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Isolation and characterization of the cytochrome domain of flavocytochrome b_2 expressed independently in *Escherichia coli*

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The cytochrome domain of flavocytochrome b_2 (L-lactate dehydrogenase) was expressed in the bacterium *Escherichia coli* and a purification procedure was developed. When expressed in *E. coli*, the b_2-cytochrome domain contains protohaem IX and has an electronic absorption spectrum identical with that of the cytochrome b_2 'core' produced by proteolytic cleavage of the enzyme isolated from yeast. The b_2-cytochrome domain isolated from *E. coli* has an M_r of 10500 and a redox potential of -31 ± 2 mV. High-field n.m.r. studies indicate pK_a values for the haem propionate groups to be 4.8 and 4.6, consistent with these groups being exposed to solvent rather than buried inside the protein. Using n.m.r. spectroscopy, we have determined an electron self-exchange rate constant for the b_2-cytochrome domain of 2.3×10^6 $M^{-1} \cdot s^{-1}$, which is more than two orders of magnitude larger than the value obtained for microsomal cytochrome b_5, a homologue of b_2-cytochrome domain.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a tetramer of identical subunits, with M_r 57500 [1]. The enzyme is a soluble component of the mitochondrial intermembrane space [2], where it catalyses the oxidation of L-lactate to pyruvate and transfers electrons to cytochrome *c* [3]. The crystal structure of flavocytochrome b_2 has been solved to 0.24 nm resolution [4] and reveals that each subunit consists of two distinct domains: an *N*-terminal haem-containing, or cytochrome, domain; and a *C*-terminal flavin mononucleotide-containing domain. Various attempts have been made to isolate these functionally distinct domains independently by using proteolytic methods [5–7]. Labeyrie *et al.* [5] isolated a tryptic fragment of M_r 11000 that contained haem but was devoid of flavin and had no lactate dehydrogenase activity. The fragment, referred to as the 'cytochrome b_2 core', was found to have spectral properties very like those of microsomal cytochrome b_5 [5]. The isolated cytochrome b_2 core was subsequently shown to consist of residues 8–103 of the mature flavocytochrome b_2 amino acid sequence [6].

An alternative, 'cleaner', method of obtaining the b_2-cytochrome domain alone would be to express this domain independently in *Escherichia coli*. In the present paper we describe the expression of the b_2-cytochrome domain in *E. coli* and its purification, and report initial biophysical characterization of this protein by spectroscopic and other methods.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

We have previously described a system for efficient expression of active flavocytochrome b_2 in *E. coli* [8]. To express the b_2-cytochrome domain alone we have used the same parent vector, pDS6 [9], and have modified the flavocytochrome b_2 gene by site-directed mutagenesis, introducing a TGA stop codon immediately after the codon for Gly-100. An *Eco*RI-cleavage site was also introduced overlapping with the TGA codon so that the DNA encoding the haem domain could be excised from a mutant clone as an *Eco*RI restriction fragment. Mutagenesis was performed as previously described [10] with the use of the oligonucleotide *074*A (CTCCTGGTTGAATTCA-TGGAAACTAAG) with single-stranded DNA from an M13mp19 clone containing the entire flavocytochrome b_2 coding sequence. Resulting clones were screened for the introduction of the novel *Eco*RI-cleavage site, and one positive clone was then subjected to DNA sequencing, which showed that the stop codon had been introduced as expected and that no further, unwanted, mutations had been introduced into the coding sequence. The *Eco*RI fragment containing the b_2-cytochrome domain coding sequence was inserted in the *Eco*RI site of pDS6, and a recombinant was selected with the correct orientation of the insert for expression.

E. coli MM294 harbouring the recombinant pDS-core was found to express the b_2-cytochrome domain at a high level. Standard methods for growth of *E. coli*, plasmid purification, DNA manipulation and transformation were performed as described in Maniatis *et al.* [11].

b_2-cytochrome domain isolation

Frozen *E. coli* cells were resuspended in 100 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-EDTA. Lysozyme (Sigma) was added to approx. 0.2 mg/ml and the mixture was incubated, with stirring, for 30 min at 4 °C. The solution was centrifuged at 39000 *g* for 20 min. The red supernatant was retained, and the pellet, consisting of cell debris and unlysed cells, was resuspended and subjected to a second lysis followed by a second centrifugation. The supernatant from this second lysis (again red in colour) was combined with that from the first and the solution was adjusted to 30% $(NH_4)_2SO_4$ saturation and centrifuged at 39000 *g* for 10 min. The pellet from this was discarded, and the supernatant was adjusted to 70% $(NH_4)_2SO_4$ saturation, at which point the b_2-cytochrome domain was precipitated. The precipitate was collected by centrifugation at 39000 *g* for 10 min. The red precipitate was redissolved in a minimum volume of 10 mM-sodium phosphate buffer, pH 7.0, and dialysed against this same buffer overnight at 4 °C.

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Table 1. Purification data for isolation of the b_2-cytochrome domain from *E. coli*

A detailed description of the procedure is given in the Materials and methods section.

Purification step	Total protein (mg)	b_2-cytochrome domain (mg)	Recovery (%)	A_{275}/A_{413} ratio
Supernatant after cell lysis	500	8	100	> 6
After $(NH_4)_2SO_4$ and dialysis	160	7.1	89	1.9
After hydroxyapatite column chromatography	28	4.3	54	0.56
After DE-52 DEAE-cellulose column chromatography	3.2	2.2	27	0.27
After Sephadex G-75 column chromatography	2.0	2.0	25	0.19

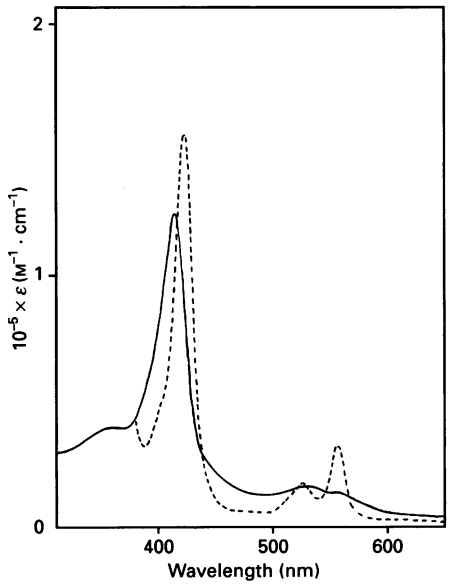

Fig. 1. Visible absorption spectrum of the b_2-cytochrome domain after isolation from *E. coli*

The oxidized and reduced states are denoted by the continuous and broken lines respectively.

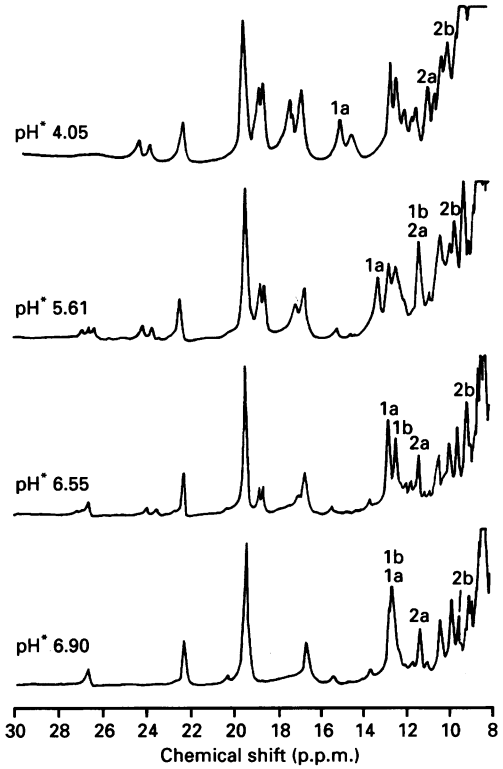

Fig. 2. N.m.r. pH* titration of 2 mM-b_2-cytochrome domain at 25 °C

Spectra were recorded over a frequency range of 25000 Hz with 16 k data points and transformed with an exponential line-broadening function of 2 Hz. They are the sum of 1000 scans.

After dialysis, the solution was centrifuged at 39000 *g* for 2 min, to remove any insoluble material, and then loaded on to a hydroxyapatite (Fluka, fast-flow) column (80 cm × 2.5 cm) previously equilibrated in 10 mM-sodium phosphate buffer, pH 7.0. The column was washed with 2 column volumes of the phosphate buffer and the protein was then eluted by using a gradient of 0–5 %-saturated $(NH_4)_2SO_4$ in the 10 mM-phosphate buffer. Fractions were collected and their purity was evaluated by measuring the ratio of u.v.–visible absorptions at 275 nm and 413 nm. Those fractions with A_{275}/A_{413} ratios < 1.0 were pooled and precipitated at 70 % $(NH_4)_2SO_4$ saturation. After centrifugation at 39000 *g* for 10 min, the precipitate was re-dissolved in 2 mM-sodium phosphate buffer, pH 8.5, and dialysed overnight against this buffer at 4 °C.

After dialysis, the solution was centrifuged at 39000 *g* for 2 min to remove insoluble material and then loaded on to a DE-52 DEAE-cellulose (Whatman) column (50 cm × 2.5 cm) equilibrated in 2 mM-sodium phosphate buffer, pH 8.5. The column was washed with 2 column volumes of the same buffer and the protein was eluted with a gradient of 2–20 mM-sodium phosphate buffer, pH 8.5. Fractions were collected as before and those with A_{275}/A_{413} ratios < 0.3 were pooled. This protein solution was adjusted to 70 % $(NH_4)_2SO_4$ saturation and centrifuged at 39000 *g* for 10 min. The resulting precipitate was dissolved in a minimum volume of 20 mM-sodium phosphate buffer, pH 7.0, and passed through a Sephadex G-75 gel-filtration column (130 cm × 2.5 cm) in the same buffer. Fractions with A_{275}/A_{413} ratio 0.2 ± 0.01 were pooled. Protein purified to this stage showed a single band of M_r 10500 on SDS/PAGE.

The b_2-cytochrome domain concentration was calculated from the absorbance at 413 nm by using the previously published molar absorption coefficient, ε_{413}, of 121500 $M^{-1} \cdot cm^{-1}$ [12].

Further details of the purification procedure, including yields etc., are given in Table 1.

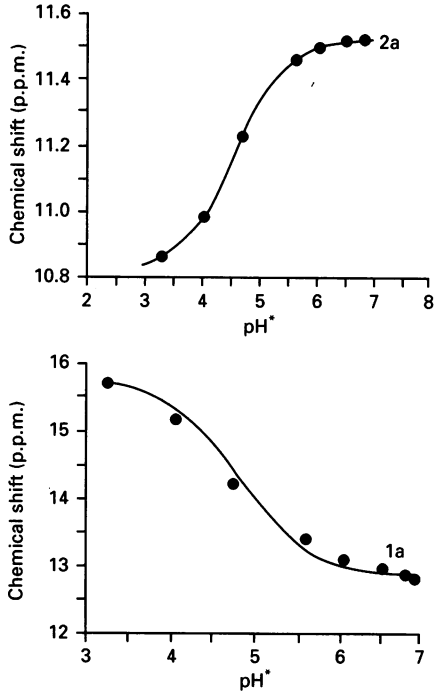

Fig. 3. Shifts of the haem propionate resonances with change in pH*

The points are experimental data obtained from spectra of the type shown in Fig. 2.

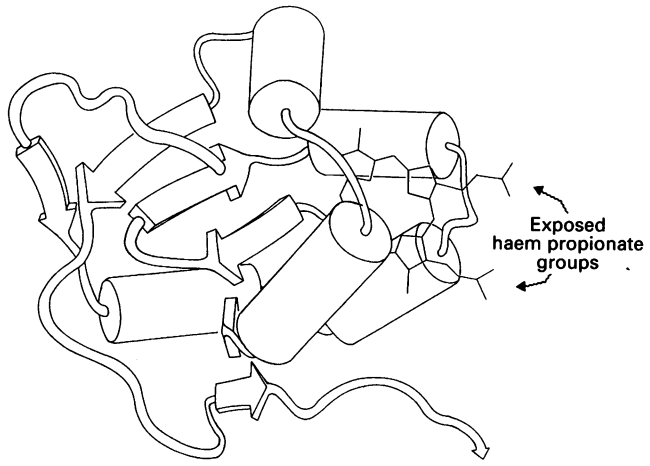

Fig. 4. Schematic diagram of the b_2-cytochrome domain based on the three-dimensional structure of flavocytochrome b_2 determined by Xia & Mathews [4]

Cylinders represent helices and arrows represent β-strands. The exposed propionate groups are indicated.

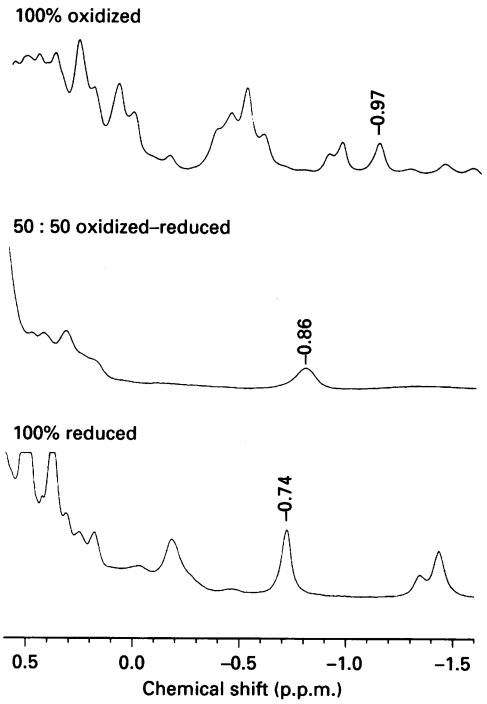

Fig. 5. Oxidation–reduction titration of b_2-cytochrome domain

A 1.1 mM solution of oxidized b_2-cytochrome domain in 5 mM-phosphate buffer, pH 7, was reduced in stages by the addition of $Na_2S_2O_4$ solution (3%, w/v) in ^2H_2O. The electron self-exchange rate was calculated from the excess linewidth at half-height ($\Delta\nu_{\frac{1}{2}}^e$) of peaks in the 50:50 oxidized–reduced mixture by using eqn. (1)

$$\Delta\nu_{\frac{1}{2}}^e = \frac{2\pi(\nu_A - \nu_B)^2}{k_e} \quad (1)$$

where ν_A is the chemical shift of a peak in the oxidized spectrum, ν_B the chemical shift of a peak in the reduced spectrum and k_e is the first-order self-exchange rate constant [16]. The Figure shows the linewidth change for one of the peaks used to determine k_e.

Measurement of redox potential

The midpoint potential of the cytochrome domain was determined spectrophotometrically by using a previously published redox potentiometry method [13]. The mediators *N*-ethylphenazonium sulphate, *N*-methylphenazonium sulphate, 2,3,5,6-tetramethylphenylenediamine and 2-hydroxy-1,4-naphthoquinone were used as previously described [13]. The cytochrome domain was reduced by titrating with $Na_2S_2O_4$ under anaerobic conditions and oxidized by titrating with $K_3Fe(CN_6)$. Changes in the haem absorbance at 557 nm were measured with changing electrode potential. The system was buffered with 100 mM-sodium phosphate buffer, pH 7.0. The Nernst plots for both reductive and oxidative sequences showed no hysteresis, implying that the system was at equilibrium.

N.m.r. measurements

^1H n.m.r. spectra were recorded with a JEOL GX-400 n.m.r. spectrometer operating at 400 MHz. 1,4-Dioxan was used as an internal standard, but chemical shifts are reported in p.p.m. downfield from the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulphonate. Samples were prepared for n.m.r. with Amicon Centricon devices for exchange of solvent. Three to five cycles of dilution and concentration were used to obtain samples with solvent > 98% ^2H_2O. ^2HCl and NaO^2H were used to adjust pH values. Reported pH values are direct meter readings uncorrected for the small isotope effect and they are designated as pH*. Other conditions are given in Figure legends.

RESULTS AND DISCUSSION

By introducing a termination codon immediately after the Gly-100 codon of the *S. cerevisiae* flavocytochrome b_2 coding region, it has been possible to express the b_2-cytochrome domain with the pDS–b_2 vector previously used for efficient expression of the holoenzyme in *E. coli* [9]. As in the case of the holoenzyme, the cytochrome domain expressed in *E. coli* commences at the amino acid position equivalent to Met-6 of the mature yeast protein (confirmed by determination of the *N*-terminal sequence

with an Applied Biosystems 477 sequencer by the WELMET protein characterization facility at the University of Edinburgh). A purification procedure (see Table 1 and description in the Materials and methods section) has been developed and this has provided good yields of the cytochrome domain. After purification a single band of M_r 10500 can be seen on an SDS/PAGE gel. The electronic absorption spectrum of the b_2-cytochrome domain expressed in *E. coli* (Fig. 1) was superimposable on the spectrum for the cytochrome b_2 core produced by trypsin digest of the holoenzyme from yeast [5], with peaks at 557, 528 and 423 nm in the reduced form and 560, 530 and 413 nm in the oxidized form. The reduction potential (at pH 7.5) of the b_2-cytochrome domain was measured as -31 ± 2 mV, which is very close to the previously published value of -28 mV determined for the proteolytically produced cytochrome b_2 core. These results are consistent with the idea that the b_2-cytochrome domain (residues 6–100 of the mature protein) expressed in *E. coli* and the cytochrome b_2 core (residues 8–103 of the mature protein [6]) produced by proteolysis of the holoenzyme from yeast are essentially identical in terms of their physical properties. This is supported by analysis of the n.m.r. spectra of the two proteins. The general appearance of the b_2-cytochrome domain n.m.r. spectrum resembles that of the proteolytically produced b_2 core as reported by Keller *et al.* [14], particularly with respect to the chemical shift of the haem methyl resonance at 19.5 p.p.m. However, other peaks in the high-frequency region with chemical shifts greater than 14 p.p.m., which were assigned to methyl groups by Keller *et al.* [14], do not have sufficient intensity to arise from methyl groups.

The isolated b_2-cytochrome domain is stable over a wide pH range with no significant change in the electronic absorption spectrum of the oxidized protein between pH 4.0 and 10.0. This pH stability made it possible to use high-field n.m.r. to determine the pK_a values for the ionization of both of the haem propionate groups. Fig. 2 shows n.m.r. spectra of the ferricytochrome domain between 8 and 30 p.p.m. at various pH* values. The peaks assigned to the propionate are labelled 1a, 1b, 2a and 2b. The positions of these peaks shift significantly with pH*. Plots of chemical shift against pH* for peaks 1a and 2a are shown in Fig. 3; these give rise to pK_a values of 4.8 ± 0.1 (1a) and 4.6 ± 0.1 (2a). These values are close to that for free haem propionic acid groups [15], indicating that the propionate groups of the isolated b_2-cytochrome domain must be exposed to solvent rather than buried inside the protein. In the X-ray crystal structure of the holoenzyme, the haem propionate groups point directly into the flavin-binding domain [4]. Thus the isolated b_2-cytochrome domain, independently of the flavin-binding domain, should have exposed propionate groups, as illustrated in Fig. 4 and consistent with the n.m.r. observations.

The electron self-exchange rate constant, k_{11} (at relatively low ionic strength, 5 mM-phosphate), for the b_2-cytochrome domain was determined from a redox titration as described in Fig. 5. At 25 °C and pH 7.4, k_{11} was found to be 2.3×10^6 $M^{-1} \cdot s^{-1}$. This value is around 600 times larger than the value found for the homologous protein cytochrome b_5 [17]. Cytochrome b_5 and the b_2-cytochrome domain both share a common folding topology, the so-called 'cytochrome b_5 fold' [18]. The two proteins have a high degree of sequence similarity, with 13 residues invariant between the two proteins. One significant difference, however, is the overall charge; this is around -8 in cytochrome b_5 but close to zero in the b_2-cytochrome domain. This difference in charge may be one of the reasons for the large difference in the values of the self-exchange rate constants for the two proteins. In the case of cytochrome b_5 a larger coulombic barrier would have to be overcome to allow electron transfer to take place, and thus one might expect the electron self-exchange rate in cytochrome b_5 to be slower than in the b_2-cytochrome domain, and this is indeed reflected in the values of the measured rate constants.

In conclusion, we have demonstrated an expression system for the b_2-cytochrome domain in *E. coli* and have described an efficient purification procedure for the protein. Biophysical and biochemical properties of the b_2-cytochrome domain, determined by spectroscopic and other methods, have been reported.

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