Epitope mapping for the monoclonal antibody that inhibits intramolecular electron transfer in flavocytochrome *b₂*

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Flavocytochrome *b₂* (yeast L-lactate dehydrogenase) carries one FMN and one protohaem IX on each of its four subunits. The prosthetic groups are bound to separate domains, the haem domain (residues 1–99) and the flavin domain (residues 100–485), which interact for electron transfer between lactate-reduced FMN and haem *b₂*; *in vivo*, the latter reduces cytochrome *c*. In the crystal structure, one haem domain out of two is mobile. Previously we have described a monoclonal antibody, raised against the tetramer, that only recognizes the native haem domain and prevents electron transfer between flavin and haem, while having no effect on flavin reduction by the substrate [Miles, Lederer and LÊ (1998) *Biochemistry* 37, 3440–3448]. In order to understand the structural basis of the uncoupling between the domains, we proceeded to site-directed mutagenesis, so as to map the epitope on the surface of the haem domain. We analysed the effects of 14 mutations at 12 different positions, located mostly in the

domain interface or at its edge; we also analysed the effect of replacing protohaem IX with its dimethyl ester. We used as criteria the antibody-mediated inhibition of cytochrome *c* reduction by flavocytochrome *b₂*, competitive ELISA tests and surface plasmon resonance. We have thus defined a minimal epitope surface on the haem domain; it encompasses positions 63, 64, 65, 67, 69 and 70 and one or both haem propionates. When the haem and flavin domains are docked for electron transfer, the 65, 67 and 70 side chains, as well as the haem propionates, are excluded from solvent. The present results thus indicate that, when bound, the antibody acts as a wedge between the domains and constitutes a physical barrier to electron transfer.

Key words: domain mobility, electron transfer, epitope mapping, flavocytochrome *b₂*, surface plasmon resonance.

INTRODUCTION

Flavocytochrome *b₂* (Flb2) is a haem- and FMN-containing L-lactate dehydrogenase, present in the intermembrane space of yeast mitochondria, with cytochrome *c* as the physiological acceptor. Its crystal structure shows that, in each subunit of the tetrameric protein, protohaem IX binds to a domain encompassing the first 99 residues, while FMN is bound to a $\beta_8\alpha_8$ barrel made up of the next 385 amino acids; the last residues successively contact the three other subunits [1]. The haem domain belongs to the family of cytochrome *b₅*-like proteins [2], and its fold is very similar to that of microsomal cytochrome *b₅* [1,3]. During the catalytic cycle, substrate reduces the flavin in a two-electron reaction; reduced FMN then transfers electrons one at a time, in two successive steps, to haem *b₂*, which is reoxidized by cytochrome *c* (for a review, see [4]).

With respect to redox partners, the same question arises for Flb2 as for cytochrome *b₅*: does the same surface area of the haem-*b₂* domain or do different ones come into play for electron transfer to and from the partners, namely the flavin domain on one hand, cytochrome *c* on the other? For cytochrome *b₅* as for cytochrome *c*, it is generally believed that it is the opening of the haem crevice that interacts with partners, with possibly a series of slightly different docking modes [2,5–7]. In the Flb2 crystal structure, this area is in contact with the FMN domain, and the distance between FMN N-5 and the closest haem atom is about 10 Å, with no intervening side chain [1] (Figure 1). Actually, this picture is given by only one out of the two subunits in the crystal asymmetric unit. In the other subunit, the haem domain is invisible, which indicates mobility in the crystal. Mobility in solution was also suggested by NMR experiments [8]. The

interaction forces between flavin and haem domains appear to be weak, since electron transfer between the two prosthetic groups requires the covalent link between the domains; indeed, when they are separated, either by proteolysis or by genetic engineering, the flavin cannot reduce the haem any more [9,10]. Moreover, in the past, no influence of a domain on the properties (such as visible spectra, redox potential or EPR signals) of the other domain was detected [4]. In view of these findings, it can be wondered what role, if any, the mobility of the haem domain plays in the Flb2 catalytic cycle. It could be required to let the substrate access the active site, or to let cytochrome *c* access its binding area for productive electron transfer, if this area overlaps with that of the flavin domain, or both.

This problem has been studied by protein engineering, through either site-directed mutagenesis [11] or manipulation of the length and sequence of the peptide segment linking the two domains [12–14]. These studies demonstrated the importance, for flavin to haem electron transfer, of the Tyr-143 phenol group and of the integrity of the peptide linker between the domains. However, although the effects observed could be rationalized in terms of haem domain mobility, other interpretations could not be excluded. For our part, we developed an immunological tool that turns out to be adapted to the study of the mobility problem. In a previous paper, we described the effect of a monoclonal antibody (mAb) on the Flb2 enzymic activity [15]. This antibody was raised against the holoenzyme, but only recognized a conformational epitope on the native haem domain. Kinetic studies showed that its binding abolished electron transfer from flavin to haem, which entailed the inhibition of cytochrome *c* reduction by haem *b₂*. In contrast, the flavodehydrogenase (FDH) activity was normal in the presence of the small non-physiological acceptor ferricyanide.

Abbreviations used: DME, dimethyl ester; Flb2, flavocytochrome *b₂*; FDH, flavodehydrogenase; mAb, monoclonal antibody; SPR, surface plasmon resonance; WT, wild type; TBS, Tris-buffered saline.

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Figure 1 The F1b2 subunit (C α representation with haem and FMN)

This image was created using TURBO-FRODO [53] and the F1b2 co-ordinates in the Protein Data Bank (FCB1) [1]. The other subunit in the asymmetric unit only shows the flavin domain, with pyruvate bound at the active site.

These results led us to attempt to delineate the mAb epitope on the haem domain, so as to understand in structural terms the functional uncoupling between flavin and haem domains. We now report the results of site-directed mutagenesis, which have led us to map the epitope.

EXPERIMENTAL

Site-directed mutagenesis and protein production

Plasmid pDSb2 was used as a starting point for mutagenesis [16], as it carries the entire F1b2 coding sequence. The sequence corresponding to the haem-binding domain was excised between the polylinker *Eco*RI and the internal *Bam*HI restriction sites, and subcloned into the Bluescript vector between the same sites. After mutagenesis, the fragment was cloned back into pDSb2, so as to replace the wild-type (WT) sequence. The entire coding sequence was verified using an Applied Biosystems 377 Prism DNA sequencer.

Two methods were used for mutagenesis. The first one, for mutations R38E, N69K and D72A, was the overlap-extension method [17]. Briefly, two fragments in opposite directions were produced by PCR, both overlapping the site to be mutagenized at their 5' ends. The sequences of the oligonucleotides used for mutagenesis are shown in Table 1. For PCR, they were used together with the reverse primer and the T7 primer. The fragments were then combined so that the 5' ends annealed and the 3' overlaps of each strand served as a primer for the 3' extension of the complementary strand in a PCR reaction. The whole fragment was then further amplified by PCR, using the reverse primer and the T7 primer. The second method, using the QuikChange Site-Directed Mutagenesis kit (Stratagene), involved copying the whole plasmid followed by digestion of the original strand with the *Dpn*I restriction enzyme. Mutations F39A, P44A, P64R, P64Q, L65A, A67L, A67Q, V70M, K73A and Y74F were introduced with this method using the oligonucleotides shown in Table 1. The mutagenized plasmids were propagated in *Escherichia coli* XL1-Blue. The plasmid bearing the E63K mutation was given kindly by Dr G. A. Reid (University of Edinburgh, Edinburgh, U.K.) [18].

Table 1 Sequence of the mutagenic oligonucleotides

Mutagenic codons are underlined. For each mutation, the second line gives the inverse complementary sequence, shown 5' to 3'.

Mutation	Primer sequence
R38E	5'-GACTTAACGGAGTTCCTACCA-3' 5'-TGGTAGGAACTCCGTTAAGTC-3'
F39A	5'-GACTTAACGCGAGCCCTACCAATCATCC-3' 5'-GGATGATTTGGTAGGGCTCGCGTTAAGTC-3'
P44A	5'-CTACCAAATCATGCCGGTGGGCAGGA-3' 5'-TCCTGCCACCGGCATGATTTGGTAG-3'
P64R	5'-CTGCTATTTTGAACGGTACATGCTCC-3' 5'-GGAGCATGTAACCGTTCAAAAATAGCAG-3'
P64Q	5'-CTGCTATTTTGAACAGTTACATGCTCC-3' 5'-GGAGCATGTAACGTTTCAAAAATAGCAG-3'
L65A	5'-CTATTTTGAACAGCACATGCTCCTAATG-3' 5'-CATTAGGAGCATGTGCTGGTTCAAAAATAG-3'
A67L	5'-CCATTACATCTGCCCTAATGTC-3' 5'-GACATTAGGCAGATGTAATGG-3'
A67Q	5'-CCATTACATCAGCCCTAATGTC-3' 5'-GACATTAGGCTGATGTAATGG-3'
N69K	5'-TGCTCCTAAGGTCATCGAT-3' 5'-ATCGATGACCTTAGGAGCA-3'
V70M	5'-CATGCTCCTAATATGATCGATAAG-3' 5'-CTTATCGATCATATTAGGAGCATG-3'
D72A	5'-CCTAATGTCATCGCTAAGTATATAGCTCCC-3' 5'-GGGAGCTATATACTTGAGCGATGACATTAGG-3'
K73A	5'-CTAATGTCATCGATCCGTATATAGCTCCCGA-3' 5'-TCGGGAGCTATATACGCATCGATGACATTAG-3'
Y74F	5'-GTCATCGATAAGTTCATAGCTCCCGA-3' 5'-TCGGGAGCTATGAACCTATCGATGAC-3'

F1b2 expression and purification

The WT and the mutagenized proteins were constitutively expressed in *E. coli* HB101 in Terrific Broth medium or sometimes in LB (Luria-Bertani) medium [19], and purified according to [20]. All the enzymes were concentrated by (NH₄)₂SO₄ precipitation (70 % saturation), redissolved in 10 mM L-lactate-containing standard buffer (0.1 M phosphate buffer/1 mM

EDTA, pH 7.0) and stored as such at -80°C . Their purity was confirmed by SDS/PAGE. Enzyme concentrations were expressed relative to one haem ($\epsilon_{413}^{\text{ox}} = 129.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{423}^{\text{red}} = 183 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The flavin content of the preparation, when necessary, was determined by differential spectral titration in the presence of sulphite [21].

The haem-binding domain was prepared from the holoenzyme by tryptic proteolysis as follows. Trypsin (1 mg; Merck) was added to 0.4 μmol of Flb2 in 1 ml of 0.1 M Na^+/K^+ phosphate buffer, pH 8.2. After a 2-h incubation at 37°C , digestion was complete and the reaction mixture was separated on a Sephadex G-100 column (2.5 cm $\times$ 100 cm) equilibrated in 0.1 M phosphate buffer, pH 7. Fractions presenting an absorbance ratio at 280 nm/423 nm of ≤ 0.18 were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80% saturation). The yield was about 50%.

For the BIAcore experiments, the preparation was submitted to chromatofocusing on a MonoP HR 5/20 column (Pharmacia). The column was first treated with 1 ml of 5 M NaOH solution, then equilibrated with 25 mM Bistris/HCl buffer, pH 6.7. The protein was concentrated and dialysed against the equilibration buffer on a Centricon 10 cartridge (Amicon); 2 mg of protein in 200 μl was eluted with Pharmacia Polybuffer 74 (diluted 10 times), pH 5, at a flow rate of 0.5 ml/min. The major fraction was concentrated on a Centricon 10 cartridge. Isoelectric focusing showed a single band with a pI of 6.5. The overall yield was about 50%.

Preparation of the haem-binding-domain derivative with the haem dimethyl ester (DME)

To 800 μl of 220 μM haem-binding domain after dialysis against water was added 6 ml of acid acetone (0.2% conc. HCl) at 4°C . After 10 min, the precipitated protein was collected by centrifugation at 12000 g for 10 min and taken up in 500 μl of water. The haem-extraction procedure was repeated twice. Residual acetone in the final pellet was removed in a stream of nitrogen and the apoprotein was dissolved in 150 μl of water. The protein yield, as estimated from the absorbance at 280 nm, was about 90%. The reconstitution with normal and esterified haem was carried out using a protocol adapted from [22]. To 50 μl of the apo domain was added 30 μl of DMSO; the protein concentration was then about 0.6 mM; 20 μl of a haemin chloride solution (2 mg/ml in 30% DMSO) was added and the solution was gently stirred overnight at 4°C , although reconstitution was almost immediate. The reconstitution yield was 100%. For reconstitution with the DME, 100 μl of the apo domain solution was diluted with 60 μl of DMSO and treated with 40 μl of ferriprotoporphyrin DME (2 mg/ml in DMSO). The solution was gently stirred overnight at 4°C . At that point, the visible spectrum showed a 60% reconstitution yield; 7 μl of the DME solution was added and the solution was stirred for another 24 h. This increased the reconstitution yield to only 65%. The reconstituted domains were separated from excess free haem by filtration on a Sephadex G-75 column (1 cm $\times$ 25 cm) equilibrated in 0.1 M Na^+/K^+ phosphate buffer, pH 7. The fractions with a 280 nm/411 nm absorbance ratio of ≤ 0.2 were pooled and concentrated with a Centriprep 10 cartridge (Amicon). The final yield of DME haem-binding domain was about 50%, and the preparation contained about one-third non-reconstitutable protein.

Fab fragment preparation

The production and purification of the B2B4 antibody were described in [15]. The Fab fragment was obtained by papain proteolysis as follows. To 1 mg of purified IgG in 100 μl of

0.1 M Na^+/K^+ phosphate buffer/2 mM EDTA, pH 7.4, were added cysteine to 10 mM and 0.05 mg of papain (Boehringer Mannheim). After 2 h at 37°C the reaction was stopped by adding iodoacetamide to 40 mM. After a 10-fold dilution in 0.1 M Tris/HCl/1 M NaCl, pH 8.9, the reaction mixture was chromatographed on a Protein A-Sepharose column (0.5 cm $\times$ 1 cm) equilibrated with the same buffer. Uncleaved IgG and Fc fragment were retained, and the Fab fragment flowed through and was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation). The precipitated protein was diluted 10-fold in 5 mM Na^+/K^+ phosphate buffer, pH 7, and loaded on to a DEAE-cellulose column (0.5 cm $\times$ 1 cm) equilibrated in the same buffer. The proteins were eluted with successive 2-ml volumes of 50, 100, 150, 200 and 300 mM phosphate buffer. The Fab fragment was eluted between 200 and 300 mM buffer. It was aliquoted and stored as such at -80°C . Its affinity decreased with storage time, and a preparation was not used for more than 6–8 weeks. For the BIAcore experiments, the fragment was additionally submitted to chromatofocusing on a MonoP HR 5/20 column as described above for the haem-binding domain, except that concentration steps were carried out with a Centricon 30 cartridge. Isoelectric focusing showed a band with pI around 6. The overall yield was about 50%.

Steady-state kinetics

Enzymic assays were carried out at 30°C with a Uvikon 930 spectrophotometer, in standard buffer (0.1 M Na^+/K^+ phosphate buffer/1 mM EDTA, pH 7) containing an electron acceptor and the required substrates or inhibitors. The acceptors were ferricyanide ($\epsilon_{420}^{\text{ox-red}} = 1.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) or horse heart cytochrome c (Sigma; $\epsilon_{550}^{\text{red-ox}} = 20.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Before the assays, the enzyme was transferred to lactate-free standard buffer by filtration on a minimal Sephadex G-15 or G-25 column. Enzymic activities were expressed relative to one haem.

Competition ELISA tests

They were carried out as described in [23], with a few modifications. Briefly, the ELISA plates (polystyrene; Dinex) were coated with 0.5 μg of Flb2 in 200 μl of 0.1 M phosphate buffer, pH 7.4, overnight at 4°C , then washed with 200 μl of Tris-buffered saline (TBS; 0.1 M Tris/HCl/0.9% NaCl/0.1% Tween 20) after a 3-min incubation in this buffer at room temperature (this washing and incubation step was repeated three times). Remaining sites were saturated with 0.5% BSA in TBS for 1 h at 37°C ; the wells were then washed three times with TBS as before. In parallel, 1 nM Fab was incubated in 1 ml with a range of Flb2 concentrations (0.03 nM–5 μM , according to the case, in 0.1 M phosphate buffer, pH 7.4, containing 0.02% BSA) for 30 min at 20°C . Each mixture (200 μl) was added to the well in triplicate. After a 30-min incubation at room temperature, the wells were washed three times with TBS. Coupling was achieved with 200 μl of a goat anti-mouse λ chain antibody-alkaline phosphatase conjugate (1/1000 dilution; Southern Biotechnology Associates). After 30 min at room temperature and three washes in TBS, the p -nitrophenyl phosphate solution was added and the plates were read at 405 nm with a Dynatech 5000 apparatus. The results were plotted as Scatchard representations, and the plots were generally linear. The K_d values determined in this work are lower than those given in our previous paper, by about an order of magnitude [15]. A possible cause for the discrepancy could be that we were not aware at the time of the relative instability of the Fab fragment (see above).

BIAcore analyses

All surface plasmon resonance (SPR) experiments were performed using a BIAcore 1000 equipped with a Pioneer sensor chip F1. The ligand was the Fab fragment which had been purified on a MonoP column after separation from the Fc fragment on Protein A–Sepharose, as described above. The surfaces of two flow cells were activated with 20 μ l of a 1:1 mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M 3-(*N,N*-dimethylamino)propyl-*N*-ethyl-carbodi-imide ('EDC') at a flow rate of 5 μ l/min. The ligand (50 μ g/ml in 10 mM sodium acetate, pH 3.8) was immobilized on one of the flow cells to a density of about 1000 response units after two 30- μ l injections at a flow rate of 5 μ l/min. The second surface was treated identically in the absence of Fab, to act as a control. After ligand immobilization, the surfaces were blocked for 4 min with 20 μ l of 1 M ethanolamine, pH 8.5. Regeneration was carried out with 10 μ l of 1 M aqueous NaCl at a flow rate of 5 μ l/min. The analytes were the WT and mutant haem-binding domains prepared as described above, in 0.1 M Na⁺/K⁺ phosphate buffer/1 mM EDTA/0.005 % Tween 20, pH 7.4. They were injected at concentrations varying between 50 and 5400 nM, according to the case, at a flow rate of 30 μ l/min (20 °C) for 50–90 s. The dissociation was allowed to proceed for 120 s. The data were fitted to a simple 1:1 interaction model (Langmuir) using Bioevaluation 3.1 software. Values of k_{obs} were obtained by individual fitting of the curves at each concentration. These values were then plotted according to eqn 1:

$$k_{\text{obs}} = k_{\text{on}} \times C + k_{\text{off}} \quad (1)$$

where C is the analyte concentration. In parallel, the K_d values were also obtained independently from a plot of the plateau level reached at each concentration versus the analyte concentration, which was fitted by non-linear regression analysis to eqn 2:

$$R_{\text{eq}} = (R_{\text{max}} \times C)/K_d + C \quad (2)$$

RESULTS

Scheme 1 depicts the Flb2 catalytic cycle. Lactate reduces the flavin in a two-electron reaction (Scheme 1, 1→2). Flavin then yields one electron to haem b_2 in the same subunit (2→3) and the latter is reoxidized by an external electron acceptor (3→4). The flavin semiquinone (Scheme 1, Fl_{sq}) then yields the second electron to the haem (4→5), which is again reoxidized by the electron acceptor (5→1). This scheme depicts the electron transfer through the enzyme from substrate to the physiological acceptor cytochrome *c*. When a non-physiological acceptor such as ferricyanide is used, other steps have to be added (3→5 and 4→1), because, although ferricyanide cannot compete with the

haem for an electron from reduced flavin (Scheme 1, Fl_{red}), it can compete for the flavin semiquinone electron [24]. However, with the recombinant flavodehydrogenase domain, or mutants with impaired flavin to haem electron transfer [11], ferricyanide can react with reduced flavin.

In our previous work describing the effect of bound mAb on the Flb2 catalytic cycle, we showed that, in the antigen–antibody complex, flavin-to-haem electron transfer was completely inhibited, whereas the FDH domain could normally turn lactate over in the presence of ferricyanide. Furthermore, uncoupling was not due to inhibition of mobility by freezing the conformation of the peptide-chain segment linking the two domains; indeed, a mutant enzyme, in which residues 85–116 had been replaced with the equivalent linker segment from a homologous enzyme [14], showed the same affinity as the WT enzyme for the mAb. The antibody was found to bind equally well to oxidized or lactate-reduced enzyme. We concluded that functional uncoupling between the domains could possibly be due to the antibody binding to a region close to or at the interface between the domains, thus preventing the formation of a competent interface [15]. We decided to map the epitope using site-directed mutagenesis, to shed light on the structural basis of the phenomenon.

Mutagenesis and enzyme production

Our hypotheses thus led us to choose, for a first mutation round, three residues bordering the buried interface, namely Glu-63, Asn-69 and Asp-72, and another one away from the interface as a control, Arg-38. We reversed the charge for residues 63 and 38, introduced a bulkier, charged side chain at position 69 and replaced Asp-72 with a small non-polar side chain (Table 2). As, from the start, Glu-63 and Asn-69 appeared to play an important role in complexation with the antibody, we extended our mutagenesis work to other haem-domain surface residues located in the interface area or bordering it. Alanine was introduced at positions 39, 44, 65 and 73 to reduce the size of the side chain; in contrast, bulkier side chains were introduced at positions 64, 67 and 70; Tyr-74 was minimally mutated to Phe. The complete list of mutations is presented in Table 2, and includes two different mutations at positions 64 and 67. The structural location of the mutated residues is shown in Figure 2. We avoided mutating the two glycines in the segment His-Pro-Gly-Gly (residues 43–46), which is the consensus sequence for probing the presence in databases of b_3 -like sequences. His-43 is the first iron ligand, and as such is buried; the three other residues of the consensus sequence are accessible in the isolated domain, but all are invariant, and we feared that mutating the glycines would have dramatic structural consequences. The crystal structure of the first

Scheme 1 The Flb2 catalytic cycle

Fl_{sq}, flavin semiquinone; Fl_{red}, reduced flavin; Fl_{ox}, oxidized flavin; Lac, lactate; Pyr, pyruvate; H_{ox}, oxidized haem; H_{red}, reduced haem.

Table 2 Enzymic tests: effect of Flb2 mutations on antibody-mediated inhibition of cytochrome *c* reduction

Assays were carried out in the presence of 20 mM L-lactate either with 13 mM ferricyanide in 0.1 M phosphate buffer, pH 7, or with 500 μ M cytochrome *c* in 0.05 M phosphate buffer, pH 7. Enzymes (2 μ M) were incubated with or without the Fab fragment (10 μ M) at 30 °C for 10 min, and diluted 10-fold in the lactate- and acceptor-containing buffer in the assay cuvette. Activity units are electrons/mol per s. Data are means from duplicate assays with at least two independent enzyme preparations, except for mutations at positions 38, 73 and 74 (one preparation).

Mutation	<i>k</i> _{obs} (s ⁻¹)			
	Acceptor . . .		Cytochrome <i>c</i>	
	Free Flb2	Flb2 + Fab	Free Flb2	Flb2 + Fab
WT	420 ± 15	432 ± 17	364 ± 13	24 ± 13
R38E	432 ± 27	423 ± 33	380 ± 44	18 ± 7
F39A	413 ± 13	430 ± 24	85 ± 7	5 ± 2
P44A	423 ± 23	418 ± 14	157 ± 17	7 ± 3
E63K	437 ± 35	430 ± 46	268 ± 25	271 ± 30
P64Q	414 ± 18	398 ± 24	354 ± 32	116 ± 33
P64R	423 ± 23	417 ± 25	348 ± 24	113 ± 23
L65A	378 ± 26	351 ± 14	100 ± 7	30 ± 3
A67Q	375 ± 26	372 ± 22	75 ± 5	111 ± 18
A67L	355 ± 20	352 ± 15	8 ± 2	13 ± 2
N69K	395 ± 21	407 ± 21	241 ± 22	288 ± 19
V70M	436 ± 19	417 ± 19	352 ± 17	94 ± 23
D72A	446 ± 37	430 ± 28	316 ± 15	29 ± 9
K73A	423 ± 30	408 ± 25	328 ± 13	19 ± 13
Y74F	447 ± 22	421 ± 30	335 ± 10	31 ± 5

known cytochrome *b*₅ bacterial homologue has been published [25]. This protein presents the sequence His-Pro-Gly-Pro; the Gly-to-Pro mutation is obviously not deleterious to the structure, but induces a local peptide-chain reorganization [25].

All the mutant enzymes expressed well, with a yield of between 0.5 and 1 μ mol of purified protein/l of culture; this was also true, to our surprise, for the P44A mutation in the consensus sequence. The F39A mutant enzyme appeared to have little enzyme activity when it was first purified, but activity could be recovered by adding FMN. This was surprising because a mutation in the haem domain might not be expected to affect the structurally independent flavin domain. In the past, a few mutations in the latter domain had led to partly flavinylated proteins, but these would not bind additional flavin, and this was ascribed to a folding problem during expression [26]. For further F39A mutant enzyme preparations, FMN was added throughout purification. Because of this problem, the flavin to haem stoichiometry was verified also for the mutations at positions 44 and 65, and was found normal.

The haem spectra, which dominate the visible region, were identical with the wild-type one. As we also wanted to use the isolated haem domains in some experiments, we planned at first to introduce the same mutations into the expression plasmid for this domain. The first attempt with the N69K mutant domain was a failure, because of its instability in the low-ionic-strength buffers required for the chromatographic steps leading to pure haem domain. The columns turned brown, indicating denaturation with haem release from the protein. From then on, the haem domains were prepared from the respective holoenzymes by tryptic proteolysis, the whole procedure being compatible with the use of 0.1 M phosphate buffer; it yields a major fragment ending at Lys-103 of the mature enzyme sequence and a minor component with an additional glutamic acid [27]. In conclusion, although some of the mutations may have led to instability of the isolated haem domain at low ionic strength, the tetrameric mutant enzymes gave no indication of particular instability or

improper folding under the experimental conditions we used, and the tryptic domains were also stable in 0.1 M phosphate buffer upon long-term storage at -80 °C, with the exception of the F39A domain.

Effect of the mutations on antibody-antigen interactions

We analysed the effect of the mutations on the mAb affinity using three different approaches. We first tested the enzymic activity in the presence and absence of the Fab fragment using two acceptors: ferricyanide, for probing the FDH activity, and cytochrome *c*, the reduction of which includes the flavin-to-haem *b*₂ and haem *b*₂-to-cytochrome *c* electron-transfer steps (Scheme 1). Affinity of the Fab fragment for the holoenzyme was then tested using classical competition ELISA tests. Finally, we also carried out SPR measurements, using the isolated haem-binding domains after having attached the Fab fragment to the reaction chips.

Inhibition kinetics for assessing antibody binding to the mutant proteins

The results are shown in Table 2. Steady-state kinetics at saturating lactate concentration and 13 mM ferricyanide, which should be saturating for most if not all mutant enzymes [11], indicated that their activities were, within the limits of error, identical with that of the WT enzyme. As expected, no rate alteration was observed in the presence of an arbitrary 10-fold molar excess of the Fab fragment. With respect to cytochrome *c* reduction, a few mutant enzymes displayed activities and inhibition levels similar to those of the WT enzyme (R38E, D72A, K73A and Y74F). A few others, with again normal cytochrome *c* reduction rates, were definitely less inhibited than the WT enzyme under the conditions used (P64Q/R, V70M). In contrast, for a number of mutations, cytochrome *c* reduction (at a constant cytochrome *c* concentration which is saturating for the WT enzyme) was found slower than reduction by the WT enzyme, indicating an impairment of one of the monoelectronic transfer steps, either flavin-to-haem *b*₂ transfer, or haem *b*₂-to-haem *c* transfer (Scheme 1); this was the case for the F39A, P44A, E63K, L65A, A67Q/L and N69K mutations. Among these, the enzymes mutated at positions 39 and 44 showed clear inhibition of cytochrome *c* reduction in the presence of antibody, the L65A mutant was only partly inhibited, whereas those mutated at positions 63, 67 and 69 were not inhibited at all under the chosen conditions. This first series of analyses therefore suggested that positions 63, 64, 65, 67, 69 and 70 were located in the epitope and that the substitutions interfered with antibody binding. The five side chains are located on one side of the haem-binding crevice (Figure 2).

Binding experiments: ELISA tests

Competition ELISA tests were carried out with WT and mutant enzymes. In addition, we analysed a chemical mutant of the haem-binding domain. Indeed, Reid et al. [22] described the replacement of the normal protohaem IX of cytochrome *b*₅ with its DME, so as to suppress the propionates' negative charges. We had to adapt the method to the Flb2 isolated haem domain. Whereas reconstitution with normal haem ran smoothly and rapidly, reconstitution with the DME was slow and gave a lower yield, as detailed in the Experimental section. The mixture analysed was devoid of free haem but contained some uncomplexed protein; this was of no consequence for the binding experiments, since we showed before that the haem-free domain has no affinity for the antibody [15]. No attempt was made to reconstitute the holoenzyme rather than the haem domain, because the acidic conditions used for removing

Figure 2 Residues mutated on the surface of the haem-binding domain of the F1b2 tetramer

The haem is coloured red, residues 85–99 (hinge region) in yellow; the residues in green are those for which the mutations had no effect on the antibody affinity. Residues in blue are those for which the mutation decreased affinity by more than three orders of magnitude; those in blue-green have binding constants decreased by less than one order of magnitude with the mutations introduced. The Figure was created using TURBO-FRODO [53] and the F1b2 co-ordinates in the Protein Data Bank (FCB1) [1].

the haem are much harsher than those used for reversibly removing the FMN, conditions under which the haem domain is not affected [28]. The binding constants determined in the ELISA tests are shown in Table 3. For the WT enzyme and all the mutant proteins that had undergone efficient inhibition of cytochrome *c* reduction, the K_d was in the order of 10 nM. But, for mutations at positions 63, 67 and 69, no value could be obtained, because no competition was apparent up to 5 μ M mutant enzymes in the preincubation step. In addition, the P64Q, P64R and V70M mutant proteins showed a 3–5-fold K_d increase. With only a 2-fold K_d value increase, the L65A case was somewhat ambiguous. Interestingly, for the haem-domain DME derivative, there was an about 20-fold decrease in affinity. These results confirm that positions 63, 64, 67, 69 and 70 lie in the epitope, to which should be added one or both haem propionates and possibly Leu-65. It can be noticed that the K_d value determined for the isolated haem domain is about three times higher than that for the tetramer. This result was reproducible, but the origin of this difference is unclear, since we showed before that the mAb has no affinity for the FDH, and its K_d is identical for the haem domain or a mixture of FDH and haem domain [15].

Binding experiments: SPR

The purified Fab fragment was attached to the chip as described in the Experimental section. We first tested the tetrameric holoenzyme, which had been used in the ELISA tests, with CM5 chips. It turned out to be very difficult to reach a plateau during the binding phase, even at high concentrations and with a prolonged exposure time. Moreover, desorption was exceedingly slow. When F1 chips, with shorter carboxymethylated dextran chains, were used instead, the results were somewhat better, but still not satisfying, in contrast with those for the haem-binding domain

Table 3 Binding constants of mutant enzymes for mAb B2B4

Langmuir: data obtained from k_{on} and k_{off} values obtained by a fit to eqn 1 of a plot of k_{obs} values versus analyte concentration (see the Experimental section and Table 4). Equilibrium: data obtained from a plot of response-unit values at equilibrium versus analyte concentration according to eqn 2 (see the Experimental section). nd, not determined; nm, not measurable.

Mutation	K_d (nM)		
	ELISA (tetramer)	BIAcore (haem domain)	
		Langmuir	Equilibrium
WT	11 $\pm$ 5	68 $\pm$ 19	46 $\pm$ 4
R38E	15 $\pm$ 9	nd	nd
F39A	9 $\pm$ 5	nd	nd
P44A	10 $\pm$ 4	65 $\pm$ 12	75 $\pm$ 17
E63K	> 10 ⁴	nm	nm
P64Q	42 $\pm$ 15	490 $\pm$ 220	495 $\pm$ 90
P64R	55 $\pm$ 9	325 $\pm$ 85	338 $\pm$ 57
L65A	19 $\pm$ 5	468 $\pm$ 57	518 $\pm$ 59
A67Q	> 10 ⁴	nm	nm
A67L	> 10 ⁴	nm	nm
N69K	> 10 ⁴	nm	nm
V70M	30 $\pm$ 13	600 $\pm$ 105	605 $\pm$ 126
D72A	13 $\pm$ 4	nd	nd
K73A	9 $\pm$ 5	nd	nd
Y74F	10 $\pm$ 3	nd	nd
WT domain	34 $\pm$ 2	–*	–*
Reconstituted domain	30 $\pm$ 3	99 $\pm$ 19	71 $\pm$ 11
DME domain	680 $\pm$ 35	860 $\pm$ 102	500 $\pm$ 123

* For BIAcore data for the WT domain, see the BIAcore entries for WT in the same column.

Figure 3 Representative sensorgrams

The Fab fragment was immobilized on F1-chip surfaces. The tracings for the WT tetramer, the WT haem domain and the N69K haem domain were obtained with a 45 μ l injection of 900 nM protein at 30 μ l/min. The buffer was 0.1 M phosphate/0.005 % Tween 20, pH 7.4. Inset: injections of 90 μ l of WT tetramer at various concentrations up to 3 μ M. RU, response units.

(Figure 3). We ascribe the behaviour of the holoenzyme to its tetrameric nature; presumably, after dissociating from the surface, the protein carried away in the analyte-free buffer would be able to readsorb via another subunit as it would roll on the surface. Decreasing the Fab density on the chip surface was of no avail. Adding antibody to the dissociation buffer was not tried [29].

The results shown in Table 3 were acquired with the F1 chips using the haem domains of those mutant proteins that had shown a modest affinity decrease in the ELISA tests, as well as for the DME derivative. For the E63K, A67Q/L and N69K mutations, no significant binding to the chip could be observed up to 5 μ M analyte (Figure 3). The mutant proteins that had behaved similarly

Table 4 Kinetic parameters for haem-domain association to immobilized Fab fragment

The response curves were fitted individually and the derived k_{obs} values were plotted versus the analyte concentration, according to eqn 1 (see the Experimental section).

Domain	k_{on} ($\text{M}^{-1} \cdot \text{s}^{-1}$; $\times 10^{-3}$)	k_{off} (s^{-1} ; $\times 10^{-3}$)
WT		
Native	281 ± 50	19 ± 2
Reconstituted	210 ± 25	21 ± 2
DME	23 ± 1	20 ± 1
P44A	215 ± 28	14 ± 1
P64Q	110 ± 23	54 ± 12
P64R	104 ± 14	33 ± 4
A65L	93 ± 4	52 ± 4
V70M	110 ± 8	66 ± 6

to the WT enzyme, both with respect to inhibition of cytochrome c reduction and in the ELISA tests, were not examined, with the exception of the P44A mutant domain as a check of the WT value. Table 3 shows the binding constants calculated in two ways. The Langmuir column values are the ratio of the k_{on} and k_{off} values. The equilibrium column gives the values calculated using only the plateau values at each analyte concentration. It can be seen that the agreement between the two columns is generally quite good.

Comparison of the ELISA and SPR results

The values derived from ELISA tests for the tetramer and from SPR measurements for the haem-binding domain differ by factors of 5–20. Part of the difference may be ascribed to the somewhat higher ELISA value determined for the domain compared with the tetramer. It can thus be expected that, had the ELISA values all been obtained for the domain and not for the tetramer, the difference between the two sets of values would be smaller. The competitive ELISA procedure we used determines affinity at equilibrium [23], whereas with SPR, one of the partners is immobilized; therefore the difference between the two sets of values is not entirely unexpected. An abundant literature discusses the potential problems met with in the SPR methodology (see for example [29]). The good agreement between the kinetic and equilibrium determinations of the dissociation constants (Table 3) does not suggest a systematic bias in our SPR analyses and thus does not help rationalize the differences observed between the two methodologies. A number of other studies also reported discrepancies between BIAcore values and affinity values determined by other methods [29].

Whatever the case, the important point is that the two sets of results provide a coherent picture of the effect of the various mutations. Those at positions 63, 67 and 69 alter the affinity for the antibody by at least three orders of magnitude; those at positions 64, 65 and 70 by less than one order of magnitude, and replacing the haem propionate carboxyl groups by their methyl ester induces a 10–20-fold fall in affinity. Moreover, for all the mutations, the combined results are in agreement with the conclusions from the kinetic data. Therefore, when the difference between the domain and the tetramer in the ELISA tests was noticed, it was deemed unnecessary to repeat all the ELISA experiments with the haem-binding domain.

Table 4 presents the kinetic constants determined by SPR. It appears that the mutations have a dual effect: they decrease the on rate and increase the off rate, both by factors of two or three.

In contrast, the DME derivative dissociates at the same rate as the WT domain, but associates about 10-fold more slowly. This suggests that an electrostatic component may be at play in the binding process [30]. In contrast, in a number of other situations in the literature, it has been reported that mutations mainly affected the dissociation rate (see for example [31–33]).

DISCUSSION

The results presented above define a minimum area for the mAb epitope on the haem b_2 domain. It encompasses positions 63, 64, 65, 67, 69 and 70 as well as one or both haem carboxylates. The epitope area thus defined is shown in Figure 4(A) for the isolated haem domain, in an orientation different from that of

Figure 4 The epitope on the haem-binding domain as mapped in this study

(A) Isolated haem-binding domain; in black, all the positions where mutations decreased the antibody affinity, including the haem. (B) The domain, in the same orientation as in (A), capped with the flavin-binding domain. Created using TURBO-FRODO [53] and the Flb2 co-ordinates in the Protein Data Bank (FCB1) [1].

Figure 2. With the data at hand, it cannot be decided if other parts of the haem group than the propionates' carboxyl groups influence binding to the antibody. Furthermore, it seems probable that more residues than those we singled out are engaged in the interface between antigen and antibody, residues that we did not mutate, or some of those we mutated without any influence on the affinity. For example, the Tyr-74 phenol group does not appear to be engaged in an important hydrogen bond, but the aromatic group may contribute hydrophobic interactions that the mutation to phenylalanine would not detect. Moreover, it is known that contacts in antibody–antigen interfaces may be mediated by water molecules, and that sometimes solvent molecules can compensate interactions lost upon mutagenesis [34–41].

The energetic contribution to affinity between antigen and antibody of the side chains we mutated cannot be immediately deduced from our results. The mutations with most spectacular effects at positions 63, 67 and 69 all introduced steric bulk, in addition to introduction of a charge (N69K) or a charge reversal (E63K). The effect of the Gln and Leu substitutions for Ala-67 suggest that this side chain must make close contact in the antibody-binding site, and that steric complementarity may be as important, or more, than a hydrophobic interaction of the methyl group. In contrast, the equivalent effect of the two very different side chains (Gln and Arg) substituted for Pro-64 suggest that bulk is not too important at that position. The L65A mutation suggests a hydrophobic interaction of the leucine side chain. The substitution of Val-70 with Met may disrupt steric complementarity and/or introduce undesirable polarity due to the sulphur atom. Finally, one or both haem propionates contribute a polar or electrostatic interaction. Altogether, as our results stand, they give evidence that is in line with a number of published mutational analyses of epitopes: the minimal surface is continuous, and not all residues appear to contribute equally to the affinity [33,37,42–46]. Moreover, the present results confirm that the epitope is conformational, and explain why the antibody is unable to bind to the haem-free domain [15]. In the Flb2 crystal structure, two helices encompassing residues 59–66 and 69–74 cover one haem face [1]. In homologous cytochrome *b₅*, with a very similar structure [3], haem removal leads to a partly folded protein in which residues 40–69 form a largely disordered loop [47,48]. In the sequence and structural alignments, these residues correspond to Flb2 positions 45–72. It is probable that reversible haem removal from the latter protein also leads to disorder in that area, and one could have expected that the peptide chain would refold to a more native conformation upon interaction with the antibody-binding site. Our results show that either this is not the case or that interactions with the haem, those detected with our chemical mutation as well as others, are indispensable for binding to the antibody. The present work also rationalizes the absence of haem domain's cross-reactivity between *Saccharomyces cerevisiae* and *Hansenula anomala* [15]. The segment corresponding to the *S. cerevisiae* positions 63–69 presents four substitutions in the *H. anomala* protein [49,50].

Our aim was not to try to establish a complete map of the functional epitope, but to understand the structural basis of the inhibition of flavin to haem electron transfer upon antibody binding. Crystals of the domain-Fab complex have recently been obtained (K.H.D. Lê and B. Golinelli, unpublished work) and will hopefully enable a comparison between the functional and the structural epitope. Figure 4(B) shows the haem domain docked to the flavin domain, in the same orientation as the free domain in Figure 4(A). It is clear that some of the residues are well shielded from solvent upon docking, and that the epitope encompasses part of the domain interface. In particular, in the crystal structure, one haem propionate is engaged in a water-

mediated hydrogen bond to flavin O-4, and the other propionate is in an electrostatic interaction with Lys-296 of the flavin domain. With the two domains docked for electron transfer, the water accessibility of residues 65, 67 and 70 is 13.1, 5.9 and 10.1 Å², respectively, and the side chains at positions 63, 64 and 69 are the only ones that are partly or fully solvent accessible. It is thus clear that antibody binding can only occur during the haem-domain movements; the weak interactions between the functional domains are insufficient to displace the strongly bound antibody, and thus productive docking for electron transfer cannot occur any more. Since electrons can tunnel through space in a protein matrix given favourable conditions, the structural basis of the inhibition of electron transfer between flavin and haem in Flb2 was intriguing. Our results clearly indicate that the antibody acts as a physical barrier to formation of a functionally competent complex between the partner domains. An IgG molecule (150 000 kDa) is large compared with the FDH domain (45 000 kDa); its binding to the haem domain or even the binding of the Fab fragment (50 000 kDa) will prevent access to the haem, and force the FDH to remain at too large a distance and in the wrong orientation.

Besides leading us to provide the first direct proof for haem-domain mobility in solution, the results presented here have given preliminary evidence that electron transfer between the domains in the absence of the antibody, during normal enzyme functioning, is impaired by a number of mutations, namely those at positions 39, 44, 63, 65, 67 and 69 (Table 2). Short et al. [51] found a decreased cytochrome *c* reduction rate with the E63K mutant enzyme, as well as with the D72K mutation, whereas our D72A mutant enzyme appears normal at this stage. Work is in progress to analyse these effects and will hopefully lead us to defining other important interdomain contacts than those already analysed [11,12,14]. Furthermore, it will be worthwhile to study the effect of the mutations on electron transfer between reduced enzyme and cytochrome *c*. Two models were proposed for the complex between the two proteins [51,52]. Site-directed mutagenesis subsequently appeared to invalidate the first model [51]. Both models have in common that they propose a binding mode for cytochrome *c* in which it straddles the two domains. In such a case, some of the interface mutations we introduced may affect the Flb2–cytochrome *c* interaction by affecting the surface complementarity and thus increasing the distance between the cytochrome *c* anchor points on the two domains from each other, without directly taking part in the interaction.

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