Isolation and characterization of the flavin-binding domain of flavocytochrome *b₂* expressed independently in *Escherichia coli*

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Flavocytochrome *b₂* consists of two distinct domains. The N-terminal domain contains protohaem IX and the larger, C-terminal domain contains flavin mononucleotide (FMN). We describe here the isolation of the flavin-binding domain expressed in *Escherichia coli* independent of the cytochrome domain. The isolated domain is an efficient lactate dehydrogenase with ferricyanide as electron acceptor but reduces cytochrome *c*, the

physiological oxidant for flavocytochrome *b₂*, extremely poorly; electron transfer from the flavin-binding domain to the separately expressed cytochrome domain is undetectable. FMN reduction by lactate occurs as a single exponential process in the isolated flavin-binding domain, in contrast to the biphasic kinetics observed with native flavocytochrome *b₂*.

INTRODUCTION

Flavocytochrome *b₂* (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) from *Saccharomyces cerevisiae* is a homotetramer of subunit *M*, 57 500 [1]. The enzyme is a soluble component of the mitochondrial intermembrane space [2] where it catalyses the oxidation of L-lactate to pyruvate with subsequent electron transfer to cytochrome *c* [3]. The crystal structure of flavocytochrome *b₂* has been solved to 0.24 nm resolution [4] and shows that each subunit consists of two distinct domains: an N-terminal cytochrome domain containing protohaem IX and a C-terminal domain containing flavin mononucleotide (FMN). Various attempts have been made to isolate these domains using controlled proteolysis of the intact holoenzyme [5–7] and the cytochrome domain has been successfully isolated after trypsin digestion. This domain has more recently been prepared by expression in *Escherichia coli*, independent of the flavin domain [8]. In contrast, the flavin domain has proven difficult to isolate using proteolytic methods, particularly because of a highly proteinase-sensitive region within this part of the protein [9]. Celerier et al. [7] succeeded in isolating the flavodehydrogenase domain of *Hansenula anomala* flavocytochrome *b₂* after mild proteolysis of the intact enzyme by an *H. anomala* protease preparation.

Expression of the flavin domain, independent of the cytochrome domain, would facilitate the investigation of its biochemical properties and would also enable the redox changes at the flavin to be monitored spectrophotometrically without interference from the haem. We have therefore developed a system for the expression in *E. coli* of the flavin domain of flavocytochrome *b₂*, purified this flavoprotein and investigated its spectroscopic and kinetic properties.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

The flavocytochrome *b₂* coding sequence was modified by site-directed mutagenesis as described previously [8,10] using the oligonucleotide 074A (CTCCTGGTTGAATTCATGGAACTAAG). This oligonucleotide introduces an *EcoRI* cleavage

site between the sequences encoding the cytochrome and flavin-containing domains and places an ATG initiation codon immediately preceding the codon for Glu-101, the first residue of the flavin domain. Resulting clones were screened for the introduction of the novel *EcoRI* site and one positive clone was then subjected to DNA sequence analysis to verify the nature of the modification and to ensure that no further, unwanted mutations had been introduced.

The DNA encoding the flavin-containing domain was transferred to the expression vector pRC23 [11] as an *EcoRI*–*HindIII* fragment. This vector contains the λP_L promoter, enabling high levels of transcription through the inserted sequence. *E. coli* NF1, which expresses a temperature-sensitive *cl* repressor, was used to enable tight regulation of transcription. Transformants grown to mid-exponential phase at 30 °C and then shifted to 37 °C for 2 h expressed the flavin-containing domain as 5–10 % of total cell protein. We found also during the course of this work that high levels of expression could be achieved simply by growing at 37 °C using an inoculum of 1 ml per litre of medium.

E. coli MM294 was used as a host for recombinant plasmids and all bacterial growth was carried out in Luria broth, containing ampicillin at 50 mg/l where appropriate. Standard methods for growth of *E. coli*, plasmid purification, DNA manipulation and transformation were performed as described in Sambrook et al. [12].

Purification of the flavin domain

Frozen *E. coli* cells were suspended in 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM lactate [lithium L-(+)-lactate, Sigma] and 10 mM EDTA. Lysozyme was added to 0.2 mg/ml and the mixture was incubated with stirring for 30 min at 0–4 °C. The suspension was centrifuged at 39 000 *g* for 20 min. The pale brownish-yellow supernatant was retained and the pellet, consisting of cell debris and unbroken cells, was subjected to a second cycle of lysis and centrifugation as above. The two supernatants were combined, brought to 30 % satn. with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 39 000 *g* for 10 min. The supernatant was brought to 70 % satn. $(\text{NH}_4)_2\text{SO}_4$ and the pale yellow

precipitate was collected by centrifuging for 10 min at 39000 *g*. The precipitate was dissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.0, containing 10 mM L-(+)-lactate and dialysed against this same buffer overnight at 4 °C under an atmosphere of nitrogen. The solution was dialysed for a further hour against 10 mM sodium phosphate buffer, pH 7.0, allowing the enzyme to oxidize in the absence of lactate so that its yellow colour could be seen in the subsequent column chromatography.

Following dialysis the solution was centrifuged at 39000 *g* for 10 min to remove any insoluble debris and loaded on to a DE-52 cellulose (Whatman) anion-exchange column (15 cm × 2.5 cm) that had been equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The column was washed with 100 ml of this same buffer and bound proteins were eluted with a gradient of 10–50 mM sodium phosphate buffer, pH 7.0, in 10 mM steps. The yellow band containing the flavin-binding domain of flavocytochrome *b*₂ began to elute between 30 and 50 mM phosphate. Fractions were collected and assayed for lactate dehydrogenase activity using ferricyanide as the electron acceptor and monitoring the decrease in absorbance at 420 nm. Active fractions with A_{280}/A_{460} of 8.56 or less were pooled, concentrated and stored in the presence of 10 mM lactate in liquid nitrogen. Protein thus purified migrated as a single detectable band of 47000-*M_r* on SDS/PAGE.

Molecular mass determination

A Sepharose S300 (Pharmacia LKB) gel-filtration column (150 cm × 3 cm) was used to determine the native molecular mass of the enzyme using the following markers: wild-type flavocytochrome *b*₂ (*M_r* 230000), β-amylase (*M_r* 200000), alcohol dehydrogenase (*M_r* 150000), BSA (*M_r* 66000) and carbonic anhydrase (*M_r* 29000).

Kinetic analysis

Kinetic experiments were performed with enzyme purified as described above. Steady-state experiments were performed at 25 ± 0.1 °C in Tris/HCl, pH 7.5, and 1.0 M. The buffer concentration was 10 mM in HCl, with *I* adjusted to 0.10 M by addition of NaCl. Ferricyanide (potassium salt, BDH) reduction was monitored by the decrease in absorbance at 420 nm (ϵ_{420} 1010 M⁻¹·cm⁻¹) using a Beckman DU-62 spectrophotometer. The reduction of cytochrome *c* (horse heart, Sigma type-VI) was followed by the increase in absorbance at 550 nm using published absorption coefficients for the reduced and oxidized forms [13]. Enzyme concentrations were estimated spectrophotometrically for the oxidized enzyme using published molar absorption coefficients for haem-free flavocytochrome *b*₂ [14].

Stopped-flow experiments were carried out with an Applied Photophysics SF.17MV stopped-flow spectrofluorimeter using the same buffer and temperature as with steady-state measurements. Flavin reduction was monitored by the absorbance decrease at 452 nm and the data were collected and analysed as described previously [15].

RESULTS

Isolation of the flavin-binding domain

By introducing a translation initiation codon immediately preceding the DNA sequence encoding the flavin-binding domain of flavocytochrome *b*₂ we have developed a system for the independent expression of this domain in *E. coli*, allowing its isolation in large quantities. The protein can be purified with good yields by (NH₄)₂SO₄ fractionation and one column chromatography

step (Table 1) resulting in a preparation that is homogeneous, migrating as a single band of *M_r* 47000 on SDS/PAGE and with an A_{280}/A_{460} ratio of 8.56. The enzyme behaves as a species of *M_r* 175000 ± 30000 on gel filtration. The calculated monomer relative molecular mass is 45704 and each native monomer contains one molecule of FMN (456-*M_r*). The native flavin-binding domain as isolated from *E. coli* thus behaves as a tetramer. This is to be expected from analysis of the crystal structure of the intact flavocytochrome *b*₂ since its tetrameric nature is maintained by intersubunit contacts between the flavin-binding domains, with no contribution from the cytochrome domains [4].

In the oxidized state the isolated flavin-binding domain is bright yellow in colour and has an electronic absorption spectrum with maxima at 452 nm and 375 nm (Figure 1).

Steady-state kinetic analysis

The isolated flavin domain of flavocytochrome *b*₂ is capable of oxidizing L-lactate when ferricyanide is used as an electron acceptor but shows a cytochrome *c* reductase activity that is about 10⁴-fold lower than that observed with intact flavocytochrome *b*₂. This observation of activity, albeit extremely low, is in contrast with previous reports that the haem domain of flavocytochrome *b*₂ from *Hansenula anomala* is essential for electron transfer to cytochrome *c* [7,14]. We have, however, been unable to observe electron transfer between the separately expressed and purified flavin and cytochrome [8] domains of flavocytochrome *b*₂, even at high concentrations of protein, emphasizing the requirement for covalent attachment between the domains for efficient electron transfer to take place. We estimate that the sensitivity of our measurements would have allowed us to detect a rate 10⁶-fold lower than that seen with the intact enzyme.

We have analysed the steady-state kinetic behaviour of the flavin-binding domain, monitoring the reduction of ferricyanide in the presence of substrate (L-lactate or L-[2-³H]lactate). The results of these experiments are summarized in Table 2, where they are compared with previously determined kinetic parameters for intact flavocytochrome *b*₂. The *k_{cat}* value for L-lactate:ferricyanide reductase activity measured for the isolated flavin domain is about one-third lower than with the wild-type flavocytochrome *b*₂. The *K_m* value for the flavin domain is about half that of the wild-type enzyme so that the values for the catalytic efficiency (*k_{cat}*/*K_m*) differ only slightly and this difference is within experimental error. It thus appears that removal of the cytochrome domain has a negligible effect on catalysis at the active site of the flavin domain.

The rate-limiting step in lactate oxidation catalysed by intact flavocytochrome *b*₂ is the removal of the C-2 hydrogen of the substrate [16] and this is reflected in the measured kinetic isotope effect (KIE) of 4.7 ± 0.3. The KIE observed with the isolated flavin-binding domain is of a similar magnitude to that observed with the wild-type, intact enzyme but is slightly lower, indicating that C-2 proton abstraction may not be solely rate-limiting and that other processes may contribute. Our recent work indicates that this contribution most probably comes from the relatively slow electron transfer from flavin semiquinone to ferricyanide.

The L-lactate:ferricyanide reductase activity of wild-type flavocytochrome *b*₂ is effectively saturated at very low acceptor concentrations (*K_m* ≪ 0.1 mM) so that the observed rate of reaction is independent of ferricyanide concentration over the experimentally useful concentration range (sufficient must be present to allow the absorbance change on reduction to be

Table 1 Purification of the flavocytochrome *b₂* flavin-binding domain from *E. coli*

Details of the purification procedure are described in the Materials and methods section. The results are shown for a typical preparation starting with 18 g of bacterial wet weight.

Purification stage	Total volume (ml)	Total activity (μmol of ferricyanide reduced/s)	Recovery (%)	Total protein (mg)	Purification (fold)
Supernatant from cell lysis	330	265	100	1653	1
After $(\text{NH}_4)_2\text{SO}_4$ fractionation and dialysis	122	225	85	1159	1.2
After DE-52 column	222	122	46	101	7.5

Figure 1 U.V./visible absorption spectrum of the isolated flavin domain

The flavin-binding domain was dissolved in Tris/HCl buffer, pH 7.5.

observed). In contrast, the isolated flavin domain exhibits typical saturation kinetics with a K_m for ferricyanide of 0.58 mM. Similar behaviour was reported by Iwatsubo et al. [14] who measured a K_m value of 0.5 mM, at 15 °C, for *H. anomala* flavocytochrome *b₂* from which the haem had been removed.

Preliminary experiments failed to detect cytochrome *c* reductase activity with the separately expressed flavin-binding domain [17]. By using relatively high enzyme concentrations we have been able to increase the sensitivity of our assays and to

Table 3 Steady-state kinetic parameters for the isolated flavin domain and intact, wild-type flavocytochrome *b₂* with cytochrome *c* as electron acceptor

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 ($I = 0.10$), using 10 mM L-lactate as the electron donor and cytochrome *c* as acceptor.

Enzyme	k_{cat} (s^{-1})	K_m cyt <i>c</i> (μM)	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Flavin domain	0.020 ± 0.002	23 ± 7	$(8.7 \pm 5.3) \times 10^2$
Wild type	207 ± 2	10 ± 1	$(2.1 \pm 0.2) \times 10^7$

consistently measure activity, albeit at very low rates. The data shown in Table 3 allow a comparison of the steady-state kinetic parameters with cytochrome *c* as electron acceptor between the isolated flavin-binding domain and intact flavocytochrome *b₂*. The maximum rate of reaction has decreased by a factor of 10^4 and the K_m has increased more than twofold so that the catalytic efficiency (k_{cat}/K_m) for this reaction has decreased 24000-fold.

Stopped-flow kinetics

Reduction by L-lactate of FMN in the isolated flavin domain of flavocytochrome *b₂* was followed by stopped-flow spectrometry. Monophasic kinetics were observed throughout the range of lactate concentrations used (0.1–30 mM). Although monophasic kinetics were observed for fully active flavin domain we found that enzyme which had lost activity exhibited biphasic behaviour; however, this is most likely due to heterogeneity in the enzyme. The monophasic reduction of fully active enzyme contrasts dramatically with the behaviour of wild-type flavocytochrome *b₂* which exhibits biphasic FMN reduction, notably at high concentrations of lactate [15,16,18,19]. The fast phase corresponds to the initial two-electron reduction of FMN but subsequent reduction of the haem and interprotomer electron transfer

Table 2 Steady-state kinetic parameters and ^2H kinetic isotope effects for the isolated flavin domain and intact, wild-type flavocytochrome *b₂*

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 ($I = 0.10$), using ferricyanide as the electron acceptor, at a concentration of 10 mM. The values for the wild-type enzyme are taken from [15] and were obtained under identical conditions except that the concentration of ferricyanide was 1 mM. k_{cat} is expressed as mol electrons transferred/s per mol enzyme. Abbreviations: WT, wild-type flavocytochrome *b₂*; KIE, deuterium kinetic isotope effect; $[^1\text{H}]\text{lac}$, L- $[^1\text{H}]\text{lactate}$; $[^2\text{H}]\text{lac}$, L- $[^2\text{H}]\text{lactate}$.

Enzyme	k_{cat} (s^{-1})		K_m (mM)		k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)		KIE
	$[^1\text{H}]\text{lac}$	$[^2\text{H}]\text{lac}$	$[^1\text{H}]\text{lac}$	$[^2\text{H}]\text{lac}$	$[^1\text{H}]\text{lac}$	$[^2\text{H}]\text{lac}$	
Flavin domain	273 ± 6	72 ± 2	0.22 ± 0.05	0.41 ± 0.06	$(1.2 \pm 0.4) \times 10^6$	$(1.4 \pm 0.2) \times 10^5$	3.8 ± 0.2
Wild type	400 ± 10	86 ± 5	0.49 ± 0.05	0.76 ± 0.06	$(8.2 \pm 1.1) \times 10^5$	$(1.1 \pm 0.2) \times 10^5$	4.7 ± 0.3

Table 4 Stopped-flow kinetic parameters and ^2H kinetic isotope effects for isolated flavin domain and wild-type flavocytochrome b_2

All experiments were carried out at 25 °C Tris/HCl buffer, pH 7.5 ($I = 0.10$). Stopped-flow data for FMN reduction were analysed as described in the Materials and methods section. The values for wild-type flavocytochrome b_2 relate to the fast phase of flavin reduction and are taken from [15]. k_{cat} is expressed as the number of prosthetic groups reduced/s. Abbreviations: WT, wild-type flavocytochrome b_2 ; KIE, kinetic isotope effect; $[^1\text{H}]\text{lac}$, L- $[^1\text{H}]\text{lactate}$; $[^2\text{H}]\text{lac}$, L- $[^2\text{H}]\text{lactate}$.

Enzyme	k_{cat} (s^{-1})		K_{m} (mM)		KIE
	$[^1\text{H}]\text{lac}$	$[^2\text{H}]\text{lac}$	$[^1\text{H}]\text{lac}$	$[^2\text{H}]\text{lac}$	
Flavin domain	400 ± 40	69 ± 3	0.26 ± 0.07	0.38 ± 0.09	5.8 ± 0.9
Wild type	604 ± 60	75 ± 5	0.84 ± 0.2	1.33 ± 0.28	8.1 ± 1.4

between flavins allows the entry of a third electron per protomer (slow phase). In the isolated flavin domain there is no opportunity for reoxidation of the flavin, thus no interprotomer electron transfer occurs and only one phase of FMN reduction is observed.

The kinetic parameters measured by stopped-flow are summarized and compared with previously determined values for the wild-type flavocytochrome b_2 in Table 4. The observed k_{cat} value for FMN reduction in the isolated flavin domain is only slightly lower than the corresponding figure for the wild-type enzyme and the kinetic isotope effects are identical within experimental error.

DISCUSSION

The independent expression of the flavin-binding domain of *S. cerevisiae* flavocytochrome b_2 opens up many possible experimental approaches that had hitherto been inaccessible. Previous workers had attempted to study the properties of the flavin domain in a dehaemo-enzyme prepared by denaturation of the holoenzyme to remove both prosthetic groups followed by reconstitution in the presence of FMN [14]. It has since been suggested that conformational differences exist between native and reconstituted flavocytochrome b_2 [20]. In the native holoenzyme, the flavin absorbance is swamped by the much more intense haem absorbances so that it is extremely difficult to monitor the spectroscopic properties of the flavin. This problem is clearly overcome in the isolated flavin domain. Flavocytochrome b_2 is a member of a family of 2-hydroxy-acid dehydrogenases, most of which consist of a single domain and transfer electrons to molecular oxygen rather than a cytochrome [21,22]. The isolated flavin domain is therefore of interest from two viewpoints: as a tool to examine the properties and function of the flavin domain within the intact enzyme and as an L-lactate dehydrogenase in its own right.

The lack of observable electron transfer from the purified flavin-binding domain to the isolated cytochrome domain demonstrates the importance of the interdomain interaction for electron transfer. In the intact enzyme, the cytochrome domain is mobile relative to the flavin domain [4,23,24]. It is likely that the covalent linkage between the two domains serves not only to restrict this mobility, maintaining a high collision frequency, but also to achieve the correct relative orientation of the prosthetic groups for electron transfer.

Kinetic studies have shown that the isolated flavin-binding domain of flavocytochrome b_2 is almost as efficient an L-lactate dehydrogenase as is the intact, wild-type enzyme but transfers electrons to cytochrome c 10^4 -fold less well. Previous reports

Figure 2 Linear representation of the catalytic cycle of lactate oxidation and ferricyanide reduction by flavocytochrome b_2

The overall catalytic cycle is shown schematically at the top with the experimentally measured steps below: A, L-lactate $\rightarrow$ FMN electron transfer (stopped-flow); B, L-lactate $\rightarrow$ ferricyanide electron transfer. The k_{cat} values shown for the isolated flavin-binding domain and intact, wild-type flavocytochrome b_2 were determined at 25 °C.

[7,14,17] have indicated that the flavin-binding domain of flavocytochrome b_2 is unable to reduce cytochrome c . The contrast with the observations reported here are almost certainly due simply to the fact that we have been able to detect very low activities by using very large amounts of the overexpressed protein, whereas most previous work was performed with relatively small amounts of the purified domain released by partial proteolysis of the intact enzyme.

The proposed catalytic cycle for the lactate:ferricyanide reductase activity of the isolated flavin domain is shown diagrammatically in Figure 2. Although the rate-determining step is unaltered in the isolated flavin domain, the first step in the catalytic cycle, FMN reduction, is somewhat slower in the isolated domain than in the intact protein, even though all of the catalytically important, active-site residues [21,25] are located within the flavin-binding domain. One possible reason for the observed decrease in the rate of substrate oxidation is that removal of the cytochrome domain increases accessibility of the active site to solvent, thereby affecting the dielectric nature of the catalytic centre.

The lactate:ferricyanide oxidoreductase activity of the isolated flavin-binding domain, in contrast with native flavocytochrome b_2 , exhibits a marked dependence on the concentration of acceptor, a feature shared with the Y143F mutant enzyme, in which flavin to haem electron transfer has been disrupted [15]. In the native enzyme, ferricyanide is essentially reduced exclusively by the b_2 haem, with electron transfer from the flavin hydroquinone being considerably slower. In the Y143F mutant it was concluded that the rate of haem reduction by the flavin was lowered such that the rate of electron transfer from flavin to ferricyanide became more significant, bypassing the haem. Similarly, it was shown that the observed K_{m} for ferricyanide observed with dehaemo-flavocytochrome b_2 [14] arises from a combination of the steps involved in electron transfer from substrate to flavin, and from the hydroquinone and semiquinone forms of flavin to ferricyanide. The isolated flavin domain, like the dehaemo-enzyme, can only reduce ferricyanide via the flavin since haem is absent and the observed K_{m} values for ferricyanide in these two forms of the enzyme are very similar.

Perhaps the most striking difference in the kinetic

Figure 3 Schematic representation of the reduction of the flavocytochrome b_2 tetramer

(a) 1, The wild-type enzyme undergoes an initial phase of FMN reduction by four molecules of L-lactate (eight electrons); 2, electron transfer from FMNH $_2$ to haem yields the flavin semiquinone; 3, intramolecular redistribution of the electrons between protomers yields fully oxidized FMN which is then a substrate for further reduction; 4, full reduction of the enzyme by a further two molecules of lactate (four electrons). Steps 3 and 4 correspond to the slow phase of FMN reduction observed in stopped-flow experiments. This scheme is based on that proposed by Pompon [19]. (b) In the isolated flavin domain, full reduction occurs by electron transfer from four molecules of lactate (eight electrons), the equivalent of step 1 in the scheme for wild-type flavocytochrome b_2 and no further reduction is possible. A single phase of FMN reduction is therefore observed in stopped-flow experiments.

characteristics of the isolated flavocytochrome b_2 flavin domain and the intact, native enzyme is seen in stopped-flow experiments. FMN in the isolated domain is reduced as a single exponential process, as found also in the dehaemo-enzyme [14], whereas FMN reduction in the intact holoenzyme is biphasic [15,16,18,19]. The slow phase has been attributed to an intramolecular redistribution of electrons after haem reduction (Figure 3a). In the isolated flavin domain, this interprotomer electron transfer

cannot occur since haem is absent and thus only one phase of flavin reduction is observed (Figure 3b).

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