Expression in *Escherichia coli* of the flavin and the haem domains of *Hansenula anomala* flavocytochrome b_2 (flavodehydrogenase and b_2 core) and characterization of the recombinant proteins¶

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The flavin and haem domains of *Hansenula anomala* flavocytochrome b_2 have been independently expressed in *Escherichia coli*. The flavin domain activity, studied only in the total cellular extract, owing to its instability, has characteristics very similar to those of the flavin domain obtained by proteolysis. The haem domain (r-core) has been purified to homogeneity and characterized in detail from spectroscopic and functional points of

view. Spectral differences with respect to the domain produced by proteolysis (p-core) were found using resonance Raman and c.d. spectroscopy and have been interpreted in terms of changes in haem–protein interactions. However, this structural difference is functionally silent, since the r-core is able to reduce cytochrome c with the same efficiency as the proteolytic domain.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase, EC 1.1.2.3) is a bifunctional enzyme which constitutes an alternative pathway for electrons in yeast mitochondria, catalysing the direct electron transfer from L-lactate to cytochrome c (Pajot and Claisse, 1974; de Vries and Marres, 1987). The protein is a homotetramer; each subunit (~58 kDa) contains two non-covalently bound prosthetic groups, one b -type haem and one flavin mononucleotide (FMN).

Two electrons provided by the substrate, L-lactate, are accepted by FMN and rapidly transferred intramolecularly one by one to the monoelectronic acceptor, the b haem, with the formation of a semiquinone intermediate (Capeillère-Blandin et al., 1975). Subsequently, an intermolecular electron transfer occurs from the b haem to the external acceptor, ferricytochrome c (Capeillère-Blandin, 1982).

Biochemical investigations using controlled trypsin lysis on *Hansenula anomala* and *Saccharomyces cerevisiae* flavocytochrome b_2 have previously shown that the FMN and the b haem are located in different structural domains (Gervais et al., 1977; Gervais and Tegoni, 1980). More recently, the determination at 0.24 nm (2.4 Å) resolution of the three-dimensional structure of the enzyme from *S. cerevisiae* neatly confirmed the presence of these separate domains (Xia and Mathews, 1990).

The flavin domain was shown to be itself highly sensitive to internal proteolysis (Gervais et al., 1977). However, under particular proteolytic conditions (digestion with *Staphylococcus aureus* V8 proteinase I (Gervais et al., 1983) or an *H. anomala* proteinase (Célerier et al., 1989), the flavin domain remains intact. After this type of proteolysis, the flavodehydrogenase moiety (FDH), which retains a tetrameric structure (molecular mass 4×39 kDa), and the monomeric cytochrome domain (b_2 core) (molecular mass 11 kDa), dissociate spontaneously under non-denaturing conditions. The two domains have been purified

and their kinetic properties partially characterized; they maintain, with minor differences, the ability to react with the external substrates, L(+)-lactate and ferricytochrome c respectively, but, strangely enough, they do not associate, either to form a thermodynamically stable complex or to form a catalytically competent kinetic complex (Labeyrie, 1982). This lack of function could be due to the loss, during proteolysis, of a few residues (10–14 amino acids, depending on the proteinase (Célerier et al., 1989) crucial for the recognition and/or the correct orientation of the prosthetic groups in the active kinetic complex, or directly involved in the intramolecular transfer. A thorough investigation of this problem and a deeper insight on the kinetic properties of the domains have been impaired by the low yield after proteolysis and purification (Gervais et al., 1983; Célerier et al., 1989) and by the presence of microheterogeneity of the proteolytic fragments (Vicente Risler, 1991).

A possible method for the production of homogeneous protein domains, with well defined amino acid sequence and without peptide loss, is given by recombinant DNA techniques. These methods have indeed been used to produce FDH and b_2 core from *S. cerevisiae* flavocytochrome b_2 (Pallister et al., 1991; Brunt et al., 1992).

The great amount of kinetic results collected over the past years on the *H. anomala* enzyme and the significant functional differences between the latter enzyme and the *S. cerevisiae* one (Labeyrie, 1982), make the proportion of *H. anomala* flavocytochrome b_2 isolated domains particularly interesting. In addition, the production of these isolated domains could give new possibilities for crystallization attempts, unsuccessful until now on the holoenzyme.

In the present paper we describe the expression of the two isolated domains of *H. anomala* flavocytochrome b_2 in a heterologous system. The spectroscopic and kinetic characterization of the recombinant b_2 core, purified to homogeneity, is also presented.

Abbreviations used: r.R., resonance Raman; FDH, flavodehydrogenase; IPTG, isopropyl β -D-thiogalactopyranoside.

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¶ This paper is dedicated to the memory of Françoise Labeyrie, who died suddenly in September, 1993, and who introduced us to the study of flavocytochrome b_2 .

MATERIALS AND METHODS

Flavocytochrome b_2 was purified from *H. anomala* as already described (Gervais et al., 1983). Flavocytochrome b_2 core was prepared by proteolytic cleavage of the *H. anomala* enzyme and subsequent purification (Silvestrini et al., 1986). Hereafter it will be called 'p-core', to recall its proteolytic origin, while flavocytochrome b_2 core produced by recombinant DNA techniques (see below) will be called 'r-core'.

Spectroscopic measurements

Spectrophotometric measurements were carried out on a Perkin–Elmer spectrophotometer. Flavocytochrome b_2 and b_2 core concentrations were determined using the following absorption coefficients: $\epsilon_{423} = 183 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for dithionite-reduced flavocytochrome b_2 and $\epsilon_{413} = 121 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for oxidized b_2 core (Pajot and Groudinsky, 1970). L-Lactate dehydrogenase activity was measured at 30 °C in the presence of ferricyanide or horse heart cytochrome *c* (type VI, Sigma) as electron acceptors. Catalytic-centre activities were calculated on the basis of a $\Delta\epsilon_{420}$ of $1.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ or $\Delta\epsilon_{460}$ of $0.168 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ferri-ferricyanide and a $\Delta\epsilon_{550}$ of $18.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ferro-ferricytochrome *c* (Margoliash and Frowhrt, 1959).

Fluorescence measurements were carried out on a Perkin–Elmer LS-5 spectrofluorimeter. Tryptophan residues were determined as described by Pajot (1976), with excitation at 295 nm and emission measured at 354 nm, in 6 M guanidinium chloride. In this medium, owing to the standardization of the environment of the aromatic chromophores, the quantum yield values of tryptophan residues were normalized. The fluorescence-intensity scale was calibrated using free tryptophan under the same conditions and taking into account as correcting factor a correlation coefficient (Pajot, 1976) between the fluorescence of protein tryptophan residues and of free tryptophan.

Resonance Raman (r.R.) spectra were recorded at room temperature (20 ± 1 °C) on a Jobin Yvon spectrometer (Ramanor HG2S-UV), with the 441.6 nm excitation from a helium–cadmium laser (Liconix model 4050). Grazing incidence of the excitation laser beam was used (Desbois et al., 1979). The signal-to-noise ratio was improved by summation of individual spectra in a multichannel analyser (Tracor Northern TW 1710). Under these conditions the frequency precision is from 0.5 to 2 cm^{-1} , depending on both the band intensity and the signal-to-noise ratio. The r.R. spectra were recorded over the frequency range 150–1650 cm^{-1} . The frequencies of the r.R. bands of the oxidized and reduced *H. anomala* cytochrome b_2 core had previously been assigned (Desbois et al., 1989).

C.d. measurements were recorded in the visible and Soret region at 20 °C with a Jasco J500 spectropolarimeter, equipped with a Jasco model DP 500 N data processor, on p- and r-core in the native state or after a 'pH jump' which should induce rotational disorder of the haem (Aojula et al., 1988). For the 'pH jump' the protein was equilibrated in 10 mM sodium/potassium phosphate buffer, pH 7.0, adjusted to pH 2.3 by addition of 1 M HCl, and left to stir for 15 min. The pH was then re-adjusted rapidly to 7.0 by addition of 1 M NaOH; then the sample was centrifuged to remove any denatured protein and held on ice. Each spectrum was accumulated four times. The data are expressed as molar ellipticities ($\text{degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ of protein $^{-1}$).

Recombinant DNA techniques

All DNA manipulations were performed by using standard protocols (Sambrook et al., 1989). Oligonucleotides to be used as primers for *TaqI* polymerase in PCR (Mullis and Faloona, 1987)

were synthesized by Mrs. D. Menay (Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette, France), and purified by DEAE-Sephacel chromatography as described by Sambrook et al. (1989). PCR was carried out on a Hybaid thermal reactor, with an hybridation temperature of 45 °C for three cycles and 55 °C for 30 cycles. The samples were then treated with DNA polymerase (Klenow fragment) and DNA kinase in order to ensure correct filling of the ends of the newly synthesized fragments. DNA fragments of the correct size (approx. 350 bp for b_2 core and 1240 bp for FDH) were purified from preparative agarose-gel electrophoresis for subsequent cloning into the *SmaI* site of the plasmid pUC19. DNA sequencing was performed as described by Sanger et al. (1977).

Purification of recombinant core (r-core)

Transformed and wild-type *E. coli* cells were harvested at stationary phase and resuspended in 100 mM sodium/potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 5 mM MgCl_2 (8–10 ml/g of wet cells), and were disrupted by prolonged treatment (4–6 h at 4 °C) with lysozyme (1 mg/g of wet cells) in the presence of RNAase (0.05 mg/g) and DNAase (0.05 mg/g). Crude extracts were assayed for the presence of L-lactate:ferricyanide oxidoreductase activity as described above and centrifuged (30 min, 39000 g).

r-core was purified from the supernatant using the method described by Silvestrini et al. (1986) for p-core, with minor modifications. The crude soluble extract was adjusted to 45% $(\text{NH}_4)_2\text{SO}_4$ saturation, centrifuged (10 min, 18000 g), the pellet discarded and the supernatant adjusted to 80% saturation. The pellet resulting from the second centrifugation (10 min, 18000 g) was resuspended in a minimum volume of 100 mM sodium/potassium phosphate buffer, pH 7.0, and dialysed overnight. These steps were followed by molecular filtration on a Sephadex G-75 column in the same buffer and chromatography on a DEAE-cellulose column equilibrated in 35 mM sodium/potassium phosphate buffer, pH 7.0. r-core was eluted with an ionic strength gradient between 35 and 200 mM in sodium/potassium phosphate buffer, pH 7.0. The purified protein was stored at 4 °C in 100 mM sodium/potassium phosphate buffer, 80% saturated with $(\text{NH}_4)_2\text{SO}_4$, pH 7.0. Before each experiment, an appropriate amount of r-core was centrifuged (10 min, 18000 g), the pellet redissolved, and the solution filtered through a Sephadex G-25 column. The concentration of r-core was determined using the absorption coefficient reported above for the proteolytic core (p-core).

Rapid kinetic experiments

Rapid kinetic experiments were performed at 5 °C on a Gibson–Durrum stopped-flow apparatus equipped with a 1.9 cm-light-path cell (dead time 3 ms) and connected to a Tracor NS 570 analog-to-digital converter. Reduction of horse heart cytochrome *c* was monitored at 416.5 nm, an isosbestic wavelength for b_2 core oxidoreduction [$\Delta\epsilon_{416}$ $51 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ferro-ferricytochrome *c* (Capeillère-Blandin, 1982)], under two experimental set-ups, that differ in the way of obtaining reduced b_2 core. In the first series of experiments, b_2 core (either r-core or p-core) was degassed, reduced with a small amount of solid sodium dithionite and mixed with various concentrations of cytochrome *c*, dissolved in aerated buffer. This kind of approach has already been applied to study the oxidation of reduced p-core by azurin and stellacyanin (Silvestrini et al., 1986). In the second series of experiments, r-core was degassed and reduced by addition of a catalytic amount of flavocytochrome b_2 in the presence of

1 mM L-lactate, as reducing substrate, and 3 mM oxalate, a competitive inhibitor, and then mixed with anaerobic cytochrome c solutions, thus reproducing the conditions used for studying the reduction of cytochrome c by p-core (Capeillère-Blandin and Albani, 1987). In all these experiments the buffer was 100 mM phosphate, pH 7.0.

RESULTS AND DISCUSSION

PCR amplification, cloning and expression of the isolated domains

Sequencing of the *H. anomala* flavocytochrome b_2 gene showed that the molecule is synthesized as a pre-protein, with a 73-residues-long leader peptide to address the protein to the mitochondrial compartment (Risler et al., 1989; Gervais et al., 1991). A pre-protein had already been shown (Gasser et al., 1982) and sequenced (Guiard, 1985) in the case of *S. cerevisiae* flavocytochrome b_2 . The three-dimensional structure of the *S. cerevisiae* enzyme (Xia and Mathews, 1990) shows that the haem domain is located at the N-terminus of the mature protein, while the flavin domain starts at residue 100. On the basis of *H. anomala* flavocytochrome b_2 gene sequence (Risler et al., 1989), and in order to amplify separately the two domains, the following oligonucleotides [(1) and (2) for b_2 core; (3) and (4) for FDH] were designed:

CCCCATGGATGTTCTCATTGGAAAGATATAG (1)
NcoI

CCCGGATCCCTATTCTTCATCACTCAATTC (2)
BamHI

CCCCATGGATAGATTGGAAAGAATCGAAA (3)
NcoI

CCGGATCCCTAATCCTCTATACCTGGTCTAAATTCAGC (4)
BamHI

Oligonucleotides (1) and (3) are identical (**bold** characters) to the coding sequence, from base positions 220 to 244 and from 517 to 538 respectively; their starting sequence has been designed to insert an *NcoI* site (underlined), including an ATG codon at the 5' end of the gene sequences to be amplified. Oligonucleotides (2) and (4) are complementary (**bold** characters) to the non-coding strand, from positions 513 to 496 and from 1719 to 1692 respectively; their sequence allows the insertion of a stop codon and a *BamHI* site (underlined) at the 3' end of the sequences to be amplified.

After PCR amplification and subsequent purification (see the Materials and methods section), the fragments were cloned in the *SmaI* site of pUC19. The recombinant plasmids were digested with *NcoI* and *BamHI* and the inserts, purified from preparative agarose-gel electrophoresis, were cloned in the *NcoI* and *BamHI* sites of the expression vector pUHE24-2, $\Delta 1$ downstream a promoter inducible by isopropyl β -D-thiogalactopyranoside (IPTG). This vector derives from pUHE24-2 (kindly provided by Professor H. Bujard; H. Bujard, unpublished work), by deletion of the region between the *NheI* site at position 261 and the *XbaI* site at position 1118, to eliminate a second unwanted *NcoI* site (position 815). The recombinant plasmids, called pUHE24-2, $\Delta 1$ -core and pUHE24-2, $\Delta 1$ -FDH, were used to transform *E. coli* strain MC1061, where expression can occur without induction, or *E. coli* strain BMH71-18, where expression can be induced by

IPTG. Expression in both cases occurred at the same level in the two different strains, so that the strain requiring no induction was used throughout.

In view of the differences observed in the r.R. spectra of r-core with respect to p-core (see below), the sequence of r-core DNA obtained by PCR amplification was determined, in order to exclude amino acid differences due to polymerase mistakes. The sequence was found to be exactly as expected, i.e. identical with that of the flavocytochrome b_2 gene from base 220 to base 538, with an extra ATG at the beginning.

r-FDH

The *E. coli* clone transformed with pUHE24-2, $\Delta 1$ -FDH shows a L-lactate dehydrogenase activity, totally absent in the non-transformed control cells and in cells transformed with pUHE24-2, $\Delta 1$ -core. This activity is associated to the soluble crude extract obtained after lysozyme treatment and centrifugation (see the Materials and methods section). This L-lactate dehydrogenase activity, recovered with a yield of approx. 0.2 mg/g (wet weight) of cells, appears to decrease significantly with time and has not been further purified, but characterized as such under steady-state conditions in the presence of ferricyanide. Experiments carried out at different concentrations of L-lactate (10 and 60 mM) showed the presence of excess substrate inhibition, (40% inhibition at 60 mM L-lactate), which is a characteristic feature of flavocytochrome b_2 holoenzyme and of the FDH purified after proteolysis (Gervais et al., 1983; Célerier et al., 1989). Experiments carried out at different concentrations of ferricyanide permitted the determination of the K_m as 3 mM, in line with previous results [K_m 2–3 mM for p-FDH obtained by digestion with *S. aureus* V8 proteinase I (Gervais et al., 1983) and K_m 1.1 mM for p-FDH obtained by treatment with *H. anomala* proteinases (Célerier et al., 1989)].

We have tested the ability of the FDH domain to transfer electrons to the isolated haem domain (r-core) under steady-state conditions. In line with the hypothesis that polar interactions could be present at the interface of the two domains in the holoprotein (Xia and Mathews, 1990), the activity has been tested at two different ionic strengths (10 and 500 mM phosphate buffer, pH 7.0). No reduction of the r-core has been detected in the presence of the r-FDH, as previously occurred with p-core and p-FDH (Labeyrie, 1982).

r-core

The *E. coli* clone transformed with pUHE24-2, $\Delta 1$ -core is able to express b_2 core at high levels; intact cells are in fact deeply red. The amount of recombinant protein in crude extracts is approx. 5 mg/g (wet weight) of cells; after the purification procedure (see the Materials and methods section) the yield is halved.

The visible and u.v. absorption spectra of purified r-core (Figure 1) are identical in both oxidation states with those already described for the same domain obtained by proteolysis (p-core), with a u.v./Soret_{red} ratio of 0.2, typical of a species more than 95% pure. The purified protein migrates in fact as a single band on SDS/PAGE (results not shown), with a migration behaviour consistent with that expected for its molecular mass.

The tryptophan content of the molecule, determined by emission fluorescence spectroscopy (see the Materials and methods section), is 2.5 tryptophan residues per haem; two tryptophan residues are expected on the basis of the amplified sequence. It must be recalled that p-core contains one (Haumont et al., 1987) or two (Gervais et al., 1980) tryptophan residues per haem, depending on the proteolysis conditions.

Figure 1 U.v. and visible absorption spectra of r-core

The Figure shows the spectra of oxidized (dotted line) and dithionite-reduced (continuous line) r-core.

Table 1 High-frequency modes (1000–1650 cm⁻¹) of reduced and oxidized forms of p- and r-core

Mode	Reduced		Oxidized		Assignment
	p-core	r-core	p-core	r-core	
ν_{10}	1620	1621	1640	1641	ν_a (CaCm)
	1635	1632	1636	1638	ν (CC _{vinyl})
				1628	
ν_{37}	1603	1603	1597	1599	ν_a (CaCm)
ν_2	1580	1579	1580	1579	ν (CbCb)
ν_{38}	1556	1556	1538	1542	ν (CbCb)
ν_{11}	1536	1539	1557	1558	ν (CbCb)
ν_3	1493	1493	1505	1505	ν_s (CaCm)
ν_{28}	1475	1473	1469	1473	ν_s (CaCm)
			1457	1462	
	1430	1434	1431	1435	δ (CH _{2vinyl})
ν_{20}	1389	1390	1395	1409	ν (Pyr quater-ring)
ν_4	1361	1361	1374	1374	ν_s (Pyr half-ring)
			1332	1331	δ (CH _{2vinyl})
ν_{21}	1303	1305	1308	1310	δ (CmH) + δ (CH _{vinyl})
ν_{42}	1285	1282		1290	δ (CmH)
$\nu_5 + \nu_9$	1253	1250	1253	1252	
	1241	1240			
ν_{13}	1225	1228	1233	1237	δ (CmH)
ν_{30}	1174	1173	1172	1172	δ (CbC _{vinyl})
	1170	1170			
			1181	1180	
ν_{43}	1146	1147	1154	1153	ν_s (CbS)
ν_{44}	1129	1132	1127	1133	ν_a (Pyr half-ring)
$\nu_6 + \nu_8$	1119	1120		1122	
		1087	1088		CH _{2vinyl} rock
		1056			
		1047	1042		
	1010	1011	1004	1008	(CH _{vinyl})

Figure 2 r.R. spectra in the 1350–1650 cm⁻¹ regions

r.R. spectra of reduced [Fe(II)] and oxidized [Fe(III)] forms of p- and r-core are shown. Experimental conditions: excitation, 441.6 nm; laser power, 40 mW; summation of four to six scans. For clarity, the band corresponding to the mode of reduced and oxidized p-core (1361 and 1374 cm⁻¹ respectively) has been omitted.

In order to probe the structure of the chromophore in the purified haemoprotein by a method more sensitive than optical spectroscopy, we have obtained the r.R. spectra of r-core in the high-, medium- and low-frequency regions in order to obtain a comparison with the r.R. spectra of p-core, which has already been characterized (Desbois et al., 1989).

Figure 2 and Table 1 show that the frequencies of well-characterized skeletal porphyrin modes of the oxidized and dithionite-reduced derivatives of r-core are the same as those previously obtained for the corresponding species of p-core (Desbois et al., 1989). This close identity points to a similar structure of the haem macrocycle and indicates that a low-spin hexa-co-ordination is maintained in both oxidation states of r-core. However, the ν_{11} mode is upshifted by 3 cm⁻¹ in the spectrum of the reduced r-core.

The C=C vinyl stretching mode has been previously located at 1635 cm⁻¹ in the spectra of oxidized and reduced p-core (Desbois et al., 1989). In the r.R. spectrum of ferric r-core, a change in shape of overlapping bands in the 1620–1650 cm⁻¹ region is seen (Figure 2). This spectral modification is attributable to a downshift of a ν (C=C vinyl) component from ~1636 to 1628 cm⁻¹.

Figure 3 r.R. spectra in the 1050–1390 cm^{-1} regions

r.R. spectra of reduced [Fe(II)] and oxidized [Fe(III)] forms of p- and r-core are shown. Experimental conditions were as in Figure 2.

On the other hand, a broad band corresponding to a deformation mode of vinyl groups [$\nu(\text{CH})$] (Choi et al., 1982) is upshifted from 1430 to 1434 cm^{-1} in both oxidation states (Figure 2; Table 1). The high-frequency regions of r.R. spectra of cores therefore suggest that one of the vinyl groups of protohaem is the subject of a conformational change in r-core. Compared with the p-core, the medium-frequency regions of r.R. spectra of r-core also reveal spectral modifications. In particular, the most pronounced differences concern the upshifts of the 1127–1133 and 1303–1310 cm^{-1} bands (3–6 and 2 cm^{-1} respectively) (Figure 3; Table 1). The former band may be assigned to a mode involving an asymmetric stretching mode of pyrrole rings (ν_{44}) (Li et al., 1990), since the equivalent band of the bis(imidazole) complex of Fe(II)-etioporphyrin (1136 cm^{-1}) is strongly sensitive to the [^{14}N]pyrrole $\rightarrow$ [^{15}N]pyrrole isotopic substitution (Desbois et al., 1981). On the other hand, the 1306 cm^{-1} line clearly originates from a CmH stretching mode (Abe et al., 1978; Li et al., 1990).

In the low-frequency regions, significant differences are again observed between the spectra of p-core and r-core (Figure 4). A 233 cm^{-1} band is shifted by $\sim 6 \text{ cm}^{-1}$ in both oxidation states. The origin of this band is not clear (Choi and Spiro, 1983). However, it is noteworthy that its frequency is sensitive to the haem environment (Desbois and Lutz, 1991). The frequency of this band is indeed observed at 242 cm^{-1} for the bis(imidazole) complex of iron(II) protoporphyrin in water and at 232 cm^{-1} when this compound is dispersed in an aqueous detergent. The relative high frequency observed for r-core (238–240 cm^{-1}) would imply a higher degree of hydrophilicity in its haem environment.

In the low-frequency r.R. spectra of p-core, we assigned a band at 205 (± 1) cm^{-1} to the symmetric stretching of the His-Fe-His grouping (Desbois et al., 1989). This band presents a slight downshift in frequency (203 $\pm 1 \text{ cm}^{-1}$) in the spectrum of r-core (Figure 4). This change could be related to the upshift of the ν_{11} mode (see above), and may indicate slight modifications of the H-bond network around the axial histidine (Desbois and

Figure 4 r.R. spectra in the 200–450 cm^{-1} regions

r.R. spectra of reduced and oxidized forms of p- and r-core are shown. Experimental conditions were as in Figure 2.

Lutz, 1992). Both the narrowing (by $3 \pm 1 \text{ cm}^{-1}$) and the upshift of the ν_8 r.R. band (346–350 cm^{-1}) in the spectra of r-core confirm changes in the structure of some peripheral haem groups (Figure 4; Table 2).

On the basis of the assignments of r.R. bands over a wide frequency range (150–1650 cm^{-1}), we have therefore characterized structural differences on the haem in p- and r-cores. These changes are essentially identified for modes involving the peripheral haem groups and/or parts of the pyrrole rings (Tables 1 and 2). These spectral alterations could have their origin in modification of the immediate haem-binding environment. Problems of polymerase infidelity during the PCR step, forming a polypeptide different from that expected from the parental gene, and thus different haem-protein contacts, have to be excluded, taking into account the DNA sequence. The only difference in primary structure between p- and r-core is the presence of an extra methionine residue at the N-terminus of the latter molecule. Looking at *S. cerevisiae* core structure by molecular graphics, it can be seen that the N-terminal is very flexible and located at least 0.7 nm (7 Å) from the haem edge (results not shown). On

Table 2 Low-frequency modes (150–1000 cm⁻¹) of reduced and oxidized forms of p- and r-cores

Mode	Reduced		Oxidized		Assignment
	p-core	r-core	p-core	r-core	
ν_{45}	990	991	991	987	ν (CaN)
$\nu_{32} + \nu_{35}$	972	970	974	970	
ν_{46}	927	928	928	932	ν (CbS)
			901	908	
			885	884	
	839	841	832		
ν_6	814	813	806	805	δ (CaCmCa)
ν_{32}	793	794	797	797	δ (CbS)
ν_{16}	748	749	744	748	δ (CaNCa)
ν_{47}	720	720	716	717	δ (CbS)
			693	694	
ν_7	676	675	679	678	δ (CbCaN)
	637	644			
	608	609			
ν_{49}	558	556	545		
	547	545			
	491	491	490	490	Pyr fold
	425	427	425	426	δ (CbCC _{vinyl})
	419	421	415	414	
$2\nu_{35}$	382	381	379	380	
ν_8	346	349	349	350	δ (CbS)
ν_{50} or ν_{51}	309	310			
	303	302	302	309	
	295	292	293	289	(CmCa)
ν_9	265	265	270	270	Pyr tilt + ν (FeN)
	233	238	233	240	
	205	203	204	203	ν (His–Fe–His)
	195	194	196	198	
			188	189

Figure 5 C.d. spectra of p-core and r-core in the haem absorption region

The Figure shows the c.d. spectra of p-core and r-core in both redox states. —, Reduced p-core; ----, oxidized p-core; ·····, reduced r-core; - · - · -, oxidized r-core. (a) Visible region; (b) Soret region.

the other hand, the observed spectral differences may be accounted for by different haem insertions in the protein matrix, causing changes in orientation and/or conformation of the haem substituents. Indeed, ¹H n.m.r. studies have shown that haemoglobin and myoglobin prepared by reconstitution of the apoprotein with protohaem, the natural prosthetic group also present in flavocytochrome *b*₂, contain a mixture of two interconvertible isomers. These isomers, referred to as 'native' and 'disordered' forms, arise from a 180° rotation of the haem around the α - γ meso axis (La Mar et al., 1978, 1980; Lecomte et al., 1985); they are also present in the native haemoproteins in a proportion which is generally in favour of the 'native' isomer and characteristic of the species (La Mar et al., 1980). The two isomers were shown to be functionally equivalent (Aojula et al., 1987).

More recently it has been shown that the c.d. signal in the Soret region for sperm-whale myoglobin and human haemoglobin is inversely related to the proportion of the 'disordered' isomer present (Aojula et al., 1988). This finding suggested the use of c.d. spectroscopy to verify the hypothesis that the frequency differences observed in several r.R. modes among the two cores are due to different orientations of the haem in the pocket. Thus we have characterized the c.d. spectra in the visible and Soret regions of both r- and p-core, in the native state as well as after a 'pH jump' (see the Materials and methods section). Indeed, a rapid and strong acidification of the solvent has been described to induce rotational disorder in myoglobin and haemoglobin with no need for reconstitution (Aojula et al., 1988). Figure 5 shows the c.d. spectra of p- and r-core in both oxidation states in

the visible and Soret regions. The spectra look very similar to the already characterized spectra of *S. cerevisiae* p-core (Iwatsubo and Risler, 1969), with positive peaks centred at the level of the absorption bands, around 557, 525 and 425 nm in the reduced form and 412 nm in the oxidized species; the negative peak detected at 550 nm in the reduced form has a lower intensity than that observed for *S. cerevisiae* p-core (Iwatsubo and Risler, 1969). Throughout the spectrum, the molar ellipticity of r-core is lower, in agreement with our working hypothesis; the difference is particularly evident in the Soret region for the reduced species, where a 20% difference is observed. For this reason, the molar ellipticity of the Soret band was measured for reduced p- and r-core before and after a 'pH jump' (see the Materials and methods section). The molar ellipticity of p-core decreases after the 'pH jump', down to the level of r-core, while the latter is not influenced by the 'pH jump' (Table 3). These results are in agreement with the assignment of the differences observed in the r.R. spectra to different proportions of the two isomers in the two populations of core and indicate that r-core is constituted by an equal proportion of 'native' and 'disordered' isomers; however, we cannot determine from the c.d. data how far the p-core is from this equilibrium position. The insertion of protohaem in the core pocket is different for the r-core, either because it occurs in a heterologous organism, lacking a specific enzymic system for haem insertion, or because the presence of the polypeptide chain of the entire protein is necessary to control this process.

As a functional test of r-core integrity, we have characterized its cytochrome *c* reductase activity under pre-steady state conditions, following two different experimental protocols (see the

Table 3 Effect of the pH jump on the c.d. of p- and r-cores in the Soret region

C.d. spectra of p- and r-cores in the reduced state were recorded before and after the 'pH jump' (see the Materials and methods section). The molar ellipticity at the Soret peak maximum ($[\theta]_{425}$) is reported for the four species. Results are means $\pm$ S.D.

Core	$10^{-3} \times [\theta]_{425}$ (degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$)
p-core	
Before pH jump	67 ± 4
After pH jump	50 ± 3
r-core	
Before pH jump	51 ± 5
After pH jump	49 ± 5

Figure 6 Reduction of ferricytochrome c by reduced p- and r-cores

The Figure shows the analysis in terms of a single exponential process ($k = 16 \text{ s}^{-1}$), obtained with standard fitting procedures, of the reaction observed at 416.5 nm upon mixing $2.5 \mu\text{M}$ core, degassed and reduced, with $5 \mu\text{M}$ ferricytochrome c . (a) p-core; (b) r-core. The insets show the actual experimental traces.

Materials and methods section). Figure 6 shows the time courses of cytochrome c reduction by p-core (a) and r-core (b), previously reduced by solid sodium dithionite, and the analysis of the traces according to a pseudo-first-order model, in view of the difference in redox potentials of the reactants [approx. 250 mV

Figure 7 Reduction of ferricytochrome c by reduced r-core

The Figure shows the analysis in terms of a single exponential process ($k = 30 \text{ s}^{-1}$), obtained with standard fitting procedures, of the reduction of $1 \mu\text{M}$ ferricytochrome c by $4 \mu\text{M}$ r-core observed at 416.5 nm. For the experimental conditions, see the Materials and methods section. The inset shows the actual experimental traces (upper trace, total time 500 ms; lower trace, total time 200 ms).

(Henderson and Rawlinson, 1956; Silvestrini et al., 1986)]. From these experiments, carried out as a function of cytochrome c concentration, the same second-order rate constant, $k = 6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, is obtained for r-core and p-core.

Figure 7 shows the time course observed when studying the reduction of cytochrome c by reduced r-core under the conditions used by Capeillère-Blandin and Albani (1987) for the p-core. The pseudo-first-order behaviour shown in Figure 7 is fully consistent with that previously observed by these authors over the same range of concentration of reactants. The dependence of the pseudo-first-order rate on r-core concentration gives a second-order rate constant (k) of $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value is in reasonable agreement with the value determined for p-core at the same ionic strength [$k = 8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; (Capeillère-Blandin and Albani (1987))] and with the value obtained by us using a different experimental approach (see above). Thus, as far as cytochrome c reduction is concerned, r-core and p-core show substantially identical behaviour.

Concluding remarks

We have successfully expressed the isolated domains of *H. anomala* flavocytochrome b_2 . The characterization of FDH, albeit preliminary, owing to its instability in the heterologous organism, indicates that this isolated domain shows the functional properties of that obtained by proteolysis. Attempts to increase the stability and the yield after purification are in progress.

The haem domain has been purified at homogeneity with good yields. Its u.v.-visible spectroscopic properties look identical with those of the proteolytic core, while small differences are observed using both resonance r.R. and c.d. spectroscopy. These differences are interpretable in structural terms at the level of the haem, but do not influence the rate of electron transfer to cytochrome c .

The finding that the r-core is not able to oxidize r-FDH under steady-state conditions clearly indicates that, in order to have rapid electron transfer among the two prosthetic groups, they

have to be in a particular orientation and relative position that only the correct folding in one polypeptide chain can provide.

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