High-level expression of fully active yeast flavocytochrome b_2 in *Escherichia coli*

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Wild-type flavocytochrome b_2 (L-lactate dehydrogenase) from the yeast *Saccharomyces cerevisiae* and three singly substituted mutant forms (F254, R349 and K376) have been expressed in the bacterium *Escherichia coli*. The enzyme expressed in *E. coli* contains the protohaem IX and flavin mononucleotide (FMN) prosthetic groups found in the enzyme isolated from yeast, has an electronic absorption spectrum identical with that of the yeast protein and an identical M_r value of 57 500 estimated by SDS/polyacrylamide-gel electrophoresis. N-Terminal amino-acid-sequence data indicate that the flavocytochrome b_2 isolated from *E. coli* begins at position 6 (methionine) when compared with mature flavocytochrome b_2 from yeast. The absence of the first five amino acid residues appears to have no effect on the enzyme-catalysed oxidation of L-lactate, since K_m values for the yeast- and *E. coli*-expressed wild-type enzymes were identical within experimental error. The F254 mutant enzyme expressed in *E. coli* also showed kinetic parameters essentially the same as those found for the enzyme from yeast. The R349 and K376 mutant enzymes had no activity when expressed in either yeast or *E. coli*. The yield of flavocytochrome b_2 from *E. coli* is estimated to be between 500- and 1000-fold more than from a similar wet weight of yeast (this high level of expression results in *E. coli* cells which are pink in colour). The increased yield has allowed us to verify the presence of FMN in the R349 mutant enzyme. The advantages of *E. coli* as an expression system for flavocytochrome b_2 are discussed.

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 57 500 [1]. Each subunit consists of two functionally distinct domains, one containing protohaem IX and the other flavin mononucleotide (FMN) [2]. The protein is a soluble component of the mitochondrial intermembrane space [3], where it catalyses the oxidation of L-lactate to pyruvate and transfers electrons directly to cytochrome c [2]. The protein is synthesized as a precursor form with an 80-amino-acid leader sequence [4] which is proteolytically removed during translocation to the mitochondrial intermembrane space [5]. The crystal structure of flavocytochrome b_2 has been solved [6,7], allowing structural information to be correlated with models of the catalytic mechanism. It was on this basis that site-directed mutagenesis of the flavocytochrome- b_2 gene was carried out to probe the active site of the enzyme [8]. Among the mutations generated were Y254→F, K349→R and R376→K [8]. Kinetic characterization of the wild-type and mutant enzymes gave results consistent with a mechanism for L-lactate oxidation as proposed by Lederer & Mathews [9]. One technical limitation with this work, however, was that yields of the mutant enzymes expressed in yeast were low, preventing extensive characterization. This was a particular problem for one mutant enzyme, namely K349→R, which was obtained in low yield, preventing verification of the presence of the

FMN prosthetic group [8]. Clearly a better expression system, offering improved yields of enzyme, was desirable for further work to proceed. In the present paper we describe the expression of wild-type and mutant forms of flavocytochrome b_2 in *Escherichia coli* and the characterization of these enzymes with respect to those isolated from yeast.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

E. coli strains MM294 [10] or HB101 [11] were used for plasmid propagation and expression of flavocytochrome b_2 . Plasmid-bearing cells were grown at 37 °C in Luria Broth [11] supplemented with ampicillin at 100 µg/ml. The entire flavocytochrome b_2 coding sequence, including the region encoding the presequence, was excised from the plasmid pGR401 [8] on a 1.8 kb *EcoRI*–*HindIII* restriction fragment and inserted between the *EcoRI* and *HindIII* sites of the plasmid pDS6 [12] to generate pDSb₂ (see Fig. 1). Mutant forms of the flavocytochrome- b_2 gene were excised from appropriate pGR401 derivative plasmids [8] as *EcoRI*–*BglII* fragments and inserted into pDSb₂ after cleavage with *EcoRI* and *BglII*. *E. coli* were transformed by the CaCl₂ method [11], and positive transformants were selected according to ability to grow in the presence of ampicillin and by the presence of a pink coloration due to high-level expression of a haem-containing protein, flavocytochrome b_2 .

Abbreviations used: Y254→F (etc.), a mutation from the wild-type-enzyme's tyrosine-254 to phenylalanine (one-letter amino acid notation); PAGE, polyacrylamide-gel electrophoresis.

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Fig. 1. Construction of a plasmid for expression of flavocytochrome b_2 in *E. coli*

The DNA encoding flavocytochrome b_2 was excised from pGR401 [8] on an *EcoRI*–*HindIII* restriction fragment and inserted into pDS6 [12]. Mutant flavocytochrome b_2 DNA sequences were transferred as *EcoRI*–*BglII* restriction fragments as described in the Materials and methods section. Abbreviations: p, promoter; t, terminator; rbs, ribosome-binding site; *cat*, chloramphenicol acetyltransferase gene; *bla*, β -lactamase gene; ori, origin of replication.

Enzyme isolation

From yeast. Flavocytochrome b_2 was prepared either from commercial baker's yeast (United Yeast Co. Ltd., Menstrie, Scotland) or from GR20 transformants [8] as described previously [13]. Phenylmethanesulphonyl fluoride (1 mM) was present throughout the preparation to yield intact enzyme [13].

From *E. coli*. Frozen *E. coli* cells were resuspended in 10 mM-Tris/HCl buffer (*I* adjusted to 0.10 with NaCl), pH 7.5, containing 10 mM-EDTA at 4 °C. Lysozyme (Sigma) was added to approx. 0.2 mg/ml and the mixture was incubated on ice for 30 min. The solution was centrifuged at 39000 *g* for 10 min to remove cell debris etc. The supernatant, which had a strong orange-red colour due to the presence of flavocytochrome b_2 , was adjusted to 30% $(\text{NH}_4)_2\text{SO}_4$ saturation (the pH was maintained at 7.5 if necessary by addition of NaOH) and centrifuged at 39000 *g* for 10 min. The pellet was discarded, and the supernatant was adjusted to 70%

$(\text{NH}_4)_2\text{SO}_4$ saturation to precipitate the flavocytochrome b_2 and centrifuged at 39000 *g* for 10 min. The pellet was redissolved either in the appropriate buffer (10 mM-Tris/HCl, pH 7.5; *I* adjusted to 0.1 with NaCl), for preliminary kinetic measurements, or in 0.1 M-phosphate buffer, pH 7.0, containing 1 mM-EDTA and 1 mM-D,L-lactate, for further purification. The purity of the enzyme after the 70%-satd. $(\text{NH}_4)_2\text{SO}_4$ -precipitation step was such that a characteristic flavocytochrome b_2 spectrum in the visible region could be obtained (see Fig. 2 below). All subsequent purification steps, involving dialysis and chromatography on hydroxyapatite and derivatized-Sepharose columns, were as previously described [13]. Enzyme prepared from *E. coli* or yeast was stored under N_2 at 4 °C as a 70%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate and maintained in the reduced state, where possible, by the presence of L-lactate (5 mM). When fully purified the enzyme showed a single band at M_r 57500 on SDS/PAGE and a protein/haem ratio (A_{269}/A_{423}) when reduced of 0.5 [13]. Further details of the purification procedure are given in Table 1.

Table 1. Purification data for a typical isolation of wild-type flavocytochrome b_2 from *E. coli*

Values are based on a preparation from 1 litre of culture (this typically yields 5–7 g wet weight of cells). Note that flavocytochrome b_2 expressed in *E. coli* accounts for approx. 5% of the total soluble protein in the cell lysate. This Table may be compared with that for isolation of the enzyme from yeast previously described in [13].

Step	Total protein (mg)	Flavocytochrome b_2 (mg)	Yield of b_2 (%)	Purification (fold)
Supernatant from cell lysate	~ 300	~ 14	100	1
(NH ₄) ₂ SO ₄	~ 170	9–10	65–70	1.1–1.3
Hydroxyapatite	~ 15	8–9	55–65	11–13
Derivatized Sepharose	4–6	4–6	30–40	21

Flavin isolation and verification

The presence of flavin could be detected by monitoring the change in absorbance at 438.3 nm (which is a haem isosbestic point [15]) upon reduction of the flavocytochrome b_2 with sodium dithionite.

To further verify the presence of flavin, it was isolated from the R349 mutant enzyme by using procedures similar to those previously reported [14]. Precipitated [70%-satd. (NH₄)₂SO₄] mutant flavocytochrome b_2 was solubilized in a minimum volume of 35 mM-phosphate/4 M-urea/100 mM-2-mercaptoethanol/1 mM-phenylmethanesulphonyl fluoride at pH 7.0. The mixture was incubated at 0 °C for 50 to allow complete dissociation of flavin. The mixture was then passed through a Sephadex G-25 column (2 cm × 20 cm) equilibrated in the same solution. Two coloured peaks were eluted from the column; the protein which still contained haem was eluted first and was red in colour, followed by FMN, which was in the oxidized form and was thus yellow in colour.

Kinetic analysis

Kinetic experiments involving enzymic oxidation of L-lactate were carried out on fully purified enzyme from yeast and *E. coli*, and on enzyme from *E. coli* purified only to the 70%-satd.-(NH₄)₂SO₄ stage. These experiments were carried out under steady-state conditions at 25 ± 0.1 °C in 10 mM-Tris/HCl, pH 7.5, with *I* adjusted to 0.10 by addition of NaCl. Flavocytochrome b_2 concentrations were determined spectrophotometrically by using published molar absorption coefficients [13]. Enzyme activity was measured with ferricyanide as electron acceptor. The decrease in absorbance was monitored at 420 nm (ϵ_{420} 1010 M⁻¹ cm⁻¹) with a Pye-Unicam SP.800 spectrophotometer. Assays were performed with a ferricyanide concentration of 1 mM and L-lactate concentrations varying from 0 to 20 mM.

RESULTS AND DISCUSSION

The parent expression vector pDS6 contains a strong promoter derived from coliphage T₅ (P_{N25 x/o}), but does not contain a ribosome-binding site proximal to the *EcoRI* site into which the flavocytochrome b_2 gene was inserted. Notwithstanding this fact, *E. coli*, when transformed with pDSb₂, produces large quantities of the protein, resulting in pink-coloured cells (1–2 mg/100 ml of cell culture, which is 500–1000-fold more than we have obtained from either wild-type or transformed baker's

yeast). The factors which affect translational initiation have been recently reviewed [16], and it has been proposed that five components provide high-level translational initiation in *E. coli*. In this case three factors appear to be particularly relevant: a Shine–Dalgarno region (consensus sequence AGGAGG) of four or five nucleotides is probably sufficient for complementary base-pairing with the 3' end of 16S RNA; an ATG initiation codon is required, and a spacing of seven to nine nucleotides between these two components is probably optimal. The flavocytochrome b_2 gene contains a hexanucleotide sequence ⁵ACGAGC³ commencing at nucleotide 239 and extending over the region corresponding to the first amino acid of the mature-protein sequence; four out of the six nucleotides match the consensus Shine–Dalgarno sequence. An ATG codon, corresponding to methionine-6, is present 11 bases downstream from the putative Shine–Dalgarno sequence. It is therefore likely that the nucleotides which code for amino acids 80–86 of the precursor protein (positions –1 to +6 of the mature sequence) act as a translational initiation site for flavocytochrome b_2 in *E. coli*. N-Terminal amino-acid-sequence data (which were obtained by using an Applied Biosystems 477 Sequencer by Dr. J. Van Beeumen) confirm that flavocytochrome b_2 , when expressed in *E. coli*, commences at the amino acid position equivalent to methionine-6 of the mature yeast protein.

The absence of the first five amino acids appears to have little or no effect on the physical properties of the enzyme. From the crystal structure these five N-terminal amino acids are seen to be on the surface of a haem domain at a large distance from the flavin active site. They show no significant interactions with other amino acid side chains. The electronic absorption spectrum of flavocytochrome b_2 expressed in *E. coli* was identical with that of the protein from yeast (Fig. 2), with peaks at 269, 328, 423, 550 and 557 nm. The protein/haem (A_{269}/A_{423} when reduced = 0.5) and flavin/haem (A_{328}/A_{423} when reduced = 0.21) ratios are identical, indicating correct incorporation of protohaem IX and FMN in the *E. coli*-expressed protein. On an SDS/12%-polyacrylamide gel the *E. coli*- and yeast-expressed proteins show bands with apparently identical M_r values of ~ 57000.

The kinetics of the oxidation of L-lactate to pyruvate by wild-type flavocytochrome b_2 from *E. coli* and yeast were determined under steady-state conditions. Experimental data were fitted to a non-linear least-squares program [17] to yield the K_m and k_{cat} values shown in Table 2. The values determined for the enzymes from yeast and *E. coli* are identical within error limits. Kinetic

Fig. 2. Visible spectra of (A) fully purified flavocytochrome b_2 expressed in yeast and (B) flavocytochrome b_2 expressed in *E. coli* and purified up to the 70%-satd.-(NH₄)₂SO₄ step as described in the Materials and methods section

determinations carried out on the enzyme isolated from *E. coli* immediately after purification to the 70%-satd.-(NH₄)₂SO₄ step always showed slightly higher k_{cat} values. It is known that flavocytochrome b_2 does lose some activity with time, and it would appear that the 2 days required for further purification result in this small loss of activity. This observation shows the benefit of obtaining kinetic parameters at as early a stage as possible. K_m and k_{cat} values for the F254 mutant enzyme are also shown in Table 2. Again the k_{cat} value for the *E. coli*-expressed enzyme after the 70%-satd.-(NH₄)₂SO₄ step is slightly higher than that after full purification. For a detailed discussion of the role of tyrosine-254 in the mechanism of L-lactate oxidation and the effect of replacing this residue with a phenylalanine residue, see [8].

The K376 and R349 mutant enzymes show no detectable activity whether isolated from yeast or *E. coli*. Possible explanations for this total loss of activity have already been put forward [8]. One such explanation in the case of the R349 mutant was that replacing the original lysine residue with an arginine residue in a tightly packed region close to the flavin group may have prevented incorporation of the flavin itself. The yield of this mutant enzyme from yeast was so small that it was

Table 2. Kinetic properties of wild-type and mutant flavocytochrome b_2 for the oxidation of L-lactate to pyruvate

All constants were determined from steady-state kinetic measurements at 25 ± 0.01 °C, pH 7.5, with ferricyanide (1 mM) as electron acceptor. The K349→R and R376→K mutant enzymes expressed in either yeast or *E. coli* had no activity.

Enzyme and source	k_{cat} (s ⁻¹)	K_m (mM)
Wild-type		
Yeast	192 ± 5	0.49 ± 0.03
<i>E. coli</i>		
Fully purified	189 ± 5	0.44 ± 0.05
After 70%-satd.-(NH ₄) ₂ SO ₄ step	204 ± 10	0.46 ± 0.03
Y254→F mutant		
Yeast	4.3 ± 0.1	0.54 ± 0.03
<i>E. coli</i>		
Fully purified	3.8 ± 0.1	0.52 ± 0.04
After 70%-satd.-(NH ₄) ₂ SO ₄ step	6.5 ± 0.2	0.51 ± 0.03

impossible to verify the presence or absence of flavin. However, with the improved yields from expression in *E. coli* we have now been able to show unequivocally that flavin is indeed present in the R349 enzyme. Spectrophotometric measurements at haem isosbestic points show the expected content of flavin, and this has been further verified by isolating and assaying the FMN after denaturing the protein as described in the Materials and methods section. Both experiments indicate the expected flavin content in the R349 mutant enzyme within experimental error.

In conclusion, we have shown that *E. coli* transformed with pDSb₂ provides an improved expression system for the yeast protein flavocytochrome b_2 . Advantages over expression of the protein in yeast include vastly improved yields and quicker and easier purification. Wild-type and mutant proteins expressed in *E. coli* have been shown to exhibit the same kinetic properties as the protein from yeast. The increased yields have allowed more extensive characterization of these enzymes and make possible experiments requiring large quantities of protein, including crystallographic structure determination of the mutant enzymes.

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REFERENCES

- Jacq, C. & Lederer, F. (1974) *Eur. J. Biochem.* **41**, 311–320
- Appleby, C. A. & Morton, R. K. (1954) *Nature (London)* **173**, 749–752
- Daum, G., Böhni, P. C. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13028–13033
- Guiard, B. (1985) *EMBO. J.* **4**, 3265–3272

5. Reid, G. A. (1985) *Curr. Top. Membr. Transp.* **24**, 295–334
6. Xia, Z.-X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F. & Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2629–2633
7. Mathews, F. S. & Xia, Z.-X. (1987) in *Flavins and Flavoproteins* (McCormick, D. B. & Edmondson, D. E., eds.), pp. 123–132, Walter de Gruyter, Berlin
8. Reid, G. A., White, S., Black, M. T., Lederer, F., Mathews, F. S. & Chapman, S. K. (1988) *Eur. J. Biochem.*, in the press
9. Lederer, F. & Mathews, F. S. (1987) in *Flavins and Flavoproteins* (McCormick, D. B. & Edmondson, D. E., eds.), pp. 133–142, Walter de Gruyter, Berlin
10. Backman, K., Ptashne, M. & Gilbert, W. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4174–4178
11. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY
12. Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. & Bujard, H. (1984) *EMBO. J.* **3**, 3143–3148
13. Labeyrie, F., Baudras, A. & Lederer, F. (1978) *Methods Enzymol.* **53**, 238–256
14. Silvestrini, M. C., Brunori, M., Tegoni, M., Gervais, M. & Labeyrie, F. (1986) *Eur. J. Biochem.* **161**, 465–472
15. Capeillere-Blandin, C., Barber, M. J. & Bray, R. C. (1986) *Biochem. J.* **238**, 745–756
16. Gold, L. & Stormo, G. (1987) *Am. Soc. Microbiol.* **2**, 1302–1307
17. Gamp, H., Maeder, M. & Zuberbuhler, A. D. (1980) *Talanta* **27**, 313–518

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