain of P450 BM3,
Cvtochrome c₃ modules as electron transfer nanowires

5'- TCACTCATTAGGCACCCCAG (forward) and 5'-
CAGCTGGCGAAAGGGGGATG (reverse).

2.4 Transformation of plasmids into *Shewanella oneidensis* MR-1

2.4.1 Chemical transformation of plasmids into *Shewanella oneidensis* MR-1

Previous experimental procedures indicated that the chemical transformation of plasmid DNA into *E. coli* strains, JM109 and TG1, worked very well. Actually, this strategy had been proven to perform efficiently for the one-step transformation of DNA into most *E. coli* strains [142-143]. This method was developed and applied to the transformation of DNA into *Shewanella* species. The cell membrane of *Shewanella oneidensis* is thicker than that of *E. coli*, although they are both classified as γ-proteobacteria. This physiological property makes *Shewanella oneidensis* difficult to be transformed. The commonly used transformation methods are electroporation and conjugation. Electroporation is highly efficient and quick, but it needs expensive equipment and special cuvettes. The conjugation is a time-consuming way of transformation and it will normally take a couple of days. The following transformation protocol, with some modifications to the literature method, was fully supported by the experimental evidence.

5 ml of LB broth with 10 μg / ml rifampicin was inoculated from the stock of wild type *Shewanella oneidensis* MR-1 in the -80 °C freezer using a sterile inoculating loop and grown at 37 °C in an incubator at 225 rpm overnight. A new 5-ml LB broth with the same antibiotics was inoculated with 200 μl overnight culture and grown until the optical density at 600 nm reached approximate 0.5 ~ 0.7. The cells were harvested by centrifugation at 5,000 rpm for 5 min and resuspended in 500 μl of ice-cold transformation and storage solution (TSS). 200-μl aliquots of cells were dispensed into pre-chilled eppendorfs.
5 μl of plasmid DNA, extracted from 5-ml overnight culture of *E. coli* containing pKFC3k or pSLn (n = 1 ~ 13), was added to an aliquot of cells and incubated for 30 min at 4 °C. 800 μl of SOC broth was added to the mixture and the entire mini-culture was shaken at 225 rpm at 37 °C for 1 hour. The cells were pelleted at 6,000 rpm for 4 minutes and resuspended in 200 μl of SOC broth. Then, the cells were spread onto an LB Agar plate supplemented with 50 μg / ml kanamycin and 10 μg / ml rifampicin, and incubated at 37 °C overnight.

A single colony was picked up for the growth of 5-ml overnight culture with antibiotics. A DMSO storage stock was made. The plasmid DNA was extracted from the rest of the culture and checked by agarose gel electrophoresis.

2.4.2 Electrical transformation of plasmids into *Shewanella oneidensis* MR-1

2.4.2.1 Preparation of electro-competent cells of *Shewanella oneidensis*

5 ml of LB broth with 10 μg / ml rifampicin was inoculated from the stock of wild type *Shewanella oneidensis* MR-1 in the -80 °C freezer using a sterile inoculating loop and grown at 30 °C in an incubator at 225 rpm. A 250-ml flask, filled with 100 ml LB Broth, was inoculated with 2-ml overnight culture and incubated for approximately 3 hours at 30 °C in an incubator at 180 rpm until the optical density at 600 nm reached approximately 0.5 ~ 0.7. The flask was placed on ice for 20 minutes. Then the culture was transferred into 4 sterile, ice-cold 50-ml Falcon tubes and the cells were harvested by centrifugation at 4000g (5000 rpm in a Sorvall GSA rotor), 8 min, 4 °C. The supernatant was discarded, and the cell pellets were gently resuspended in 200 ml of ice-cold 10 % glycerol. The cells were harvested by centrifugation at 4000g, 8 min, 4 °C, and the supernatant was discarded. The wash of the cell pellets was repeated with 100 ml and 40 ml of ice-cold 10 % glycerol. Finally the cell pellets were resuspended in 1 ml cold 10 % glycerol. 40-μl aliquots of the cell suspension were dispensed into sterile, ice-cold 1.5-ml
2.4.2.2 Electrotransformation of plasmids into *Shewanella oneidensis* competent cells

The competent cells of *Shewanella oneidensis* MR-1 were thawed on ice. A 1.5-ml eppendorf and 0.2 cm electroporation cuvette were placed on ice as well. In a cold 1.5-ml eppendorf, 40 µl of the competent cell suspension was mixed with 2 µl of plasmid well and incubated on ice for approximately 1 minute. The MicroPulser (Bio-Rad) was set to “Ec2”, and the mixture of competent cells and plasmid was transferred into a cold 0.2 cm electroporation cuvette and tapped to the bottom. The slide was placed into the chamber and the “Pulse” button was pressed once. The cuvette was removed from the chamber and immediately 1 ml SOC medium was added. The cells were resuspended quickly but gently, and then transferred into a 1.5-ml eppendorf and incubated at 30°C for 1 hour, shaking at 225 rpm. The miniculture was spun down at 6,000 rpm for 4 minutes. The supernatant was discarded and the cell pellet was resuspended gently with 200 µl of SOC medium. And then the mixture was plated out on an LB-agar plate supplemented with 50 µg / ml of kanamycin and 10 µg / ml of rifampicin, and incubated at 30 °C overnight.

2.5 Protein Expression

2.5.1 The expression of all cytochrome $c_3$ mutants in *Shewanella oneidensis* MR-1

Starter cultures, 5 ml of LB broth supplemented with 50 µg / ml of kanamycin and 10 µg / ml of rifampicin, were inoculated with the stock of pKFC3k|MR-1 or pSLn|MR-1 (n = 1 ~ 13) in the -80 °C freezer using a sterile inoculating loop and grown at 30 °C in an incubator at 225 rpm
overnight. 2-litre flasks, filled with 1000 ml of TB broth supplemented with 200 µg / ml of kanamycin and 10 µg / ml of rifampicin per flask, were inoculated with 1 ml of starter culture and grown for 48 hrs at 30 °C, 150 rpm. Cells were harvested by centrifugation (16000 g for 12 minutes) and stored at -20 °C.

2.5.2 The expression of cytochrome P450 BM3 heme domain mutants

Starter cultures, consisting of 5 ml of LB broth supplemented with 100 µg / ml of ampicillin, were inoculated with the stock of pSLx[TG1 (x = a, b, c) in the -80 °C freezer using a sterile inoculating loop and grown at 30 °C in an incubator at 225 rpm overnight. 2-litre flasks, filled with 1000 ml of TB broth supplemented with 25 µg / ml of carbenecillin and 1-ml hemin solution (hemin 7mM and NaOH 1mM) per flask, were inoculated with 1 ml of starter culture and grown for 48 hrs at 37 °C, 150 rpm. Cells were harvested by centrifugation (16000 g for 12 minutes) and stored at -20 °C.

2.6 Protein Purification

2.6.1 The purification of cytochrome c₃ cysteines mutants
Table 2.37: buffers for the purification of cytochrome c₃ cysteine mutants

<table>
<thead>
<tr>
<th>Name of buffer</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer I</td>
<td>MOPS 10 mM, pH 7.0</td>
</tr>
<tr>
<td></td>
<td>DTT 5 mM</td>
</tr>
<tr>
<td></td>
<td>Streptomycin 1% (wt/vol)</td>
</tr>
<tr>
<td></td>
<td>PMSF 200 μM</td>
</tr>
<tr>
<td>Buffer A1</td>
<td>MOPS 10 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td>Buffer B1</td>
<td>MOPS 10 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl 500 mM</td>
</tr>
<tr>
<td>Buffer C1</td>
<td>MOPS 10 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl 200 mM</td>
</tr>
</tbody>
</table>

Generally, the purification of untagged cytochrome c₃ cysteine mutants mainly utilized the unusual positive electrostatic surface while most of other proteins have negative surfaces. The cytochromes c₃ could bind very tightly to the cation exchange resin, SP Sepharose because the exchanging functional group of SP Sepharose is the sulfopropyl anion (−CH₂CH₂CH₂SO₃⁻). Elution from the SP Sepharose column followed by gel filtration on Superdex 75 [19]. Gel filtration, also called size exclusion, separates proteins according to the difference in their sizes.

Approximately 30 g frozen cell pellets were defrosted, resuspended in 100 ml of Lysis Buffer I, and lysed by sonication (MSE soniprep 150, 10 ~ 15 miron) for 20s × 10 on ice. Cell debris were removed by centrifugation at 4000 g (20000 rpm in a Sorvall GSA rotor) for 1 hour at 4 °C. Further purification was carried out in the 4 °C cold room. The supernatant was loaded onto an SP-Sepharose gravity column (2.6 cm by 10 cm, Sigma) previously equilibrated with Buffer A1. Cytochrome c₃ bound to the column forming a red band at the top and the column was washed by 10 column
volumes of Buffer A1. A gradient from Buffer A1 to Buffer B1 was then used to wash out the cytochrome c3, which was eluted at 150 mM NaCl. The cytochrome c3 fraction was concentrated to approximately 2.5 ml by centrifugation at 3000 rpm (MSE centaur 2) using a concentrator (MWCO 5,000, Vivascience) and further purified by gel filtration though fast protein liquid chromatography (FPLC) using a Hiload Superdex 75 column (2.6 cm by 60 cm, Amersham) on an ÄKTApurifier (GE healthcare) equilibrated with Buffer C1. The cytochrome c3 fractions were pooled, and concentrated to approximate 2 ml. 200-μl aliquots were dispensed into sterile eppendorfs, dropped into a bath of liquid nitrogen, and stored in a -80°C freezer.

2.6.2 The purification of cytochrome c3 mutants with Calmodulin binding peptide (CBP)
Generally the purification of CBP tagged cytochromes \(c_3\) mainly utilized the tight binding activity of CBP and Calmodulin (CaM) to a CaM agarose resin (Stratagene) or a CaM Sepharose resin (Sigma) [83].

Approximately 30 g frozen cell pellets were defrosted, resuspended in 100 ml of Lysis Buffer II, and lysed by sonication (MSE soniprep 150, 10 ~ 15 miron) for 20s × 10 on ice. Cell debris was removed by centrifugation at 4000 g (20,000 rpm in a Sorvall GSA rotor) for 1 hour at 4 °C. Further purification was carried out in the 4 °C cold room. The supernatant was loaded onto a Calmodulin (CaM) affinity column (1.6 cm by 5 cm, Stratagene or Sigma) previously equilibrated with Buffer A2. CBP tagged cytochromes

### Table 2.38: buffers for the purification of CBP tagged cytochrome \(c_3\)

<table>
<thead>
<tr>
<th>Name of buffer</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer II</td>
<td>Tris 50 mM, pH 8.0, DTT 1 mM, NaCl 150 mM, Mg(AcO)(_2) 1 mM, Imidazole 1 mM, CaCl(_2) 2mM, Streptomycin 1% (wt/vol), PMSF 200 µM</td>
</tr>
<tr>
<td>Buffer A2</td>
<td>Tris 50 mM, pH 8.0, DTT 1 mM, NaCl 150 mM, Mg(AcO)(_2) 1 mM, Imidazole 1 mM, CaCl(_2) 2mM</td>
</tr>
<tr>
<td>Buffer B2</td>
<td>Tris 50 mM, pH 8.0, DTT 1 mM, NaCl 150 mM, EGTA 2mM</td>
</tr>
</tbody>
</table>
were supposed to bind to the top of the CaM affinity column and were eluted out with Buffer B2. However, all red proteins in the supernatant simply went through the CaM affinity column and none of them were bound to the column. All three CBP tagged cytochromes \( c_3 \) did not show any CaM-binding. The CBP tagged cytochromes \( c_3 \) were then purified with the protocol in section 2.6.1. The CBP tagged cytochromes \( c_3 \) were eluted out at approximately 400 mM NaCl from SP Sepharose column and then purified by gel filtration through FPLC. The CBP tagged cytochrome \( c_3 \) fractions were pooled and concentrated. 200-μl aliquots were dispensed into sterile eppendorfs, dropped into a bath of liquid nitrogen, and stored in a -80°C freezer.

### 2.6.3 The purification of cytochrome \( c_3 \) mutants with Strep Tag II or New Strep Tag

**Table 2.39: buffers for the purification of Strep tagged cytochrome \( c_3 \)**

<table>
<thead>
<tr>
<th>Name of buffer</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer W</td>
<td>Tris 100 mM, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>NaCl 150 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td>Buffer E</td>
<td>Tris 100 mM, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>NaCl 150 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td></td>
<td>Desthiobiotin 2.5mM</td>
</tr>
<tr>
<td>Buffer R</td>
<td>Tris 100 mM, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>NaCl 150 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 1 mM</td>
</tr>
<tr>
<td></td>
<td>HABA 2.5 mM</td>
</tr>
</tbody>
</table>
Generally the purification of strep tagged cytochromes c³ utilized the tight binding activity of Strep Tag II / New Strep Tag to a commercial streptavidin derivative resin (IBA) [144].

Approximately 30 g frozen cell pellets were defrosted, resuspended in 100 ml of Lysis Buffer I, and lysed by sonication (MSE soniprep 150, 10 ~ 15 mirons) for 20s x 10 on ice. Cell debris were removed by centrifugation at 4000 g (20000 rpm in a Sorvall GSA rotor) for 1 hour at 4 °C. Further purification was carried out in the 4 °C cold room. The supernatant was loaded onto an SP-Sepharose column (2.6 cm by 10 cm, Sigma) previously equilibrated with Buffer A1. Cytochrome c³ bound to the column forming a red band at the top and was eluted out with Buffer B1. The cytochrome c³ fractions were pooled, adjusted to pH 8.0 with Tris solution (1M, pH 8.0), and loaded onto a Strep-Tactin Sepharose column (10 ml, IBA) previously equilibrated with Buffer W. The strep column was washed by 10 column-volume Buffer W and the strep tagged cytochrome c³ was eluted out with Buffer E. The red fractions were pooled, and concentrated to approximately 2.5 ml. 200-µl aliquots were dispensed into sterile eppendorfs, dropped into a bath of liquid nitrogen, and stored in a -80°C freezer.

In case of cytochromes c³ with New Strep Tag, the elution from strep column was further purified by gel filtration (Section 2.6.1). The fractions of the protein’s monomer were collected, concentrated to approximately 2.5 ml. 200-µl aliquots were dispensed into sterile eppendorfs, dropped into a bath of liquid nitrogen, and stored in a -80°C freezer.

The strep column was regenerated with Buffer R and Buffer W.

2.6.4 The purification of cytochrome P450 BM3 heme domain mutants
Table 2.40: buffers for the purification of mutants of P450 BM3 heme domain

<table>
<thead>
<tr>
<th>Name of buffer</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer III</td>
<td>Tris 50 mM, pH 8.0 NaCl 300 mM TCEP 1 mM Imidazole 10 mM PMSF 200 μM Egg white lysozyme 1mg/ml</td>
</tr>
<tr>
<td>Buffer A3</td>
<td>Tris 50 mM, pH 8.0 NaCl 300 mM TCEP 1 mM Imidazole 20 mM</td>
</tr>
<tr>
<td>Buffer B3</td>
<td>Tris 50 mM, pH 8.0 NaCl 300 mM TCEP 1 mM Imidazole 250 mM</td>
</tr>
<tr>
<td>Buffer C3</td>
<td>Tris 50 mM, pH 8.0 TCEP 1 mM</td>
</tr>
</tbody>
</table>

Generally the purification of His\textsubscript{6} tagged P450 BM3 heme domain utilized the tight binding activity of His\textsubscript{6} Tag to Nickel-Nitrilotriacetic acid (Ni\textsuperscript{2+}-NTA) resin (Qiagen). The excess imidazole introduced by Ni\textsuperscript{2+}-NTA column purification was removed by passing the protein solution through a G-25 medium, which is a gel filtration column resin separating biomacromolecules from small molecules very well.

Approximate 30 g frozen cell pellets were defrosted, resuspended in 100 ml of Lysis Buffer III, and lysed by sonication (MSE soniprep 150, 10 - 15 mirons) for 20s × 10 on ice. Cell debris were removed by centrifugation at 4000 g (20000 rpm in a Sorvall GSA rotor) for 1 hour at 4 °C. Further purification was carried out in the 4 °C cold room. The supernatant was loaded onto a Ni\textsuperscript{2+}-NTA agarose gravity column (2.6 cm by 10 cm, Qiagen)
previously equilibrated with Buffer A3. The heme domain of P450 BM3 bound to the column forming a red band at the top and the column was washed by 10 column volumes of Buffer A3. The heme domain was washed out with Buffer B3 directly. The red fraction was concentrated to approximate 2.5 ml by centrifugation at 3000 rpm (MSE centaur 2) using a concentrator (MWCO 30,000, Vivascience) and further purified by a G-25 desalting gravity column (2.6 cm by 10 cm, Sigma) equilibrated with Buffer C3. The imidazole-free heme domain solution was concentrated to approximately 2 ml. 200-μl aliquots were dispensed into sterile eppendorfs, dropped into a bath of liquid nitrogen, and stored in a -80°C freezer.

2.7 Determination of Protein purity and Concentration

2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) is an effective method for addressing protein purity by the separation of proteins in complex mixtures. The principle is that different proteins of different sizes run at different rates in an electric field. Small proteins will run faster than large ones, and globular-shaped proteins will run more quickly than linear-shaped proteins. Sodium dodecyl sulphate (SDS) is used to dissociate the proteins into individual chains, which are easy to be separated according to molecular weights in polyacrylamide gel electrophoresis. Pre-cast NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen) were used to determine the purity of the protein throughout the purification procedures. Samples were prepared by adding 10 μl of NuPAGE LDS sample buffer (4x, Invitrogen) to 30 μl of protein sample. They were then boiled for 5 minutes to denature the protein before being loaded onto the gel. SeeBlue Plus 2 pre-stained protein standard (Invitrogen) was used to mark the molecular weight. The running buffer was prepared by diluting 50 ml of NuPAGE MES SDS
(20×, Invitrogen) stock solution to 1 litre with deionised water (millipore). The gel was run for 1 hour at 150 V.

(1) Coomassie Brilliant Blue (CBB) staining

Coomassie Brilliant Blue (CBB) staining is widely used for detection of proteins in SDS-polyacrylamide gel electrophoresis. The blue dye can attach to polypeptides on the gel and form blue bands displaying the existence and abundance of proteins in the gel sample of complex mixture.

Table 2.41: solutions for CBB staining

<table>
<thead>
<tr>
<th>Name of solution</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBB staining solution</td>
<td>Coomassie Brilliant Blue 1 %</td>
</tr>
<tr>
<td></td>
<td>Methanol 40 %</td>
</tr>
<tr>
<td></td>
<td>Acetic acid 10 %</td>
</tr>
<tr>
<td>CBB destaining solution</td>
<td>Methanol 40 %</td>
</tr>
<tr>
<td></td>
<td>Acetic acid 10 %</td>
</tr>
</tbody>
</table>

The gel was stained with CBB staining solution for approximate 20 minutes. The dye was removed with CBB destaining solution until clear protein blue bands appeared on the gel.

(2) Heme staining (3,3',5,5'-tetramethylbenzidine (TMBZ)/H$_2$O$_2$ staining)

The staining method is always used to detect $c$-type cytochromes, in which the $c$-type hemes are covalently bound to polypeptide and will not lost during the sample preparation of gel electrophoresis in tough conditions.
Table 2.42: solutions for heme staining

<table>
<thead>
<tr>
<th>Name of solution</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>heme staining buffer I</td>
<td>Methanol 30 %</td>
</tr>
<tr>
<td></td>
<td>Sodium acetate 70 %, pH 5.2</td>
</tr>
<tr>
<td>TMBZ solution</td>
<td>Methanol 30 %</td>
</tr>
<tr>
<td></td>
<td>Sodium acetate 70 %, pH 5.2</td>
</tr>
<tr>
<td></td>
<td>TMBZ solution (in Methanol, Sigma), 1 mg/ml</td>
</tr>
<tr>
<td>heme staining buffer II</td>
<td>Isopropanol 30 %</td>
</tr>
<tr>
<td></td>
<td>Sodium acetate 70 %, pH 5.2</td>
</tr>
</tbody>
</table>

All the staining steps were performed in a black box without any lights, which would degrade TMBZ. The gel was soaked in heme staining buffer I for 30 minutes. Then the gel was soaked in TMBZ solution for 30 minutes. 150 μl of H₂O₂ (AR Grade, 30%, 100 volumes, Fisher) was added to develop bands for 15 minutes and the dyes were then poured. Heme staining buffer II was used to fix the bands.

2.7.2 UV/Vis spectrophotometry

UV/Vis spectrophotometry was performed at room temperature using a Shimadzu UV-1601 UV/Vis scanning spectrophotometer with the UV-1601 software. The characteristic absorbances and extinction coefficients for Desulfovibrio cytochrome c₃ in both its oxidized and reduced forms are shown in Table 2.43.
Table 2.43: characteristic absorbances and extinction coefficients for Desulfovibrio cytochrome c₃ in both its oxidized and reduced forms

<table>
<thead>
<tr>
<th>Soret band</th>
<th>Visible band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λₘₐₓ / nm</td>
</tr>
<tr>
<td>Oxidised state</td>
<td>410</td>
</tr>
<tr>
<td>Reduced state</td>
<td>419</td>
</tr>
</tbody>
</table>

For cytochrome c₃ of Desulfovibrio vulgaris Miyazaki F, ε₅₅₂ = 116 mM⁻¹ cm⁻¹ in the reduced state or ε₄₁₀ = 416 mM⁻¹ cm⁻¹ in the oxidized state was used to determine the concentration [145].

The concentration of P450 was determined by producing the CO-bound reduced P450 BM3 heme domain, calculating out the difference spectra [Fe(II)-CO] – [Fe(II)], using the extinction coefficient of ε₄₅₀₄₉₀ = 92 mM⁻¹ cm⁻¹ [146].

Generally, the proteins were diluted 200 fold (5 µl protein stock solution in 995 µl of the appropriate buffer). Spectra of protein samples were taken in the oxidised form, the reduced form, and the CO-bound reduced form only for P450 BM3 heme domain. The protein was reduced by addition of excess sodium dithionite (Na₂S₂O₄), and on addition bubbled with CO (Sigma) only for P450 BM3 heme domain. The protein concentration could be determined using the Beer-Lambert Law (A = εcl, l = 1 cm) and the above extinction coefficients.

2.8 Thiol Counting

The number of cysteine residues on the surface of the protein can be determined by reaction with 2,2’ -dipyridyl disulphide (2-PDS). 2-PDS reacts with the thiol of the cysteine group to yield 2-pyridinethione, the presence of which was measured spectrophotometrically (Fig. 2.3)
Figure 2.3: the reaction scheme of a 2-PDS with a thiol yielding a chromophore, 2-pyridinethione (in yellow)

The accumulation of the chromophore, 2-pyridinethione, was detected spectrophotometrically at 343 nm ($\varepsilon_{343} = 8.08 \text{ mM}^{-1} \text{ cm}^{-1}$) \cite{52}. The concentration change of 2-pyridinethione can therefore be used to calculate the concentration of thiol groups in the protein and hence the number of cysteine residues on the protein.

<table>
<thead>
<tr>
<th>Table 2.44: solutions for thiol counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of solution</td>
</tr>
<tr>
<td>2-PDS solution (oxygen free)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NaOAc buffer (oxygen free)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Generally, the protein stock solution from -80 °C freezer was defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes. Then, the sample was delivered into an anaerobic glove box (Belle Technology) and the following steps were carried out. The protein sample was passed through a gravity G-25 column (1.6 cm $\times$ 5cm) that had been equilibrated with the NaOAc buffer to remove the DTT. The concentration of the protein was determined by UV/Vis Spectroscopy and the protein was then diluted to 10 $\mu$M.

900 $\mu$l of the protein solution was added to a 1cm pathlength quartz crystal cuvette and a background reading of the protein sample was taken. 100 $\mu$l of
2-PDS working solution was added to the cuvette and the change in absorbance at 343nm was measured immediately and every minute for 30 minutes. The total absorbance change corrected with baseline and protein dilution was converted to the quantity of free cysteines in the cysteine mutants of cytochrome $c_3$.

2.9 Fluorescent Labelling of Cysteine on the protein

Fluorescein-5-maleimide (F5M) was used to determine the accessibility of mutation sites of cysteine 40 (K40C) and cysteine 107 (S107C) to the bis-maleimide crosslinkers.

F5M is a water-soluble and thiol-reactive fluorescent probe. There is a maleimide group that is connected to a rhodamine-like fluorescent group (Fig. 2.4). The increased photostability and pH insensitivity of the rhodamine-like component is useful in imaging experiments.
Table 2.45: solutions for F5M labelling

<table>
<thead>
<tr>
<th>Name of solution</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5M solution</td>
<td>stock solution: 1 mM</td>
</tr>
<tr>
<td>(in MOPS buffer)</td>
<td>working solution: 100 µM</td>
</tr>
<tr>
<td>MOPS buffer</td>
<td>MOPS 10 mM, pH 7.0</td>
</tr>
</tbody>
</table>

Generally, the protein stock solution from -80°C freezer was defrosted and incubated with 10 mM DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes. Then, the protein sample was passed through a gravity G-25 column (1.6 cm x 5 cm) column that had been equilibrated with the MOPS buffer to remove the DTT.

100 µl of F5M stock solution was added to 900 µl of protein sample (approximate 10 µM) and the mixture was incubated on ice in the dark for 5 minutes. The reaction was quenched by adding DTT (1 M) to a final concentration of 10 mM for 5 minutes. The mixture was analyzed with SDS-PAGE for evaluating the result of fluorescent labelling, and the gel was photographed under UV light.

2.10 Substrate binding to the P450 BM3 heme domain mutant

UV/Vis spectra were recorded at room temperature over the range 250 – 800 nm using a Shimadzu UV-2101PC spectrophotometer and quartz cuvette with a path length of 1 cm.

Due to the insoluble nature of sodium laurate and palmitic acid/palmitate, the saturated solutions of them were made by dissolving the substrates in the hot Buffer C3 (Section 2.6.4) initially. And then the precipitates were removed by centrifugation while the hot solution cooled to the room temperature. The clear solution for each substrate was taken as a baseline correction on the UV-Vis spectrophotometer.
The stock solution of P450 BM3 heme domain mutant from storage was defrosted and 10 μl of the stock was added into 990 μl of the substrate-saturated solution. Then, the absorption spectrum was recorded.

2.11 Chemical Crosslinking

The crosslinker was 1,8-bis-Maleimidodiethyleneglycol (BM(PEO)2, Pierce). BM(PEO)2 can irreversibly crosslink to thiol groups of cysteine. It has a non-cleavable flexible PEG(polyethylene glycol)/PEO spacer arm, which can enhance the aqueous solubility of the crosslinker, causing a reduced tendency toward protein aggregation, increased stability of conjugate, and reduced immunogenicity. Generally, the conjugation environment was pH 7.0 maintained by an XL Buffer (MOPS 50mM, pH 7.0; glycerol 5%) and all crosslinking reactions were performed at room temperature.

2.11.1 Homodimers of K40C and S107C cytochrome c₃

The frozen stock of K40C (or S107C) cytochrome c₃ was defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface cysteines. Then, the protein stock was passed through a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. The protein was diluted to approximate 10 μM. 10 μl of BM(PEO)2 (0.5 mM in DMSO) was added to 990 μl of the diluted protein solution to a final concentration of 5 μM and mixed thoroughly. Every 100-μl solution taken out from the mixture was quenched by 1 M DTT at 0, 1, 2, 3, 4, 5, 24 hours. The time-dependent crosslinking reaction samples were analyzed by SDS-PAGE. The gel was stained by heme staining, photographed, and analyzed by an image processing and analysis tool, ImageJ (NIH) (see appendix II).
2.11.2 Heterodimer of K40C and S107C cytochrome c₃

The cytochrome c₃ modules were reacted at a molar ratio of 1:1. Frozen stocks of K40C and S107C cytochromes c₃ were defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface cysteines. Then, the protein stock of S107C was passed through a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. The eluted S107C was diluted to approximately 10 µM. 1 ml of the diluted S107C solution was slowly added to 1 ml of BM(PEO)₂ solution (0.1 mM in XL Buffer, 1 mM in DMSO stock) with mixing. The reaction mixture was incubated for 1 hour and loaded onto a syringe-driven SP FF column (1 ml SP Sepharose column, Amersham) previously equilibrated with XL Buffer to remove excess BM(PEO)₂ by washing the column with 10 ml of XL Buffer. All the red proteins were eluted out from the SP FF column with 0.5 M NaCl in XL Buffer. The protein stock of K40C was passed though a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. The eluted K40C was diluted to approximate 10 µM. 1 ml of K40C solution was mixed with the SP FF column's red elution and the mixture was concentrated by centrifugation to ~ 200 µl and incubated at room temperature overnight. The crosslinking reaction was quenched with 1M DTT. The samples taken from every steps were analyzed by SDS-PAGE/heme staining.

2.11.3 Polymers of K40C:S107C cytochrome c₃

2.11.3.1 Polymers formed by disulfide bonding

The frozen stock of K40C:S107C cytochrome c₃ was defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface cysteines. Then, the protein
stock was passed though a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. CuSO4 (1 mM) was added to the K40C:S107C cytochrome c3 solution (~ 20 μM) to a final concentration of 20 μM and the reaction mixture was incubated at room temperature overnight. EDTA (stock concentration of 10 mM and working concentration of 1 mM) was used to quench the metal-induced disulfide bond crosslinking. The reaction mixture was analyzed by SDS-PAGE/heme staining and FPLC (see 2.12).

2.11.3.2 Polymers formed by bismaleimide crosslinking

The frozen stock of K40C:S107C cytochrome c3 was defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface cysteines. Then, the protein stock was passed though a syringe-driven SP FF column (1 ml SP Sepharose column, Amersham) previously equilibrated with XL Buffer to remove excess DTT. K40C:S107C cytochrome c3 was absorbed on the top of the mini SP Sepharose column and the column was washed with 10 ml of XL Buffer to remove excess DTT. The column was further washed 50 ml of BM(PEO)2 (0.1 mM in XL Buffer made from 1 mM DMSO stock solution). Then, all the red proteins were eluted out from the SP FF column with 1 M NaCl in XL Buffer. The elution was incubated at room temperature overnight and the crosslinking reaction was quenched with 1M DTT. The reaction mixture was analyzed by SDS-PAGE/heme staining and FPLC.

2.11.4 Heterotrimer of cytochrome c3 modules

All three cytochrome c3 modules were reacted at a molar ratio of 1:1:1.  
(1) Preparation of K40C-BM(PEO)2 or S107C-BM(PEO)2  
Frozen stocks of K40C or S107C cytochromes c3 were defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface
Cysteines. Then, the protein stock was passed through a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. 100 μl of BM(PEO)$_2$ solution (10 mM in DMSO) was added to the eluted cytochrome $c_3$ solution (~2 ml) with gentle stirring. The reaction mixture was incubated for 0.5 hour and loaded onto a syringe-driven SP FF column (1 ml SP Sepharose column, Amersham) previously equilibrated with XL Buffer to remove excess BM(PEO)$_2$ by washing the column with 10 ml of XL Buffer. All the red protein fractions containing K40C-BM(PEO)$_2$ or S107C-BM(PEO)$_2$, were eluted out from the SP FF column with 0.5 M NaCl in XL Buffer.

(2) Production of S107C-BM(PEO)$_2$-nstK40C

Frozen stocks of K40C cytochromes $c_3$ with a New Strep Tag (nstK40C) was defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to reduce the thiols of the surface cysteines. Then, nstK40C was loaded onto a gravity Strep-Tactin column (1 ml, IBA) previously equilibrated with DTT-free Buffer W (Section 2.6.3). nstK40C was bound to the strep column and the column was washed with 10 ml of DTT-free Buffer W. The column was drained out and S107C-BM(PEO)$_2$ was added to the nstK40C bound resin. The mixture was kept shaking for 0.5 hour and the unreacted S107C-BM(PEO)$_2$ was drained out. The strep column was washed with 5 ml of DTT-free Buffer W and the red protein fractions were eluted out with DTT-free Buffer E (Section 2.6.3).

(3) Production of S107C-BM(PEO)$_2$-nstK40C-BM(PEO)$_2$-K40C

The strep column elution was added to K40C-BM(PEO)$_2$. The mixture was concentrated to ~500 μl and incubated at room temperature overnight. The crosslinking reaction was quenched with 1M DTT. The samples taken from every step were analyzed by SDS-PAGE/heme staining.
2.11.5 Heterodimer of cytochrome c₃ and the heme domain of P450 BM3

The proteins were reacted at a molar ratio of 1:1. The frozen stock of K40C cytochrome c₃ with Strep Tag II (stK40C) and C62S:C156S:Q387C P450 BM3 heme domain (C387P450) were defrosted and incubated with DTT (stock concentration of 1 M and working concentration of 10 mM) for 30 minutes to free the thiols of the surface cysteines. Then, the protein stock of C387P450 was passed though a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. 100 μl of BM(PEO)₂ solution (10 mM in DMSO) was added to the eluted C387P450 solution (~ 2 ml) with gentle stirring. The reaction mixture was incubated for 0.5 hour and loaded onto a syringe-driven Q FF column (1 ml Q Sepharose column, strong anion exchange column, Amersham) previously equilibrated with XL Buffer to remove excess BM(PEO)₂ by washing the column with 10 ml of XL Buffer. All the red protein, containing C387P450-BM(PEO)₂, were eluted out from the Q FF column with 0.5 M NaCl in XL Buffer. The protein stock of nstK40C was passed though a gravity G-25 column (1.6 cm × 5 cm) previously equilibrated with XL Buffer to remove excess DTT. The eluted nstK40C protein was mixed with the Q FF red elutent and the mixture was concentrated by centrifugation to ~ 200 μl and incubated at room temperature overnight. The crosslinking reaction was quenched with 1M DTT. The samples taken from every step were analyzed by SDS-PAGE/heme staining.

2.12 Analysis of crosslinking products by Fast Protein Liquid Chromatography (FPLC)

Fast Protein Liquid Chromatography (FPLC) on a ÄKTApurifier (GE healthcare) was utilized to analyse the crosslinking mixture. A size exclusion column, Superdex S200 10/30 (Amersham), was attached to the
ÄKTApurifier and the column was equilibrated with Buffer C1 (Section 2.6.1) running at a flow rate of 0.5 ml/min. 500μl of protein was loaded onto the column and the size distribution in the crosslinking products was then determined by the elution profile.

2.13 OTTLE Potentiometric Titrations

OTTLE (Optically Transparent Thin Layer Electrode) potentiometric titration is a powerful electrochemical technique to determine the mid-point potentials of redox proteins.
Generally, the working system (Fig. 2.5) of the technique contains three components: OTTLE, mediators (small electro-active molecules), and the redox protein. At a certain potential, the electrode interacts rapidly with the mediators and then these activated small molecules exchange electrons with the redox protein. After a period of equilibration, the portion of redox state change can be measured by UV-Vis spectrophotometry. According to the
Nernst equation (Eq. 2.1), the mid-point potential of the redox protein can be determined.

\[ E = E_m - \frac{RT}{nF} \ln \left( \frac{[\text{red}]}{[\text{ox}]} \right) \]  

(Equation 2.1)

where \( E \) is the half-cell reduction potential, \( E_m \) is the standard half-cell reduction potential (mid-point potential), \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( n \) is the number of electrons transferred in the half-cell reaction, and \( F \) is the Faraday constant.

OTTLE potentiometric titrations were carried out at 25 °C using a Cary UV-Vis spectrophotometer and Autolab PGSTAT10 potentiostat. All samples for OTTLE titration were prepared in a Belle Technology glove box in a nitrogen atmosphere, with \( \text{O}_2 \) levels maintained at < 5 ppm.

<table>
<thead>
<tr>
<th>Name of solution</th>
<th>Reagents and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer PT1</td>
<td>MOPS 50 mM, pH 7.0</td>
</tr>
<tr>
<td></td>
<td>KCl 500 mM</td>
</tr>
<tr>
<td></td>
<td>glycerol 10 %</td>
</tr>
<tr>
<td>Buffer PT2</td>
<td>MOPS 50 mM, pH 7.0</td>
</tr>
<tr>
<td></td>
<td>KCl 500 mM</td>
</tr>
</tbody>
</table>

All buffers were degassed prior to use by bubbling with nitrogen gas for at least 1 hour and left in the glove box overnight.
Table 2.47: mediators for cytochrome c3 in OTTLE

<table>
<thead>
<tr>
<th>Name of mediator</th>
<th>( E_m (\text{mV}) ) vs. SHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavin Mononucleotide (FMN)</td>
<td>-200</td>
</tr>
<tr>
<td>Benzyl Viologen (BV)</td>
<td>-310</td>
</tr>
<tr>
<td>Methyl Viologen (MV)</td>
<td>-430</td>
</tr>
</tbody>
</table>

Mediators were introduced into the glove box as powdered samples and dissolved in degassed buffer PT1 to a concentration of approximate 2 mM and added to the protein sample at a concentration of around 2 µM.

A protein sample was concentrated to around 50 µl with a concentration of at least 300 µM and introduced into the glove box upon degassing under the nitrogen atmosphere for at least 1 hour. This sample was added to 250 µl of Buffer PT1 with mediators and mixed thoroughly. Then, the mixture was introduced into the thin part of the OTTLE cell (0.3 mm) by a micro-syringe, carefully avoiding bubble production. The remainder of the cell was filled with Buffer PT2 with care, keeping clear layers of Buffer PT1 and Buffer PT2. A three-electrode system, Pt/Rh (95/5) gauze working electrode (wire diameter 0.06 mm, mesh size 1024 cm\(^{-1}\), Engelhardt, UK), platinum wire counter electrode and a Ag/AgCl reference electrode (model MF2052, Bioanalytical Systems, IN 47906, USA) was used. Upon setup of electrodes, the OTTLE cell was taken out of the glove box and located in the Cary UV-Vis spectrophotometer so that changes in the spectrum could be observed. Then, the potential was applied using an Autolab PGSTAT potentiostat via the three-electrode system (Fig. 2.5). An initial potential more positive than the approximate reduction potential was applied and lowered in 50 mV steps until reduction was achieved. The enzyme samples were allowed to equilibrate before the potential was moved on and spectra were recorded at each applied potential. Difference spectra were calculated and the maximum absorbance changes associated with a change in the redox state were plotted against applied potential (vs. SHE). \( E_m \) values were determined by fitting the data to the Nernst equation (Microcal Origin 7.0).
2.14 OTTLE-driven electron transfer

OTTLE was also used to investigate the inter-molecular electron transfer between cytochrome c3 and P450 BM3 heme domain, and intra-molecule electron transfer within the conjugate of cytochrome c3 and P450 BM3 heme domain. At a low applied potential supplied by a potentiostat, the cytochrome c3 module was reduced by the electrode due to its direct electrochemical interaction with electrodes while P450 BM3 heme domain can not interact with the electrode due to its buried active site. The reduced cytochrome c3 module can then transfer electrons to the redox center of P450 BM3 heme domain on docking with the concave surface near the active site of P450 BM3 heme domain. In the presence of CO, the reduction of P450 BM3 heme domain can be monitored by the UV-Vis spectrophotometer with the increases in absorbance at 450 nm. Here, cytochrome c3 plays a role as an electron transfer mediator. And palmitate was also added to favour reduction of P450 BM3.

A saturated solution of palmitate was prepared in Buffer PT1 (Section 2.13) with the previous protocol (Section 2.10), degassed, and transferred into the glove box. And a protein sample (1:1 mixture of cyt c3 and P450 BM3 heme domain, or their conjugate) was concentrated to around 50 μl with a concentration of at least 300 μM and introduced into the glove box upon degassing under a nitrogen atmosphere for at least 1 hour. This sample was added to 200 μl of palmitate-saturated Buffer PT1 without any mediators and mixed thoroughly. Finally, approximate 50 μl of CO saturated Buffer PT1 (200 μM), which was generated by bubbling a sealed Sterilin tube with CO (Sigma) for ~5 minutes, was added to the reaction mixture. Then, the mixture was introduced to the thin part of the OTTLE cell (0.3 mm) by a micro-syringe, carefully avoiding bubble production. The remainder of the cell was filled with Buffer PT2 (Section 2.13) with care, keeping clear layers of Buffer PT1 and Buffer PT2. A three-electrode system (Section 2.13) was fitted to the OTTLE cell and the cell was taken out of the glove box and located in the Cary UV-Vis. Then, the potential was applied using an Autolab PGSTAT...
potentiostat via the three-electrode system (Fig. 2.5). An initial potential was set to -200 mV for 1 hour to get rid of traces of molecular oxygen in the sample. Then, the potential was set to -300 mV and absorption spectrum was recorded every 5 minutes over a long period of 24 hour.
Chapter 3

Untagged cytochrome $c_3$
cysteine mutants
3.1 Introduction
The tetraheme cytochrome $c_3$ from the bacterium *Desulfovibrio vulgaris* Miyazaki F is a small, globular, highly soluble $c$-type cytochrome. The four hemes have extremely low reduction potentials, with half exposure to the solvent and a cyclic arrangement, which makes them able to transfer electrons in every direction. The highly positively charged surface also facilitates the interaction with negatively charged redox partners. In addition, this cytochrome has great stability to protease digestion and high temperature. Due to its physical, chemical, and electronic properties, this tetraheme cytochrome $c_3$ was selected to be a fundamental module in the protein conjugation part of this project.

It was found that the area on the surface near lysine 40 is negatively charged and serine 107 (C terminus), which is opposite to lysine 40, is surrounded by positive charge (Fig. 3.1).
Figure 3.1: the electrostatic surface of cytochrome $c_3$. Panel A: the electrostatic surface of the K40C mutant; panel B: the electrostatic surface of the S107C mutant; (the cysteines are highlighted in yellow); panel C: the spatial positions of residues 40 and 107 (the cysteine is in yellow). The electrostatic surfaces were generated using Pymol [147-149].

These two amino acids were chosen as the ligation sites and mutated to cysteines. To some extent, the natural dipole may facilitate controllable ligation. The aims in this chapter are to construct three cysteine mutants, K40C, S107C, and K40C:S107C, express them in *Shewanella oneidensis*, purify and characterise them by thiol counting and thiol labelling, produce homodimers and a heterodimer, with a bismaleimide crosslinker, 1,8-bis-Maleimidodiethyleneglycol (BM(PEO)$_2$, Pierce), and generate high molecular weight polymers by disulfide and bismaleimide crosslinking of K40C:S107C.
3.2 Basic characterization of untagged cytochrome c₃ cysteine mutants

3.2.1 Protein purities and UV-Vis absorption spectra

Wild-type cytochrome c₃ and the mutants K40C, S107C and K40C:S107C were successfully over-expressed in *Shewanella oneidensis* MR-1 with yields of 1 ~ 2 mg per litre culture and purified with ion exchange and gel filtration procedures. The presence of a single band in SDS-PAGE analysis (Figure 3.2B) indicated that the purity was higher than 90%.

![Figure 3.2: SDS-PAGE/Coomassie Brilliant Blue staining analysis of wild type cytochrome c₃ during purification. Panel A: SDS-PAGE analysis of SP Sepharose eluted fractions. Lane 1, molecular weight marker; lane 2, soluble cells' extract; lane 3, column flow through; lanes 4 ~ 5, low salt elution; lane 6, cytochrome c₃ fraction. Panel B: SDS-PAGE analysis of gel filtration eluted fractions. Lane 1, molecular weight marker; lane 2 ~ 12, eluted red fractions; fractions of lanes 9 and 10 were collected. The pattern of SDS-PAGE gels of untagged mutants was similar to that of wild type.](Image)
Figure 3.3: UV-Vis spectra of wild type cytochrome c\textsubscript{3} from Desulfovibrio vulgaris Miyazaki F. The red curve represented the oxidised form of cytochrome c\textsubscript{3} while the reduced form is in black. All cytochrome c\textsubscript{3} mutants, including tagged mutants in later chapters, had the similar UV-Vis spectra.

The UV-Vis spectra of cytochrome c\textsubscript{3} of Desulfovibrio vulgaris Miyazaki F and all its mutants had two peaks (at 410 nm, 530 nm) in the ferri-form (oxidised) and three peaks (at 419 nm, 523 nm, and 552 nm) in the ferro-form (reduced). The ratio of Abs\textsubscript{552} (ferro-form) and Abs\textsubscript{520} (ferri-form), of all the cytochromes c\textsubscript{3} were about 3 (the highest ratio for this cytochrome c\textsubscript{3}), which meant the proteins were highly purified and had high heme content [16].

3.2.2 Fluorescent labelling of the cysteine thiol

Fluorescein-5-maleimide (F5M) was used to determine the accessibility of free thiols in the cysteine mutants of Desulfovibrio vulgaris Miyazaki F cytochrome c\textsubscript{3} (K40C, S107C and K40C:S107C) to the maleimide group. F5M has a maleimide group that is connected to a rhodamine-like fluorescent group (Fig. 2.4, 2.9).
The thiols of all three mutants: K40C, S107C, and K40C:S107C, were shown to be accessible to maleimide-type reagents since they reacted with fluorescein-5-maleimide forming strong fluorescent bands on a gel (Fig. 3.4). However, there were small bands shown below the cytochrome c\textsubscript{3} bands (Fig. 3.4B). It was likely to be a small free-cysteine-containing protein co-purified with the cytochrome c\textsubscript{3} mutant. It could be seen on the SDS-PAGE/CBB stained gel (Fig. 3.2B, ~ 6 kDa) and chromatography analysis (Fig. 3.12, ~ 20 mL).

![Figure 3.4](image)

**Figure 3.4**: fluorescent labelling of cysteine mutants of Desulfovibrio vulgaris Miyazaki F cytochrome c\textsubscript{3}. Coomassie Brilliant Blue Stained analysis of the SDS-PAGE gel (panel A): lane 1, molecular mass marker; lane 2, 3, K40C without / with F5M; lane 4, 5, S107C without / with F5M; lane 6, 7, K40C:S107C without / with F5M. Fluorescent lights on the same SDS-PAGE gel under UV light (panel B): 17 kDa Myoglobin is the marker (lane 1); big cytochrome c\textsubscript{3} bands and small unknown protein bands appear (lane 3, 5, 7)

### 3.2.3 Thiol counting

The number of free cysteine residues within the cytochrome c\textsubscript{3} was determined using the reagent of 2,2'-pyridyl disulfide (2-PDS). 2-PDS reacts specifically with free thiols yielding a chromophore, 2-pyridinethione (Fig. 2.3, 2.8). The accumulation of 2-pyridinethione is usually monitored spectrophotometrically at 343 nm (ε\textsubscript{343} = 8.08
mM$^{-1}$ cm$^{-1}$) to give quantification of the number of free cysteine groups within the protein.

The thiol counting was carried out on the three cysteine mutants (Fig. 3.5). The absorbance change at 343 nm was monitored over 30 minutes. There were fast initial phases followed by slow phases in all three cases. The initial mixing phase could not be recorded due to the rapid reaction and it could be corrected by the spectrum of protein before adding 2-PDS. The absorbance change was recalculated to show thiol equivalents. The mean of thiol numbers obtained from 16 ~ 30 minutes (where the reaction was almost finished) was used to represent the overall thiol number of each mutant and the standard deviation of them represented the errors.

![Thiol counting for cysteine mutants](image)

**Figure 3.5:** time-dependent traces of the absorbance change at 343 nm during the counting analysis of three cysteine mutants of Desulfovibrio vulgaris Miyazaki F cytochrome c$_3$
Experimentally, for two single cysteine mutants, K40C and S107C, the number of free cysteines was 0.80 ± 0.06 and 1.00 ± 0.02, respectively. And the double mutant, K40C:S107C had 2.04 ± 0.04 equivalent free cysteines. Generally, the results of fluorescent labelling in the qualitative view and 2-PDS counting in the quantitative view indicated that the cysteines at positions 40 and 107 of cytochrome \( c_3 \) were non-coordinated and could be accessed by small molecules.

3.3 Crosslinking of untagged cytochrome \( c_3 \) cysteine mutants

In order to determine whether cysteines 40 and 107 were exposed enough for protein conjugation, homodimers of K40C and S107C cytochrome \( c_3 \) were produced and dimer formation was analyzed with time dependence. A heterodimer of K40C and S107C cytochrome \( c_3 \) was also made in a two-step crosslinking reaction, which laid the basis for developing the multi-step crosslinking (Section 4.4). Furthermore, high molecular weight polymers were also produced by disulfide crosslinking and by bismaleimide crosslinking of K40C:S107C cytochrome \( c_3 \). This demonstrated the possibility of the formation of cytochrome \( c_3 \) "nanowires".

3.3.1 Homodimers of cytochrome \( c_3 \) modules: K40C-K40C and S107C-S107C

The production of homodimers was achieved by simply mixing the single cysteine mutant with a half-equivalent of bismaleimide (BM(PEO)$_2$, Section 2.11.1). There was no colour change in the reaction mixture and the solution retained clear during the crosslinking process. The reaction was quenched by concentrated DTT at different times allowing the time dependence of homodimer formation to be studied by SDS-PAGE (Fig. 3.6). The gels were analysed by ImageJ (see appendix II), which is a free image processing and analysis tool from NIH [150].
The analysis of crosslinking results showed that the crosslinking reaction was almost finished in 2 hours and the yields were $\sim 60\%$ for the S107C dimer and $\sim 30\%$ for the K40C dimer respectively. S107C was more efficient in forming homodimers than K40C. The C terminus of protein (S107C) was much more flexible and exposed to the solvent than K40C, which is located in the middle of the polypeptide sequence. There was still a little monomeric mutant left uncrosslinked in the reaction mixture, even after 24 hours. It might be due to hydrolysis of the maleimide group (Fig. 3.7), which makes unreactive towards cysteine thiol, and the two-step crosslinking reaction mechanism.
Figure 3.7: hydrolysis of the maleimide group. Half life is approximately 24 hours at pH 7.0 [48].

There were small bands between monomer and dimer shown on the gel of S107C homodimer formation (Fig. 3.6). They were considered as the conjugates of small free-cysteine-containing protein (Section 3.2.2) and S107C.

3.3.2 Heterodimer formation

For the production of the heterodimer of K40C-S107C cytochrome c₃, S107C was treated with 10-fold excess bismaleimide crosslinker and the maleimide-modified S107C (S107-BM(PEO)₂) was purified using a small SP Sepharose column. Then, S107-BM(PEO)₂ was mixed with the equivalent K40C (Fig. 3.8, Section 2.11.2). There was no colour change in the reaction mixture and the solution retained clear in each step.

Figure 3.8: reaction scheme for the production of the K40C-S107C heterodimer

Small amounts of solution were taken from each step of the crosslinking reaction and were prepared as gel samples to be analysed by SDS-PAGE/heme staining (Fig. 3.9).
According to the SDS-PAGE result, most S107C was converted into S107C-BM(PEO)$_2$ with a little S107C homodimer side-product (lane 2, Fig. 3.9). Upon the addition of K40C, the amount of dimer sharply increased (lane 4, Fig. 3.9), which meant that the heterodimer of K40C and S107C was successfully synthesized in this two-step crosslinking reaction with a yield of ~25%. The middle bands between monomer and dimer (lane 2, 4, Fig. 3.9) were considered as the conjugates of small free-cysteine-containing protein and S107C (Section 3.3.1). Although this heterodimer was not separated from the reaction mixture for further analysis, the two-step reaction indicated that the crucial intermediate product, the bismaleimide-modified cytochrome $c_3$ module, could be synthesized, purified, and reacted with the other reactant. In addition, the synthesis of K40C-S107C laid the basis for developing a controllable multi-step crosslinking method (4.4).
3.3.3 Polymerising cytochrome c₃ modules

The polymerization of cytochrome c₃ modules was achieved by crosslinking the double cysteine mutant of K40C:S107C, through disulfide bonding (cleavable) or bismaleimide linking (non-cleavable). Since the ligation sites were opposite to each other on the surface of cytochrome c₃, the created protein polymers could be one-dimensional long chains. The chains should have properties as bioelectronic "nanowires".

When two cysteine thiol groups are close enough to each other, a disulfide bond is formed spontaneously in an oxidising environment. That’s the reason DTT was used in the purification of cytochrome c₃ cysteines mutants to prevent protein aggregate formation. Transition metal ions, like Cu²⁺ and Ni²⁺, can catalyze disulfide bond formation, even in trace amounts. Here, disulfide-linked polymers were produced in the presence of small amount of CuSO₄. The polymerization process was also initiated by adding the bismaleimide crosslinker (BM(PEO)₂) to an equimolar amount of K40C:S107C. There was no colour change in the reaction mixture and the solution remained clear during the crosslinking process. Both kinds of cytochrome c₃ polymer were investigated by UV-Vis spectrometry, SDS-PAGE, size exclusion chromatography and OTTLE potentiometric titration.

(1) UV-Vis spectra of cytochrome c₃ polymers
The UV-Vis spectra of cytochrome c₂₃ polymers were similar to those of wild type cytochrome c₂₃, with two peaks at 410 nm, 530 nm in the oxidised form and three peaks at 419 nm, 523 nm, and 552 nm in the reduced form (Fig. 3.10). It showed that the conjugation of cytochrome c₂₃ modules, through either the short linkage of disulfide bond or long soft polyethylene glycol group, did not affect the chemical nature of the heme groups. It implied that the components of the protein conjugates retained their properties after crosslinking.

(2) SDS-PAGE/heme staining analysis of cytochrome c₂₃ polymers
Figure 3.11: SDS-PAGE/heme stained gels of cytochrome c₃ polymers.
Panel A: lane M, molecular weight marker; lane 1, disulfide-linked cytochrome c₃ polymers; lane 2, cleavage of disulfide-linked cytochrome c₃ polymers by DTT. Panel B: lane 3, unreacted K40C:S107C cytochrome c₃; lane 4, bismaleimide-linked cytochrome c₃ polymers

In the disulfide-crosslinking reaction mixture (lane 1, Fig. 3.11), most of the K40C:S107C cytochrome c₃ modules formed a large portion of high molecular weight polymers (at least 12 mer), although a little monomer was left unreacted in the mixture. The disulfide driven crosslinking reaction was reversible and the polymers could be cleaved by concentrated DTT, which was added into the gel sample to 1 M before boiling. The cleavage product was mainly K40C:S107C cytochrome c₃ monomer (lane 2, Fig. 3.11). The bismaleimide-crosslinking reaction also showed high degree polymerization (at least 8 mer) but was not as efficient as disulfide-crosslinking reaction. The portion of bismaleimide-linked high molecular weight polymers in the reaction mixture was not as much as that of disulfide-linked polymers (lane 4, Fig. 3.11). And the crosslinking progress was not reversible because there was already concentrated DTT (1 M) in the gel sample. Possible reasons for lower crosslinking efficiency include the hydrolysis of bismaleimide...
crosslinker and the two-step reaction mechanism being more complex than the one-step reaction progress of disulfide crosslinking. Although disulfide crosslinking is efficient, the fact that they are cleaved in a reducing environment limits their application.

(3) Size exclusion chromatography analysis of the cytochrome \( c_3 \) polymers

Size exclusion chromatography (gel filtration chromatography) separates proteins based on their size. Usually proteins with large size will go through the gel filtration column faster than proteins with small size. Besides being a common tool for protein purification, size exclusion chromatography was applied here to analyze the crosslinking reaction mixture.

![The elution profile of cytochrome \( c_3 \) polymers on Superdex S200](image)

**Figure 3.12: Elution profile of cytochrome \( c_3 \) polymers on the size exclusion column, Superdex S200, flow rate 0.5 ml/min. The blue curve represents the elution of bismaleimide-linked polymers; the black curve represents the elution of disulfide-linked polymers; the red curve represents the elution of DTT cleaved disulfide-linked polymers (mainly K40C:S107C); the green vertical line highlights the position of the monomeric cytochrome \( c_3 \) module (16.4 ml).**
There was a large portion of high molecular weight polymers in the disulfide-crosslinking reaction (Fig. 3.12, black curve). The bismaleimide driven polymerization was not so efficient and had less high molecular weight polymers (Fig. 3.12, blue curve). The results of size exclusion chromatography analysis were consistent with that of SDS-PAGE/heme staining analysis.

(4) OTTLE potentiometric titration analysis of cytochrome c₃ polymers

The results of UV-Vis spectroscopy showed that heme groups in the cytochrome c₃ modules retained their chemical properties before and after polymerization. However, the conjugation of cytochrome c₃ modules may modify the microscopic electronic environments of the hemes. OTTLE potentiometric titration analysis was used to study the change in the reduction potentials of the hemes before and after polymerization. And the protein samples remained soluble during the potentiometric titration. Difference spectra were calculated and the maximum absorbance (410 nm) changes associated with a change in the redox state were plotted against applied potential (vs. SHE). One-electron form Nernst equation (Eq. 2.1) was transformed to equation 3.1 (note that [Ox]+[Red] = 1) and equation 3.1 was extended to four-electron form (Eq. 3.2). Eₘ values of four hemes were determined by fitting the data to the equation 3.2 using Microcal Origin 7.5.

\[
[\text{Red}] = A \times \frac{10^{\frac{E_x - E}{59}}}{1 + 10^{\frac{E_x - E}{59}}}
\]

(Eq. 3.1)

\[
[\text{Red}] = A \times \frac{10^{\frac{E_1 - E}{59}}}{1 + 10^{\frac{E_1 - E}{59}}} + A \times \frac{10^{\frac{E_2 - E}{59}}}{1 + 10^{\frac{E_2 - E}{59}}} + A \times \frac{10^{\frac{E_3 - E}{59}}}{1 + 10^{\frac{E_3 - E}{59}}} + A \times \frac{10^{\frac{E_4 - E}{59}}}{1 + 10^{\frac{E_4 - E}{59}}}
\]

(Eq. 3.2)
Figure 3.13: potentiometric titration data and 4-electron Nernst fits for wild type cytochrome c₃ (black solid square), disulfide-linked polymers (red solid circle), and bismaleimide-linked polymers (blue solid triangle)
Table 3.1: macroscopic reduction potentials of wild type cytochrome c<sub>3</sub> and two crosslinked polymers

<table>
<thead>
<tr>
<th></th>
<th>E&lt;sub&gt;1&lt;/sub&gt; / mV</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; / mV</th>
<th>E&lt;sub&gt;3&lt;/sub&gt; / mV</th>
<th>E&lt;sub&gt;4&lt;/sub&gt; / mV</th>
<th>$\overline{E}$* / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type cytochrome c&lt;sub&gt;3&lt;/sub&gt; (literature value [151])</td>
<td>-242</td>
<td>-296</td>
<td>-313</td>
<td>-358</td>
<td>-302</td>
</tr>
<tr>
<td>wild type cytochrome c&lt;sub&gt;3&lt;/sub&gt; (this work)</td>
<td>-261&lt;sup&gt;§&lt;/sup&gt;</td>
<td>-261&lt;sup&gt;§&lt;/sup&gt;</td>
<td>-261&lt;sup&gt;§&lt;/sup&gt;</td>
<td>-433</td>
<td>-304±11</td>
</tr>
<tr>
<td>disulfide-linked polymers</td>
<td>-247±10</td>
<td>-321&lt;sup&gt;§&lt;/sup&gt;</td>
<td>-321&lt;sup&gt;§&lt;/sup&gt;</td>
<td>-456±9</td>
<td>-336±11</td>
</tr>
<tr>
<td>bismaleimide-linked polymers</td>
<td>-261±8</td>
<td>-355±9</td>
<td>-428±10</td>
<td>-507±11</td>
<td>-388±11</td>
</tr>
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</table>

* $\overline{E} = \frac{E_1 + E_2 + E_3 + E_4}{4}$

§ Errors could not be determined due to interdependency.

Although the individual macroscopic reduction potentials were different from the literature values (Table 3.1), the overall mid-point reduction potential of wild type cytochrome c<sub>3</sub> ($\overline{E} = -304$ mV) was very close to the literature value ($\overline{E} = -302$ mV).

Generally, the reduction of the three species occurred over a range of -100 mV to -600 mV (Fig. 3.13). However, the electrochemical behaviours of the three species were a little different. Wild type cytochrome c<sub>3</sub> had the least negative mid-point reduction potential ($\overline{E}$) whilst those of the two polymers were shifted towards the negative. Actually, disulfide bonds are reduced at potentials < 300 mV and the real active species in the potential titration is likely to be the K40C:S107 cytochrome c<sub>3</sub> monomer. The bismaleimide-linked polymers had the lowest overall mid-point potential ($\overline{E} = -388$ mV). A possible reason is that the interaction among hemes in the cytochrome c<sub>3</sub> modules makes the polymer more difficult to reduce.
3.4 Conclusions

Three cysteine mutants of cytochrome $c_3$, K40C, S107C, and K40C:S107C, were genetically constructed, successfully expressed in *Shewanella oneidensis* MR-1, and purified. Fluorescent labelling showed that the thiols were accessible towards the maleimide group and the 2-PDS counting of cysteine thiol groups confirmed the number of free thiols in the mutants.

Protein crosslinking was performed with a bismaleimide crosslinker, 1,8-bis-Maleimidodiethyleneglycol (BM(PEO)$_2$) on the cysteine mutants and the heme environment remained unchanged during reaction. The following crosslinking products were successfully produced:

1. **K40C and S107C cytochrome $c_3$ homodimers**

   S107C was more efficient in forming homodimers than K40C probably because it is the flexible and exposed C terminus of the protein. The yields in two hours were ~60% for the S107C dimer and ~30% for the K40C dimer respectively. The incomplete dimerization might be due to the hydrolysis of the maleimide group and two-step crosslinking mechanism. The trace amount of thiol containing protein also affected the production of homodimers in conjugating with the monomeric cytochrome $c_3$ mutant.

2. **K40C and S107C cytochrome $c_3$ heterodimer**

   In order to make a heterodimer, a bismaleimide-modified S107C intermediate was prepared, purified, and reacted with K40C with a yield of ~25%. The two-step reaction shows that protein ligation can be performed in a controllable multi-step way. However, the maleimide group hydrolysis during reaction and the impurity in the protein sample were still the problems limiting the production of the heterodimer.

3. **Disulfide-linked and bismaleimide-linked cytochrome $c_3$ polymers**

   Cytochrome $c_3$ polymers are long chains of heme proteins. It is possible that electron transfer could occur along the length forming bioelectronic nanowires. Two methods were used for the polymer production: disulfide crosslinking and bismaleimide crosslinking. The polymers generated through disulfide crosslinking were cleavable whereas bismaleimide crosslinked polymers were stable in the reducing environment. Polymerization was conducted by adding Cu$^{2+}$/bismaleimide directly. Both gave high
degree polymerization yielded up to 12mer at least for disulfide crosslinking and up to 8mer at least for bismaleimide crosslinking. The UV-Vis spectroscopy of both polymers showed that the chemical nature of heme groups in cytochrome c₃ remained unchanged before and after polymerization. OTTLE potential titration shows that the polymerisation of the cytochrome c₃ modules leads reduction potentials shift towards the negative from –304 mV (wild type) to -336 mV (disulfide-linked cytochrome c₃ polymers) and -388 mV (bismaleimide-linked cytochrome c₃ polymers). A possible reason is that the interaction among hemes in the cytochrome c₃ modules makes the polymer more difficult to reduce.
Chapter 4

Using affinity tags
to control protein conjugation
4.1 Introduction

Many different peptides, domains and proteins, have been developed as affinity tags for one-step purification of recombinant proteins. Generally, the tags should have only a minimal effect on the target proteins’ tertiary structure and biological activity. In some applications, it is required to specifically remove the tag from the recombinant protein. There is not a single tag suitable for all proteins and it is difficult to decide on the best affinity tag for a specific protein of interest.

In this project, the affinity tag not only aided the purification of recombinant proteins but was also used as “protective groups” in the controllable crosslinking strategy (Figs. 1.20 ~ 1.22). Two common commercially available affinity tag systems were applied: Calmodulin binding peptide (CBP) / Calmodulin (1.9.1) and Strep Tag II / Streptavidin (1.9.2).

The initial aims in this chapter were to construct a series of CBP tagged K40C:S107C cytochromes $c_3$, express them in Shewanella oneidensis, purify them with the Calmodulin affinity column, and characterize them by thiol counting, and produce a cytochrome $c_3$ heterotrimer by controlled crosslinking. Upon failure of the CBP tagged cytochromes $c_3$ to bind to the Calmodulin affinity resin, the Strep Tag II / Streptavidin derivative tag system was exploited. Furthermore, a new strep tag based on Strep Tag II was developed specifically for protein crosslinking.

4.2 Calmodulin binding peptide (CBP) tagged cytochrome $c_3$

Calmodulin (CaM) is a small, Calcium-binding protein constituting of 148 amino acids. The apo-Calmodulin is dumb-bell-shaped. In the presence of Ca$^{2+}$, Calmodulin undergoes a major conformational change and wraps around its target peptide or protein (Fig. 1.13). The commonly used CaM binding peptide (CBP) affinity tag is a 28 amino acid long sequence with a size of about 3 kDa (Table 1.1).

In order to achieve maximum blocking effect on the cysteine 107, the length of the linker between protein and CBP should be as short as possible. Therefore, three CBP-tagged cytochromes $c_3$ with different linkers were constructed mainly by overlap PCR (2.3.3). They were K40C:S107C-Gly-Thr-CBP, K40C:S107C-Thr-CBP, and K40C:S107C-thrombin-CBP ($thrombin$ = Met-Tyr-Pro-Arg*-Gly-Asn, a thrombin-cleavable linker). They were all expressed in Shewanella oneidensis for
purification on CaM affinity column. Unfortunately, no red proteins bound the column. Two CaM affinity columns from different companies, Stratagene and Sigma, were tested using three CBP tagged cytochromes $c_3$ and the results were negative.

Figure 4.1: SDS-PAGE/heme staining analysis of the eluted fractions of the SP Sepharose column for purification of CBP tagged cytochromes $c_3$. All the three tagged cytochromes $c_3$ had similar patterns of SDS-PAGE/heme staining gels. Lane M: molecular weight marker; lane 1: 150 mM NaCl elution; lane 2: 400 mM NaCl elution.

The CBP tagged cytochromes $c_3$ were then purified by SP Sepharose column chromatography (cation exchange). They were eluted out from SP Sepharose column at about 400 mM NaCl concentration, which is higher than the elution conditions of untagged cytochromes $c_3$ (150 mM, Fig. 4.1). A possible reason is that the total charge of CBP is mainly positive (Arg + Lys = 8).

The UV-Vis spectra of the purified CBP-tagged cytochromes $c_3$ were identical to that of the wild type (Fig. 3.3). Therefore, the attachment of a short peptide to the C terminus did not affect the coordination environments of the heme groups.

Possible reasons for CBP-tagged cytochromes $c_3$ without CaM binding activity might be that the linker was not long enough or that the CBP was partly proteolysed during expression. The problems might be solved by genetically lengthening the linker or co-expressing CBP-tagged proteins with Calmodulin in *Shewanella oneidensis*. 

Chapter 4
However, the long linker might not block the cysteine 107 very well and the expression of Calmodulin in *Shewanella oneidensis* had not been tested yet. Hence, the work was moved onto the following affinity system.

### 4.3 Tagging cytochrome c₃ with Strep Tag II

After the failure of the CBP/CaM affinity system, another affinity tag, Strep Tag II, was utilized. Strep Tag II is a small peptide with only 8 amino acids and its associated protein is Strep-Tactin, a streptavidin derivative. Due to the size and neutral charge of Strep Tag II, it is less likely to affect the folding and biological activity of proteins. Streptavidin, a small tetrameric protein, has high physical and chemical stability and can survive various protease digestions. The special properties of Strep Tag II and Strep Tactin make them very popular in affinity purification.

The strep-tagged K40C:S107C cytochrome c₃ was constructed by overlap PCR (Section 2.3.4), expressed in *Shewanella oneidensis*, and purified by a Strep-Tactin Sepharose column chromatography (IBA). The strep-tagged K40C:S107C cytochrome c₃ was successfully bound to the Strep-Tactin Sepharose column and formed a red band on the top of the column. The red band was eluted by desthiobiotin buffer E (Section 2.6.3) and was analyzed by SDS-PAGE/heme staining (Fig. 4.2). Interestingly, the bands of strep tagged cytochrome c₃ on the SDS-PAGE gel could not be developed by Coomassie Brilliant Blue (CBB) staining.
Besides the monomeric strep tagged K40C:S107C cytochrome c$_3$ (stKS), there were oligomers of stKS formed. The oligomers were covalently linked since the gel sample was prepared in presence of 1 M DTT. The reason of the oligomer formation remains unclear. Ferguson and co-workers [152] showed that a cysteine, within or next to the c-type heme binding motif in the sequence of cytochromes c (CXXCH or CXXXXCH, where X is any amino acid), could be activated during the cytochrome c maturation progress. Cysteine 107 in the stKS was just next to the heme binding motif (Fig. 4.3)
Cys 107

Figure 4.3: protein sequence of stKS near the C-terminus. The cytochrome c₃ portion is highlighted in red; Strep Tag II is highlighted in yellow after the linker of serine and alanine. Cysteine 107 is in italic and -Cys-Lys-Gly-Ser-Lys-Cys-His is a typical cytochrome c binding motif (CXXXCH).

Therefore, cysteine 107 might be activated during expression and connected to another cytochrome c₃ module forming covalent linked oligomers. However, the untagged S107C mutant of cytochrome c₃ did not encounter this problem, although the carboxyl group of the C-terminal cysteine might affect the process.

The UV-Vis spectra of the stKS remained the same as those of wild type cytochrome c₃ (Fig. 3.3).

4.4 Tagging cytochrome c₃ with New Strep Tag

To counteract the self oligomerization of strep-tagged K40C:S107C cytochrome c₃, a mutation was introduced the Strep Tag II, where the second amino acid, serine, was changed to cysteine; and the cysteine 107 was changed back to serine (Fig. 4.4).

Figure 4.4: mutagenesis of K40C:S107C cytochrome c₃ with Strep Tag II (stKS) into K40C cytochrome c₃ with New Strep Tag (nstK40C). The cytochrome c₃ portion is highlighted in red; Strep Tag II and New Strep Tag are highlighted in yellow after the linker of serine and alanine. The mutation sites are in italic and underlined.
The mutated Strep Tag II is now named New Strep Tag. The physical similarity of serine and cysteine should minimise the disruption of the Strep-Tactin binding activity. The cysteine in the new strep tag was also more exposed than the cysteine at the C terminus of cytochrome \( c_3 \) module. Upon interaction with Strep-Tactin, the cysteine in the New Strep Tag would be inside the Strep-Tactin binding pocket and crosslinking reaction at this site should be fully blocked (Fig. 1.21).

(1) Purification of nstK40C cytochrome \( c_3 \)

The nstK40C cytochrome \( c_3 \) was constructed by point mutagenesis of the plasmid encoding stKS cytochrome \( c_3 \) (Section 2.3.5). It was expressed in \textit{Shewanella oneidensis}, and purified by Strep-Tactin Sepharose column chromatography (IBA). The nstK40C cytochrome \( c_3 \) was bound to the Strep-Tactin Sepharose column and formed a red band on the top of the column. The red band was eluted by the desthiobiotin buffer E (Section 2.6.3) and was analyzed by the SDS-PAGE/heme staining (Fig. 4.5). The bands of nstK40C cytochrome \( c_3 \) on the SDS-PAGE gel also could not be developed by Coomassie Brilliant Blue (CBB) staining.
Figure 4.5: SDS-PAGE/heme staining analysis of the fractions of the Strep-Tactin Sepharose column. Lane M: molecular weight marker; lane 1: column flow though; lane 2 ~ 7: eluted fractions of nstK40C cytochrome c₃

The gel analysis showed that there were no high molecular weight oligomers formed at this time. Most nstK40C was monomeric with minor dimer and trace trimer bands. It also supported the hypothesis that the cysteine at position 107 next to the heme binding motif might involved in stKS cytochrome c₃ oligomerization.
The nstK40C cytochrome \( c_3 \) was further purified by size exclusion chromatography to separate the monomeric protein. The elution from the Strep-Tactin column was concentrated to about 0.5 ml and loaded on to a Superdex S200 column at a flow rate of 0.5 ml/min on the ÄKTApurifier (Section 2.12). The biggest peak on the elution curve (Fig. 4.6) was nstK40C monomer at approximately 16.5 ml elution volume. The peaks of monomer and dimer overlapped together. The nstK40C monomer fractions were collected.

The UV-Vis spectra of nstK40C were the same as those of the wild type cytochrome \( c_3 \) (Fig. 3.3) and were used to determine the concentration.

2) Thiol counting of nstK40C cytochrome \( c_3 \)

The previous studies of thiol counting on the untagged cytochrome \( c_3 \) (Section 3.2.3) indicated that there were free cysteines at positions 40 and 107 on cytochrome \( c_3 \) mutants. The cysteine on the soft New Strep Tag might be expected to be more solvent exposed. The number of free thiols within nstK40C cytochrome \( c_3 \) was determined using the reagent of 2,2'-pyridyl disulfide (2-PDS) as described earlier. 2-PDS reacts specifically with free thiols to yield a chromophore, 2-pyridinethione (Fig. 2.3, 2.8). The accumulation of 2-pyridinethione is usually monitored spectrophotometrically at 343 nm \((\varepsilon_{343} = 8.08 \text{ mM}^{-1} \text{ cm}^{-1})\) to give quantification of the number of free cysteine groups within the protein. The data were processed using the previous method.
Figure 4.7: time-dependent traces of the absorbance change at 343 nm during the counting analysis of nstK40C cytochrome c₃

According to the results (Fig. 4.7), the number of free thiols was estimated to be 1.52 ± 0.06, but should be 2 theoretically. The error may be due to the existence of small amount of nstK40C dimer (also refer to lane 5 in Fig. 4.8). Size exclusion chromatography can not totally separate monomer and dimer because their peaks overlap with each other.

(3) Production of cytochrome c₃ heterotrimer (controlled protein crosslinking)
The production of homodimers of K40C and S107C cytochromes c₃ showed that the cysteines at position 40 and 107 were reactive in protein crosslinking, and the time-dependence studies of homodimer formation also showed that their rate of reaction is fast (Section 3.3.1). K40C-S107C heterodimer was generated by two-step crosslinking (Section 3.3.2). The reactive cytochrome c₃-bismaleimide (1:1) intermediate made during the reaction process implied a way of developing multi-step crosslinking. Furthermore, the high molecular weight soluble polymers linked by disulfide bonds or bismaleimides of K40C:S107C cytochrome c₃ indicated the
possibility of the formation of the long chain cytochrome \( c_3 \) “nanowires” without any precipitation (3.3.3).

On the basis of the previous work, a controllable protein crosslinking strategy was developed and production of a short well-defined wire of cytochrome \( c_3 \) modules was attempted (Fig. 1.22). All three cytochrome \( c_3 \) modules were reacted at a molar ratio of 1:1:1 (Section 2.11.4). Firstly, the K40C-bismaleimide and S107C-bismaleimide intermediates were produced by the reaction of the mutant cytochromes \( c_3 \) with 10 fold excess bismaleimide crosslinker, BM(PEO)\(_2\). Then, the excess BM(PEO)\(_2\) was removed by cation exchange column chromatography. Secondly, nstK40C cytochrome \( c_3 \) was bound to the Strep-Tactin column, where the cysteine in the New Strep Tag should be buried in the Strep-Tactin binding pocket and cysteine 40 exposed to the environment. S107C-BM(PEO)\(_2\) was loaded onto the Strep-Tactin column to produce S107C-BM(PEO)\(_2\)-nstK40C. After the unreacted S107C-BM(PEO)\(_2\) had been washed away and the S107C-BM(PEO)\(_2\)-nstK40C was eluted from the Strep-Tactin column. Finally, K40C-BM(PEO)\(_2\) was added to the Strep-Tactin elution to make S107C-BM(PEO)\(_2\)-nstK40C-BM(PEO)\(_2\)-K40C. There were no precipitates formed throughout the entire reaction progress. SDS-PAGE gel samples were taken from each reaction step and the gel was analyzed by heme staining analysis (Fig.4.8).
There was a little homodimer formed during the production of S107C-BM(PEO)$_2$ (lane 2, fig. 4.8) and a side product (middle band in lane 2), the molecular weight of which was between 14 kDa (monomer) and 28 kDa (dimer). It is probably the crosslinking product of a small (~6 kDa) non-heme protein with an exposed cysteine and S107C (Section 3.3.1). This side product only appeared when the sample of S107 was very concentrated, due to its trace existence in the purified S107C. Although nstK40C was purified by size exclusion chromatography, there was still certain amount of homodimer in the reaction sample of nstK40C (lane 5, Fig 4.8). Upon the addition of S107C-BM(PEO)$_2$ to nstK40C, the amount of dimer increased with a yield of ~20% (the dimer band was more intensive in lane 6, Fig 4.8). In the last step of the reaction, K40C- BM(PEO)$_2$ was added to the S107C-BM(PEO)$_2$-nstK40C and the heterotrimer of cytochrome $c_3$ was generated with a yield of ~12% (a new band appeared in lane 7, fig 4.8).
The creation of a heterotrimer of cytochrome $c_3$ validated the feasibility of controlled protein crosslinking. However, it was difficult to get pure heterotrimer. The main reason is probably the low yield of the bismaleimide crosslinking reaction, especially the crosslinking on the Strep-Tactin column, due to the hydrolysis of the maleimide group and the multi-step reaction mechanism. Also the size difference among the monomer, dimer, and trimers too small to allow total separation by size exclusion chromatography. Because the peaks of them would overlap with each other.

4.5 Conclusions

Three CBP-tagged cytochrome $c_3$ mutants were constructed and expressed in *Shewanella oneidensis* but did not bind to the Calmodulin (CaM) affinity resin during purification. Possible reasons might be that the linker between protein and CBP was not long enough or that the CBP was partly proteolysed during expression. A strep-tagged K40C:S107C cytochromes $c_3$ was constructed and expressed in *Shewanella oneidensis*. This mutant bound to the Strep-Tactin affinity resin but formed oligomers. The cysteine 107 residue next to the heme binding motif may have caused this protein self-oligomerisation. In order to prevent oligomerization, a K40C cytochromes $c_3$ with New Strep Tag containing a cysteine (nstK40C) was constructed, expressed and purified.

Strep-Tactin was used to block the cysteine in New Strep Tag of nstK40C cytochromes $c_3$ allowing the cysteine 40 reactive in order to control the ligation process. nstK40C was reacted with bismaleimide-modified S107C to produce a heterodimer (yield: ~20%) while bound to the Strep-Tactin column. This dimer was purified and reacted with bismaleimide-modified K40C to generate a heterotrimer (yield: ~12%). The results validated the feasibility of multi-step controlled protein crosslinking. The yield of the wire was low. It was likely due to the hydrolysis of bismaleimide crosslinker and the multi-step reaction mechanism. It was also difficult to get the pure form of the final product because peaks of components in the reaction mixture would overlap with each other in the size exclusion chromatography separation. However, a controlled protein ligation strategy was successfully developed and a short well-defined wire was generated using three different cytochrome $c_3$ modules.
Chapter 5

The conjugation of

cytochrome c₃ and

P450 BM3 heme domain
5.1 **Introduction**

The cytochromes P450 (CYP or P450) are a huge superfamily of heme containing proteins from various biological species, including humans. The reduced P450s have extremely high affinity towards carbon monoxide (CO), forming characteristic Soret peaks at 450 nm in the UV-Vis absorption spectra. That's why they are named P450 (Pigment at 450 nm). Usually, P450s all contain a b-type heme axially coordinated by the thiol group of a conserved cysteine residue, leaving another axial coordination position free for substrate binding.

Monooxygenation, illustrated simply below, is the typical catalytic reaction of P450s, which have a broad range of substrates.

\[
\text{RH} + \text{O}_2 + 2\text{H}^+ + 2e^- \xrightarrow{\text{P450}} \text{ROH} + \text{H}_2\text{O}
\]

In addition to the above chemical reagents, P450s need the support of their associated redox partners to supply two electrons to drive every catalytic cycle. The redox partners are commonly small polypeptides containing redox active species like FAD, FMN, and [2Fe-2S] clusters.

Flavocytochrome P450 BM3 from *Bacillus megaterium* (Fig 1.13) is a well-studied P450. It is a large soluble protein with only a single polypeptide including a heme domain (cytochrome P450 oxygenase domain) and a flavin domain (cytochrome P450 reductase domain) binding FAD and FMN. Hence, flavocytochrome P450 BM3 is an ideal model for P450 systems consisting of a single component enzyme.

The b-type heme of P450 BM3 is deeply buried in the protein (Fig. 1.22) and it naturally accepts electrons from the flavin domain. The aim of the chapter is to attach a stable electro-active protein, e.g. cytochrome c₃, to P450 BM3 near the active site and to study the conjugate with an electrode driving catalytic reactions. This strategy replaces the NADPH and flavin domain, which are both expensive and fragile species.
Figure 5.1: potential crosslinking sites in P450 BM3 heme domain (PDB:2IJJ2). Panel a: cartoon view; panel b: molecular surface view. Cysteines are represented in yellow; glutamine is represented in blue; the possible docking area of cytochrome c\textsubscript{3} on the P450 BM3 heme domain surface is highlighted by the red dashed circle.

There is a concave pocket located near the active site of P450 BM3 heme domain (Fig. 5.1). It might be a suitable binding area for the globular cytochrome c\textsubscript{3}. Hence, the residues in the pockes were good candidates as crosslinking sites. There are three native cysteines in the P450 BM3 heme domain at positions 62, 156, and the conserved cysteine 400, the fifth ligand of heme (Fig. 5.1a). Cysteine 156 is buried in the protein and cannot be accessed by other molecules. Cysteine 62 is half exposed to solvent in the concave pocket, and might be a possible site for protein crosslinking. In addition, the most exposed amino acid in the pocket, glutamine 387 (Fig. 5.1), was considered as an alternative crosslinking site in case of failure of crosslinking at cysteine 62. Therefore, two mutants of P450 BM3 heme domain were constructed, C156S (C62P450) and C62S:C156S:Q387C (C387P450). A short six-histidine tag was attached to the C terminus of the mutants facilitating their purification.

The cytochrome c\textsubscript{3} module used for conjugation with P450 BM3 heme domain was K40C cytochrome c\textsubscript{3} with Strep Tag II. Hence, the conjugate of them could be easily separated using two affinity columns, Strep-Tactin Sepharose column (IBA) and Ni\textsuperscript{2+}-NTA Agarose column (Qiagen). In fact, S107C cytochrome c\textsubscript{3} had better
performance in the crosslinking reaction than K40C (Section 3.3.1). However, after conjugation to P450, strep-tagged S107C might not be purified by the Strep-Tactin resin because Strep Tag II might be blocked by P450. It would make the crosslinking product separation difficult.

P450 BM3 mutants were expressed, characterized with UV-Vis spectroscopy and substrate binding, crosslinked with a cytochrome $c_3$ module near the active site. The electrochemistry of the conjugate was studied using an OTTLE and UV-Vis spectroscopy combinatory technique (Fig. 1.23).

5.2 Protein purification and basic characterization

(1) SDS-PAGE and UV-Vis spectroscopic analysis of P450 BM3 heme domain mutants

Two mutants of P450 BM3 heme domain were over expressed in *E. coli* TG1, and purified by Ni$^{2+}$-NTA Agarose column chromatography. Both mutants were successfully bound to the Ni$^{2+}$-NTA column and formed a red tight band on the top of the column. After several washes of binding buffer, the red band was eluted with 250 mM Imidazole buffer. The purification progress was analyzed by SDS-PAGE/CBB staining (Fig. 5.2). During the preparation of the gel samples, the $b$-type heme of P450 BM3 was dissociated and hence the gel could not be developed by heme staining.
Figure 5.2: SDS-PAGE/CBB staining analysis of the fractions of the Ni\textsuperscript{2+}-NTA Agarose column. Lane M: molecular weight marker; lane 1: cell extract; lane 2: Ni\textsuperscript{2+}-NTA column flow through; lane 3: Ni\textsuperscript{2+}-NTA column wash; lane 4: Ni\textsuperscript{2+}-NTA column elution of the target protein. Both mutants of P450 BM3 heme domain had the same pattern of SDS-PAGE gel.

The single band on the SDS-PAGE/CBB stained gel (lane 4, fig.5.1), showed good correlation with the expected molecular mass of P450 BM3 heme domain at approximate 55 kDa, indicated that the mutant had a high purity.
Figure 5.3: UV-Vis spectra of C62P450. The red curve represents the oxidised form of the mutant [Fe(III)]; the blue curve represented the CO bound reduced form [Fe(II)-CO]. The insert plot showed the different spectrum of the CO bound reduced form and the reduced form ([Fe(II)-CO]- [Fe(II)]).

Both mutants had the identical UV-Vis spectra since the mutation sites were far away from the active centre (Fig. 5.3). Typically, there were four peaks (363, 420, 535 and 565 nm) in the fern-form (oxidised). Upon binding CO to the ferro-form P450 heme domain, a “famous” Soret peak at 450 nm appeared. The concentration of mutants could be determined by the difference spectrum of the CO bound ferro-form and the ferro-form with the extinction coefficient $\varepsilon_{450-490} = 92 \text{ mM}^{-1} \text{ cm}^{-1}$ [90].

(2) SDS-PAGE and UV-Vis spectroscopic analysis of K40C cytochrome c$_3$ with Strep Tag II (stK40C)

This strep-tagged cytochrome c$_3$ was constructed on the basis of the plasmid (pSL9) encoding K40C cytochrome c$_3$ with New Strep Tag (Section 2.3.7), expressed in *Shewanella oneidensis* MR-1, and purified on a Strep-Tactin column.
Figure 5.4: SDS-PAGE/heme staining analysis of the fractions of the Strep-Tactin Sepharose column. Lane M: molecular weight marker; lane 1: cell extract; lane 2: SP Sepharose column flow through; lane 3: 500mM NaCl elution of SP Sepharose; lane 4: Strep-Tactin column flow though; lane 5 - 10: eluted fractions of stK40C cytochrome c₃.

The SDS-PAGE/heme stained gel (Fig. 5.4) showed a similar pattern to previous gels (Figs. 4.2 & 4.5). More importantly, compared with the purification result of stKS (Section 4.3), this strep-tagged cytochrome c₃ massively reduced the self oligomerization of the protein, with only a minor dimer band observed (lane 7, fig. 5.4). It supported the previous hypothesis (4.3) that the cysteine 107 next to the heme binding motif could be activated towards self oligomerization of the cytochrome c₃ modules.

Again, the UV-Vis spectra of stK40C cytochrome c₃ were identical to those of wild type cytochrome c₃ (Fig. 3.3).
5.3 Substrate binding of the mutants of P450 BM3 heme domain

In order to check the active site functionality of the mutants, they were treated with the common substrates of P450 BM3, laurate and palmitate. Upon binding, the substrates of P450 BM3 heme domain displace the water ligand at the sixth coordination position leading to a change in geometry and a low- to high-spin shift of the ferric form (Fig. 5.5).

![Figure 5.5: substrate binding to P450 BM3 heme domain. The heme group is represented by a purple rectangle and a red ball.](image-url)
Substrate binding of C387P450

**Figure 5.6: UV-Vis spectra showing substrate binding to C387P450.** Substrate free P450 was represented in black curve; laurate bound P450 was represented in red curve; palmitate bound P450 was represented in blue curve.

Both mutants gave similar UV-Vis spectra (Fig. 5.6) and exhibited high spin-state conversion in the presence of excess palmitate. However, laurate resulted in a considerably lower proportion of high-spin protein. This is the same as observed for the wild-type enzyme, as laurate is not long enough to be an ideal substrate.

### 5.4 Conjugation of the P450 BM3 and cytochrome c₃ mutants

(1) **Crosslinking capability of P450 BM3 heme domain mutants**

Two mutants of the P450 BM3 heme domain were made: C62P450 and C387P450. The native cysteine 62 is close to the active and has half exposure to solvent (Fig. 5.1), which might limit its activity in the crosslinking reaction. Cysteine 387 should have much higher exposure than cysteine 62 so that it should be more reactive. According to the previous study (Section 3.3.1), S107C cytochrome c₃ had better performance in the crosslinking reaction than K40C. Hence, it was used to test the crosslinking capability of the two mutants of the P450 BM3 heme domain (C62P450 and C387P450).
The small scale crosslinking reaction between the P450 mutants and S107C followed the procedure described in section 2.11.1, in which K40C was replaced by C62P450 or C387P450 in overnight reactions. The gel samples were taken from the reactants and crosslinking mixtures and analyzed by SDS-PAGE/heme staining (Fig. 5.7).

Figure 5.7: SDS-PAGE/heme staining analysis of the crosslinking reactions of P450 BM3 heme domain mutants (C62P450 & C387P450) and S107C cytochrome c₃.

Lane M: molecular weight marker; lane 1: S107C; lane 2: C62P450; lane 3: C387P450; lane 4: S107C+C62P450; lane 5: S107C+C387P450

The bands of P450 BM3 heme domain mutants could not be developed by the heme staining due to the dissociation of heme during the sample preparation (lane 2 & 3, fig. 5.7). However, it did not affect the analysis of crosslinking reaction. The band of the P450-S107C conjugate could be highlighted without the interference of the side products (P450 monomers and homodimers) in the high molecular weight area. Only the cytochrome c₃ modules (monomer, homodimer, and heterodimer) appeared on this heme staining gel. The band (> 62 kDa) in lane 5 of figure 5.7 indicated the mutant C387P450 performed well in conjugation with cytochrome c₃ whereas the mutant C62P450 could not be crosslinked.
The conjugation of C387P450 and stK40C

The two reactants, C387P450 and stK40C, were selected to make a heterodimer on a large scale. To get a good control of the reaction, the crosslinking progress underwent two steps. Firstly, C387P450 was reacted with 10 fold excess BM(PEO)$_2$ to produce C387P450-BM(PEO)$_2$. Secondly, C387P450-BM(PEO)$_2$ was purified by anion exchange chromatography. It then was mixed with an equivalent of stK40C cytochrome c$_3$ producing C387P450-BM(PEO)$_2$-stK40C with a yield of ~ 28%. The reaction mixture was purified via a Strep-Tactin Sepharose column and a Ni$^{2+}$-NTA Agarose column. Proteins remained soluble throughout the reaction and each step of reaction was analyzed by SDS-PAGE/heme staining (Fig. 5.8). The purified conjugate was studied by the size exclusion chromatography (Fig. 5.9) and UV-Vis spectroscopy (Fig. 5.10).

![Figure 5.8: SDS-PAGE/heme staining analysis of the crosslinking reactions of P450 BM3 heme domain mutants (C387P450) and stK40C cytochrome c$_3$. Lane M: molecular weight marker; lane 1: C387P450; lane 2: C387P450-BM(PEO)$_2$; lane 3: stK40C; lane 4: C387P450-BM(PEO)$_2$-stK40C; lane 5: the reaction product after Strep-Tactin and Ni$^{2+}$-NTA columns.](image)
Although the crosslinking process was a two-step controlled reaction, there were still a few side products. Apart from the homodimer formed by stK40C, a minor band (lane 4, fig. 5.8) appeared with a higher molecular weight than the target conjugate (C387P450-BM(PEO)₂-stK40C). Probably an additional cytochrome c₃ module was attached to C387P450-BM(PEO)₂-stK40C. After purification on the two affinity columns, there were still some cytochrome c₃ modules (monomer and homodimer) in the product (lane 5, fig. 5.8).

The purified conjugate was further analyzed by size exclusion chromatography with a Superdex S200 column (Fig. 5.9).

**Figure 5.9:** the elution profile of Superdex S200 for the analysis of the purified conjugate of C387P450 and stK40C. The blue curve represents the elution of stK40C; the black curve represents the elution of C387P450; the red curve represents the elution of purified conjugate. The stK40C and C387P450 monomers are highlighted by green and magenta vertical lines, respectively; the C387P450 dimer is highlighted by the orange vertical line.

The unreacted proteins, C387P450 and stK40C, were passed through the Superdex S200 column as molecular markers and the elution curves showed that both proteins were very pure. There were still small amounts of cytochrome c₃ modules (monomer...
and homodimer) in the product solution. This result was consistent with the analysis of SDS-PAGE/heme staining. The situation was more complicated in the high molecular weight area. The peak of the target conjugate (C387P450-BM(PEO)$_2$-stK40C) was overlapped by the peak of the C387P450 dimer.

![UV-Vis spectra of C387P450-BM(PEO)$_2$-stK40C](image.png)

**Figure 5.10:** The UV-Vis spectra of C387P450-BM(PEO)$_2$-stK40C. The black curve represented the oxidized form of the conjugate; the red curve represented the reduced form; the blue curve represented the CO bound reduced form.

The UV-Vis spectrum of the conjugate seemed like the combined spectra of the individual components. There are four hemes in cytochrome $c_3$ and only one heme in P450 BM3. Hence, in the oxidized form of the conjugate, the Soret peak of the cytochrome $c_3$ module (410 nm) was so intensive that the Soret peak of P450 BM3 heme domain (420 nm) was covered. The spectrum of the reduced form also only showed the characteristic peaks of the cytochrome $c_3$ at 419 nm, 523 nm and 552 nm. Upon CO binding to the reduced conjugate, a typical peak at 450 nm belonged to P450 component appeared. The peaks of reduced cytochrome $c_3$ at 523 nm and 552 nm decreased and the Soret peak (419 nm) was shifted to 415.5 nm, which were consistent with the literature description of the CO-binding of cytochrome $c_3$ [153]. The concentrations of cytochrome $c_3$ module and P450 BM3 heme domain module
were figured out with their own extinction coefficients and the ratio of them was about 1:2.5 (stK40C : C387P450).

5.5 OTTLE driven electron transfer between cytochrome c₃ and P450 heme domain mutant

OTTLE (Optically Transparent Thin Layer Electrode) was used to investigate the electron transfer between cytochrome c₃ and P450 BM3 heme domain. The samples would be the equimolar mixture of uncrosslinked cytochrome c₃ and P450 BM3 heme domain, and their conjugate. At a low applied potential (-300 mV), the cytochrome c₃ would be half reduced by OTTLE because its first two microscopic reduction potentials are -242 mV and -296 mV [150], lower than -300 mV. P450 BM3 heme domain can not be directly reduced by the electrode due to its buried heme group. The reduced cytochrome c₃ module is expected to transfer electrons to the active site of P450 BM3 heme domain when it diffuses to the concave pocket near the active site of P450 BM3 heme domain. In the presence of CO, the reduction of P450 BM3 heme domain can be monitored by the UV-Vis spectrophotometer with the increases in absorbance at 450 nm. Here, cytochrome c₃ plays a role as an electron transfer mediator. Palmitate, a substrate of P450 BM3, is also added to favour the reduction of P450 BM3 heme domain. This technique can test the possibility of the electron transfer between cytochrome c₃ and P450 BM3 heme domain. Furthermore, it would show whether cytochrome c₃ and P450 BM3 heme domain are crosslinked at the right postion for productive electron transfer.

(1) Inter-molecular electron transfer between the stK40C cytochrome c₃ and P450 BM3 heme domain (C387P450)

Upon the application of -300 mV to the equimolar mixture of stK40C cytochrome c₃ and P450 BM3 heme domain mutant (C387P450) in the presence of CO and palmitate, the UV-Vis spectrum of the sample in the OTTLE cell were recorded every 5 minutes over a period of 24 hours. The selected spectra (Fig. 5.11) showed that the Soret peak at 410 nm of the mixture was shifted to 415.5 nm and a small peak at 552 nm appeared. These were typical changes of cytochrome c₃, when reduced and bound with CO. The other characteristic increase was at 450 nm
indicating that the heme of the P450 BM3 mutant had been reduced and coordinated by CO.

![Graph](image)

Figure 5.11: UV-Vis spectra of OTTLE driven inter-molecular electron transfer between stK40C cytochrome c3 and C387P450. The red curve represents the initial spectrum of the sample; the magenta curve represents the final spectrum of the sample.

The time dependence of the absorbance changes at 450 nm and 552 nm (Fig. 5.12) showed that the cytochrome c3 species was reduced by the electrode in ~ 35 min while P450 BM3 heme domain took ~ 10 hours to fully reduce. The possible reason for the asynchronous reduction of the two species (~ 20 fold difference) was that, upon reduction of cytochrome c3 module, it took some time to diffuse to the right position for transferring electrons into the active centre of P450 BM3 heme domain.
Figure 5.12: the absorbance changes during OTTLE driven inter-molecular electron transfer between stK40C cytochrome c<sub>3</sub> and P450 BM3 heme domain mutant (C387P450). The red curve represents the absorbance change at 450 nm; the black curve represents the absorbance change at 552 nm.

As a control, the P450 BM3 heme domain mutant (C387P450) with CO and palmitate was also applied with -300 mV potential in the OTTLE cell. No CO-bound reduced P450 was observed over the 24-hour experiment (Fig. 5.13). This meant that the P450 BM3 heme domain could not directly interact with the electrode.
Figure 5.13: the absorbance change at 450 nm of P450 BM3 heme domain mutant (C387P450) upon the application of -300 mV

(2) Intra-molecular electron transfer between the stK40C cytochrome c3 and P450 BM3 heme domain (C387P450)

The same experiment was performed on the conjugate of cytochrome c3 and P450 BM3 heme domain, C387P450-BM(PEO)2-stK40C. Unfortunately, only the reduction of the cytochrome c3 component was observed. Taking the baseline shift into account, there were almost no changes at 450 nm over a 24-hour period (Fig. 5.14), even at a lower applied potential (-500 mV).
The reason for the failure of the electron transfer might be that cytochrome C3 was not crosslinked to the right position of P450 BM3 close enough to the P450 BM3 heme. The movement of the crosslinked cytochrome C3 might have been limited or blocked so that it could not access the heme of the P450.

5.6 Conclusions
Cytochrome C3 was used to pass electrons to P450 in a conjugate for immobilization on an electrode or for further conjugation. In order to crosslink cytochrome C3 close to the heme of P450, two mutants of P450 BM3 heme domain (C62P450 and C387P450) with suitable ligation sites were constructed, expressed, and purified. The mutant C387P450 was more reactive with cytochrome C3. C387P450 was crosslinked to a newly constructed cytochrome C3 mutant (stK40C) with a yield of ~28% and the conjugate was purified by two affinity columns. The UV-Vis spectrum of the CO-bound reduced conjugate indicated that the heme of P450 had the similar environment as the heme of wild type P450. The electron transfer between cytochrome C3 and P450 BM3 heme domain was studied by OTTLE. P450 heme domain can not be reduced directly by the electrode due to its buried active site and it
was slowly reduced by cytochrome $c_3$ (~ 10 hours). However, there was no electron transfer observed at the same condition for the conjugate of cytochrome $c_3$ and P450 BM3 heme domain. Probably cytochrome $c_3$ was not crosslinked to the right position of P450 BM3 close enough to the P450 BM3 heme.
Chapter 6

Conclusions and future work
In order to utilize the cytochrome $c_3$ for protein conjugation, three cysteine mutants (K40C, S107C, and K40C:S107C) were constructed, successfully expressed in *Shewanella oneidensis* MR-1, purified and characterized by fluorescent labelling and thiol counting. Protein crosslinking was performed with a bismaleimide crosslinker, 1,8-bis-Maleimidodiethyleneglycol (BM(PEO)$_2$) on the cysteine mutants to produce cytochrome $c_3$ dimers and polymers. S107C was more efficient in forming homodimers than K40C probably because it is the flexible and exposed C terminus of the protein. The yields in two hours were ~ 60 % for the S107C dimer and ~ 30 % for the K40C dimer respectively. The incomplete dimerization might be due to the hydrolysis of the maleimide group and two-step crosslinking mechanism. A trace amount of an impurity thiol containing protein also affected the production of homodimers in conjugating with the monomeric cytochrome $c_3$ mutant. The K40C and S107C heterodimer (yield: ~ 25 %) was generated upon the production of a bismaleimide-modified S107C intermediate. This two-step reaction shows that protein ligation can be performed in a controllable multi-step way. However, the maleimide group does hydrolyse during the reaction and the impurity in the protein sample still limited the production of the heterodimer. Two methods were used for the polymer production: disulfide crosslinking and bismaleimide crosslinking. The polymers generated through disulfide crosslinking were cleavable whereas bismaleimide crosslinked polymers were stable in a reducing environment. Both gave a high degree of polymerization, yielding up to the 12mer at least for disulfide crosslinking and up to the 8mer at least for bismaleimide crosslinking. The UV-Vis spectroscopy of both polymers showed that the chemical nature of heme groups in cytochrome $c_3$ remained unchanged before and after polymerization. OTTLE potential titration shows that the polymerisation of the cytochrome $c_3$ modules leads to a reduction potential shift towards the negative from - 304 mV (wild type) to -336 mV (disulfide-linked cytochrome $c_3$ polymers) and -388 mV (bismaleimide-linked cytochrome $c_3$ polymers). A possible reason is that the interaction among hemes in the cytochrome $c_3$ modules makes the polymer more difficult to reduce.

The affinity tags, Calmodulin Binding Peptide (CBP) and Strep Tag II, were used not only to aid protein purification but also to control the protein ligation. Three cytochrome $c_3$ mutants tagged with CBP were constructed and expressed in
*Shewanella oneidensis* but did not bind to the Calmodulin (CaM) affinity resin during purification. Possible reasons might be that the linker between protein and CBP was not long enough or that the CBP was partly proteolysed during expression. A K40C:S107C cytochrome *c*₃ tagged with Strep Tag II formed oligomers after affinity purification. The cysteine 107 residue next to the heme binding motif may have caused this protein self-oligomerisation. In order to prevent oligomerization, a mutant K40C cytochrome *c*₃ with New Strep Tag containing a cysteine (nstK40C) was constructed, expressed and purified. Strep-Tactin was used to block the cysteine in New Strep Tag of nstK40C cytochromes *c*₃ allowing the cysteine 40 to be reactive in order to control the ligation process. nstK40C was reacted with bismaleimide-modified S107C to produce a heterodimer (yield: ~ 20%) while bound to the Strep-Tactin column. This dimer was purified and reacted with bismaleimide-modified K40C to generate a heterotrimer (yield: ~ 12%). The yield of the wire was low. It was likely due to the hydrolysis of bismaleimide crosslinker and the multi-step reaction mechanism. It was also difficult to get the pure form of the final product because peaks of components in the reaction mixture would overlap with each other in the size exclusion chromatography separation. However, the controlled protein ligation strategy was successfully developed and a short well-defined wire was generated using three different cytochrome *c*₃ modules.

Cytochrome *c*₃ was used to pass electrons to P450 in a conjugate for immobilization on an electrode or for further conjugation. In order to crosslink cytochrome *c*₃ close to the heme of P450, two mutants of P450 BM3 heme domain (C62P450 and C387P450) with suitable ligation sites were constructed, expressed, and purified. The mutant C387P450 was more reactive with cytochrome *c*₃. C387P450 was crosslinked to a newly constructed cytochrome *c*₃ mutant (stK40C) with a yield of ~ 28 % and the conjugate was purified by two affinity columns. The UV-Vis spectrum of the CO-bound reduced conjugate indicated that the heme of P450 had a similar environment to the heme of wild type P450. The electron transfer between cytochrome *c*₃ and P450 BM3 heme domain was studied by OTTLE. The P450 heme domain can not be reduced directly by the electrode due to its buried active site, but it was slowly reduced by cytochrome *c*₃ (~ 10 hours). However, there was no electron transfer observed under the same conditions for the conjugate of cytochrome
cytochrome $c_3$ and P450 BM3 heme domain. Probably cytochrome $c_3$ was not crosslinked to the right position of P450 BM3 close enough to the P450 BM3 heme.

It would be interesting to carry out the following work:

1. Optimise the crosslinking reaction by varying conditions and stoichiometries to increase yield of crosslinking products;
2. Produce the conjugates of P450 BM3 heme domain and cytochrome $c_3$ with new suitable crosslinking sites;
3. Produce the conjugates of P450 BM3 heme domain and other small redox-active proteins, e.g. ferredoxin;
4. Study the effects of cytochrome $c_3$ on P450 catalysis.
Chapter 7

References


66. Shiro, Y; Fuji, M; Iizuka, T; Adachi, S; Tsukamoto, K; Nakahara, K; Shoun, H. *J. Biol. Chem.* 1995 270, 1617-23.


95. Ghosh, S. S.; Kao, P. M.; McCue, A. W.; Chappelle, H. L. Bioconjugate Chem. 1990 1, 71-76.


APPENDIX I

CYTOCHROME C₃ PEPTIDE SEQUENCE

1 APKAPADGLK MDKTKQPVVF
21 NHSTHKAVKC GDCHHPVNGK
41 EDYQKCATAG CHDNMDKKDK
61 SAKGYYHAMH DKGTKFKSCV
81 GCHLETAGAD AAKKKeLTGC
101 KGSKCHS
1 TIKEMPQPKT FGELKNLPLL NTDKPVQALM
31 KIADELGEIF KFEPAPGRVTR YLSSQRLIKE
61 ACDESREFDKN LSQALKFVRD FAGDGLFTSW
91 THEKNWKKAH NILLPSFSQQ AMKGYHAMMV
121 DIAVQLVQKW ERLNADEHIE VPEDMTRLTL
151 DTIGLCGFNY RFNSFYRDQP HPFITSMVRA
181 LDEAMNKLQR ANPDDPAYDE NKRQFQEDIK
211 VMNDLVDKII ADRKASGEQS DDLLTHMLNG
241 KDPETGEPLD DENIRYQIIT FLIAGHETTS
271 GLLSFALYFL VKNPHVQLQA AEEAARVLVD
301 PVPSYKQVKQ LKYVGMVLNE ALRLWPTAPA
331 FSLYAKEDTV LGGEYPLEKG DELMVLPQL
361 HRDKTIWGDD VEEFRPERFE NPSAIPQHAF
391 KPFNGNRQAC IGGQFALHEA TLVLGMLLKH
421 FDFEDHTNYE LDIKETTLK PEGFVKAKS
451 KKIPLOGGIPS PSTEQSAKKV RK
Quantification of Gels by ImageJ

ImageJ could be downloaded freely from http://rsbweb.nih.gov/ij/. It is available for Windows, Mac OS X and Linux platforms. After installation (Mac OS X here), ImageJ was calibrated by the method provided online (http://rsbweb.nih.gov/ij/docs/examples/calibration/index.html).

The procedure of gel analysis:

1. Open a gel picture by File/Open;
   Invert the color of picture by Edit/Invert, the background will become black;
   Set bands to white color only by Image/Type/8-bit.
2. Use the rectangular selection tool to outline the first lane. This should be the left most lane if the lanes are vertical or the top lane if the lanes are horizontal. Note that lanes are assumed to be vertical unless the width of the initial selection is at least twice its height.
2. Select Analyze/Gels/Select First Lane and "Lane 1 selected" will be displayed in the status bar. ImageJ will also duplicate the image and outline and label the lane if 'Outline Lanes' is checked in the Analyze/Gels/Gel Analyzer Options.

3. Move the rectangular selection right to the next lane (or down if the lanes are horizontal) and select Analyze/Gels/Select Next Lane. "Lane 2 selected" will be displayed in the status bar. ImageJ will also outline and label the lane if "Outline Lanes" is checked.

4. Repeat the previous step for each remaining lane.

5. Check the 'Invert Peaks' in the Analyze/Gels/Gel Analyzer Options.

6. Select Analyze/Gels/Plot Lanes (or press "3") to generate the lane profile plots.

7. Use the straight line selections tool to draw base lines and/or drop lines so that each peak of interest defines a closed area. To get to all the lanes, it may be necessary to scroll the image vertically using the "Hand" tool. (Hold down the space bar to temporarily switch to this tool).

8. For each peak, measure the size by clicking inside with the wand tool. If necessary, scroll the image by holding down the shift key and dragging.

9. Select Analyze/Gels/Label Peaks to label each measured peak with its size as a percent of the total size of the measured peaks.
APPENDIX III

MEETINGS ATTENDED

10th and 11th Redox Enzymes Meeting, Firbush Point Field Centre, Perthshire
  June 2006  Delegate
  June 2007  Speaker

Chemical Biology Section, Firbush Point Field Centre, Perthshire
  April 2007  Poster
  April 2008  Speaker

Transition Metals in Biochemistry, Norwich
  June 2008  Poster

2nd Joint German/UK Bioenergetics Conference, Edinburgh
  April 2008  Poster

13th International Conference on Biological Inorganic Chemistry, Vienna, Austria
  August 2007  Poster

Scottish Protein Group, Glasgow
  March 2006  Delegate

Chemistry Biology Section Seminars

School of Chemistry Colloquia