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REVIEW

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Another look at the interaction between mitochondrial cytochrome *c* and flavocytochrome b_2

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Abstract Yeast flavocytochrome b_2 tranfers reducing equivalents from lactate to oxygen via cytochrome *c* and cytochrome *c* oxidase. The enzyme catalytic cycle includes FMN reduction by lactate and reoxidation by intramolecular electron transfer to heme b_2. Each subunit of the soluble tetrameric enzyme consists of an N terminal b_5-like heme-binding domain and a C terminal flavodehydrogenase. In the crystal structure, FMN and heme are face to face, and appear to be in a suitable orientation and at a suitable distance for exchanging electrons. But in one subunit out of two, the heme domain is disordered and invisible. This raises a central question: is this mobility required for interaction with the physiological acceptor cytochrome *c*, which only receives electrons from the heme and not from the FMN? The present review summarizes the results of the variety of methods used over the years that shed light on the interactions between the flavin and heme domains and between the enzyme and cytochrome *c*. The conclusion is that one should consider the interaction between the flavin and heme domains as a transient one, and that the cytochrome *c* and the flavin domain docking areas on the heme b_2 domain must overlap at least in part. The heme domain mobility is an essential component of the flavocytochrome b_2 functioning. In this respect, the enzyme bears similarity to a variety of redox enzyme systems, in particular those in which a cytochrome b_5-like domain is fused to proteins carrying other redox functions.

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Abbreviations

Cyt. b_5	Cytochrome b_5
Cyt. *c*	Cytochrome *c*
CCP	Cytochrome *c* peroxidase
ET	Electron transfer
Fcb2	Flavocytochrome b_2
FDH	Flavodehydrogenase
FeCya	Ferricyanide
Fl_{red}	Two-electron reduced flavin
Fl_{sq}	Flavin semiquinone
Fl_{ox}	Oxidized flavin
IET	Intermolecular electron transfer
H_{red}	Reduced heme
H_{ox}	Oxidized heme
WT	Wild type

Introduction

Electron transfer (ET) processes are essential to all living organisms. ET chains can comprise several protein-bound redox cofactors, as well as several redox-active amino acid side chains, (Brettel and Byrdin 2010; Jensen et al. 2010; Seyedsayamdost et al. 2006; Shih et al. 2008) maintained at a certain distance and orientation in the same molecular edifice, often a stable assembly of several polypeptide chains. Alternatively, they can require the formation of transient complexes between proteins carrying different redox prosthetic groups. These transient complexes have been attracting increasing attention in recent years. Indeed, while they have been studied for a long time in solution,

they are difficult to crystallize, because of the nature of the weak interactions between partners, without which turnover would not be possible. When they are finally crystallized, the question arises as to the functional significance of the interactions observed in the crystal.

The first structure of such a transient complex was that of the complex between CCP and yeast iso-1 cytochrome *c* (Pelletier and Kraut 1992). Subsequent studies, using for example cross-linking (Guo et al. 2004; Pappa et al. 1996) and site-directed mutagenesis (Miller et al. 1994; Pearl et al. 2008), concluded that the structure seen in the crystal was the one giving rise to physiological ET. More recently, NMR studies of that complex in solution added the notion of an equilibrium between a dynamic encounter complex and a dynamic ET complex (Volkov et al. 2006). After the structure of the CCP-cyt. *c* complex, that of the cyt. *c*-cytochrome bc_1 complex was determined (Lange and Hunte 2002; Solmaz and Hunte 2008) as well as that of several other transient redox complexes (Crowley and Carrondo 2004; Jensen et al. 2010; Nojiri et al. 2009; Sevrioukova et al. 2010; Sukumar et al. 2006). The nature of the interactions between the partners, in the crystal and in solution, was analyzed (Crowley and Carrondo 2004; Prudencio and Ubbink 2004). The conclusion was that the surface hidden upon complex formation is smaller than that in stable protein complexes (Lo Conte et al. 1999); furthermore, the core of the interface is non-polar, surrounded by oppositely charged residues, so as to allow a rapid dissociation. The NMR studies of several transient complexes between redox enzymes led to the idea that the electrostatic interactions orient the formation of the encounter complexes, within which dynamic reorientations would produce the ET-competent interactions (Prudencio and Ubbink 2004; Ubbink 2009).

Mitochondrial cytochrome *c* exerts its redox function in the intermembrane space; it is reduced by cytochrome bc_1 and oxidized by cytochrome oxidase, two membrane-bound partners. It is also the physiological electron acceptor for sulfite oxidase (Ito 1971) and mitochondrial cyt. b_5 (Bernardi and Azzone 1981; Fukushima et al. 1972; Sottocasa et al. 1967). In yeast it is a partner of CCP and of flavocytochrome b_2 (Appleby and Morton 1954; Daum et al. 1982; Ogston and Green 1935). But cytochrome *c* can exchange electrons with a number of other proteins that are not physiological acceptors and, for example, its interactions with adrenodoxin (Worrall et al. 2003) and with microsomal cyt. b_5 have been studied, the latter in great detail (Durham et al. 1995; Mauk et al. 1995). Flavocytochrome b_2 (Fcb2), or L-lactate ferricytochrome *c* oxidoreductase, is a soluble flavohemoprotein in the yeast mitochondrial intermembrane space, and since it is easier to study than membrane proteins, its interaction with cytochrome *c* has also been the object of numerous studies. A detailed review of these interactions was published in Capeillère-Blandin (1995); it was essentially centered on kinetic and thermodynamic aspects. The present review, while recalling for clarity some of the previous results, aims at providing an update for the topic, which will be essentially centered on structural aspects and their relevance to function. The question discussed in this review is the following: is the available Fcb2 crystal structure sufficient for explaining all the aspects of the enzyme functioning? Does cyt. c react with a subunit as is seen in the crystal? Recent evidence strongly suggests that this is not the case and that domain mobility plays an essential role in the Fcb2-catalyzed intra- and inter-molecular ET reactions.

Flavocytochrome b_2, the structure

Early work on Fcb2 from *S. cerevisiae* has been reviewed (Chapman et al. 1991; Lederer 1991). The first crystal structure was obtained with the WT enzyme extracted from yeast (Xia and Mathews 1990). It was subsequently redetermined to 2.3 Å resolution with crystals cooled to 100 K (Cunane et al. 2002). It was essentially identical with the first one. The structure of the recombinant WT enzyme in complex with sulfite has been determined to 2.6 Å resolution (Tegoni and Cambillau 1994). Structures of a few variant enzymes are now known and will be mentioned when necessary. Fcb2 from *H. anomala* has also been actively studied in solution (Capeillère-Blandin 1991, 1995; Chapman et al. 1991; Lederer 1991). Its amino acid sequence presents 60% identity with its *S. cerevisiae* counterpart (Black et al. 1989; Risler et al. 1989), but its three-dimensional structure has not been determined. This review will essentially concentrate on studies of the recombinant *S. cerevisiae* enzyme.

The protein is a homotetramer. Each subunit is structured into two domains (Cunane et al. 2002; Xia and Mathews 1990) (Fig. 1a). The first 99 residues are folded around the heme, the next ~390 constitute the FMN-binding domain, and the last residues up to 511 make contacts with the other three subunits. Thus, the flavodehydrogenase (FDH) domains constitute the core of the molecule, with a fourfold symmetry, while the heme domains lie at the periphery. Actually, only two heme domains per tetramer are visible in the crystal structure, indicating that the other two are disordered in the crystal (Fig. 1b). In line with this finding, the average B factors for the visible heme domains are higher than those of the FDH domains (Cunane et al. 2002; Tegoni and Cambillau 1994; Xia and Mathews 1990). NMR evidence suggested that the heme domains are also mobile in solution (Labeyrie et al. 1988). In the crystal structure of the complete subunit, the two prosthetic groups face each other, with the heme

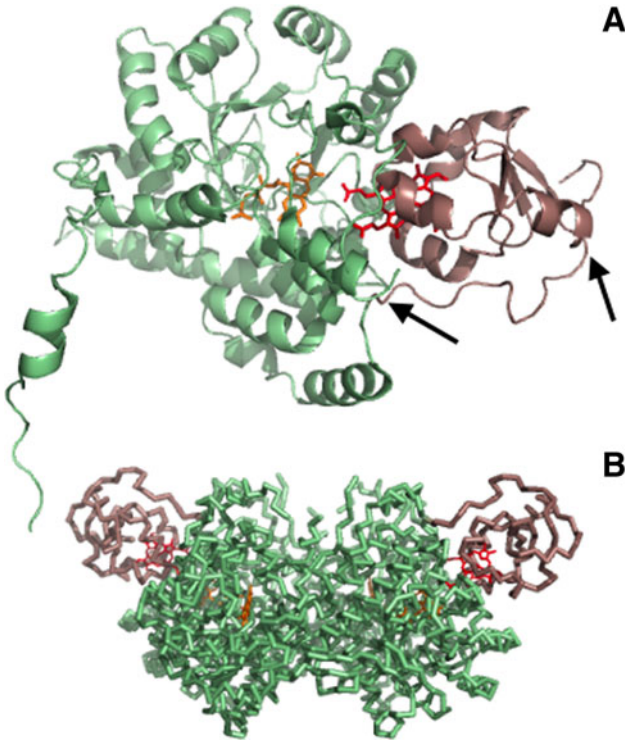


Fig. 1 The Fcb2 subunit with ordered heme domain (**a**). The FDH domain is in *green* with *yellow* FMN, the heme domain in *violet* with *red* heme.The *arrows* indicate the approximate limits of the so-called hinge region (*left arrow* at about residue 85, *right* one at position 99). **b** A view of the tetramer perpendicular to the vertical four-fold axis, showing only two heme domains at the periphery of the tetrameric FDH core. PDB code: 1KBI

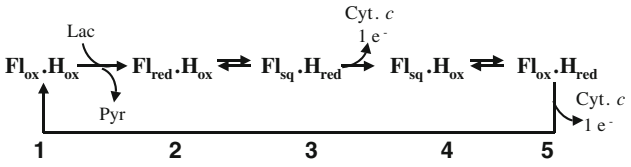
propionates pointing towards the flavin in the FDH domain. There is a tilt of about 20° between their planes. The distances from the FMN N5 atom to the closest pyrrole ring atom and to the iron are 9.7, and 13.9 Å, respectively. As detailed in Xia and Mathews (1990), the two domains share a surface area of about 400 $Å^2$; the contacts between the domains are mostly hydrophobic, with few polar ones.

The FDH domain adopts the $\beta_8\alpha_8$ barrel fold and is a member of the FMN-dependent L-2-hydroxy acid dehydrogenases family, which encompasses the well-studied lactate oxidase and monooxygenase (Maeda-Yorita et al. 1995), glycolate oxidase (Lindqvist 1992), long chain hydroxy acid oxidase (Lê and Lederer 1991) and the mandelate dehydrogenases from *P. putida* (Xu and Mitra 1999) and *R. graminis* (Illias et al. 1998). The heme domain belongs to the family of b_5-like cytochromes (Guiard and Lederer 1979; Lederer 1994). The heme porphyrin ring is embedded in a hydrophobic crevice, the walls of which are made up of two helices on each side and the floor of a ß-sheet (Durley and Mathews 1996). This evolutionary and structural relationship is of particular importance in view of the wealth of evidence now available concerning the cyt. *c*-cyt.b_5 interaction. In the Fcb2 subunit with invisible heme domain, the FDH domain chain starts at residue 100. Mobility of the heme domain could possibly arise only from movements around the bond between residues 99 and 100. Actually, evidence to be described below suggests that mobility may result from the flexibility of a piece of peptide chain (10–15 residues) at the C terminal end of the heme domain sequence, called the hinge in a broad sense. These residues appear to have few strong interactions with the body of the domain, apart from a couple of H bonds at the level of residues 92 and 94. The crystal structures of cyt. b_5 from several species show fewer than 90 residues. This suggests that these last residues in the Fcb2 heme domain may not be essential for its structural stability.

Flavocytochrome b_2, the catalytic cycle

Overall scheme

The catalytic cycle, as defined by numerous studies on the native yeast enzyme (Capeillère-Blandin 1991, 1995; Chapman et al. 1991; Daff et al. 1996a; Lederer 1991), is depicted in Scheme 1. It involves both intra- and intermolecular electron transfer steps. The substrate reduces the flavin with the transfer of two electrons and a proton (Lederer et al. 2005). Fl_{red} then transfers one electron to the heme, with formation of an anionic semiquinone (Fl_{sq}) (Capeillère-Blandin et al. 1975). As reviewed in Capeillère-Blandin (1995), and confirmed by genetic engineering (Balme et al. 1995), cyt. *c* takes electrons only from the heme. The first intermolecular electron transfer (IET) thus takes place from species 3 in Scheme 1. Completion of the catalytic cycle requires a second ET, this time from Fl_{sq} to the heme (step 4–5), and a second IET to cyt. *c*. In view of this multi-step nature, analyzing the cyt. *c*-Fcb2 interactions requires some understanding of the steps that precede cyt. *c* reduction. Non-physiological acceptors have ben used in the past (reviewed in Lederer 1991), but the usual one is FeCya, which takes electrons from both H_{red} (species 3 and 5) and Fl_{sq} (species 3 and 4), but not from two-electron reduced flavin (Fl_{red}) (species 2) in the WT enzyme (Iwatsubo et al. 1977). Nevertheless, it can also oxidize (Fl_{red}) when ET to the heme has been slowed down by a mutation (Miles et al. 1992), as it can in the heme-free



Scheme 1 Steps succession in the Fcb2 turnover

enzyme and in the isolated FDH domain (Balme et al. 1995; Gervais and Tegoni 1980; Iwatsubo et al. 1977). In the latter cases, the K_M^{app} for FeCya rises from less than 0.1 mM to about 1 mM. This parameter provides a useful tool for checking the coupling between the domains.

During turnover, a subunit is not supposed to carry more than two electrons at any time, due to the relative rate of the various steps (see below). But, when flavin and heme reductions are analyzed in a stopped-flow apparatus in the absence of acceptor, full enzyme reduction ensues, with three electrons per subunit. As lactate gives two electrons at a time, the tetramer would be stuck at stage 3 (Scheme 1) if an electron reshuffling did not occur between prosthetic groups so as to regenerate one fully oxidized flavin per dimer. Stopped-flow traces of both flavin and heme reductions are biphasic with a rapid and a slow phase. The latter is clearly irrelevant during turnover. Before the enzyme crystal structure was determined, Pompon et al. (Pompon 1980; Pompon et al. 1980) rationalized the fast phase as corresponding to ET between a privileged flavin and heme pair, and the slow phase to the electron reshuffling between non-privileged pairs. It is now clear that the privileged pair belongs to the same subunit, and that electron reshuffling is slow because inter-subunit distances between individual prosthetic groups vary between 38 and 73 Å (Xia and Mathews 1990). In what follows, the pre-steady-state rates given as k_{red}^F and k_{red}^H (the latter includes the first two steps in scheme 1) are those of the fast phases for flavin and heme reduction, respectively, unless otherwise mentioned.

The first steps

Studying the cyt. *c*-Fcb2 interaction at the kinetic level meets with the problem of a rate limitation at the level of flavin reduction by lactate. This rate limitation was demonstrated by determining primary deuterium isotope effects, using L-(2-^{2}H)-lactate. This was first done with the native yeast enzyme (Pompon et al. 1980). Since then, the recombinant Fcb2 has been studied under two different buffer and temperature conditions, as illustrated in Table 1 for the steady-state with cyt. *c* as acceptor and in Table 2 for pre-steady-state analyses of flavin and heme reductions at appropriate wavelengths. Whatever the buffer and the temperature, for the WT enzyme the results indicate that the first flavin to heme ET (2–3, Scheme 1) is fast, but not

Table 1 Steady-state primary deuterium isotope effect on cytochrome *c* reduction

Enzyme	Conditions	k_{cat} (s^{-1})	$K_M^{cyt\ c}$ (μM)	^DV	References
WT	Phosphate (30°C)[a]	155 ± 15	131 ± 2	3.0 ± 0.3	Rouvière et al. (1997)
	(5°C)[a]	61 ± 4	45 ± 4	3.6 ± 0.4	Rouvière et al. (1997)
Y143F	Phosphate (30°C)[a]	61 ± 7	121 ± 2	1.8 ± 0.4	Rouvière et al. (1997)
	(5°C)[a]	20 ± 4	131 ± 7	1.5 ± 0.2	Rouvière et al. (1997)
WT	Tris-HCl (25°C)[b]	103 ± 5	10 ± 1	3.0 ± 0.6	Miles et al. (1992)
Y143F		11 ± 1	1.5 ± 0.2	1.7 ± 0.5	Miles et al. (1992)

[a] 0.1 M phosphate buffer, 1 mM EDTA, pH 7 ($I = 0.22$ M), in the presence of 10 mM L-lactate

[b] Tris-HCl, 10 mM in HCl with ionic strength adjusted to 0.1 M with NaCl, pH 7.5 at 25°C in the presence of 10 mM L-lactate. The units for k_{cat} are moles substrate s^{-1}. The results obtained in phosphate buffer at 5°C are given for an easier comparison with the stopped-flow results in Table 2

Table 2 Stopped-flow kinetic parameters for flavin and heme reduction

Enzyme	Conditions	Flavin reduction			Heme reduction			References
		k_{red}^F (s^{-1})	K_M^{Lac} (mM)	$^Dk_{red}^F$	k_{red}^H (s^{-1})	K_M^{Lac} (mM)	$^Dk_{red}^H$	
WT	Phosphate (5°C)[a]	144 ± 4	0.89 ± 0.07	7.2 ± 0.9	108 ± 5	0.54 ± 0.7	4.7 ± 0.4	Rouvière et al. (1997)
Y143F		310 ± 19	2.55 ± 0.26	4.4 ± 0.5	49 ± 3	0.40 ± 0.04	2.0 ± 0.3	Rouvière et al. (1997)
WT	Tris-HCl (25°C)[b]	604 ± 60	0.84 ± 0.20	8.1 ± 1.4	445 ± 50	0.53 ± 0.5	6.3 ± 1.2	Miles et al. (1992)
Y143F		735 ± 80	2.81 ± 0.30	4.3 ± 0.8	21 ± 2	0.19 ± 0.02	1.6 ± 0.5	Miles et al. (1992)

[a] 0.1 M phosphate buffer, 1 mM EDTA, pH 7 ($I = 0.22$ M) at 5°C

[b] Tris-HCl, 10 mM in HCl with ionic strength adjusted to 0.1 M with NaCl, pH 7.5 at 25°C. The observation wavelengths were 557 nm for the heme, a wavelength at which the flavin absorbance is negligible whatever its redox state (Čénas et al. 2007), and 438.3 nm for the FMN. The latter corresponds to a heme isosbestic point. The absorbance decrease at this wavelength corresponds to flavin reduction, with some participation from the semiquinone. Indeed, according to (Capeillère-Blandin 1991), $\varepsilon_{ox}^{438.3} = 10\ mM^{-1}\ cm^{-1}$, $\varepsilon_{sq}^{438.3} = 3\ mM^{-1}\ cm^{-1}$, $\varepsilon_{red}^{438.3} = 1\ mM^{-1}\ cm^{-1}$ for the *H. anomala* enzyme; these values are similar for the *S. cerevisiae* Fcb2 (Čénas et al. 2007)

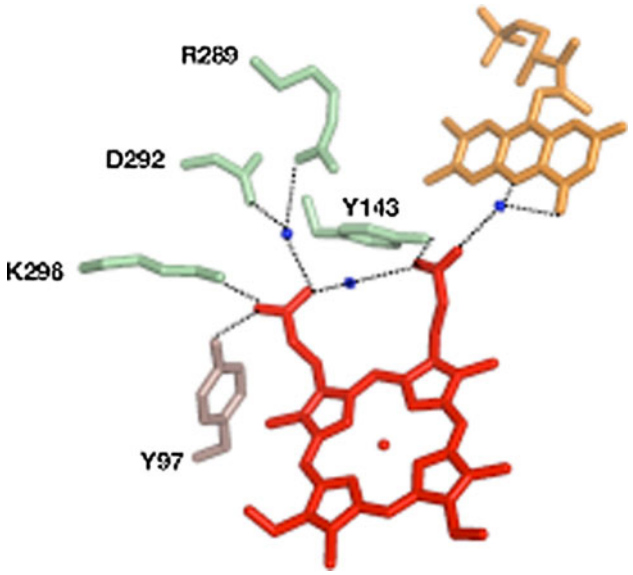


Fig. 2 Polar contacts between the domains (Xia and Mathews 1990). Colors for FMN, heme and residues are the same as in Fig. 1. The *blue spheres* are water molecules

extremely fast compared to flavin reduction, and that further steps, such as Fl_{sq} to heme ET (4–5) and/or IET from heme b_2 to heme c must introduce a further rate limitation. With the Y143F enzyme (Fig. 2) taken as an example, both conditions also indicate that the mutation induces a somewhat faster flavin reduction and slows down intramolecular ET (Table 2). But with this variant enzyme, a noticeable difference between the two sets of conditions is observed with respect to the heme overall reduction rate (k^{H}_{red} is sixfold lower than k^{F}_{red} in phosphate, but 35-fold lower in Tris, Table 2); similarly, the turnover in the presence of cyt. c is 2.5-fold slower for the Y143F enzyme than for the WT one in phosphate buffer, but 10-fold slower in Tris-HCl (Table 1). This shows that, in these experiments, the buffer nature influences flavin to heme ET more than flavin reduction, and perhaps also influences IET. The main difference between phosphate and Tris-HCl may arise from the fact that, according to studies on the recombinant FDH domain, chloride (as well as a number of other anions) binds at the active site of both oxidized and reduced enzyme, and thus lowers both flavin reduction and oxidation rates, with phosphate ions being the least inhibitory (Čénas et al. 2007). The ionic strength difference between the two buffers is essentially felt at the level of the interaction with cyt. c, as will be discussed below. Thus, when presenting kinetic parameters, one should always mention the buffer conditions under which they were obtained. Altogether, the rate of electron transfer from Fl_{red} to heme was estimated from stopped-flow studies to be 500–600 s^{-1} at 5°C (phosphate buffer) (Capeillère-Blandin et al. 1975; Pompon 1980) and ~1,500 s^{-1} at 25°C (Tris buffer) (Chapman et al. 1994). Laser flash photolysis experiments suggested a value of 1,900 s^{-1} at this same temperature (phosphate buffer) (Hazzard et al. 1994) and T-jump experiments a value of ~1,500 s^{-1} (16°C, phosphate buffer) (Tegoni et al. 1998). There is a satisfactory agreement between all these values, considering the temperature differences.

It is clear from what precedes that it is difficult to find experimental conditions for determining the rate of Fl_{sq} to heme b_2 transfer. The hemes (b_2 and c) and flavin spectra, be they in the oxidized or the reduced state, all overlap to a certain extent; isosbestic points for one prosthetic group do not necessarily offer the best observation wavelength for the partner group. Daff et al. (Daff et al. 1996a) nevertheless carried out a stopped-flow study of step 4–5 (Scheme 1), with an elaborate combination of wavelengths and the use of a slow substrate. It was concluded that this step, at 120 s^{-1}, rate-limits the overall catalytic cycle at 100 s^{-1} (Table 1, Tris-HCl, 25°C). This conclusion is however somewhat surprising in view of the value of 3 for the deuterium isotope effect on turnover, transmitted from the first step in the cycle. In T-jump experiments (from 11 to 16°C, phosphate buffer), two relaxation processes were observed; the first one was ascribed to the first ET to heme, the second one to Fl_{sq} to heme ET. The results led to the conclusion that the second flavin to heme ET is indeed slower than the first one (~200 s^{-1} vs. ~1,500 s^{-1}) (Tegoni et al. 1998). Taking into account the temperature difference between these experiments and the steady-state data of Table 1 (phosphate buffer, 30°C), it would appear that the Fl_{sq}-to-heme ET is less limiting in phosphate than in Tris buffer. In view of the value of the prosthetic groups redox potentials ($Fl_{sq}/Fl_{red} = -135$ mV, $Fl_{ox}/Fl_{sq} = -45$ mV, $H_{ox}/H_{red} = -3$ mV), Tegoni et al. (1998) concluded that intramolecular ET was most likely governed by thermodynamics and not by the dynamics of the protein.

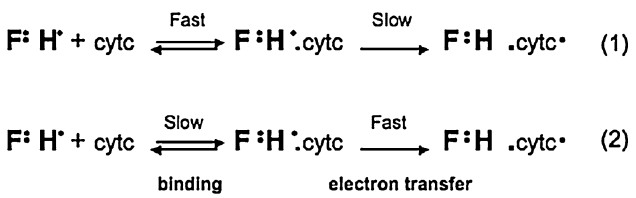
Cytochrome c reduction, influence of ionic strength

At this stage, it is easily understood that IET to cyt. c also cannot be studied in a straightforward manner. Capeillère-Blandin (1982, 1995) carried out the first detailed kinetic study of IET between Fcb2 and cyt. c using the two proteins from *H. anomala*. Cyt. c reduction by pre-reduced enzyme was followed in the stopped-flow apparatus at varying concentrations, ionic strengths and temperatures. Several conclusions were drawn. A complex is formed between the two proteins, and its stability is strongly dependent on ionic strength ($K_d = 13$ μM and 0.29 μM at $I = 0.23$ M and 0.02 M respectively, phosphate buffer, 5°C). Fitting the data with the Debye-Hückel equation led to calculate a charge factor of −5.4 and an extrapolated second order rate constant for IET at $I = 0$ of

2×10^{10} M^{-1} s^{-1}, indicating a diffusion-limited process under those theoretical conditions. Furthermore, the interaction was found to be largely entropic in nature, in other words cyt. *c* binding would displace a number of water molecules. Finally, a comparable study of IET between the isolated heme b_2 domain and cyt. *c* (Capeillère-Blandin and Albani 1987, also reviewed in Capeillère-Blandin 1995) showed a lesser dependence on ionic strength, but suggested that the presence or absence of the FDH domain had no influence on the efficiency of IET within the complex. The general conclusion was that the apparent IET rate is directly controlled by the stability of the reaction complex formed between cyt. *c* and its partner.

Daff et al. (1996b) carried out a similar kinetic study of horse heart cyt. *c* reduction by the pre-reduced recombinant enzyme from *S. cerevisiae*. At I = 0.1 M (Tris-HCl, 25°C) in the pre-steady state, the reaction was purely second order (k_{obs} = 34.8 μM^{-1} s^{-1} for the holoenzyme and half this value for the isolated heme domain). The results led the authors to conclude that the reaction obeyed equation (2) rather than (1) (Scheme 2) under the conditions used, with a rate of electron transfer in excess of 1,000 s^{-1} and a k_{off} of 280 s^{-1}, a surprisingly low value. The reaction was again ionic strength dependent, with a charge factor of −5.9 in the linear portion of the Debye-Hückel plot. However, the plot was not linear at high ionic strengths. In addition, at ionic strengths lower than ~0.08, most of the reduction occurred within the dead time of the instrument, and the presence of a slow phase led the authors to suggest the existence of IET between the 1:1 complex and another cyt. *c* molecule, as well as the existence of alternative electron transfer sites. Using the inhibition exerted by Zn-cyt. *c*, they proposed a K_d value of 8 μM for the best complex.

In conclusion, the kinetic studies carried out with Fcb2 from two different yeasts and cyt. *c* from two different species agree on the fact that the cyt. *c* reduction rate is sensitive to ionic strength. But one study analyzed the results in terms of a rapid equilibrium between the two partners (Capeillère-Blandin 1982), while the other study favored a slow equilibrium (Daff et al. 1996b). The possibility of multiple electron transfer pathways proposed by Daff et al. (1996b), using the *Saccharomyces* holoenzyme,



Scheme 2 Alternative kinetic schemes for cytochrome *c* reduction by reduced Fcb2 (Daff et al. 1996b)

does not seem to fit with the results of the comparison mentioned above for cyt. *c* reduction between the *Hansenula* holoenzyme and the corresponding isolated heme b_2 domain (Capeillère-Blandin 1995; Capeillère-Blandin and Albani 1987). Whether these differences are intrinsic to the protein species used or arise from differences or limitations in experimental conditions remains to be seen.

Binding studies and models of the complex between cytochrome *c* and Fcb2

Direct binding studies

The binding stoichiometry between the two partners has been the object of conflicting results from a variety of methods over the years, with the use of different cyt. *c* species and two different forms of the *Saccharomyces* Fcb2, as well as with the *Hansenula* Fcb2. As this problem was reviewed in detail by Blandin (Capeillère-Blandin 1995), it will only be said that a heme *b*/heme *c* stoichiometry of 1/1 would appear to be a satisfactory solution.

More recently, methods such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) were tested in the author's laboratory using the recombinant enzyme, but with little success. With H39C C102T yeast cyt. *c* (Geren et al. 1995) covalently attached to the Biacore chip via C39 on the cytochrome face opposite the heme crevice opening, there was an important non-specific Fcb2 adsorption to the matrix at low ionic strength (Mayer 1999). In addition, the binding did not obey a simple Langmuir model; fitting the same data curves with equations for different models gave rather different K_d values for some variants, so that reaching a rigorous conclusion was difficult. This non-ideal behavior was possibly due, at least in part, to the Fcb2 tetrameric nature. This series of experiments (at I = 150 mM) at least suggested that E63 is involved in the interaction with cyt. *c*, as proposed by Short et al. (1998) (see below). The results with a D72A variant were not as clear (Mayer 1999). With ITC, the signals were weak, whatever the conditions, and the apparent stoichiometry was uninterpretable, on the order of 0.1 per subunit or less (K.H.D. Lê and F. Lederer, unpublished experiments).

Models of the cytochrome *c*-flavocytochrome b_2 complex

Early experiments had shown that diffusion of cyt. *c* into Fcb2 microcrystals led to its reduction by lactate, which could have meant a physiological binding (Tegoni et al. 1983). In view of these observations, using molecular modeling and energy minimization, a model was later proposed on the basis of the crystal packing, which showed

large solvent channels between the molecules (Tegoni et al. 1993; Xia and Mathews 1990). In the best model, the b_2 and yeast *c* hemes were coplanar, with an edge-to-edge distance of 14.2 Å and an iron-to-iron distance of 25.6 Å. The contact surface area was ca. 800 Å^2. Several electrostatic interactions involving the two domains stabilized the complex. With a 1/1 heme *c*/heme b_2 stoichiometry, each cyt. *c* interacted with three subunits. Several ion pairs and hydrogen bonds between the partners were listed, among them an ion pair between R13(*c*) and E91(b_2). An ET route was proposed, involving in particular F52(b_2). Daff et al. (1996b) showed that the mutations F91K and F52A did not affect the pre-steady state rates of horse heart cyt. *c* reduction by pre-reduced enzyme and concluded that the model was unlikely to be correct.

In contrast, with the E63K, D72K and E63K:D72K variants, the stopped-flow cyt. *c* reduction rate was about 3-fold, 1.5-fold and 6-fold lower, respectively, than the WT rate (Tris-HCl, 25°C) (Short et al. 1998), suggesting the involvement of E63 and D72 in complex formation. Based on this information, Short et al. (1998) tried to model docking sites for cyt. *c* that would implicate these two residues. It was concluded that there was not one single best configuration. Two possible complexes were described, both of them involving ionic bonds with E63, D72 and with E237 on the FDH domain; one of the complexes presented an additional interaction with E240. Thus, the interaction areas were rather similar in terms of overall localization, but the individual partners in ion pairs and hydrogens bonds differed between the two complexes. Each cyt. *c* interacted with both domains of a single subunit, and the partner's porphyrin rings were perpendicular, with Fe–Fe distances on the order of 20–22 Å. There are at present no published experimental data proving a direct role of E237 and/or E240 in the interaction between cyt. *c* and Fcb2.

Interdomain molecular contacts: genetic engineering approach

In the two studies just discussed (Short et al. 1998; Tegoni et al. 1993), mention was made of the homology between the heme b_2 domain and cyt. b_5, but the possibility of a b_2/*c* interaction similar to the one proposed at the time for the b_5/*c* complex (Salemme 1976) was either not considered, or rejected. Since then, the important features of the b_5/*c* interaction have been delineated in great detail, and new results concerning Fcb2 in contrast lend credit to this hypothesis, as will be discussed below. This possibility entails that there is a more or less extensive overlap on the heme b_2 domain between its interdomain surface and the cyt. *c* binding area. Although the interdomain interactions are well described in the Fcb2 crystal structures (Cunane et al. 2002; Xia and Mathews 1990), it is interesting in this context to first analyze the functional importance of these interactions.

Manipulation of the hinge region

To probe the importance of the heme domain mobility, several modifications were brought to the hinge sequence. Residues 87–116 of the *S. cerevisiae* sequence were replaced with the equivalent segment from the *H. anomala* enzyme (residues 86–109), which is more acidic and shorter by 7 residues (hinge-swap variant, White et al. 1993). The flavin reduction rate was threefold lower, but most spectacularly the heme reduction rate was about 300-fold lower (Tris-HCl, 25°C). Subsequently, progressive deletions were introduced into the hinge region of the *Saccharomyces* enzyme (HΔ3(98–100), HΔ6(95–100) and HΔ9(92–100) (Sharp et al. 1994, 1996a;). Finally, it was also lengthened by 3 and 6 residues after residue 100 (Sharp et al. 1996b). These hinge length modifications had no effect on the enzyme charge state and no significant effect on the heme redox potential. They hardly affected flavin reduction by lactate. In contrast, Fl_{red} to heme ET was slowed down, whether there was lengthening or shortening. The k^{H}_{red} value for the shortest variant (HΔ9) was 9 s^{-1} compared to 445 s^{-1} for the WT enzyme (Tris-HCl, 25°C). No conclusion about subsequent steps in the catalytic cycle (in particular the IET step) could be drawn since this first ET step was entirely rate-limiting during turnover with cyt. *c*, except for the HΔ3 variant. For the latter, the stopped-flow study of cyt. *c* reduction by pre-reduced Fcb2 showed that the mutation had also affected the IET step (Sharp et al. 1994). The essential interpretation of these interesting studies is that altering in particular the hinge length makes recognition between the domains difficult, probably by introducing strain when the tether is too short, or by letting the domain sample too much space when the tether is too long. Similar conclusions were reached for the linker between the reductase and heme domains in cytochrome P450 BM3 (Govindaraj and Poulos 1995) and the sulfite oxidase linker (Johnson-Winters et al. 2010).

Manipulation of interdomain ionic interactions

In the original structural work (Xia and Mathews 1990), the relatively small number of interdomain polar interactions was underlined (Fig. 2). They consist mainly of one salt bridge between heme propionate D and K296, a direct H bond between propionate A and Y143, another one between propionate B and Y97, and water-mediated H bonds.

Neither the K296M (Chapman et al. 1998; Pike et al. 1995) nor the Y97F mutation (Chapman et al. 1991)

appeared to significantly alter the enzyme kinetics. Invariant R289 was also mutated, and it turns out to have a dual role (Mowat et al. 2000). In the WT enzyme, as shown in Fig. 2, it forms an ion pair with invariant D292 and is connected to heme propionate D via a water molecule. But its side chain also stacks above that of R376, which is an active site residue. In the crystal structures of the enzyme-sulfite complex (2.6 Å) (Tegoni and Cambillau 1994) and of the isolated FDH domain (Cunane et al. 2002), it can also adopt the so-called proximal orientation, in which it is directed towards the active site. In all structures of other FDH family members, except one (Lindqvist 1989), it is also seen in the proximal orientation and interacts with an active-site ligand. In the crystal structure of the Fcb2 R289K variant (2.75 Å), K289 is oriented towards the active site (proximal orientation), and not towards D292 (Mowat et al. 2000). The solution properties of the R289K variant indeed prove a role for this residue in catalysis of lactate dehydrogenation. However, since flavin reduction appears to be rate-limiting both in turnover and for heme reduction, the available kinetic results do not give a clear indication about a possible role of R289 in interdomain interactions and/or interactions with cyt. *c* (Mowat et al. 2000). Nevertheless, it is interesting to note that in the crystal structure of this variant, no heme domain is visible. This is also the case for the structure of two other variants L230A (3 Å resolution) and A198G-L230A Fcb2 (2.7 Å resolution) (Mowat et al. 2004). Since A198 and L230 are in contact with the heme (Xia and Mathews 1990), it is tempting to think that the total absence of the heme domain from the crystal structures results from the weakening of interdomain contacts because of the mutations. By extension, the absence of heme domains from the R289K structure suggests that R289 could play a role in these interactions.

In contrast, the Y143F mutation has clear consequences (Miles et al. 1992; Rouvière et al. 1997; Rouvière-Fourmy et al. 1994). Y143 also has a dual role. Indeed, in the crystals, the subunit devoid of heme domain shows pyruvate, the reaction product, forming an H bond between a carboxylate oxygen and Y143 as well as several other interactions with active site residues. The Y143F properties clearly indicated that Y143 plays a catalytic role in substrate oxidation. But the most spectacular effect was observed on the intramolecular ET, as shown in Tables 1 and 2. The crystal structure of the Y143F variant was determined at a resolution of 2.9 Å in the presence of lactate and phenyllactate (Tegoni et al. 1995). The F143 aromatic ring was superimposable onto that of Y143, but the interdomain contacts were reported to be fewer than in the recombinant WT enzyme-sulfite complex, suggesting some role for the phenolic group in recognition between the domains. Mathews and collaborators (Cunane et al. 1999; Mathews et al. 2000), using the GREENPATH software, calculated that the most favorable ET pathway went from flavin O4 to heme propionate A via the connecting water molecule (Fig. 2). The next best, with a fivefold lower coupling, went from flavin C6 to propionate A through the Y143 phenol group, and was seriously impaired in the calculation upon the replacement with a phenylalanine. At the time, the route via the water molecule appeared somewhat of a curiosity, but in recent years increasing attention has been given to the role of water in mediating long-range ET (for references, see, e.g., de la Lande et al. 2010).

Mutations on the heme domain

A number of substitutions were introduced at positions around and close to the exposed heme edge (Lê et al. 2003), and their effect on the enzyme kinetics was studied (Lê et al. 2003, 2009). Figure 3 shows the location of the positions that were modified. Neither stopped-flow flavin reduction rates nor turnover in the presence of high FeCya concentrations were altered, which constitutes another piece of evidence concerning the independence of the domains. In contrast, heme reduction and consequently turnover in the presence of cyt. *c* were slower for a number of the variants, as shown in Fig. 3. Since the flavin reduction was unimpaired, it is clear

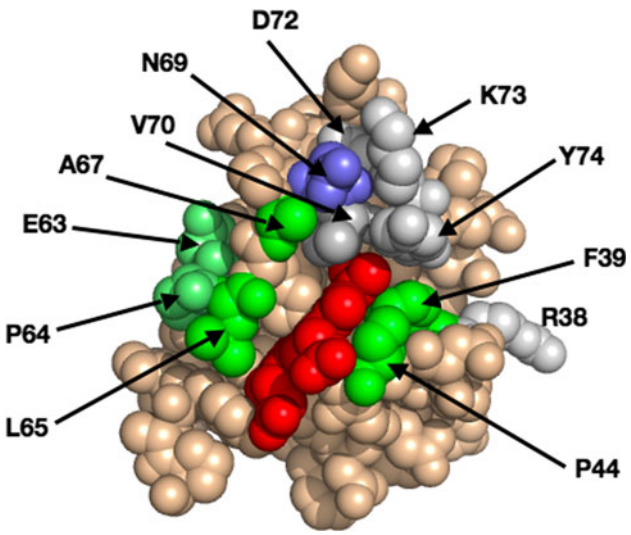


Fig. 3 Positions mutated on the heme domain, and their effect on flavin-to-heme electron transfer. The heme is in *red*. Positions in *grey*: no effect of the substitution on heme b_2 and cyt. *c* reduction (R38E, V70M, D72A, K73A, Y74F). Substitutions at the positions in darker (F39A, P44A, L65A, A67Q/L) and lighter green (E63K, P64Q/R): more or less important lowering of the heme b_2 and cyt. *c* reduction rates (Lê et al. 2009). The N69K substitution (*blue*) affects turnover in the presence of cyt. *c* but not heme b_2 reduction. The crystal structures of the WT enzyme (Cunane et al. 2002; Xia and Mathews 1990) present more contacts than those defined here with the available substitutions

that for these variants it was at least the first ET rate (step 2–3) that was modified. The most striking effects were observed for the F39A, P44A, L65A and A67Q/L mutations, and weaker effects for the E63K and P64Q/R mutations. Only for the F39A variant was the redox potential seriously altered (lowered by ~55 mV, Lê et al. 2009). Thus, the origin of the kinetic consequences of the mutations at the other positions was structural rather than thermodyamic. In the WT structures, the L65 side chain forms hydrophobic interactions with L199 and L202 of the flavin domain. The A67 methyl group is in hydrophobic contact with C233 and A231, and its peptidic O and N atoms form hydrogen bonds with the S232 peptidic N and O atoms, respectively (Cunane et al. 2002; Xia and Mathews 1990). It seems clear that replacing the methyl group by a bulkier side chain should prevent the intimate contact between the domains, and should thus impair other contacts as well, preventing productive docking. This explains the dramatic effect of the A67L mutation ($k_{red}^{H} = 1\ s^{-1}$ compared to 108 s^{-1} for the WT enzyme, Table 2). Actually, even for the F39A variant, the effect may in part be structural. Indeed, the WT structures show F325 of the FDH domain to be tucked into a hydrophobic pocket formed by P44, F39 and Y74 (Fig. 4). In the crystal structures of the subunits devoid of heme domain (WT and variants) and in the recombinant FDH tetramer, the F325 side chain protrudes into solution and shows signs of disorder, with high B factors (Cunane et al. 2002; Mowat et al. 2004; Tegoni and Cambillau 1994; Xia and Mathews 1990). Altogether, since lowering the heme reduction rate automatically slows down the steady-state cyt. *c* reduction, the results of the study of slow variants did not provide direct information as to the effects of the mutations on the IET between hemes b_2 and *c* (Lê et al. 2009). In contrast, the N69K variant (Fig. 3), showing no effect on heme b_2 reduction but a definitely slower turnover with cyt. *c*, may be affected in its interaction with the acceptor.

As another means of testing the role of mobility in the Fcb2 functioning, in the hope of favoring the formation of cross-links via disulfide bridges (Drewette et al. 2005; McFarlane et al. 1994), mutations were introduced on both heme and FDH domains. The N42C-K324C and particularly the A67C-S234C showed a decreased lactate dehydrogenase activity and a much stronger lowering of the heme reduction rate. This effect can now be easily understood since important interdomain recognition elements had been impaired by the side chain substitutions. The results were interpreted as indicating that domain movement did not play a role in cyt. *c* recognition, because IET between the pre-reduced N42C-K324C double mutant and cyt.*c* went at nearly identical rates, whether the cross-link was formed or not. Nevertheless, since that rate was more than 10-fold lower than that observed between WT enzyme and cyt.*c*, this interpretation can be questioned.

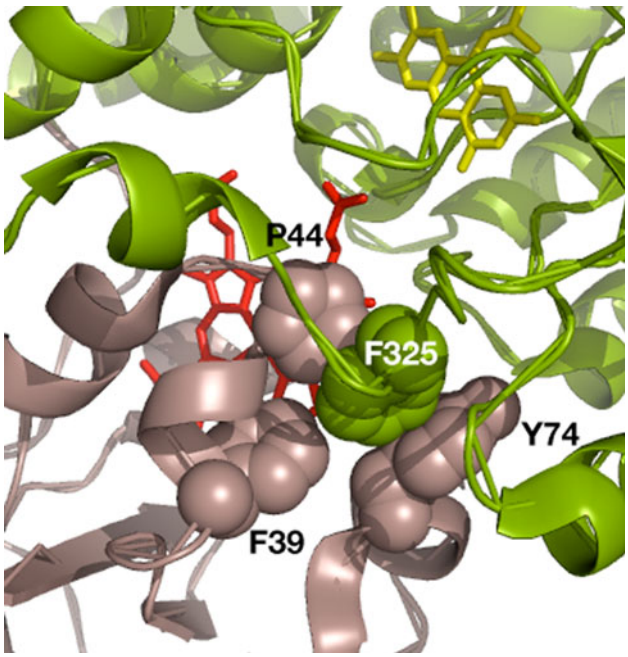


Fig. 4 The interaction of F325 (FDH domain) with P44, F39 and Y74 (heme domain)

Several conclusions can be drawn from the studies described thus far. Firstly, not unexpectedly, they confirm that the relative orientation of Fcb2 flavin and heme domains in the crystal is competent for intramolecular electron transfer in solution, an idea that was accepted from the start in view of the orientation and distance between the prosthetic groups. More importantly, they prove that the interactions between the domains are weak ones, involving essentially hydrophobic forces and steric complementarity. The covalent link between the domains plays an essential role in interdomain recognition by limiting the conformational space sampled by the domain in its movements. Indeed, separation of the domains by selective proteolysis (Gervais and Tegoni 1980) or genetic engineering (Balme et al. 1995) suppresses electron transfer between them, without severely affecting structure, redox potentials or lactate oxidation. The existence of the covalent link probably compensates for the very limited number of ionic interactions between the domains. Combined with the small contact area between them, this general description fits very well with what is now considered the hallmark of transient interactions between redox proteins (Crowley and Carrondo 2004; Prudencio and Ubbink 2004), as was mentioned in the introduction.

The monoclonal antibody approach

Unexpected information concerning the cyt. *c*/Fcb2 interaction was obtained from the study of the enzyme

interaction with a monoclonal antibody (mAb). Monoclonal antibodies against Fcb2 were elicited by injecting the WT recombinant protein into mice. One of them, mAb B2B4, had interesting effects on the enzyme kinetic properties and was selected for further study (Miles et al. 1998). The mAb-enzyme complex showed a normal lactate oxidizing activity in pre-steady-state kinetics, except that the slow phase was practically absent. Turnover was normal only in the presence of high FeCya concentrations, showing that this acceptor was taking electrons only from the reduced flavin and not from the heme. Most spectacularly, heme reduction was entirely suppressed and therefore also turnover in the presence of cyt. *c*. This total uncoupling between the domains was intriguing, so that efforts were dedicated to locating the mAb binding site.

Identification of the epitope

The Fab affinity was identical within error for the isolated heme domain, and for the holoenzyme, better than 0.1 μM. There was no binding to the recombinant tetrameric FDH. The affinity for the HΔ3 variant was unchanged, which excluded the hinge region as the epitope. The series of substitutions engineered on the heme domain, at positions around and close to the exposed heme edge, shown in Fig. 3, were tested for their effect on the Fab affinity (Fig. 5) (Lê et al. 2003). The E63K, A67Q/L and N69K mutations decreased the K_d values by at least three orders of magnitude. The P64Q/R, L65A and V70M decreased them by up to 1 order of magnitude. Finally, replacing the heme with its dimethyl ester had about the same effect as these latter mutations. These results indicated that the antibody was binding somewhat asymmetrically at the exposed heme edge to several residues and one or two heme propionates that belong to the interface between the domains, as illustrated in Fig. 3.

Crystal structure of the heme domain-Fab complex

The structure of the complex (Fig. 6) confirms the location of the epitope, while providing a more detailed picture of the contacts between the two proteins (Lê et al. 2010). In particular, it shows that only one propionate carboxylate is involved in contacts with the Fab, and that N30 is also part of the epitope. In addition, when one superimposes the chain tracings of the heme domains in the subunit and in the complex with the Fab, it is clear that binding of the Fab induces some deformation of the peptide chain between residues 65 to 74, with a maximum displacement of 1.9 Å of the P68 Cα (Cunane et al. 2002; Lê et al. 2010; Xia and Mathews 1990). More unexpectedly, it also induces a rotation of the heme in its pocket by about 20°, more or less in the same plane, as well as a translation of 0.3 Å (Fig. 7).

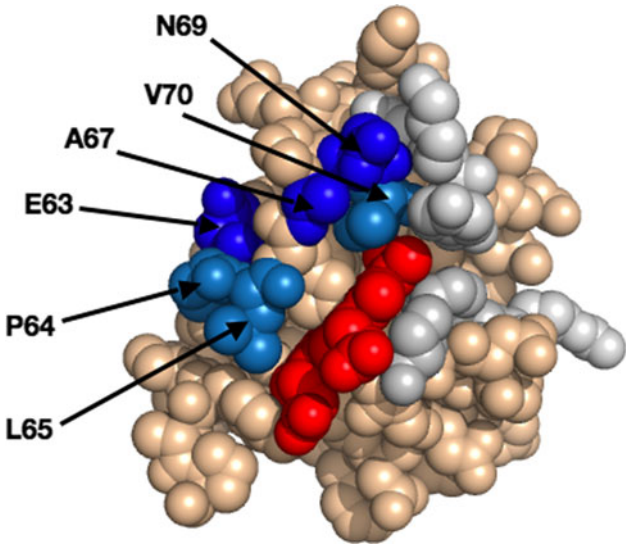


Fig. 5 Epitope mapping by site-directed mutagenesis. All the substitutions are at the same positions as in Fig. 3. Residues in *grey*: the substitutions did not affect the affinity for the mAb. Their numbering can be found in Fig. 3. For residues in *dark blue*, the substitutions lowered the affinity by more than three orders of magnitude, for those in lighter blue by about one order of magnitude, as did the substitution of protoheme IX by its dimethyl ester (Lê et al. 2003)

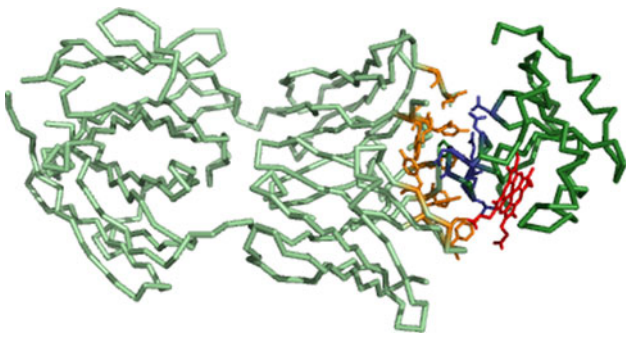


Fig. 6 Structure of the complex between the Fcb2 heme domain and the Fab of anti-Fcb2 monoclonal antibody B2B4 showing the asymmetric binding of the Fab to one side of the exposed heme edge (PDB code: 3KS0). Heme domain in *dark green*, Fab in *pale green*, heme in *red*. Side chains of important contact residues are shown as sticks, *blue* on the heme domain, *orange* on the Fab

Interestingly, in the the recombinant WT enzyme-sulfite complex, the heme showed a rotation of 10° and a translation of 0.34 Å (Tegoni and Cambillau 1994). Heme movements somewhat similar to the latter are observed in the structures of Y43F Fcb2 in complex with pyruvate and with phenylglyoxylate (Tegoni et al. 1995). It could thus be that the heme position in its hydrophobic crevice is slightly different between the yeast and the recombinant enzymes, possibly because of differences in the biosynthetic processes between yeast and *E. coli*. But the rotation in the complex with the mAb is more important, and, strikingly, the propionate orientations are different from

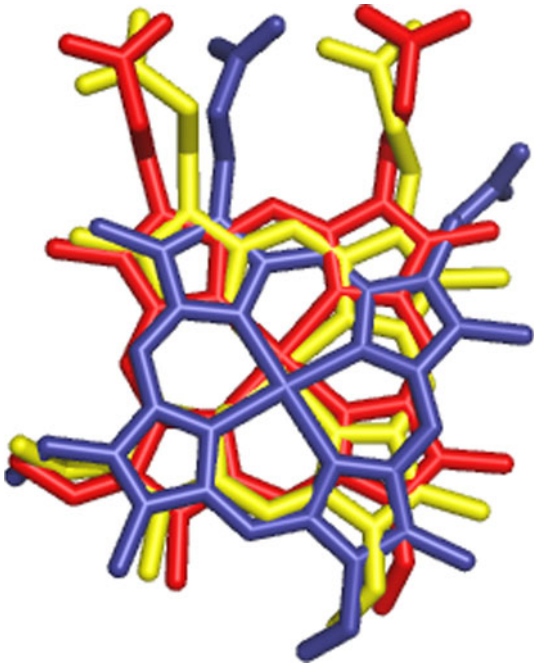


Fig. 7 Heme relative positions after superimposing the peptide chains of the heme domains of WT yeast Fcb2 (*red*, PDB code 1KBI) of WT recombinant Fcb2 in complex with sulfite (*yellow*, PDB code 1LTD) and of the domain in complex with the Fab (*blue*, PDB code 3KS0)

those in the heme domains facing the flavin domain, as shown in Fig. 7.

The crystal structure unambiguously confirms that the mAb binds when the heme domain is not docked to the FDH domain, since a structural superposition via the heme domains of the subunit (Cunane et al. 2002) and of the complex with the Fab (Lê et al. 2010) shows a severe steric clash between the FDH domain and the Fab (Fig. 8). A possible minimal movement of the heme domain by a rotation of about 63° around the 98–99 peptide bond was modeled, allowing binding of the Fab in the presence of the FDH domain. This manipulation appeared to allow the binding of four Fab molecules per enzyme, when adding the two missing heme domains to the experimental structure of the tetramer. Nevertheless, titrations had indicated only two Fab binding sites per tetramer (Miles et al. 1998). This suggests that the heme domain movements are actually of larger amplitude than the modeled one, so that the bulk of the Fab prevents its binding simultaneously to all subunits. In addition, since cyt. *c* reduction is essentially suppressed at Fab saturation, one has to conclude that, in the complex, steric hindrance prevents the two uncomplexed heme domains from docking to their respective FDH domains so that they cannot be reduced by the flavin.

Thus, altogether, if the heme domain mobility allows the binding of a ~150 kDa antibody at the exposed heme edge, there should be no steric hindrance to prevent the

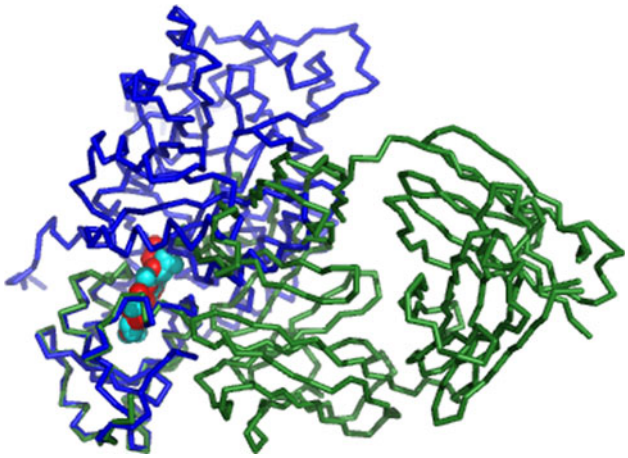


Fig. 8 Superposition via the heme domains of the WT Fcb2 subunit, in *blue* with *red* heme (PDB code: 1KBI) and in the complex with the Fab (PDB code 3KS0), in *green*, showing the steric clash between the Fab and the FDH domain

binding of a 13-kDa molecule such as cyt. *c*. When one considers the homology between cyt. b_5 and the Fcb2 heme domain, the hypothesis of a functional docking of cyt. *c* to this exposed heme edge acquires even more weight.

The cytochrome *c*-cytochrome b_5 interaction as a model

Although not physiological, the interaction between cyt. *c* and microsomal cyt. b_5 has been intensively studied as a model for IET, both at the functional and structural level. Salemme (1976) proposed a hypothetical model of the complex between tuna cyt. *c* and the soluble domain of bovine cyt. b_5, based on the only two cytochrome structures determined at the time. Its elaboration was based on the known ionic strength dependence of their interaction. The model involved specific ion pairs between positively charged cyt. *c* residues and negative ones on cyt. b_5, as well as hydrophobic interactions and steric complementarity. This model was subsequently submitted to molecular dynamics simulations, which introduced the notion of a flexible association between the two cytochromes (Wendoloski et al. 1987). Numerous studies, which cannot all be reviewed here (Durham et al. 1995; Guillemette et al. 1994; Mauk et al. 1995; Ren et al. 2004; Vergères and Waskell 1995), in particular recent NMR ones (Banci et al. 2003; Deep et al. 2005; Shao et al. 2003; Volkov et al. 2005), probed the validity of the model. After some controversy, it is now accepted that the encounter between the two cytochromes is oriented by electrostatics, that in the ET complex hydrophobic interactions and steric complementarity play a major role and that the two proteins interact essentially via the surfaces around the respective exposed heme edges, as proposed by Salemme, although several different

relative orientations within the dynamic complex, and not a single one, may lead to electron transfer.

Concerning its interaction with Fcb2, some experimental evidence exists that it is this same area on cyt. *c* that is involved. The horse heart protein, when chemically modified at single lysines, was more slowly reduced by Fcb2 when the modified lysines were those on the front face (Matsushima et al. 1986). Mutation of yeast iso-1 cyt. *c* K27 (mammalian numbering), one of these lysines, reduced the cell growth on lactate by 15 to 35% depending on the substitution (Das et al. 1988). Replacement of conserved (trimethylated in yeast) K72 by arginine, which conserves the charge, led to no or minor changes both in vivo and in vitro (Holzschu et al. 1987). With respect to the heme b_2 domain interaction area, it is not possible to directly extrapolate to Fcb2 from cyt. b_5 in the proposed b_5/*c* complexes. Indeed, the heme b_2 domain is much less negatively charged than cyt. b_5. Using the structures of the domain in complex with the Fab and that in the subunit, models of the complex between the heme b_2 domain and yeast iso-1 cyt.*c* were calculated using the docking program HADDOCK (de Vries et al. 2007; Dominguez et al. 2003; Lê et al. 2010). For cyt.*c*, the residues used in the computations were the basic ones generally found in models of the b_5/*c* complex. A number of residues around the exposed edge of the heme b_2 domain were probed *in silico* for the docking. With the same assumptions concerning the residues involved in the docking, the calculated models were somewhat different when the b_2 domain structure was that of the Fcb2 subunit or that in complex with the Fab (Lê et al. 2010).

Preliminary results for the reduction of horse heart cyt. *c* by pre-reduced Fcb2 were obtained in the author's laboratory, using the WT recombinant enzyme and a few of the variants carrying the mutations on the heme domain (Figs. 3, 5) (Lê et al. 2008). The P44A, E63K, L65A and N69K variants were roughly half as efficient as the WT enzyme, while the F39A, A67L, D72A and K73A behaved identically to the WT, within error. Noticeably, the reduction kinetics, in phosphate buffer, were not second order, as Daff et al. (1996b) had observed in Tris buffer, but showed saturation at high enzyme concentrations, with K_M values in the μM range. That mutations at positions 44 and 65, which slow down flavin to heme ET, probably by impairing the interdomain recognition, should lower the IET rate is a result compatible with a b_2/*c* model in which cyt. *c* interacts with both domains as they are seen in the crystal structures, although this does not prove the model (Short et al. 1998). The effect of the E63 K mutation is also compatible with such a model, and in agreement with previous kinetic results with this variant (Short et al. 1998). But the A67L variant, which is the most disruptive in terms of interdomain recognition, appears to behave as the WT enzyme in terms of IET. This strongly suggests that cyt. *c* binds to the heme b_2 domain when the latter is not docked to the FDH domain as it is seen in the crystal of the complete subunit. In view of the differential ionic strength sensitivity displayed by the holoenzyme and the heme domain in their interaction with cyt. *c* (Capeillère-Blandin 1995), it cannot be ruled out that the FDH domain could play a role in the formation of the encounter complex and provide interactions with the cyt. *c* side. More work is required in order to better define the respective interaction areas, using kinetic and spectroscopic methods, in particular NMR.

Conclusion

The recent results reviewed here strongly suggest that cyt. *c* binds to Fcb2 when the heme domain movements have made available the surface around the exposed heme edge and that mobility is an essential component of the enzyme functioning. One could even consider that Fcb2 by itself belongs to the category of transient ET complexes, in spite of the covalent link between the domains. These results also raise several questions. Does the plasticity of the heme domain, highlighted in the Fab-heme domain crystal structure, play a functional role? Could cyt. *c* binding induce a conformational change relative to the domain conformation when docked to the FDH domain? Could IET be actually limited by the heme domain movements rather than by thermodynamics? Furthermore, flavin reduction by lactate could itself depend on the heme domain mobility, since in the crystal structures Y143 forms an H bond either with a heme propionate (Fig. 2) or with the pyruvate carboxylate in the subunit with disordered domain. Site-directed mutagenesis showed that Y143 is indeed involved in substrate binding (Rouvière-Fourmy et al. 1994). Lactate binding could require the heme domain to be away from its docking site, or be achieved by a simple modification of the heme propionate conformation, as is observed in the crystal structure of the Y143F Fcb2 variant in complex with phenylpyruvate (Tegoni et al. 1995).

Finally, in terms of mobility, one should also mention here loop 4, at the active-site end of the $\beta_8\alpha_8$ barrel. From 9 to 23 residues of that loop are invisible in the structures of WT Fcb2 and its variants. It was noted that in one of the WT structures, the visible part of the loop encroaches into the space that would be ocupied by the heme domain, were it not disordered in that subunit (Cunane et al. 2002). Moreover, while part of loop 4 is also disordered in most structures of the FDH family members, it covers the active site in a lid-like fashion in some structures of lactate oxidase (Leiros et al. 2006; Li et al. 2007) and glycolate oxidase (Murray et al. 2008). Since proteolytic cleavage in

Fcb2 loop 4 at a single position leads to a threefold decrease of the lactate oxidation rate (Lederer 1991), it does not seem improbable that, during turnover, loop 4 could also cover the active site, which is only possible when the heme domain is not docked to the FDH domain. When it is docked, the visible part of the loop lies on the side of the heme domain. Could there be alternating movements of loop 4 and the heme domain during the catalytic cycle? Could loop 4 play a role in the interaction with cyt. *c*?

Members of the cyt. b_5 family present interesting examples of the functional role of domain mobility. Microsomal cyt. b_5 itself is anchored to the membrane by a C terminal hydrophobic tail, as are its physiological reductases, cyt. b_5 reductase and cytochrome P450 reductase and its oxidizing partners, in particular cytochromes P450 and fatty acyl desaturases (Im and Waskell 2011; Vergères and Waskell 1995). While a soluble form of this cytochrome in erythrocytes, reduced by a soluble form of the microsomal reductase, acts as a methemoglobin reductase (Hultquist and Passon 1971), b_5 homologues are found fused to other proteins besides the Fcb2 lactate dehydrogenase, such as a soluble form of cyt. b_5 reductase (Zhu et al. 1999), a number of fatty acyl desaturases (Sperling and Heinz 2001), the molybdopterin domains of sulfite oxidase and assimilatory nitrate reductases (Lederer 1994). In the latter case, the fusion also includes the flavoreductase. Chemical modifications, cross-linking studies and numerous site-directed mutagenesis studies combined with a variety of kinetic and physical methods have been carried out in order to define the interactions between the cyt. b_5 module and one or the other of its partners (Im and Waskell 2011; Prudencio and Ubbink 2004; Strittmatter et al. 1990; Vergères and Waskell 1995). The interacting surfaces have been explored by Brownian dynamics (Kidd et al. 2002; Liang et al. 2002; Liang et al. 2004; Wheeler et al. 2007). Models of the cyt. b_5 complex with several partners other than cyt.*c* (cyt. b_5 reductase, cytochrome P450, myoglobin, hemoglobin, sulfite oxidase, nitrate reductase), based on individual crystal structures with or without additional experimental evidence, have also been proposed (Bando et al. 2004; Bridges et al. 1998; Gao et al. 2006; Lu et al. 1995; Nishida and Miki 1996; Utesch and Mroginski 2010; Vergères and Waskell 1995). It was generally concluded that it is the surface around the exposed heme edge that interacts with the partners for ET, as it does with cyt. *c,* even when the interaction is found to be particularly dynamic (Vergères and Waskell 1995; Worrall et al. 2002). It thus appears that in the fused structures, the cyt. b_5 module has to be mobile in order to present the opening of the heme crevice alternatively to its reducing parner and to the oxidizing one. Thus far, only two crystal structures are known that have a cyt. b_5 module fused to a partner, namely those of Fcb2 and of sulfite oxidase (Kisker et al. 1997). For the latter, the distance between the prosthetic groups is such that it is clear that the crystal structure does not display an ET-competent interface. The cytochrome domain must clearly reorient itself via a flexible linker in order to interact productively with the molybdopterin domain. Thus, at present, Fcb2 is the only b_5-family member for which an actual structure of the physiological interaction with its cognate reductase exists. Future work will be devoted to delineating how similar its interactions with its physiological acceptor yeast cyt. *c* are to the interactions defined in particular by NMR (Deep et al. 2005; Shao et al. 2003; Volkov et al. 2005) for the non-physiological interaction between microsomal cyt. b_5 and mitochondrial cyt. *c*. A better definition of these interactions should enable an understanding of the electron transfer process itself.

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