Flavocytochrome \( b_2 \): an ideal model system for studying protein-mediated electron transfer

S. K. Chapman*, G. A. Reidt, C. Bell*, D. Short* and S. Daif*

*Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JL, Scotland, UK, and †Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JF, Scotland, UK

Introduction

Most of the recent intense scientific effort towards providing an understanding of electron transfer in proteins has focused on intraprotein electron transfer and on the pathway between the donor (D) and acceptor (A) redox centres. These centres are usually fixed within the protein matrix as in the case of the photosynthetic reaction centre [1], or involve attaching an artificial redox centre onto the surface of a protein at a fixed distance from the natural centre, e.g. in ‘ruthenated’ proteins [2]. Such studies usually reduce to an analysis of whether the electron travels from D to A directly through space (i.e. treating the intervening protein medium as homogeneous like an ‘organic glass’), or whether it travels through a distinct \( \sigma \)-tunelling pathway involving specific covalent bonds, hydrogen bonds, etc. (i.e., treating the protein medium as heterogeneous). As well as the distance between D and A, the rate of electron transfer is also influenced by the driving force of the reaction, \( \Delta G^\circ \), and the reorganization energy, \( \lambda \) [1].

In addition to intraprotein electron transfer, in which the redox centres are fixed within one protein, there is also intense interest in biomolecular reactions between proteins which result in interprotein electron transfer. Here, one must consider the dynamics of the interactions between the two proteins involved. Do the two proteins form one defined complex with a specific electron transfer path between redox centres? Are there a number of possible sites on the proteins where binding followed by electron transfer can occur? An interesting example is the complex between cytochrome \( c \) and cytochrome \( c \) peroxidase for which there is now a crystal structure [3]. Based on this structure a \( \sigma \)-tunelling pathway linking the two haem groups has been proposed [3]. However an NMR study of the dynamics of the cytochrome \( c \)–cytochrome \( c \) peroxidase interaction indicates that the complex in solution is highly mobile and probably does not have a discrete architecture with one specific electron transfer pathway [4].

It is apparent then that to probe fully protein-mediated redox processes a model system is needed that can allow both the study of intraprotein electron transfer, between redox centres within the same protein, and interprotein electron transfer between centres in separate protein partners. Flavocytochrome \( b_2 \) is arguably the ideal model system for such studies for the following reasons: (i) it is soluble and easily obtained; (ii) the enzyme has been expressed at a high level in \textit{Escherichia coli} [5] and a number of mutant enzymes have been generated [6–8]; (iii) crystal structures of the native and recombinant enzymes are available [9,10]; (iv) the crystal structure of the natural redox partner cytochrome \( c \) is also available [11]; (v) the structure of a hypothetical complex between flavocytochrome \( b_2 \) and cytochrome \( c \) has been proposed [12]; (vi) the redox potentials of all the prosthetic groups have been determined and there is a wealth of data on the mechanism of action of the enzyme [13,14].

Background to the flavocytochrome \( b_2 \) system

Flavocytochrome \( b_2 \) (\( t \)-lactate:cytochrome \( c \) oxidoreductase) from \textit{Saccharomyces cerevisiae} is a homotetramer with subunit \( M \), of 57500. It is a soluble component of the mitochondrial intermembrane space, where it catalyses the transfer of electrons from \( t \)-lactate to cytochrome \( c \) [13]. We have cloned the DNA encoding the enzyme and expressed it at a high level in \textit{E. coli}. (Note that the kinetic properties of flavocytochrome \( b_2 \) from yeast and the recombinant enzyme from \textit{E. coli} are identical [5]). This has given us the ability to produce large amounts of fully active wild-type and mutant enzyme, which has facilitated our studies on the mechanism of action of the enzyme [6–8]. The X-ray crystal structures have been determined for the native enzyme from \textit{S. cerevisiae} [9], and for the recombinant enzyme from \textit{E. coli} [10]. These are isomorphic and clearly show that each subunit is composed of two distinct domains as illustrated in Figure 1. One of these contains haem (the cytochrome domain) and the other flavin mononucleotide.
(the flavin domain) [9]. The edge-to-edge distance between the flavin and haem groups is about 9.7 Å. The two domains are connected by a single segment of polypeptide chain, which constitutes an interdomain ‘hinge’. This raises the question of whether or not mobility permitted by the hinge influences the interdomain electron transfer rate.

Although there is no X-ray structure available for the flavocytochrome b2-cytochrome c complex there is now a hypothetical model for how these two proteins interact [12]. This model predicts that the b2 and c haem groups are coplanar with an edge-to-edge distance of around 14 Å and with a possible σ-tunnelling electron-transfer pathway linking the two haems, involving residues 50–52 of flavocytochrome b2 (Figure 2). The model also predicts a number of key electrostatic interactions between the two proteins, the most prominent of which is a salt bridge between Glu-91 of flavocytochrome b2 and Arg-13 of cytochrome c [12].

We address in this paper two specific aspects of electron transfer in flavocytochrome b2: (i) how can we probe the effect of domain mobility on interdomain electron transfer and (ii) is the published hypothetical complex between flavocytochrome b2 and cytochrome c correct?

**How can we probe the effect of interdomain mobility on electron transfer in flavocytochrome b2?**

In many cases, redox centres in proteins are rigidly locked in the protein matrix, an obvious example being the photosynthetic reaction centre [1]. In such cases it is relatively easy to measure the distance between donor and acceptor redox centres, since this distance will have a definite fixed value. However, what happens if the redox centres within a protein are not at a fixed distance, i.e., there is intraprotein fluxionality? A classic example would be where two domains in a protein (one containing D and the other A) are mobile with respect to each other. Is the rate of electron transfer in such a case regulated by the frequency of motion between the two domains?

Flavocytochrome b2 is an ideal system to address this question since the cytochrome and flavin domains of the enzyme are connected by an interdomain ‘hinge’ (arrowed in Figure 1) that allows mobility of the domains with respect to each other. The occurrence of Interdomain mobility in flavocytochrome b2 is supported by crystallographic [9] and NMR evidence [15] as follows.

1. Crystallographic evidence. The crystal structure of flavocytochrome b2 shows two crystallographically distinguishable subunits in the asymmetric unit [9]. In subunit 1 the electron density map shows the presence of both

![Figure 1](image1.png)

A single subunit of wild-type flavocytochrome b2. The α-helices are shown as ribbons, β-sheets as arrows and the remaining α-carbon backbone as wire.

The prosthetic groups are shown as ball and stick representations. The interdomain hinge is arrowed.

![Figure 2](image2.png)

The proposed σ-tunnelling pathway for electron transfer from the flavocytochrome b2 haem to the cytochrome c haem, based on the hypothetical complex proposed by Tegoni et al. [12]
cytochrome and flavin domains. However in subunit 2, no electron density for the cytochrome domain is resolved owing to positional disorder (Gly-100 is the first visible residue), indicating that the haem domain can move with respect to the flavin domain. (2) NMR evidence. The linewidths of haem proton resonances in the intact enzyme are far sharper than would be expected if the cytochrome domain had no free motion relative to the flavin domain [15]. This implies that the hinge region allows the cytochrome domain a considerable degree of mobility. From a number of studies on site-directed mutant forms of flavocytochrome b2 with alterations in the hinge [7,8] and interface residues [6], it has now become clear that the rate of electron transfer from the fully reduced flavin to the haem is governed by the mobility of the two domains and the frequency of productive encounters between them.

To analyse further the effect of interdomain mobility on the rate of intramolecular electron transfer in flavocytochrome b2, a methodology is needed that can be used to prevent interdomain mobility reversibly. For this reason we decided to introduce a disulphide bridge between the two domains to act as a 'reversible lock' on mobility. The power of this methodology has already been beautifully demonstrated in the case of some non-redox proteins. For example in the case of the sulphate-binding protein (SBP) from E. coli [16], two cysteines have been introduced into the protein to form a disulphide bond across the ligand binding site cleft that lies between two domains. This disulphide bond dramatically reduces domain flexibility in the protein [16]. Similarly, to regulate catalytic activity, a disulphide bond has been introduced across the active site cleft of T4 lysozyme. This disulphide link lowers mobility and completely removes catalytic activity [17]. Reductive cleavage of the disulphide link restores the enzyme to full activity, thus demonstrating the possibility of using an artificial disulphide bridge as a reversible lock.

In the case of flavocytochrome b2, a suitable location needed to be identified to introduce cysteine residues that might form a disulphide bridge linking the domains and restricting mobility. Analysis of the three-dimensional structure of the enzyme using molecular graphics allowed us to identify possible locations at the interface of the two domains where suitable disulphide linkages might be introduced. One such analysis indicated that a cysteine introduced at residue 67 (normally an alanine) in the cytochrome domain could form a disulphide bridge with an existing cysteine in the flavin domain at position 233. Using site-directed mutagenesis we have generated the Ala-67→Cys (A67C-b2) mutant enzyme. Our preliminary studies on A67C-b2 indicate that a disulphide bridge does indeed form in oxidizing conditions and that this bridge can be broken using reductants such as dithiothreitol. For example, Ellman assays on A67C-b2 isolated from E. coli under oxidizing conditions revealed one fewer free cysteine than for wild-type enzyme, whereas Ellman assays on A67C-b2 in the presence of reductants gave one more free cysteine. These results are exactly as would be expected if we had a reversible disulphide link as indicated schematically in Figure 3. We shall refer to the mutant enzyme with the disulphide bridge in place as the 'locked' form and in the absence of the bridge as the 'open' form.

Figure 3
Schematic representation of a flavocytochrome b2 subunit showing the haem- and flavin-containing domains

The cysteine at position 67 was introduced by site-directed mutagenesis, whereas C233 is a naturally occurring cysteine residue. When there is no disulphide bridge, i.e. the open form, the haem domain is mobile with respect to the flavin domain. In the presence of a disulphide bridge, the locked form, domain mobility is restricted.

Figure 3
Schematic representation of a flavocytochrome b2 subunit showing the haem- and flavin-containing domains
kinetic properties very similar to wild-type flavocytochrome \( b_2 \). For example, the rate constants for flavin and haem reduction measured under presteady-state conditions using stopped-flow spectrophotometry are the same within experimental error for both A67C-\( b_2 \) and wild-type enzyme. However, in the absence of dithiothreitol, in the locked form, the kinetic properties of A67C-\( b_2 \) are remarkably different from those of wild-type \( b_2 \). For A67C-\( b_2 \) in the locked form, the rate constant for haem reduction is some 10-fold lower than the value seen for the open form or for the wild-type enzyme. In contrast, the rate constant for flavin reduction in the locked form is only slightly affected. This is direct evidence that disulphide bridge formation has a major effect on flavin-to-haem electron transfer but not on the reduction of flavin by \( t \)-lactate. These preliminary data demonstrate the feasibility of placing a reversible lock on inter-domain mobility in flavocytochrome \( b_2 \) and open up exciting possibilities for further analyses of intraprotein electron transfer in this system.

Is the published hypothetical complex between flavocytochrome \( b_2 \) and cytochrome \( c \) correct?

As mentioned above, a hypothetical model for the complex formed between a flavocytochrome \( b_2 \) tetramer and four cytochromes \( c \) has been proposed [12]. This model predicts a number of polar contacts between the two proteins, a notable example being that between Arg-13 of cytochrome \( c \) and Glu-91 of flavocytochrome \( b_2 \). In this case, the distance between the Arg and the Glu is suggested to be around 2.6 Å [12]. In order to test the importance of this electrostatic interaction for complex formation, we have used site-directed mutagenesis to replace Glu-91 with Lys to generate E91K-\( b_2 \). Such a change introduces a coulombic barrier, which should have a large effect on the kinetics of complex formation.

The model also indicates a possible \( \sigma \)-tunnelling pathway linking the \( b_2 \) and \( c \) haems that might act as the electron transfer route (Figure 2). From Figure 2 it is clear that the aromatic side chain of Phe-52 provides the link to the cytochrome \( c \) haem. To test the validity of this proposed pathway, we mutated Phe-52 to an Ala, generating F52A-\( b_2 \). This change will effectively interrupt the tunnelling pathway and would be expected to have a marked effect on the electron transfer rate from flavocytochrome \( b_2 \) to cytochrome \( c \).

The two mutant enzymes, E91K-\( b_2 \) and F52A-\( b_2 \), have been characterized kinetically. The second-order rate constant for cytochrome \( c \) reduction by wild-type flavocytochrome \( b_2 \) has been found to be \( 35 \pm 1 \mu \text{M/s} \) (at \( 25^\circ \text{C} \), pH 7.5, \( I = 0.10 \text{ M}+10 \text{ mM} \text{ lactate} \)) and the values for the two mutant enzymes are identical to this within experimental error. This rather surprising result indicates that the catalytically competent site on flavocytochrome \( b_2 \) at which cytochrome \( c \) binds is unlikely to be the one proposed from the published hypothetical complex. The results are also inconsistent with the proposed electron transfer pathway shown in Figure 2. It would appear then that the published hypothetical complex between the two proteins [12] does not represent a catalytically competent entity. We are now re-examining the structure of flavocytochrome \( b_2 \) to try and find alternative locations at which cytochrome \( c \) might bind in a catalytically competent way.

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The family of FMN-dependent \(\alpha\)-hydroxy acid oxidizing enzymes

The oxidation of \(\alpha\)-hydroxy acids to keto acids is catalysed by both nicotinamide-linked and FMN-dependent enzymes. The two classes form distinct evolutionary families, which are believed to operate via different chemical mechanisms. Nicotinamide-dependent reactions are classically considered to proceed by transfer of a hydride ion, whereas the flavoenzymes are assumed to first abstract the substrate \(\alpha\)-hydrogen as a proton, a step followed by flavin reduction (carbanion mechanism) \[11\].

The family of FMN-dependent \(\alpha\)-hydroxy acid-oxidizing enzymes encompasses at present a dozen proteins with known sequences, including one open reading frame (ORF) in data banks \[2-7\]. The alignment, based on the comparison between the crystal structures of flavocytochrome \(b_2\) from *Saccharomyces cerevisiae* (Flb\(_2\)) and spinach glycolate oxidase (GO) \[2,8,9\], indicates 31 totally invariant residues and 8.5 strongly conserved residues, out of about 350 positions aligned. The two structures \[10-13\] show a \(\beta_2\alpha\) barrel fold for the flavodehydrogenase (FDH) domains with an rms difference of 0.93 Å for 311 superimposed Cx-atoms \[8\]. The most variable area between the two structures corresponds to \(\beta\)-barrel loop 4, which follows a different course in space over part of its length and for which, in both cases, a few residues cannot be located, owing to their mobility. In the overall sequence alignment, it is also the most variable region, including from about 45 to 75 residues with practically no similarity. This segment was assigned the role of membrane-binding anchor for mandelate dehydrogenase from *Pseudomonas putida* \[3\]. There is also indirect evidence concerning Flb\(_2\) and long-chain \(\alpha\)-hydroxy acid oxidase (HAO) \([9,14-16]\, A. Belmouden and F. Lederer, unpublished work) that loop 4 may modulate catalysis by interacting with the active site, even though the edges of the invisible segment lie some 15-20 Å away from it in the two known structures \[10-12\].

The Flb\(_2\) structure afforded a picture of pyruvate, the reaction product, bound at the active site, where FMN is most likely under the semiquinone form. This led us to propose a binding mode for the substrate, lactate, and to assign to active site residues a role compatible with a carbanion mechanism \[15,17,18\], the validity of which rests on work carried out mainly with lactate mono-oxygenase from *Mycobacterium smegmatis* (LMO) \[19\] and Flb\(_2\) \[20\]. Figure 1 shows the hypothetical substrate binding mode, which implies participation of R-376, Y-143 and Y-254. H-373 is the postulated catalytic general base, which removes the substrate \(\alpha\)-H as a proton. Carbanion formation is assisted by the electrostatic interaction between the imidazolium ion and D-282. In the next step, electron transfer to FMN is facilitated by the K-349 positive charge which stabilizes the reduced flavin anionic form, irrespective of the exact mechanism of electron transfer \[1,18\]. All the side chains mentioned above are strictly conserved in all members of the family except for a substitution in HAO of F for Y-254 (Figure 1). Their good superposition in the crystal structures indicates a similar role for these side chains in GO and hence probably in all other family members. The observed shift between Flb\(_2\) and GO in the flavin position relative to the