Introduction
Flavocytochromes are multi-centre redox proteins containing both flavin and haem [1,2]. They catalyse a wide range of biologically important redox processes, including the oxidation and reduction of organic molecules, simple electron-transfer reactions and the activation of molecular oxygen. This diversity of function is made possible by the combination of flavin and haem cofactors, which allows the direct coupling of two-electron to one-electron oxido-reductions. Thus flavins can act as molecular transceivers, as a hydride for example, and transmitting them as electrons or vice versa. Haem groups can function both as efficient one-electron relays or as catalytic centres for the activation of small molecules such as dioxygen [1]. The combination of these cofactors produces flavocytochromes with great catalytic versatility, e.g. flavocytochrome P-450 BM3 (a fatty acid mono-oxygenase), flavocytochromes b2 (lactate and mandelate dehydrogenases) and flavocytochrome c1 (a fumarate reductase). These three flavocytochromes, although very different in reactivity, have certain structural features in common. They all have subunit arrangements in which there are two distinct domains connected by a short linker region of peptide (Figure 1). Each of these domains contains either flavin or haem prosthetic groups. Here we compare the ways in which redox equivalents are transmitted through the individual centres of these flavocytochromes.

Flavocytochrome P-450 BM3
Flavocytochrome P-450 BM3 from Bacillus megaterium catalyses the subterminal mono-oxygenation of a range of fatty acids with chain lengths of 12-20 carbon atoms. The enzyme is composed of a dillavin P-450 reductase fused to a cytochrome P-450 fatty acid mono-oxygenase in a single polypeptide chain (Figure 1A). Recent potentiometric and kinetic studies have resulted in a clearer understanding of the electron flow through this flavocytochrome [3,4]. Thus the FAD is initially reduced by hydride transfer from NADPH. The driving force for electron transfer from FAD to FMN is high [3], so there is rapid electron transfer between the two flavins. The next step requires electron transfer from FMN to the P-450 haem. However, the reduction potential of the haem is very dependent on substrate binding and, in the absence of substrate, electron transfer from flavin to haem is thermodynamically disfavoured. On binding of fatty acid, the haem reduction potential is elevated by more than 130 mV [3] and electron transfer from FMN to haem occurs. In essence, this substrate-induced switch regulates electron transfer in flavocytochrome P-450 BM3 and effectively prevents the enzyme from cycling in a futile manner, which would waste reducing equivalents in the production of H2O2. Reduction of the P-450 haem is followed by dioxygen binding and this initiates a classic P-450 catalytic cycle in which the transient formation of an oxyferyl intermediate leads ultimately to the mono-oxygenation of the fatty acid substrate [1,2]. In this case, therefore, we have a flavocytochrome that couples the reducing equivalents of NADPH to the activation of molecular oxygen, resulting in the hydroxylation of a fatty acid substrate.

Flavocytochromes b2
Unlike flavocytochrome P-450 BM3, the flavocytochromes b2 show little or no reactivity towards dioxygen. These enzymes are in fact 2-hydroxyacid dehydrogenases; they are found in the intermembrane space of yeast mitochondria. Examples are the enzymes from Saccharomyces cerevisiae and Hansenula anomala, both of which are l-lactate dehydrogenases [2], and the enzyme from Rhodotorula graminis, which is an l-mandelate dehydrogenase [5]. All of these flavocytochromes b2 are homotetramers with subunit molecular masses of close to 60 kDa; each subunit contains one flavin (FMN) and one haem. A three-dimensional structure is available for the enzyme from S. cerevisiae both in native [6] and recombinant (from Escherichia coli) forms [7]. The subunit composition is as shown
schematically in Figure 1(B) with an N-terminal (100 residues) cytochrome domain connected via a short hinge region to a C-terminal (400 residues) flavo
dehydrogenase domain. Electron flow through flavocytochrome \( b_2 \) is now fairly well understood [8–10] (Scheme 1). First the FMN is reduced by \( \alpha \)-lactate (Step 1, Scheme 1). A carb-
anion mechanism has been proposed for this redox step [11], although a hydride transfer from lactate to flavin N-5 is equally plausible, as has already been suggested for \( \alpha \)-amino acid oxidase [12]. Two-electron reduction of FMN is followed by intramolecular electron transfer from flavin to haem, generating flavin semiquinone and reduced haem [8] (Step 2, Scheme 1). There follows the first of two intermolecular electron transfers from \( b_2 \) haem to cytochrome \( c \) [13] (Step 3, Scheme 1). This results in an oxidized \( b_2 \) haem, which is then re-reduced by the flavin semiquinone. The electron transfer from semi-
quione to haem (Step 4, Scheme 1) is the slow-
est step in the catalytic cycle and is approx.

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**Figure 1**

Schematic representation of the subunit structure of three flavocytochromes

All three flavocytochromes have subunits in which a flavin-containing domain is fused to a cytochrome domain via a 'hinge' or 'linker' region of peptide. The domains are shown as ovals or spheres with the appropriate cofactors indicated. The linker is shown as a curved black line. (A) Flavocytochrome P 450 BM3; (B) flavocytochrome \( b_2 \); (C) flavocytochrome \( c \).
one-tenth as fast as the electron transfer from hydroquinone to haem [8]. Finally, the second electron is transferred from the $b_2$ haem to cytochrome $c$ (Step 5, Scheme 1). Thus in this case the flavocytochrome couples the two-electron oxidation of $L$-lactate to the reduction of two molecules of cytochrome $c$.

One area of recent controversy has concerned the nature of the complex formed between flavocytochrome $b_2$ and cytochrome $c$, which must be formed to permit efficient inter-protein electron transfer. Tegoni et al. [14] reported a computer-generated model of what the flavocytochrome $b_2$–cytochrome $c$ complex might look like. Unfortunately, mutagenesis studies that examined the predictions made by this model led to the conclusion that it was not likely to represent a kinetically competent complex [10,13]. More recently a new modelling study, consistent with mutagenesis results, has suggested a cytochrome $c$ docking site on flavocytochrome $b_2$ involving the acidic residues Glu-63, Asp-72 and Glu-237 [10]. The study concluded that cytochrome $c$ could ‘sample’ a number of different, yet similar, binding modes on this docking surface and that in each of these binding modes the edge-to-edge distance for electron transfer remains essentially the same [10].

An interesting question about electron flow through flavocytochrome $b_2$ is as follows: Why is the $b_2$ cytochrome domain required for electron transfer to cytochrome $c$? This is actually quite a fundamental question because the $b_2$ flavin group is quite capable of transferring electrons singly; the driving force for electron transfer to cytochrome $c$, directly from the flavin, is more than 300 mV. There is therefore no thermodynamic problem for electron transfer from $b_2$ flavin to cytochrome $c$ and yet the individually expressed flavodehydrogenase domain (i.e. that lacking the N-terminal cytochrome domain) has virtually no cytochrome $c$ reductase activity. An explanation for this is that there is very poor molecular recognition between the flavodehydrogenase domain and cytochrome $c$.

For efficient electron transfer to occur between these two proteins their redox centres should come as close together as possible. However, an examination of the surface around the exposed haem-edge of cytochrome $c$ and the surface of the flavodehydrogenase domain closest to the flavin indicates that these two faces are almost totally incompatible. Both surfaces are predominantly positively charged and this must present a substantial coulombic barrier to complexation between the proteins. In addition to this electrostatic effect there is also a possible...
steric problem to consider. In the crystal structure of flavocytochrome $b_2$ there is a length of peptide (residues 299–318) that shows no electron density. This portion of sequence forms a proteolytically sensitive loop on the surface of the protein. In the absence of crystal structure information we have used computational methods to model the folding and location of this loop (Figure 2). Results from these modelling studies indicate that the loop might fold directly over the docking site closest to the flavin. Thus there are both electrostatic and steric explanations for the slow reactivity of the flavodehydrogenase domain with cytochrome $c$.

To try to overcome these problems we have embarked on extensive protein engineering of the flavodehydrogenase domain to remove the steric block and build in a favourable recognition site for cytochrome $c$. This will involve the replacement of residues 298–320 with a sequence of seven glycine residues coupled with a triple mutation on the surface, of Lys-210→Glu, Lys-324→Ala and Phe-325→Glu. We believe that this redesign of the flavodehydrogenase domain surface will make an excellent docking site for cytochrome $c$, which should permit efficient interprotein electron transfer to occur.

Flavocytochrome $c_1$

Flavocytochrome $c_1$ is a fumarate reductase isolated from the periplasm of the marine bacterium *Shewanella frigida marina* NCIMB400 (previously described as *S. putrefaciens*) [15]. Production of flavocytochrome $c_1$ is induced, under anaerobic growth conditions, by the addition of fumarate [16]. Flavocytochrome $c_1$ differs from the previously characterized fumarate reductases, which are multi-subunit and anchored to the inner face of the cytoplasmic membrane [17]. In contrast, flavocytochrome $c_3$ is a soluble, single-subunit enzyme found in the periplasm [18]. The flavocytochrome $c_3$ subunit is composed of two domains, shown schematically in Figure 1(C), a tetrahaem cytochrome domain (117 residues) and a flavin domain (454 residues) that contains non-covalently bound FAD [18]. The cytochrome domain, located at the N-terminus of the protein, encapsulates four, bis-His ligated, $c$-type haems [19]. It has been proposed that this domain is structurally similar to the family of cytochromes $c_3$ [18].

The mechanism of electron flow through flavocytochrome $c_3$ is far less well understood than for flavocytochrome $b_2$ and flavocytochrome $P$-450 BM3. The physiological donor to the
Enzyme has not yet been defined. One possibility might be a membrane-bound tetrahaem cytochrome c related to the Nap C family, because such a protein has been identified in the closely related organism *S. putrefaciens* MR-1 [20]. Potentiometric and voltammetric studies on flavocytochrome c3 indicate that the electrons would first flow through the haem groups (reduction potentials ranging from −240 to −100 mV) then to the FAD (two-electron reduction potential −152 mV at 25°C, pH 7.0). The fully reduced FAD would then reduce fumarate by donation of a hydride ion, as outlined elsewhere [18].

**Conclusion**

The examples described here should have made it clear that flavocytochromes represent a very versatile group of enzymes. This versatility of function is being exemplified with the isolation of new flavocytochromes with even more variations in reactivity.

We thank Rhiannon Macfie, Scott Mathews, Florence Lederer, Malcolm Walkinshaw and Fraser Armstrong for their helpful discussions. We are grateful to the BBSRC, EPSRC, the Leverhulme Trust and Zeneca for their support of this work.


Received 19 August 1998