Tyr-143 facilitates interdomain electron transfer in flavocytochrome b_2

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The role of Tyr-143 in the catalytic cycle of flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase) has been examined by replacement of this residue with phenylalanine. The electron-transfer steps in wild-type and mutant flavocytochromes b_2 have been investigated by using steady-state and stopped-flow kinetic methods. The most significant effect of the Tyr-143 → Phe mutation is a change in the rate-determining step in the reduction of the enzyme. For wild-type enzyme the main rate-determining step is proton abstraction at the C-2 position of lactate, as shown by the ^2H kinetic-isotope effect. However, for the mutant enzyme it is clear that the slowest step is interdomain electron transfer between the FMN and haem prosthetic groups. In fact, the rate of haem reduction by lactate, as determined by the stopped-flow method, is decreased by more than 20-fold, from $445 \pm 50 \text{ s}^{-1}$ (25 °C, pH 7.5) in the wild-type enzyme to $21 \pm 2 \text{ s}^{-1}$ in the mutant enzyme. Decreases in kinetic-isotope effects seen with $[2\text{-}^2\text{H}]\text{lactate}$ for mutant enzyme compared with wild-type, both for flavin reduction (from 8.1 ± 1.4 to 4.3 ± 0.8) and for haem reduction (from 6.3 ± 1.2 to 1.6 ± 0.5) also provide support for a change in the nature of the rate-determining step. Other kinetic parameters determined by stopped-flow methods and with two external electron acceptors (cytochrome c and ferricyanide) under steady-state conditions are all consistent with this mutation having a dramatic effect on interdomain electron transfer. We conclude that Tyr-143, an active-site residue which lies between the flavodehydrogenase and cytochrome domains of flavocytochrome b_2 , plays a key role in facilitating electron transfer between FMN and haem groups.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a soluble component of the mitochondrial intermembrane space [1], where it catalyses the oxidation of L-lactate to pyruvate and transfers electrons to cytochrome c [2]. The enzyme is a tetramer of identical subunits of M_r 57 500 [3], with each subunit composed of two functionally distinct domains: a flavodehydrogenase domain containing flavin mononucleotide (FMN) and a cytochrome domain containing protohaem IX [2].

The crystal structure of flavocytochrome b_2 has been solved to 0.24 nm resolution [4] and shows two crystallographically distinguishable subunits in the asymmetric unit. In subunit 1, both the cytochrome and flavodehydrogenase domains are clearly seen in the electron-density map. However, in subunit 2 there is a molecule of pyruvate, the reaction product, located at the active site of the flavodehydrogenase domain, and the cytochrome domain is not visible, owing to positional disorder. The three-dimensional structure also reveals that, in subunit 1, the side chain of Tyr-143 is hydrogen-bonded to a haem propionate, whereas in subunit 2 this residue forms a hydrogen bond with the carboxylate group of pyruvate (Fig. 1).

From the structure, then, it would appear that Tyr-143 is uniquely placed to play an important, and perhaps key, role in controlling electron transfer from substrate to flavin and from flavin to haem. In the present paper we describe work in which we have probed the role of this residue by constructing and characterizing a mutant form of the enzyme (Y143F), in which Tyr-143 has been replaced by Phe.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

Escherichia coli MM294 was used for expression of wild-type and mutant flavocytochromes b_2 as reported elsewhere [5]. Site-directed mutagenesis was performed as previously described [6] using the oligonucleotide 295A (GTGGGCCTTCTATTCCT) (Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K.). Standard methods for growth of *E. coli*, plasmid purification, DNA manipulation and transformation were performed as described in Maniatis *et al.* [7].

Enzymes

Wild-type and Y143F flavocytochromes b_2 were isolated from frozen *E. coli* cells using the purification procedure previously reported [5]. All stopped-flow experiments were carried out with enzyme purified by hydroxyapatite chromatography as described elsewhere [5]. Steady-state experiments were carried out initially on enzyme purified to the $(\text{NH}_4)_2\text{SO}_4$ precipitation step and subsequently verified with hydroxyapatite-purified enzyme [5]. Purified enzyme preparations were stored under nitrogen at 4 °C as precipitates in 70% satd. $(\text{NH}_4)_2\text{SO}_4$. Flavocytochrome b_2 concentrations were determined spectrophotometrically by using previously published molar absorption coefficients [8].

Kinetic analysis

All kinetic experiments were carried out at 25.0 ± 0.1 °C in Tris/HCl at pH 7.5 and I 0.10. The buffer concentration was 10 mM in HCl, with I adjusted to 0.10 M by addition of NaCl.

Steady-state kinetic measurements involving the enzymic oxi-

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Fig. 1. Structure of the active sites in the crystallographically distinguishable subunits [4,11]

(a) Subunit 1 (pyruvate absent); (b) subunit 2 (pyruvate present). In subunit 1, Tyr-143 can be clearly seen hydrogen-bonding to a haem propionate group and in subunit 2 to the carboxylate group of pyruvate. W664 is a water molecule, *not* tryptophan. R376 is arginine-376 (etc.).

dation of L-lactate were performed using Pye–Unicam SP.8-400 and Beckman DU62 spectrophotometers. Ferricyanide and cytochrome *c* (horse heart; Type VI; Sigma) were used as electron acceptors where appropriate and at the concentrations indicated in Tables 1 and 3 (below). Reduction of ferricyanide

was monitored at 420 nm as previously described [8]. Cytochrome *c* reduction was monitored at 550 nm, published molar absorption coefficients for oxidized and reduced forms of the protein [9] being used.

Stopped-flow measurements were carried out with an Applied Photophysics SF.17MV microvolume stopped-flow spectrophotometer with a dead time of ≤ 1 ms. Flavin reduction was monitored at 438.3 nm (a haem isosbestic point), and haem reduction was monitored at 557 nm. Analysis of stopped-flow data was performed using the SF.17MV spectrometer software, which allows non-linear regression analysis of traces to analytical equations. Traces were fitted to single or double exponentials as appropriate; for wild-type and mutant enzymes, single exponential fits were sufficient at low lactate concentrations. At least four runs were performed at each L-lactate concentration.

K_m and k_{cat} parameters were determined using the SF.17MV spectrometer software, either by directly analysing experimental data using non-linear regression analysis or by analysing the appropriate double-reciprocal plots using linear-regression analysis; both methods gave self-consistent results. Kinetic parameters reported here are those from non-linear regression analysis.

Kinetic-isotope effects were measured by using [2- 2 H]lactate prepared and purified as previously described [10]. The purity of the [2- 2 H]lactate was confirmed by 1 H-n.m.r. spectrometry.

RESULTS

Steady-state kinetic parameters

The results of steady-state kinetic measurements with L-[2- 1 H]lactate and L-[2- 2 H]lactate as substrates and with ferricyanide and cytochrome *c* as electron acceptors are presented in Table 1. It is clear that there are a number of significant differences between the kinetic properties of wild-type and Y143F enzymes. The k_{cat} value for Y143F enzyme is dramatically reduced with cytochrome *c* as electron acceptor; however, with ferricyanide as electron acceptor the k_{cat} value is essentially the same, within experimental error, as that for wild-type flavocytochrome b_2 . The fact that these differences in k_{cat} values are dependent on the nature of the electron acceptor must reflect differences in the electron flow to these acceptors.

The 2 H kinetic-isotope effect measured under steady-state conditions for wild-type enzyme with ferricyanide as electron acceptor (4.7 ± 0.4) is identical, within experimental error, with the previously published kinetic-isotope-effect value of 5 [10] (which was determined under somewhat different conditions). This value showed that the major rate-limiting step is proton abstraction at C-2 of lactate [10]. This is illustrated in Scheme 1,

Table 1. Steady-state kinetic parameters and 2 H-kinetic-isotope effects (KIE) for wild-type and Y143F flavocytochromes b_2

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (*I* 0.10). Acceptors were used at the following concentrations: [cytochrome *c*], 30 μ M (this is 75 % saturating for wild-type enzyme and 95 % saturating for Y143F enzyme); [ferricyanide], 1 mM for wild-type enzyme (> 90 % saturating) and 8 mM for Y143F enzyme (71 % saturating). k_{cat} is expressed in electrons transferred/s per mol of enzyme (since L-lactate is a two-electron donor, these values can be halved to express them in terms of mol of substrate reduced/s). Abbreviations: [1 H]Lac, L-[2- 1 H]lactate; [2 H]Lac, L-[2- 2 H]lactate; WT, wild-type enzyme; Y143F, Tyr-143 $\rightarrow$ Phe mutant enzyme.

Enzyme	Electron acceptor	k_{cat} (s^{-1})		K_m (mM)		$10^{-5} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)		KIE
		[1 H]Lac	[2 H]Lac	[1 H]Lac	[2 H]Lac	[1 H]Lac	[2 H]Lac	
WT	Ferricyanide	400 $\pm$ 10	86 $\pm$ 5	0.49 $\pm$ 0.05	0.76 $\pm$ 0.06	8.2	1.1	4.7 $\pm$ 0.4
	Cytochrome <i>c</i>	207 $\pm$ 10	70 $\pm$ 10	0.24 $\pm$ 0.04	0.48 $\pm$ 0.10	8.6	1.5	3.0 $\pm$ 0.6
Y143F	Ferricyanide	400 $\pm$ 30	195 $\pm$ 20	2.90 $\pm$ 0.23	5.14 $\pm$ 1.10	1.4	0.4	2.0 $\pm$ 0.4
	Cytochrome <i>c</i>	22 $\pm$ 2	13 $\pm$ 2	0.23 $\pm$ 0.03	0.73 $\pm$ 0.03	1.0	0.2	1.7 $\pm$ 0.5

Scheme 1. Proposed mechanism of L-lactate oxidation by flavocytochrome b_2 [11]

(a) The Michaelis complex, with His-373 poised to remove the C-2 hydrogen as a proton. (b) The carbanion intermediate; electron transfer to the FMN group could be either via two one-electron transfers or via a covalent bond between substrate C-2 and flavin N-5. (c) Enzyme-product complex.

which shows the mechanism for L-lactate oxidation by flavocytochrome b_2 proposed on the basis of the crystal structure and various mechanistic studies [11,12].

It is noteworthy that the kinetic-isotope-effect value determined with cytochrome c as electron acceptor is significantly lower than that for ferricyanide, suggesting that electron transfer to cytochrome c contributes somewhat to the overall rate limitation in wild-type flavocytochrome b_2 . For the Y143F enzyme the kinetic-isotope-effect values found with both acceptors are substantially lower than those for the wild-type enzyme, consistent with a significant change in the nature of the rate-determining step in the mutant enzyme.

Table 2. Values of K_m for the electron acceptors ferricyanide and cytochrome c

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (I 0.10). The L-lactate concentration was kept at 10 mM throughout (this is 95 % saturating for wild-type enzyme and 78 % saturating for Y143F enzyme).

Enzyme	Electron acceptor ...	K_m	
		Ferricyanide	Cytochrome c
WT		$\ll 0.1$ mM	10 ± 1 μ M
Y143F		3.3 ± 0.6 mM	1.5 ± 0.2 μ M

With respect to K_m values for L-lactate, the main difference between Y143F and wild-type enzyme is observed when ferricyanide is used as acceptor. However, whereas a knowledge of K_m values is of immediate practical importance, interpretation of these steady-state constants is not easy, and their relationship to microscopic constants is not obvious in the case of a kinetic scheme with several steps, including interactions with an electron donor and acceptor. The mere fact that K_m values depend on the nature of the acceptor indicates that steps occurring after L-lactate reaction with FMN can influence this parameter (see the Discussion section).

Another significant difference in the steady-state kinetic behaviour of the wild-type and mutant flavocytochromes b_2 is the dependence of the reaction rates of these enzymes on the concentration of acceptor. The K_m values for ferricyanide and cytochrome c with both wild-type and Y143F enzymes are reported in Table 2. The most obvious difference is found with ferricyanide. For the wild-type enzyme reported K_m values range from 5 to about 200 μ M [13–16]. Under our experimental conditions we did not observe any concentration-dependence above 100 μ M-ferricyanide. The Y143F enzyme, however, showed a substantial K_m value for ferricyanide (Table 2), at least 30-fold larger than that for wild-type. This necessitated assays being carried out at a much higher ferricyanide concentration for the mutant than for the wild-type enzyme. A possible explanation for these changes in K_m can be found in the Discussion section.

Stopped-flow kinetic parameters

The reduction of the FMN and haem groups of wild-type and Y143F flavocytochromes b_2 by L-lactate and L-[2- 3 H]lactate was monitored by stopped-flow spectrophotometry as described in the Materials and methods section. As noted in previous work [10,17,18], at high lactate concentrations biphasic traces were observed with the rapid phase corresponding to at least 80 % of the absorbance change (monophasic traces were observed at low lactate concentrations, as described in the Materials and methods section). It has been well documented that the rapid phase corresponds to the initial reduction of FMN and haem groups by one L-lactate molecule, whereas the slow phase corresponds to entry of a third electron into the flavocytochrome b_2 promoter [10,18,19]. This occurs because full reduction of flavocytochrome b_2 , in the absence of electron acceptors, requires three electrons per protomer, i.e. six lactate molecules per tetramer [10]. Rate constants for the slow phase are about 20-fold lower than those for the rapid phase and indicate, as previously reported [10,17], that the slow phase is kinetically irrelevant during catalytic turnover of the enzyme. Thus, in the presence of an electron acceptor, flavocytochrome b_2 acts as a two-electron transferase.

Since it is the rapid phase which corresponds to the initial reduction of FMN and haem groups by one lactate molecule, we measured the rapid-phase reduction of FMN and haem by L-[2-

Table 3. Stopped-flow kinetic parameters and ^2H -kinetic-isotope effects (KIE) for the rapid-phase reduction of haem and FMN in wild-type and Y143F flavocytochromes b_2

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (1/0.10). Stopped-flow data were analysed as described in the Materials and methods section. k_{cat} values are expressed as the number of prosthetic groups reduced/s. Abbreviations: [^1H]Lac, L-[2- ^1H]lactate; [^2H]Lac, L-[2- ^2H]lactate; FMN, flavin mononucleotide.

Enzyme	Prosthetic group reduction	k_{cat} (s^{-1})		K_m (mM)		KIE
		[^1H]Lac	[^2H]Lac	[^1H]Lac	[^2H]Lac	
Wild-type	FMN	604 $\pm$ 60	75 $\pm$ 5	0.84 $\pm$ 0.20	1.33 $\pm$ 0.28	8.1 $\pm$ 1.4
	Haem	445 $\pm$ 50	71 $\pm$ 5	0.53 $\pm$ 0.05	0.68 $\pm$ 0.05	6.3 $\pm$ 1.2
Y143F	FMN	735 $\pm$ 80	171 $\pm$ 10	2.81 $\pm$ 0.30	3.87 $\pm$ 0.40	4.3 $\pm$ 0.8
	Haem	21 $\pm$ 2	13 $\pm$ 2	0.19 $\pm$ 0.02	0.37 $\pm$ 0.04	1.6 $\pm$ 0.5

^1H]lactate and L-[2- ^2H]lactate, the results of which are reported in Table 3. The kinetic-isotope-effect values for FMN and haem reduction in the wild-type enzyme are 8.1 and 6.3 respectively, and are the same, within experimental error, as those previously determined at 5 °C for the enzyme isolated from *S. cerevisiae* [10], confirming that proton abstraction at C-2 of lactate (Scheme 1) is the rate-determining step for FMN reduction in wild-type enzyme. The k_{cat} value for haem reduction by lactate is lower than that for FMN reduction and, therefore, not surprisingly, the kinetic-isotope effect is eroded somewhat (from about 8 to 6) (Table 3).

Stopped-flow data for Y143F enzyme shows major differences from the values found for wild-type flavocytochrome b_2 . Values of k_{cat} for the reduction of FMN by L-[2- ^1H]lactate and L-[2- ^2H]lactate are both slightly higher in the case of the Y143F enzyme, and the kinetic-isotope effect for FMN reduction falls from 8.1 to 4.3. However, more dramatic effects are observed for the reduction of haem. The k_{cat} value for reduction of haem by lactate falls from 445 s^{-1} in the wild-type enzyme to 21 s^{-1} in the mutant (Table 3). This represents a lowering in the rate of haem reduction by more than 20-fold in the Y143F enzyme.

Additionally, K_m values for L-lactate determined by the stopped-flow method show small, but significant, differences between wild-type and mutant enzymes. For example there is a 3–4-fold difference in K_m values between wild-type and Y143F enzymes when FMN reduction is monitored. These results would suggest that Tyr-143 plays only a very minor role in formation of the Michaelis complex. The overall catalytic efficiency of the mutant enzyme, as expressed by the value of k_{cat}/K_m , is somewhat lower than that of the wild-type enzyme ($k_{\text{cat}}/K_m = 7.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for wild-type and $2.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the mutant enzyme).

DISCUSSION

A comparison of the properties of wild-type flavocytochrome b_2 and the Y143F mutant enzyme provides an insight into the role of the Y143 residue in mediating electron transfer from L-lactate through to haem.

The first step, FMN reduction, is only slightly affected by the mutation, implying that the Tyr-143 phenolic -OH is by no means essential for this step in enzyme turnover. In fact it would appear that k_{cat} for FMN reduction is slightly increased in the mutant enzyme. It has been proposed that the role of this residue might be in stabilization of the carbanion intermediate (Scheme 1) by delocalization of the negative charge on to the tyrosine itself via proton exchange (see Fig. 3 of [20]). Replacement of Tyr-143 by phenylalanine would remove this possibility and might lead to an alteration in the transition state. In terms of

catalytic efficiency (k_{cat}/K_m) of FMN reduction the mutant enzyme is some 2–3-fold worse than the wild-type primarily because of an increase in the K_m value seen for the mutant enzyme.

The physiologically relevant catalytic cycle of flavocytochrome b_2 can be represented as shown in Scheme 2 (rate constants for wild-type and Y143F enzymes are shown for the appropriate steps). From this Scheme the much lower rate of lactate $\rightarrow b_2$ -haem electron transfer in the Y143F enzyme (more than 20-fold less than in the wild-type enzyme) can be seen in the context of the overall reaction. The obvious explanation for this substantial rate lowering is that the mutation has severely disrupted electron transfer between FMN and haem. This conclusion is supported by comparison of k_{cat} values for steps 1 and 2 (Scheme 2); for the mutant enzyme, k_{cat} for lactate $\rightarrow$ haem is 21 s^{-1} , compared with 735 s^{-1} for lactate $\rightarrow$ FMN electron transfer alone. For the wild-type enzyme the difference is very much smaller (445 s^{-1} compared with 604 s^{-1}). These results are consistent with the notion that, in the Y143F enzyme, it is FMN $\rightarrow$ haem electron transfer, and not C-2 proton abstraction, which is the major rate-determining step in the flow of electrons from L-lactate to the haem group.

This effect of the mutation on the rate-determining step is also apparent from kinetic-isotope-effect values. In the case of wild-type enzyme there is a steady erosion of the ^2H -kinetic-isotope effect as electrons flow to the external electron acceptor (8.1 for FMN reduction, 6.3 for haem reduction and 3.0 for cytochrome *c* reduction). These results are entirely consistent with previous work [10]. In contrast, for the Y143F mutant enzyme the kinetic-isotope-effect values for b_2 -haem and cytochrome *c* reduction are identical within error limits (1.6 $\pm$ 0.5 and 1.7 $\pm$ 0.5 respectively). Thus, in the Y143F enzyme, cytochrome *c* reduction makes no contribution to rate limitation in the overall reaction.

It has been previously shown that cytochrome *c* can accept electrons only from the haem group of flavocytochrome b_2 [14,21,22], whereas ferricyanide can accept electrons from both the haem and FMN groups [13,23]. This knowledge allows us to explain why the Y143F mutation has affected the electron-transfer rate to cytochrome *c* to a much greater extent than it has to ferricyanide. We have shown that FMN $\rightarrow b_2$ -haem electron transfer is the slowest step in the catalytic cycle of the Y143F enzyme; therefore it follows that the rate of cytochrome *c* reduction will be limited by this step. This is in fact the case, since the k_{cat} values for b_2 -haem and cytochrome *c* reduction are identical within experimental error.

The fact that FMN $\rightarrow$ haem electron transfer is the slowest step in the catalytic cycle of the Y143F enzyme also helps to explain the ferricyanide K_m value seen in steady-state experiments with the mutant enzyme. The k_{cat} value (Table 1) for the steady-

Scheme 2. Linear representation of the catalytic cycle of lactate oxidation and cytochrome c reduction by flavocytochrome b_2 .

Rate constants (25 °C) are reported for wild-type (WT) and mutant (Y143F) enzymes. ①, L-Lactate $\rightarrow$ FMN electron transfer (stopped-flow); ②, L-lactate $\rightarrow$ b_2 -haem electron transfer (stopped-flow); ③, L-lactate $\rightarrow$ cytochrome c electron transfer (steady-state). Abbreviations: $F_{\text{red.}}$, reduced FMN; $F_{\text{ox.}}$, oxidized FMN; $F_{\text{sq.}}$, semiquinone form of FMN; $H_{\text{ox.}}$, oxidized haem; $H_{\text{red.}}$, reduced haem; Cyt $c_{\text{ox.}}$, oxidized cytochrome c ; Cyt $c_{\text{red.}}$, reduced cytochrome c ; LAC, L-lactate; PYR, pyruvate.

Scheme 3. Electron transfer pathway from L-lactate to ferricyanide as catalysed by the Y143F mutant enzyme

After reduction of FMN by lactate, electrons are transferred directly from the reduced (flavohydroquinone) form of FMN to ferricyanide, by-passing electron transfer to the haem group. The resulting semiquinone form of FMN is oxidized by electron transfer to a second ferricyanide ion. Abbreviations: LAC, L-lactate; PYR, pyruvate; $F_{\text{ox.}}$, oxidized FMN; $H_{\text{ox.}}$, oxidized haem; $F_{\text{red.}}$, the reduced (flavohydroquinone) form of FMN; $F_{\text{sq.}}$, the semiquinone form of FMN.

state reduction of ferricyanide is almost 20-fold higher than that for b_2 -haem reduction (Table 3) in the Y143F enzyme. Thus it follows that, in the mutant enzyme, ferricyanide must be removing electrons from the flavohydroquinone and flavosemiquinone, completely by-passing the haem group, as illustrated in Scheme 3. In this respect the Y143F enzyme behaves in a similar fashion to the haem-free (dehaemo-) form of flavocytochrome b_2 reported by Iwatsubo *et al.* [13]. Dehaemo-

flavocytochrome b_2 also shows a significant K_m value for ferricyanide. The K_m values for electron acceptors measured under steady-state conditions in systems such as flavocytochrome b_2 are complex and do not represent straightforward dissociation constants. Iwatsubo *et al.* [13] have proposed a scheme in which ferricyanide takes electrons from the flavohydroquinone and flavosemiquinone in the dehaemo-enzyme. They derived [13] an equation expressing the dependence of electron-transfer rate on acceptor concentration at saturating lactate. This equation yields an expression in which the K_m for ferricyanide arises from a combination of rate constants involving electron transfer from substrate to flavin, flavohydroquinone to ferricyanide, and flavosemiquinone to ferricyanide, with ferricyanide being reduced some 20 times faster by the flavosemiquinone than by the hydroquinone [13]. We believe that the rationale given by Iwatsubo *et al.* [13] to explain the difference in K_m values for ferricyanide between holo- and dehaemo-enzyme may also explain the difference seen between wild-type and Y143F enzyme, particularly since the mutant enzyme behaves like the dehaemo-enzyme with respect to this acceptor (Scheme 3).

Finally it is worth considering the distance involved for electron transfer from FMN to haem. The shortest distance between the flavin ring and the porphyrin ring is 0.97 nm (9.7 Å) from flavin N-5 to haem C-2A. Of the various factors which affect intra-protein electron transfer, Dutton and co-workers [24,25] have suggested that the edge-to-edge distance between redox centres dominates the rate of intra-protein electron transfer. Using this simple theory one can estimate rates for electron transfer based on the distance between redox centres. Rates for FMN $\rightarrow$ haem electron transfer in wild-type flavocytochrome b_2 , which have been estimated by simulation studies ($500\text{--}600 \text{ s}^{-1}$) [26,27], are orders of magnitude lower than would be expected if the electron-transfer rate were controlled by edge-to-edge distance alone [25]. There are a number of possible explanations as to why this should be so. For example, the inhomogeneous protein medium between the flavin and haem groups may mediate the long-range electronic coupling between these two redox centres, in a manner similar to that suggested by Beratan *et al.* [28], even though the most direct path from FMN to haem would involve only solvent molecules. Another explanation, and one that we favour, is that

the haem domain (which is linked to the flavodehydrogenase domain by a hinge region) is rather mobile in solution and there is therefore no 'fixed' 0.97 nm (9.7 Å) gap between the FMN and haem groups. The existence of haem domain mobility is supported by the flavocytochrome b_2 crystal structure [4] and by previously published n.m.r. studies [29]. The apparent rate of electron transfer might actually be governed by the recognition process between the two domains or the number of productive complexes existing at any one time. In the case of Y143F enzyme an important interdomain hydrogen bond is lost which might increase the haem-domain mobility and impair recognition between the two domains, thereby dramatically affecting FMN → haem electron transfer.

Conclusions

From our studies on Y143F flavocytochrome b_2 we draw the following conclusions: (i) Tyr-143 is crucial in facilitating electron transfer between the flavodehydrogenase and cytochrome domains of the enzyme; (ii) the Tyr-143 → Phe mutation shifts the rate-determining step in the reduction of the enzyme from proton abstraction at C-2 of lactate to interdomain (FMN to haem) electron transfer; (iii) in Y143F enzyme, ferricyanide removes electrons from the FMN group only, in contrast with the wild-type enzyme, where electrons are removed from both haem and FMN groups.

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REFERENCES

- Daum, G., Böhni, P. C. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13028–13033
- Chapman, S. K., White, S. A. & Reid, G. A. (1991) *Adv. Inorg. Chem.* **36**, 257–301
- Jacq, C. & Lederer, F. (1974) *Eur. J. Biochem.* **41**, 311–320
- Xia, Z.-X. & Mathews, F. S. (1990) *J. Mol. Biol.* **212**, 837–863
- Black, M. T., White, S. A., Reid, G. A. & Chapman, S. K. (1989) **258**, 255–259
- Reid, G. A., White, S. A., Black, M. T., Lederer, F., Mathews, F. S. & Chapman, S. K. (1988) *Eur. J. Biochem.* **178**, 329–333
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Labeyrie, F., Baudras, A. & Lederer, F. (1978) *Methods Enzymol.* **53**, 238–256
- Hazzard, J. T., Cusanovich, M. A., Tainer, J. A., Getzoff, E. D. & Tollin, G. (1986) *Biochemistry* **25**, 3318–3328
- Pompon, D., Iwatsubo, M. & Lederer, F. (1980) *Eur. J. Biochem.* **104**, 479–488
- Lederer, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed.), vol. 2, pp. 153–242, CRC Press, Boca Raton, FL
- Lederer, F. & Mathews, F. S. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E. & McCormick, D. B., eds.), pp. 133–142, Walter de Gruyter, Berlin
- Iwatsubo, M., Mevel-Ninio, M. & Labeyrie, F. (1977) *Biochemistry* **16**, 3558–3566
- Morton, R. K., Armstrong, J. McD. & Appleby, C. A. (1961) in *Haematin Enzymes* (Falk, J. E., Lemberg, R. & Morton, R. K., eds.), pp. 501–523, Pergamon Press, Oxford
- Hiromi, K. & Sturtevant, J. M. (1966) in *Flavins and Flavoproteins* (Slater, E. C., ed.), pp. 283–305, Elsevier, Amsterdam
- Ogura, Y. & Hasegawa, H. (1962) *Biochem. J.* **52**, 275–278
- Capeillère-Blandin, C., Bray, R. C., Iwatsubo, M. & Labeyrie, F. (1975) *Eur. J. Biochem.* **54**, 549–566
- Capeillère-Blandin, C. (1991) *Biochem. J.* **274**, 207–217
- Pompon, D. (1980) *Eur. J. Biochem.* **106**, 151–159
- Ghisla, S. & Massey, V. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S. & Zanetti, G., eds.), pp. 123–130, Walter de Gruyter, Berlin
- Ogura, Y. & Nakamura, T. (1966) *J. Biochem. (Tokyo)* **60**, 77–86
- Capeillère-Blandin, C., Iwatsubo, M., Testylier, G. & Labeyrie, F. (1980) in *Flavins and Flavoproteins* (Yagi, K. & Yamano, T., eds.), pp. 617–630, Japan Scientific Societies Press, Tokyo
- Tegoni, M., Janot, J.-M. & Labeyrie, F. (1990) *Eur. J. Biochem.* **190**, 329–342
- Gunner, M. R. & Dutton, P. L. (1989) *J. Am. Chem. Soc.* **111**, 3400–3412
- Moser, C. C., Warncke, K., Keske, J. M. & Dutton, P. L. (1991) *J. Inorg. Biochem.* **43**, 91
- Capeillère-Blandin, C. (1975) *Eur. J. Biochem.* **56**, 91–101
- Pompon, D. (1980) *Eur. J. Biochem.* **106**, 151–159
- Beratan, D. N., Onuchic, J. N., Betts, J., Bowler, B. E. & Gray, H. B. (1990) *J. Am. Chem. Soc.* **112**, 7915–7921
- Labeyrie, F., Beloeil, J.-C., Thomas, M.-A. (1988) *Biochim. Biophys. Acta* **953**, 131–141

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