Probing Intramolecular Electron Transfer within Flavocytochrome b_2 with a Monoclonal Antibody†

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ABSTRACT: Flavocytochrome b_2 or L-lactate dehydrogenase from yeast is a tetrameric enzyme which oxidizes lactate at the expense of cytochrome *c* or artificial electron acceptors. The prosthetic group FMN is reduced by the substrate and then transfers sequentially the reducing equivalents to heme b_2 in the same subunit. The latter is reoxidized by cytochrome *c*. The crystal structure of the enzyme indicates that each subunit is composed of a flavodehydrogenase domain (FDH) and a cytochrome b_2 domain; the latter, which encompasses the first 99 residues of the peptide chain, is mobile relative to the tetrameric FDH assembly. We describe here the properties of a monoclonal antibody elicited against the holoenzyme. It only recognizes the heme-binding domain, with a K_d lower than 10^{-7} M, and its epitope is conformational. In the enzyme−IgG complex, flavin is reduced normally and can be reoxidized by ferricyanide, but no longer by heme b_2. Stopped-flow experiments in the absence of electron acceptors give no indication of flavin to heme electron transfer in the enzyme−antibody complex. In other words, the two domains are functionally uncoupled. The binding stoichiometry is 1/1 for the Fab fragment with respect to the isolated, monomeric, heme-binding domain, but 2/4 with respect to the enzyme tetramer; furthermore, binding of two Fab fragments per tetramer is sufficient to cause inhibition of intra-subunit flavin to heme electron transfer in all four subunits. Altogether these results can only be rationalized by considering that mobility of the cytochrome domain with respect to the FDH is an essential component of the catalytic cycle. The first experiment designed to locate the epitope shows it does not encompass the interdomain peptide linker (so-called hinge region, centered on residues 99−100).

Over the past few years, significant advances have been made toward the understanding of the catalytic mechanism and structure of flavocytochrome b_2 or L-lactate cytochrome *c* oxidoreductase (EC 1.1.2.3) (Flb_2),[1] from the yeast *Saccharomyces cerevisiae* (*S.c.*). This tetrameric enzyme of subunit M_r 57 500 (*1*), found in the intermembrane space of yeast mitochondria (*2*), catalyzes the two-electron oxidation of L-lactate to pyruvate and subsequent reduction of cytochrome *c* (*3*). Each subunit consists of a single polypeptide chain of 511 amino acid residues (*4*), constituting 2-folding units: a cytochrome domain (residues 1−99) containing protoheme IX and a flavodehydrogenase domain (residues 100−511) containing FMN (*5*).

Combined with a large body of mechanistic evidence (reviewed in ref 6), the elucidation of the X-ray structure of flavocytochrome b_2 at 0.24 nm resolution (*5*) allowed an interpretation of the role of various active-site residues (*7*), as well as the identification of residues at the interface between the domains (*5*). The high-level expression of the gene encoding Flb_2 in *Escherichia coli* (*8*) and the construction of point mutants by site-directed mutagenesis have led to a better understanding of the mechanism of substrate dehydrogenation (*9*−*15*) and of structural factors which influence intramolecular electron transfer from flavin to heme b_2 (*11*, *16*−*18*).

Although a wide range of structural and functional studies have been carried out on Flb_2, immunological approaches have been largely ignored. Among the few exceptions is the study of the reactivity of antisera raised in rabbits against the *S.c.* isolated cytochrome domain produced by tryptic digestion and the "cleaved" WT enzyme form (proteolyzed between residues 310 and 314) (*19*).

Since the development of hybridoma technology for the production of monoclonal antibodies (mAbs) (*20*), a wealth of information has been published on their use as probes for enzyme structure and function. Their unique specificities make them ideal for such studies, especially if their epitopes can be determined. They can be used, for example, to identify a protein binding site in a complex, as in the case of cytochrome *c* binding to cytochrome *c* oxidase (*21*). Other approaches involve the use of mAbs as conformational probes of enzymes to determine if, for example, site-directed mutagenesis or chemical modifications result in a change in conformation at or distant from the altered site (*22*, *23*). Flavocytochrome b_2 is an ideal enzyme for the above types of immunological studies since its crystal structure has been solved, a large number of point and other mutants have been generated, and the two domains of the enzyme have been expressed independently in *E. coli* and can be used, along

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[1] *Abbreviations*: Flb_2, flavocytochrome b_2; FDH, flavodehydrogenase; mAb, monoclonal antibody; *S.c.*, *Saccharomyces cerevisiae*; *H.a.*, *Hansenula anomala*; PBS, phosphate buffered saline.

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with the mutant forms of Flb_2, to aid epitope mapping. The present paper describes the production of monoclonal antibodies against Flb_2 and the characterization of one of these. During selection, this mAb, termed B2B4, appeared to be directed toward the cytochrome domain of Flb_2 and to inhibit the reduction of cytochrome *c*. The aims of such a study were to acquire information on structure/function relationships within the enzyme and to examine electron-transfer pathways and possible acceptor binding sites.

MATERIALS AND METHODS

Enzyme Preparation

Wild-type and mutant flavocytochromes b_2 expressed in *E. coli* were purified essentially as described previously (*8, 10*). The recombinant cytochrome domains from *S.c.* and *Hansenula anomala (H.a.)* were purified according to published procedures (*24, 25*), a sonication step being added after cell lysis in the case of the former.

The recombinant flavodehydrogenase domain (FDH) was purified following a modified version of the protocol described in refs 26 and 27. A DEAE-Sephadex column (3.5 × 35 cm) was substituted for the DEAE-cellulose step. After being loaded in 10 mM phosphate buffer pH 7 and being washed with 50 mM phosphate buffer pH 7, the FDH (from 18 g of cell paste, 3.5-l culture) was eluted with a gradient between 100 and 600 mM phosphate buffer pH 7 (1.2 l altogether). The lactate-oxidizing fractions were pooled, and the FDH was precipitated at 80% ammonium sulfate saturation. The pellet was dissolved in 10 mM phosphate buffer at pH 7 (120 mL) and, after control of the ionic strength, loaded onto a hydroxyapatite column (2.5 × 10 cm) equilibrated in the same buffer. The column was then washed sequentially with 50 mL volumes of 50, 100, and 150 mM phosphate at pH 7. Finally, FDH was eluted with 200 mM phosphate buffer at pH 7 (300 mL) and concentrated by ammonium sulfate precipitation.

All of the above proteins were stored as concentrated solutions (0.1 M phosphate buffer at pH 7) at −80 °C. Purity was confirmed by SDS−PAGE, and, where relevant, FMN content was verified by fluorescence measurements.

Kinetic Analyses

The steady-state kinetic measurements of the enzymic oxidation of L-lactate in the presence or absence of mAb were carried out in 0.1 M phosphate buffer of pH 7.0 at 30 °C on a Uvikon 930 spectrophotometer. Two electron acceptors were used: cytochrome *c* (horse heart, Sigma) or ferricyanide (Merck). Their reductions were monitored at 550 and 420 nm, respectively, the amount of substrate oxidized being calculated using the respective molar absorbance coefficients of 22.6 and 1.04 mM^{-1} cm^{-1}. Flb_2 concentrations were determined spectrophotometrically using the molar absorbance coefficients listed in ref 28 and expressed in terms of heme (i.e., subunit) concentration; that of the mAb was determined from the absorbance at 280 nm using a molar absorbance coefficient of 210 mM^{-1} cm^{-1} (*29*) or by using the Bradford method (*30*). The enzyme and antibody were mixed and preincubated at 37 °C for 5 min; it was verified that this was a sufficient time for complex formation. Thereafter, the solution was kept over ice for the duration of the experiment. For ferricyanide reductase activity measurements, the preincubation concentrations were usually 0.6 μM Flb_2 and 2 μM mAb: for assay, 20 μL of this mixture was added to a thermostated cuvette (0.2 cm path length) containing 600 μL of 0.1 M phosphate buffer of pH 7 with the required ferricyanide and lactate concentrations at 30 °C. For cytochrome *c* reductase activity measurements, the preincubation concentrations were around 0.26 μM enzyme and 0.8 μM mAb; dilution in the assay mixture was 1/60. Kinetic isotope effects were determined with L-[2-^{2}H]lactate as described in ref 31.

Rapid kinetics were analyzed with an Applied Photophysics DX-17MV stopped-flow instrument, at 6.5 ± 0.1 °C. Traces were followed at 438.3 nm (flavin), and 557 and 423 nm (heme), as described (*31, 32*). For the kinetics in the presence of antibody, a mixture of Ig G and enzyme was preincubated for 10 min at 30 °C (160 and 40 mM, respectively, in 0.1 M phosphate buffer of pH 7). It was then stored on ice until it was diluted 4-fold with the same buffer and placed in the instrument syringe. The L-lactate concentration in the second syringe was 20 mM. The control was handled in the same way but in the absence of antibody. Results are the average of three to four traces, which were analyzed, using the manufacturer's software, according to one or two exponentials. The observation time was 1 s.

Proteolysis of the Recombinant Heme b_2 Domain

The b_2 core (*S.c.)* was denatured in 6 M guanidine hydrochloride (Merck) for 2 h at room temperature (50 nmol in 500 μL of 0.1 M *N*-ethylmorpholine acetate buffer, 1 mM EDTA, pH 8.3). After desalting on a Sephadex G25 column (1 × 20 cm) equilibrated and run with the same buffer without guanidine, the eluted protein was concentrated (but not dried) with a Savant SpeedVac centrifuge. Proteolyses with trypsin and chymotrypsin were carried out for 4 h at 37 °C, at 1 mg/mL with a substrate-to-protein ratio of 20/1 (w/w) in the *N*-ethylmorpholine buffer. The extent of proteolysis was examined by SDS−PAGE.

Heme Dissociation from and Reconstitution of the Heme b_2 Domain (S.c.)

Dissociation. The method used was primarily that described by Groudinsky (*33*). The protein was dissolved in water (36 μM, 250 μL), and 20 volumes of a cold solution of acidified acetone (0.2% fuming HCl) were added. The mixture was incubated at −20 °C for 15 min before being centrifuged at 12000 *g* for 10 min. The resulting pellet was dissolved in water, and the treatment was repeated. After the second centrifugation, the residual acetone was dried under a nitrogen stream and the pellet was resuspended in 0.1 M phosphate buffer of pH 7.3.

Reconstitution. To the above apoprotein solution (4 μM) at room temperature (22 °C) were added successively 0.8 μM aliquots of hemin chloride (Sigma) dissolved in the minimal amount of aqueous 0.1 M NaOH. Heme incorporation was followed spectrophotometrically by monitoring the absorbance increase at 413 nm.

Fluorescence Quenching Experiments

In the first series of experiments, the Fab fragment was titrated with increasing amounts of the heme domain, in 0.1

M phosphate buffer of pH 7.4, at room temperature. Aliquots of the antigen solution were added to the Fab-containing assay cuvette, and the emission was read at 350 nm (excitation at 285 nm) with a Perkin-Elmer model LS.5 fluorimeter. The values were corrected for the intrinsic emission of the heme domain.

In the second series of experiments, holo Flb_2 was titrated with increasing amounts of the Fab fragment. In a series of test tubes, enzyme was mixed with successive dilutions of a Fab solution, in 0.1 M phosphate buffer of pH 7.4, at 30 °C for 10 min (final concentrations: 20 μM Flb_2 and from 0.37 to 48 μM Fab). The tubes were then stored on ice. Before reading, the mixtures were diluted 40-fold in the same buffer. Controls were run in parallel with identical concentrations of the Fab fragment alone, so as to calculate the amount of quenching. The fluorescence value of 0.5 μM free enzyme was assumed not to change upon complexation and was taken into account.

Immunization and Fusion

Female BALB/c mice were injected intraperitoneally with 200 μg of fully purified Flb_2 emulsified with 9 parts of complete Freund's adjuvant. After 2 weeks, three booster injections in a 1:9 mixture with complete Freund's adjuvant were given at 2 week intervals. The serum antibody response for each mouse was then determined in ELISA tests. Eight weeks after the last injection, the mice were given a final injection of Flb_2 in physiological serum only. After a further 4 days, the spleen was extracted from the mouse with the best anti-serum.

The spleen cells were fused with azaguanine-resistant, nonsecretory, murine myeloma cells (Ag 8653 × 63) essentially as described by Köhler and Milstein (*20*). Hybridoma growth was maintained in RPMI 1640 medium (Gibco) supplemented with penicillin/streptomycin (1000 units and 1 mg respectively per 100 mL), pyruvate (1 mM), glutamine (2 mM), and fetal calf serum (Institut Jaques Boy). Ten days after the fusion, the culture supernatants were tested for production of anti-Flb_2 antibodies.

Selection and Cloning

For the first series of selections, indirect ELISA tests (see below) were carried out on the hybridoma culture supernatants using Flb_2 and its heme domain (*S.c.*) as antigens. ELISA competition tests were also used to ensure that only mAbs directed against the native form of the wild-type enzyme (i.e., not denatured through adsorption onto the ELISA plate (*34*)) were selected. Culture supernatants were also tested for inhibition of Flb_2 catalysis by carrying out steady-state kinetic tests with ferricyanide (1 mM) as the electron acceptor. Subsequent subcloning by limiting dilution was carried out at least twice for each batch of cells, all of the above tests being repeated after each. In the later stages, the FDH domain of Flb_2 was included as an antigen; moreover, kinetic inhibition tests were also carried out using cytochrome *c* (50 μM) as the electron acceptor.

Production of Ascites and mAb Purification

Ascites were obtained through the injection of a suspension of cloned hybridoma cells ((3−5) × 10^6 cells/mouse) in physiological serum into pristane-primed female BALB/c mice (retired breeders). The proteins in 5 mL of ascites liquid were precipitated at 45% ammonium sulfate saturation and collected by centrifugation (10 min, 30 000 *g*). The pellet was dissolved in about 1 mL of 0.1 M Tris−HCl buffer, 0.15 M NaCl, pH 8.9. Immunoglobulins were adsorbed on a Protein A−Sepharose column (Pharmacia) (1 × 4 cm), equilibrated with the same buffer at 4 °C. After washing until the OD at 280 nm returned to the base line, the mAb was eluted with 0.1 M citrate buffer of pH 6 and collected in 1 mL fractions in test tubes containing 50 μL of 0.1 M Tris. The column was regenerated by washing with citrate buffer of pH 4 and then with 0.1 M citric acid. After resuspension in 0.1 M phosphate of pH 7 and a brief dialysis against the same buffer, the mAbs were stored in small aliquots at −80 °C. Typical storage concentrations were of the order of 5 mg/mL. Purity was established by SDS−PAGE. Isotypes were determined using an antibody typing kit (Amersham).

Production of Fab Fragments

A 10 mg sample of purified IgG in 1 mL of 0.1 M phosphate buffer, 2 mM EDTA, and 10 mM L-cysteine, pH 7.4, was treated with 0.5 mg of papain (Boehringer) for 2 h at 37 °C. The reaction was stopped by the addition of iodoacetamide to a final concentration of 40 mM. The mixture was kept at room temperature for 30 min before processing. SDS−PAGE indicated that the cleavage was complete. The Fab moiety was purified on a column of Protein A-Sepharose (1 × 5 cm) equilibrated in 0.1 M Tris−HCl buffer, 0.15 M NaCl, pH 8.9, at 4 °C. Using a concentrated Tris/HCl/NaCl solution, the proteolysis mixture was brought to a final concentration of 0.05 M Tris and 0.15 M NaCl at pH 8.9 (2 mL final volume). The flow-through fraction was collected and concentrated. The yield was 80−85%. Upon SDS−PAGE in the absence of a reducing agent, the Fab fraction showed a major band at 50 kDa and a minor one (10−30%) at 25 kDa. The two bands were eluted simultaneously from a molecular sieve column, and both were adsorbed on an immobilized-Flb_2 column. It was thus assumed that both fractions contained the antigen-binding site; this was confirmed by fluorescence quenching experiments (see above).

ELISA Tests

These were carried out using 96-well microtiter plates (Nunc).

Indirect ELISA. The plate was coated with 1 mg/well of antigen in PBS pH 8.8 and incubated for 1 h at 37 °C or overnight at 4 °C. Bovine serum albumin (1.5%, Sigma) was added as a blocking agent. After washing 3 times in Tris/Tween (0.1 M Tris/0.9% NaCl, pH 7.5, 1% Tween 20), the supernatant from the cell cultures or the purified mAb or Fab fragment (diluted in 0.1 M phosphate buffer, pH 7) was added and the plate incubated for a further hour at 37 °C. Following washing, anti-mouse IgG−alkaline phosphatase conjugate (1/4000 dilution, Sigma) or anti-mouse Fab−alkaline phosphatase conjugate (1/1000 dilution, Sigma) was added and incubated (1 h, 37 °C). After substrate addition (*p*-nitrophenyl phosphate (Sigma), 7 mg/20 mL of 10 mM diethanolamine buffer of pH 9.5 + 1 mM $MgCl_2$), plates were read at 405 nm using a Dynatech MR5000 apparatus.

Table 1: Effect of mAb B2B4 on Flavocytochrome b_2 Catalyzed Ferricyanide and Cytochrome *c* Reductions[a]

	acceptor = ferricyanide			acceptor = cytochrome *c*	
	$k_{cat.}$ (s^{-1})	K_m(lactate) (mM)	K_m(acceptor) (mM)	$k_{cat.}$ (s^{-1})	K_m(acceptor) (μm)
free Flb_2	270 ± 30	0.49 ± 0.1[a]	<0.1	136 ± 18	72 ± 7
Flb_2 + IgG	275 ± 40	0.33 ± 0.02	2.2 ± 0.2	6 ± 0.2	48 ± 2

[a] For experimental details, see Materials and Methods. The lactate concentration was 10 mM when ferricyanide and cytochrome *c* concentrations were varied. The ferricyanide concentration was 10.8 mM when the lactate concentration was varied. Kinetic parameters were calculated using a nonlinear least-squares regression program. Activity units are moles of substrate oxidized per second per mole of enzyme. [b] Taken from ref 10.

Competition ELISA. These experiments were carried out essentially as described by Friguet et al. (*34*, *35*). The plates were coated as above with 1 μg/well of Flb_2. The mAb or Fab fragment at 1.75 nM in 0.1 M phosphate buffer, 0.02% BSA, pH 7.4, was preincubated with varying concentrations of antigen for 10−15 min at room temperature, in nonadsorbing 96-well plates (Polypropylene cluster, Costar Corp.) or in Eppendorf tubes. The mixtures were then distributed into the coated wells and incubated as above. IC_{50} values were calculated from a nonlinear regression fitting to a rectangular hyperbola relating inhibition percentage and competitor concentration. For binding constant determinations, the preincubation in the presence of the competitor was carried out at 30 °C for 30 min, a duration which was checked to be sufficient for reaching equilibrium. In addition, it was verified, as described in ref *34*, that the response was linear with antibody concentration and that the equilibrium reached in the preincubation was not significantly displaced by adsorption of free antibody onto the coated antigen. K_d values were obtained from Scatchard plots (*34*).

Enzyme Depletion by Antibody

Protein A−Sepharose (50 μL) equilibrated in 0.1 M phosphate buffer and 0.15 M NaCl, pH 8.9, was incubated in test tubes together with 10 μL of the same buffer containing increasing concentrations of B2B4 IgG (from 5 to 250 μM). After 30 min at room temperature, excess IgG was removed by centrifugation; the beads were washed by resuspending in 0.1 M phosphate buffer of pH 7.4 and centrifuging (3 times). To each tube was then added 10 μL of a 70 μM solution of Flb_2 in 0.1 M phosphate buffer, pH 7.4. After 30 min at room temperature, the supernatant was collected by centrifugation and assayed for lactate−ferricyanide oxidoreductase activity under the usual assay conditions for free enzyme: 10 mM L-lactate and 1 mM ferricyanide in 0.1 M phosphate buffer at pH 7, 30 °C.

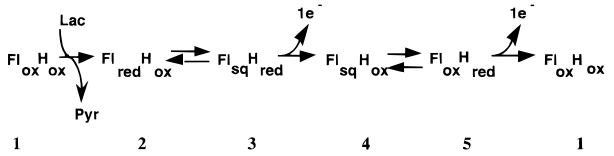
RESULTS

The panel of mAbs elicited against wild-type Flb_2 comprised, after selection and cloning, immunoglobulins directed against different regions of the enzyme. Of particular interest was the anti-native Flb_2 monoclonal antibody B2B4 (isotype IgG1l), which in the selection tests inhibited the reduction of both ferricyanide (1 mM) and cytochrome *c* (50 μM) and appeared to be directed against the heme b_2 domain. It was selected for large-scale production. The results of a more complete steady-state kinetic study of the mAb effect on activity are described below, along with the first experiments designed to map the epitope.

Kinetic Studies

In the initial selection tests during the cloning process, mAb B2B4 in culture supernatants appeared to inhibit the reduction of both acceptors. With the purified IgG, however, when the ferricyanide concentration was increased, the specific activity was found to be identical in the presence or absence of mAb B2B4 (Table 1). This was because, in the presence of the mAb, the K_m for ferricyanide was strongly raised, from <0.1 mM (see ref 6 for review) to 2.2 mM (Table 1). At saturating ferricyanide, the K_m value for lactate was practically the same as that in the absence of complexation with antibody. On the other hand, the cytochrome *c* reduction rate was less than 5% that of the uncomplexed enzyme, whereas the acceptor half-saturation concentration was hardly changed.

Scheme 1



These observations can be analyzed in the light of the existing body of knowledge concerning the sequence of steps catalyzed by flavocytochrome b_2 (Scheme 1).

This scheme holds for cytochrome *c* reduction. When the acceptor is ferricyanide, the latter is believed to also take electrons in part from Fl_{sq}, in such a case leading directly from species **3** to **5** and from **4** to **1**. In view of previous analyses of the kinetics of heme-free enzyme (*36*), of interface mutants (*11*, *14*, *16*, *18*), and of the recombinant FDH domain (*26*, *27*), the strong increase in ferricyanide K_m value is taken to indicate that this acceptor, in the presence of mAb B2B4, takes all electrons from Fl_{red} (species **2**) and then from Fl_{sq}. Thus, it would appear that complexation with the antibody has no effect on flavin reduction per se but that a later step in the cycle is inhibited. This step should most probably be flavin to heme electron transfer. If it were only the heme b_2 to heme *c* transfer step, one might expect the small reagent ferricyanide to be still capable of oxidizing heme b_2, and its K_m should remain low.

To confirm this, stopped-flow studies were carried out in the presence of the IgG. Figure 1A shows the typical traces obtained for flavin and heme reductions for the control experiment. The absorbance at 438.3 nm (a heme isosbestic point (*32*)) decreases due to flavin reduction (Scheme 1, step **1** → **2**). The absorbance at 423 and 557 nm increases due to subsequent heme reduction with flavin semiquinone formation (Scheme 1, steps **1** → **3**). When the complex between enzyme and IgG was reduced with lactate, the traces of Figure 1B were obtained. Flavin was reduced as in the absence of IgG. The heme trace at 423 nm, however, showed a decrease, of smaller amplitude than that of the FMN; the heme trace at 557 nm showed only a slight initial decrease.

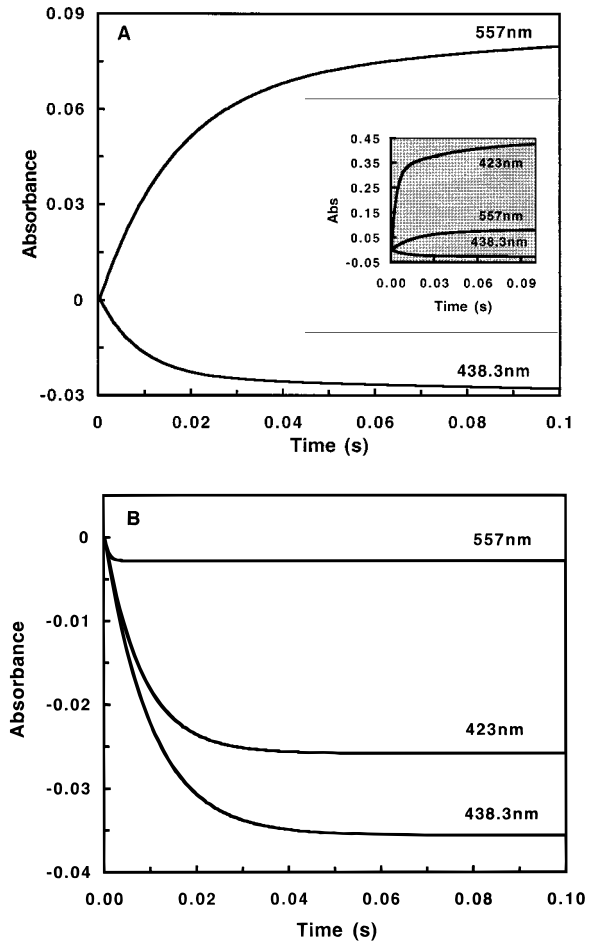


FIGURE 1: Stopped-flow traces of prosthetic group reductions by lactate in the absence (A) and the presence (B) of B2B4 IgG. Experimental details are given in Materials and Methods. The enzyme concentration was 5 μM for A and B. For B, the IgG was 20 μM. The absorbance scale is different for A and B; with an identical scale, the traces at 438.3 nm would have a similar amplitude (see Table 2). The experimental curves have been arbitrarily displaced along the vertical axis for easier visualization.

These results indicate that no intra-subunit electron transfer takes place in the Flb_2−IgG complex. The absorbance decrease in the heme trace at 423 nm should be due to the oxidized flavin decrease at this wavelength. The slight variation at 557 nm is unexpected, because the flavin absorbance does not appear to extend so far to the red, but at least shows very clearly that no heme reduction is occurring up to 1 s (the trace is only shown up to 0.1 s).

Table 2 shows the results of the stopped-flow curve fitting. For the free enzyme, all traces are biphasic, as described before (*31*, *32*). The second slow phase is due to the entry into the tetramer of four lactate-derived electrons, after a slow inter-subunit electron reshuffling which regenerates two oxidized flavins per tetramer from species **3**, Scheme 1 (*31*). This step is kinetically irrelevant in the presence of acceptors (*32*). The two heme traces show an identical time course, which is slower than that of the flavin, as expected from previous studies (*31*, *32*, *37*). For the enzyme−IgG complex, the heme traces were monophasic; for the flavin at 438.3 nm, the fit was somewhat better to the sum of two exponentials than to a single exponential, but with a very small amplitude for the second phase. The significance of this second phase is not clear. With the Flb_2 tetramer, the second phase usually has about one-third of the total amplitude. In the absence of heme, the flavodehydrogenase domain gives a monophasic curve (*36*), as expected, since only four lactate molecules are required for reducing the tetramer, and not six as in the holoenzyme. Table 2 shows that the flavin is reduced at the same rate in the presence and absence of IgG, as suggested by the steady-state studies. Furthermore, the absorbance decrease in the heme traces occurred at the same rate as flavin reduction, and the ratio of the amplitudes at 438.3 and 423 nm is in the range expected if the flavin is the only contributor at 423 nm. In conclusion, the stopped-flow results establish that, in the Flb_2−IgG complex, electron transfer from flavin to heme b_2 is suppressed. At 423 nm, the absorbance variation is 15-fold that of the flavin at 438.3 nm. We could easily have detected an absorbance increase of 0.002 at the end of the observation time, namely, 1 s, and this was not the case. Thus, if a slow heme reduction process were occurring, its rate would have to be less than 1/1000 that of the uncomplexed enzyme, much less than those for several uncoupled mutant enzymes (*16*−*18*).

The results of Table 1 indicate a residual cytochrome *c* reductase activity. In these experiments, the Flb_2 and antibody concentrations were much lower (4 and 13 nM, respectively) than in the stopped-flow study. It was possible that some uncomplexed enzyme was present in the steady-state condition. To obtain confirmatory evidence, kinetic deuterium isotope effects were determined, using L-[2-^{2}H]-lactate. As expected, the ^DV value for ferricyanide reduction (4.1 ± 0.1) was identical to that obtained in the absence of antibody (*10*, *31*). For cytochrome *c* reduction, ^DV was 2.3 ± 0.5. This figure can be compared to a ^DV of 3.0 ± 0.3 determined for cytochrome *c* reduction by the free enzyme under the same experimental conditions (*37*). The nonnegligible value of the isotope effect found here suggests that indeed the residual activity was due to some enzyme remaining uncomplexed under the given experimental conditions. This did not appear to correspond to an unreactive Flb_2 population: when a solution was depleted of enzyme by adding increasing concentrations of IgG immobilized on Protein A−Sepharose, a maximum of 0.5% of the initial

Table 2: Stopped-Flow Analysis of Flavocytochrome b_2 Reduction by Lactate in the Presence and Absence of Antibody[a]

	438.3 nm			423 nm			557 nm		
	k_{obs}^f (s^{-1})	k_{obs}^s (s^{-1})	total amplitude (OD units)	k_{obs}^f (s^{-1})	k_{obs}^s (s^{-1})	total amplitude (OD units)	k_{obs}^f (s^{-1})	k_{obs}^s (s^{-1})	total amplitude (OD units)
enzyme alone	112 (0.78)	4.4 (0.22)	−0.032	65 (0.55)	4.3 (0.45)	+0.462	63 (0.72)	4.9 (0.28)	+0.097
enzyme + IgG	118 (0.95)	7.5 (0.05)	−0.038	124 (1)	nd	−0.027	128 (1)	nd	−0.004

[a] nd = not detected. The figures in parentheses are the fraction of the total amplitude for the corresponding k_{obs}. k_{obs}^f refers to the phase; k_{obs}^s, to the slow one. Experimental conditions are described under Materials and Methods. In the observation cell, the enzyme, antibody, and substrate concentrations were 5 μM, 20 μM, and 10 mM, respectively.

Table 3: Binding of mAb B2B4 to Flavocytochrome b_2 and Its Domains

antigen	IgG IC_{50} (nM)	Fab K_d (nM)
WT holoenzyme	16 ± 10	150 ± 50
heme domain *(S.c.)*	135 ± 27	80 ± 30
flavin domain (FDH) (*S.c.*)	nd	nd
heme domain (reconstituted) (*S.c.*)	135 ± 10	80 ± 30
heme domain (after GuCl) (*S.c.*)	nd	nd
heme domain (*H.a.*)	nd	nd
hinge−swap Flb_2 *(S.c.)*	11 ± 1	140 ± 50
HΔ3−Flb_2 *(S.c)*	15 ± 2	140 ± 50

[a] nd = no binding detected. The hinge−swap flavocytochrome b_2 carries residues 83−107 of the enzyme from *H.a.* instead of its own residues 85−116; this section encompasses the hinge region proper around residues 99−100 (*16*). The HΔ3−Flb_2 is missing residues 98−100 of the hinge region (*17*). Competition ELISA assays were carried out as described under Materials and Methods. The three domains were the recombinant ones (*24*−*26*).

enzyme could be detected in the supernatant using the sensitive enzymatic activity test (see under Materials and Methods).

Binding Studies

The initial screening for clone selection eliminated antibodies which would recognize the antigen fraction that was denatured upon adsorption to the ELISA plate. That screening also indicated that mAb B2B4 was able to recognize the heme-binding domain. With the purified antibody, new competition ELISA tests were carried out. For the Fab fragment, we checked that we were working under the conditions, defined by Friguet et al. (*34*, *35*), that yield actual binding constants. With the whole IgG, the experimental conditions were not very different, so that it is probable that the IC_{50} values in Table 3, column 1, are very similar to those of dissociation constants. With the IgG, the IC_{50} value for Flb_2 was about 10-fold lower than that for the heme domain (Table 3), suggesting either that there was a conformational difference between the isolated domain and the integrated one or that the flavin domain was contributing to affinity. Nevertheless, the latter did not compete with the coated antigen up to a concentration of 2 μM. It is known that the two Flb_2 functional modules have no affinity for each other, once separated by proteolysis (*38*) or genetic engineering (*26*). Not unexpectedly, a 2 to 1 mixture of the flavin and heme domains, after a 15 min preincubation, did not compete more efficiently with Flb_2 than the heme domain alone at the same concentration. That the FDH indeed does not contribute to binding was finally demonstrated by work with the Fab fragment. Table 3 indicates it has a very similar affinity for the holoenzyme and the cytochrome domain, and the value is the same as that of the IgG for the domain. It can thus be proposed that the higher apparent affinity of the IgG for Flb_2 is due to a cooperative binding of the divalent antibody to the tetrameric protein. Finally, it is clear from Table 3 that the C-terminal end of the heme-binding domain is not part of the epitope, since holoenzymes mutated in the interdomain linker region behave identically to the wild-type enzyme (see the footnote of Table 3).

Finally, we investigated whether or not there was an overlap between the cytochrome *c* binding area and the epitope. In the competition ELISA tests, cytochrome *c* was added to the competing antigen Flb_2. When the preincubations were carried out in 0.01 M phosphate buffer of pH 7 in the presence of 1 or 2 mM cytochrome *c*, the K_d value was 12 nM, compared to 27 nM for the control run in parallel in the absence of cytochrome *c*. At a higher ionic strength, in 0.1 M phosphate buffer, 1 mM cytochrome *c* is 80% saturating in kinetic tests of lactate oxidation. We conclude from these results that the epitope and the cytochrome *c* binding areas do not appear to overlap.

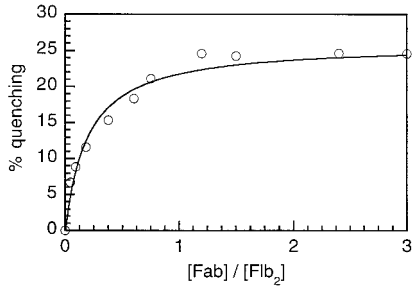


FIGURE 2: Titration of holo Flb_2 by the Fab fragment, as studied by fluorescence quenching. Experimental details are described in Materials and Methods. The Flb_2 concentration during the preincubation with the Fab was 20 μM.

The Epitope Is Conformational

Several experiments were carried out in order to try and locate the epitope. The heme-binding domain from the yeast *H.a.* shows about 60% sequence identity with that from *S.c.* (*39*, *40*) and can be obtained as a recombinant protein (*25*). After purification, it showed no competition with the coated antigen up to a concentration of 2 μM. There was no competition either, up to 2 μM, by the *S.c.* heme-binding domain after denaturation in guanidine−HCl. When the heme is extracted from the cytochrome domain by the acid acetone procedure (*41*), the polypeptide may retain some secondary structure, if one extrapolates from results obtained with cytochrome b_5 (*42*). Nevertheless, this partially denatured protein, up to 2 μM, did not compete with the coated antigen; in contrast, the protein reconstituted with hemin was indistinguishable from the native protein in competition tests. Finally, the mixtures of tryptic and chymotryptic peptides obtained from the denatured cytochrome domain did not compete with the coated antigen.

From these results, we conclude that the epitope of mAb B2B4 on the heme-binding domain of Flb_2 is conformational, and cannot be located using the overlapping peptides approach. In addition, the conformation of the epitope was insensitive to the protein redox state: identical saturation curves were obtained when the competing antigen in ELISA tests was oxidized or when it was maintained in reduced form by up to 20 mM L-lactate.

Stoichiometry of Binding

The stoichiometry of binding of the recombinant isolated heme domain to the Fab fragment was analyzed by fluorescence quenching experiments. Scatchard plots indicated a K_d of 30 nM and 1.01 sites on the Fab fragment, showing its homogeneity in terms of binding. Figure 2 gives the result of a titration of Flb_2 with the Fab fragment, where complexation was assessed again by fluorescence quenching. At half-saturation, a stoichiometry of 0.20 ± 0.03 Fab/monomer was

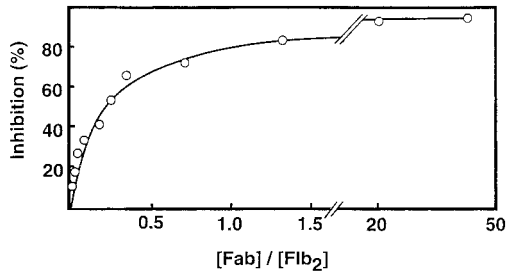


FIGURE 3: Inhibition of cytochrome *c* reduction by Flb_2 at increasing Fab/Flb_2 monomer ratios. The enzyme (632 nM) was preincubated with the antibody fragment for 5 min at 37 °C. Residual activity was determined by diluting 60-fold into an assay solution containing 10 mM L-lactate and 450 μM cytochrome *c* in 0.1 M phosphate buffer of pH 7.

obtained. In other words the Flb_2 tetramer can only bind two Fab moieties. Figure 3 shows a kinetic experiment in which cytochrome *c* reduction in the steady state was inhibited by increasing amounts of the Fab fragment. Several experiments of this kind indicated 50% of maximal inhibition for a Fab/monomer ratio of 0.26 ± 0.08. Thus, binding of only two Fab fragments per tetramer was sufficient to cause maximal inhibition.

DISCUSSION

The monoclonal antibody B2B4, which was elicited against intact flavocytochrome b_2, appears to recognize exclusively the heme-binding domain and does so with the same affinity for the holoenzyme and for the isolated domain, within error; this suggests that the epitope is equally accessible in the two structures. For the isolated domain, K_d values of 80 nM and 30 nM were determined in ELISA competition experiments and fluorescence quenching titrations, respectively. Under steady-state conditions, the antibody inhibits cytochrome *c* reduction by the enzyme, but not turnover in the presence of ferricyanide. The stopped-flow analysis reported in the preceding section indicates that, in the complex, the two functional domains are uncoupled: the FDH domain is reduced normally and can only be reoxidized at the expense of the small acceptor ferricyanide. It has become incapable of reducing the heme.

The inhibition of flavin to heme electron transfer observed in the stopped-flow experiment is sufficient in itself to explain the inhibition of cytochrome *c* reduction in the steady state. With respect to the antibody effect on heme b_2 to heme *c* transfer, our results suggest that there could be no effect, since we were unable to detect in ELISA tests any competition between cytochrome *c* and the antibody for binding to the enzyme. Nevertheless, this point should be checked in the future using other methods. Epitope mapping could contribute to delineate the cytochrome *c* binding site on Flb_2, the precise location of which is unknown at present. It was previously suggested to involve the flavodehydrogenase domain (*43*); a binding mode on the tetramer was proposed from modeling experiments on a graphics system (*44*), but the use of site-directed mutants showed it to be kinetically irrelevant and led to another proposed binding mode (*45*). In any case, even if the Fab epitope and the cytochrome *c* binding area do not overlap, steric hindrance might prevent them from binding simultaneously.

FIGURE 4: Stereodiagram of subunit S1 (residues 2−486). The cytochrome domain (residues 2−99, left), the β/α barrel (residues 192−465), and the prosthetic groups are in thick lines. The disordered loop is indicated by a dashed line. Reproduced with permission from ref 5. Copyright 1989 Portland Press.

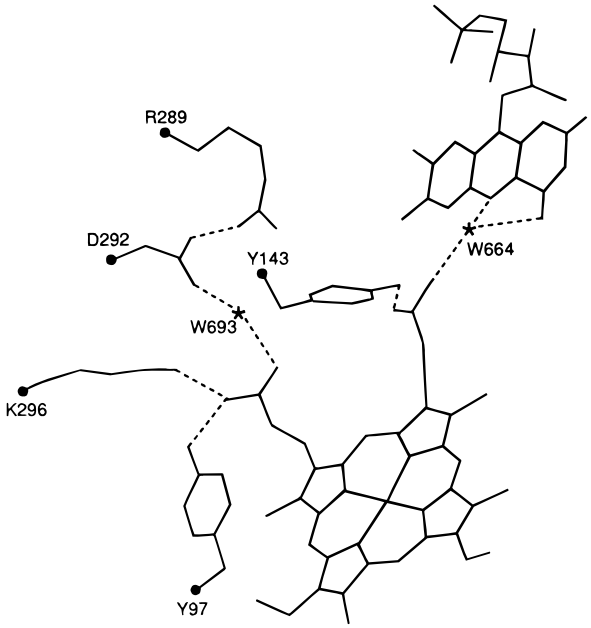


FIGURE 5: Polar contacts between the two domains in Flb_2. Tyr97 belongs to the cytochrome part, all other side chains to the FDH domain.

Our results have to be analyzed in light of the published Flb_2 crystal structure (*5*, *46*). The asymmetric unit includes two subunits, which show different features, although they are chemically identical. As shown in Figure 4, S1 encompasses the two domains, the flavin and heme planes are roughly parallel, and the distance between the FMN N5 position and the closest carbon atom of the porphyrin ring is of the order of 10 Å. There is no intervening side chain between the domains; their contacts are in part hydrophobic, in part polar, but not mainly electrostatic (*5*); Figure 5 depicts the polar contacts, which include water molecules. The second subunit, S2, only includes the FDH domain; this indicates positional mobility of the cytochrome domain, mediated by the so-called hinge region around Gly100. These observations also confirm the lack of strong interactions between the domains which was mentioned above. While a mobility of the cytochrome domain in solution was subsequently deduced from NMR experiments (*47*), neither the importance nor the extent of mobility in catalysis are fully understood. The problem has been addressed by site-directed mutagenesis. The introduction of several mutations in the hinge region indicated that a number of structural manipulations in that area lead to impaired electron transfer between the prosthetic groups (*16*−*18*). Although it seems clear that these mutations must affect the interface between the domains, the interpretation of their effects in terms of mobility is less clear. Studies of the Y143F mutant showed

the importance of the Tyr143 phenol group hydrogen bond to the heme (Figure 5) for intramolecular electron transfer (*11*, *37*). The results led us to propose that the substrate and the heme propionate carboxylates compete for making a hydrogen bond to the Y143 phenol group. This implies that when the substrate is being transformed, the heme propionate and possibly the whole domain have to move away from the active site and can dock again for electron transfer after the product has left the active site. The results described in the present paper provide a different approach to the problem which is complementary to site-directed mutagenesis.

Our goal is to define the epitope of mAb B2B4, which will require the study of site-directed mutants in the heme binding domain and/or the crystal structure of the Fab/antigen complex. These studies are underway, but meanwhile it is interesting to examine the enzyme structure and analyze the putative effects of the mAb binding to various locations on the cytochrome domain. First and foremost, we feel that the observed inhibition of the interdomain electron transfer is incompatible with a fixed structure such as that of S1 (Figure 4), given that flavin reduction occurs normally: what could prevent heme reduction from occurring at the normal rate once the flavin is reduced? It is not reasonable to invoke a possible heme redox potential modification upon mAb binding. This change would have to be very large in order to account for the suppression of intrasubunit electron transfer; it would probably have to correspond to an important structural alteration, which the known structural stability of the heme domain does not make very likely. The stoichiometry of binding and inhibition is another interesting result to be discussed in this context. The mass of a Fab fragment is slightly larger than that of the FDH. It is thus not entirely surprising that only two such moieties can bind to an F$l b_2$ tetramer. Depending on their orientation, mutual steric hindrance could prevent their binding to each subunit. What is more surprising is that electron transfer in the two uncomplexed subunits should also be suppressed, a consequence which cannot be rationalized on the basis of a fixed structure. It can be hypothesized that the steric hindrance due to the bound Fab induces a displacement of the heme domain in the second subunit, leading to an interface alteration; alternatively, and more interestingly, it prevents the cytochrome domain movements required for productive docking.

If we now consider possible binding sites for mAb B2B4 in light of a mechanism where mobility of the domain is required at certain steps of the catalytic cycle, it seems clear that some possible solutions are less attractive than others. The mAb could bind to the surface area most distal from the domain interface (Figure 4), which corresponds to the external side of the second hydrophobic core of the cytochrome domain (*5*). Complexation of the 50 kDa Fab fragment might slow the mobility of the heme-binding domain, and therefore reduce the frequency of productive docking to the flavodehydrogenase domain. While this could reduce the electron-transfer rate, it is doubtful that it would inhibit the process to such an extent. We consider, therefore, that this distal surface area is not a good candidate for the epitope.

Another possible binding site would be the hinge region. The mAb could either bind to the conformation seen in the crystal structure and prevent the movements necessary during the catalytic cycle or freeze a different conformation adopted by the hinge during movement, thus preventing normal docking of the cytochrome domain to the flavin domain. This attractive hypothesis can however be discarded, since two enzymes mutated in the hinge region behave identically to the WT enzyme in ELISA competition tests (Table 3). A final possibility would be that the mAb binds to some part of the domain close to or at the interface, capturing it while it is on the move, and then acting as a wedge to prevent productive docking. This is our preferred hypothesis, and preliminary results with site-directed mutants would appear to support it.

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