Interaction of Cytochrome *c* with Flavocytochrome b_2[†]

Simon Daff,[‡] R. Eryl Sharp,[§] Duncan M. Short,[‡] Cameron Bell,[‡] Patricia White,[‡] Forbes D. C. Manson,[||] Graeme A. Reid,[||] and Stephen K. Chapman*,[‡]

Department of Chemistry and Institute of Cell and Molecular Biology, Edinburgh Centre for Molecular Recognition, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K., and Johnson Research Foundation, B501 Richards Building, Department of Biochemistry and Biophysics, University of Pennsylvania, 37th and Hamilton Walk, Philadelphia, Pennsylvania 19104

Received September 20, 1995; Revised Manuscript Received March 11, 1996[⊗]

ABSTRACT: Flavocytochrome b_2 from *Saccharomyces cerevisiae* couples L-lactate dehydrogenation to cytochrome *c* reduction. At 25 °C, 0.10 M ionic strength, and saturating L-lactate concentration, the turnover rate is 207 s^{-1} [per cytochrome *c* reduced; Miles, C. S., Rouviere, N., Lederer, F., Mathews, F. S., Reid, G. A., Black, M. T., & Chapman, S. K. (1992) *Biochem. J. 285*, 187−192]. The second-order rate constant for cytochrome *c* reduction in the pre-steady-state has been determined by stopped-flow spectrophotometry to be 34.8 (± 0.9) μM^{-1} s^{-1} in the presence of 10 mM L-lactate. This rate constant has been found to be dependent entirely on the rate of complex formation, the electron-transfer rate in the pre-formed complex being in excess of 1000 s^{-1}. Inhibition of the pre-steady-state reduction of cytochrome *c* by either zinc-substituted cytochrome *c* or ferrocytochrome *c* has led to the estimation of a K_d for the catalytically competent complex of 8 μM, and from this the dissociation rate constant of 280 s^{-1}, a value much less than the actual electron-transfer rate. The inhibition observed is only partial which indicates that electron transfer from the 1:1 complex to another cytochrome *c* can occur and that alternative electron transfer sites exist. The cytochrome *c* binding site proposed by Tegoni et al. [Tegoni, M., White, S. A., Roussel, A., Mathews, F. S., & Cambillau, C. (1993) *Proteins 16*, 408−422] has been tested using site-directed mutagenesis. Mutations designed to affect the complex stability and putative electron-transfer pathway had little effect, suggesting that the primary cytochrome *c* binding site on flavocytochrome b_2 lies elsewhere. The combination of tight binding and multiple electron-transfer sites gives flavocytochrome b_2 a low K_m and a high k_{cat}, maximizing its catalytic efficiency. In the steady-state, the turnover rate is therefore largely limited by other steps in the catalytic cycle, a conclusion which is discussed in the preceding paper in this issue [Daff, S., Ingledew, W. J., Reid, G. A., & Chapman, S. K. (1996) *Biochemistry 35*, 6345−6350].

Over recent years there has been a huge amount of research directed at understanding the rate and specificity of interprotein electron transfer (Marcus & Sutin, 1985; McLendon & Hake, 1992; Chapman & Mount, 1995; Moser et al., 1996). To date much of the work done to address this question has been based on the reactions of the promiscuous protein cytochrome *c*, with both its physiological (e.g., cytochrome *c* peroxidase) and nonphysiological (e.g., cytochrome b_5) redox partners. In this paper, we discuss the less well characterized interaction of cytochrome *c* with another of its physiological partners, flavocytochrome b_2.

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a homotetramer of subunit molecular weight 57 500 (Jacq & Lederer, 1974). It is a soluble component of the mitochondrial intermembrane space (Daum et al., 1982) where it catalyzes the oxidation of L-lactate to pyruvate with subsequent electron transfer to cytochrome *c* (Appleby & Morton, 1954). The crystal structure of flavocytochrome b_2 determined to 2.4 Å resolution (Xia & Mathews, 1990) shows that each subunit consists of two distinct domains: an N-terminal domain containing protoheme IX ("b_2-heme domain") and a C-terminal domain containing flavin mononucleotide ("flavin domain"). It has been shown that direct electron transfer from FMN to cytochrome *c* is insignificant (Forestier & Baudras, 1971; Iwatsubo et al., 1977; Balme et al., 1995), and that the role of the b_2-heme domain is to mediate this process. Therefore, the route of electron transfer in this system is L-lactate → FMN → b_2-heme → cytochrome *c*. The intraprotein FMN → heme electron transfer reactions of *S. cerevisiae* flavocytochrome b_2 have been extensively investigated (Capeillère-Blandin et al., 1975; Pompon et al., 1980; Miles et al., 1992; Walker & Tollin, 1991; Hazzard et al., 1994; White et al., 1993; Sharp et al., 1994; Chapman et al., 1994; Daff et al., 1996). However, the rather more intractable problem of investigating the interprotein electron transfer to cytochrome *c* has been performed with flavocytochrome b_2 from the yeast *Hansenula anomala* (Capeillère-Blandin, 1982), for which the crystal structure is not yet known.

Recently, in view of the unsuccessful attempts to obtain cocrystals of *S. cerevisiae* flavocytochrome b_2 and cyto-

[†] This work was funded by research grants from the Biotechnology and Biological Science Research Council (U.K.), which also provided studentships for R.E.S., and D.M.S., and by The European Commission (FLAPS Network) and the Engineering and Physical Sciences Research Council (U.K.), which provided a studentship for S.D.

* To whom correspondence should be addressed. Fax: (44)131 650 4743. E-mail: S.K.Chapman@ed.ac.uk.

[‡] Department of Chemistry, University of Edinburgh.

[§] University of Pennsylvania.

[||] Institute of Cell and Molecular Biology, University of Edinburgh.

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0006-2960(95)02256-2 CCC: $12.00

chrome *c*, Tegoni et al. (1993) have proposed a hypothetical complex for the interaction between crystalline flavocytochrome b_2 and cytochrome *c*. The model binding site consists of an electrostatic interaction in which the positively charged side chains of cytochrome *c* (Arg13, Arg38, Lys54, and Lys79.) are positioned in the immediate vicinity of acidic flavocytochrome b_2 residues, and several hydrogen bonds are developed. Residue Glu91 of flavocytochrome b_2 is implicated in a number of these interactions and plays a key role in defining the model. As well as presenting a model binding site, Tegoni et al. (1993) suggest a possible pathway for electron transfer. This pathway proceeds from the b_2-heme through residues 50−51 and emerges on the enzyme surface at the side chain of Phe52.

In this paper we (i) report new solution studies on the flavocytochrome b_2-cytochrome *c* interaction and (ii) test the hypothetical complex using site-directed mutagenesis.

MATERIALS AND METHODS

DNA Manipulation, Strains, and Growth. Site-directed mutagenesis was performed by the Kunkel method of nonphenotypical selection (Kunkel, 1985) using the oligonucleotides G1194 (GTTATCAAGGCTAATGCCGG) and G1193b (ATGCCTCCTAAACTTGTCTG) to construct F52A and E91K, respectively (Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K.). Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation, and transformation were performed as described in Sambrook et al. (1989). *E. coli* strains AR120 and TG1 were used for expression of mutant and wild-type flavocytochromes b_2, respectively.

Enzymes. Flavocytochrome b_2. Wild-type and mutant flavocytochromes b_2 expressed in *E. coli* were isolated from cells which had been stored at −20 °C. The purification procedure was essentially as described previously (Brunt et al., 1992; Black et al., 1989). Purified enzyme preparations were either stored in the reduced state under a nitrogen atmosphere at 4 °C as precipitates from 70% saturated $(NH_4)_2SO_4$ solution or as concentrated aliquots (approximately 100 μM) in the oxidized state, snap-frozen, and stored in liquid nitrogen. Under the former conditions, the enzymes retained full activity for several weeks and indefinitely when frozen in liquid nitrogen. Enzyme concentrations were calculated using previously published extinction coefficients (Pajot & Groudinsky, 1970).

Zinc-Substituted Cytochrome c. Porphyrin cytochrome *c* was prepared essentially as described by Vanderkooi and Erecinska (1975). Approximately 100 mg of freeze-dried horse-heart cytochrome *c* (Sigma) was placed in an open PTFE test tube and cooled in liquid nitrogen. HF gas was then condensed onto the cytochrome *c* while stirring with a PTFE rod. The HF was generated by bubbling nitrogen gas through a HF-saturated pyridine solution (Sigma) contained in a sealed PTFE vessel, the only exit from which led directly to the cytochrome *c* via a length of PTFE tubing. This apparatus was contained within a well ventilated fume hood. Once the cytochrome *c* was covered entirely by solid HF, the flow was stopped and reaction tube removed from liquid nitrogen. While stirring, the HF was evaporated in a steady stream of nitrogen gas. Purification was conducted as described by Vanderkooi and Erecinska (1975). The porphyrin cytochrome *c* was converted entirely to zinc-substituted cytochrome *c* as described by Vanderkooi et al. (1976). The Zn-cytochrome *c* was dialyzed into 10 mM Tris, HCl buffer, pH 7.5, *I* 0.10, and concentrated using Centricon tubes (Amicon) to approximately 250 μM. Aliquots were stored at −20 °C. Concentrations were calculated from previously reported extinction coefficients (Vanderkooi et al., 1976).

Kinetic Analysis. All kinetic experiments were carried out at 25 ± 0.1 °C in 10 mM Tris-HCl at pH 7.5, *I* 0.10. The buffer consisted of 10 mM HCl titrated against Tris solution to pH 7.5 and adjusted to *I* 0.10 by addition of NaCl. For experiments involving ionic strength effects, *I* was adjusted by addition of the appropriate amount of NaCl. Steady-state kinetic measurements involving the enzymatic oxidation of L-lactate were performed using a Shimadzu UV2101PC or a Beckman DU62 spectrophotometer. Horse-heart cytochrome *c* (type VI, Sigma) was used as the electron acceptor. Collection and analysis of the steady-state data was as previously described (Miles et al., 1992). The K_m and k_{cat} parameters were determined using nonlinear regression analysis.

Pre-steady-state kinetics were performed on an Applied Photophysics SF.17 MV stopped-flow spectrofluorimeter as previously described (Miles et al., 1992; Sharp et al., 1994). Cytochrome *c* reduction by prereduced flavocytochrome b_2 was monitored at 416.5 nm, a flavocytochrome b_2-heme isosbestic point. To ensure that the reduction occurred under pseudo-first-order conditions, flavocytochrome b_2 was always present in excess. Reduction was carried out over a range of flavocytochrome b_2 concentrations, typically 2−10 μM. The cytochrome *c* concentration was 1 μM after mixing. The traces were fitted to monophasic exponentials by nonlinear regression analysis. At least five runs were performed at each flavocytochrome b_2 concentration. Throughout each set of experiments involving ionic strength variation, L-lactate was added to the flavocytochrome b_2 solution to a concentration of 2 mM (before mixing), to fully reduce the enzyme. Over the time scale of the experiment (5 min) no autooxidation of the enzyme occurred and it remained fully reduced. For all other experiments the L-lactate concentration was 10 mM after mixing. For cytochrome *c* reduction by b_2-core, 0.1 μM wild-type flavocytochrome b_2 (a catalytic amount) was added to achieve full b_2-core reduction prior to mixing. Precautions were taken to prevent aerobic oxidation of b_2-core by handling all solutions under an N_2 atmosphere. Second-order-rate constants were determined by plotting k_{obs} for cytochrome *c* reduction against flavocytochrome b_2 concentration and fitting the data to a linear regression analysis.

Inhibition of pre-steady-state cytochrome *c* reduction by reduced or zinc-substituted cytochrome *c* was performed essentially as described above, except that the pre-reduced flavocytochrome b_2 was mixed with a known concentration of reduced or zinc-substituted cytochrome *c*. Flavocytochrome b_2 was kept at approximately 8 μM and inhibitor concentration was varied from 2 to 100 μM (after mixing). Cytochrome *c* reduction (2 μM after mixing) was monitored at 544.8 nm, a b_2-heme isosbestic point. The inhibition constant for cytochrome *c* reduction was determined by plotting the rate of cytochrome *c* reduction against the concentration of inhibiting cytochrome *c* and the data fitted to the equation shown in Chart 1.

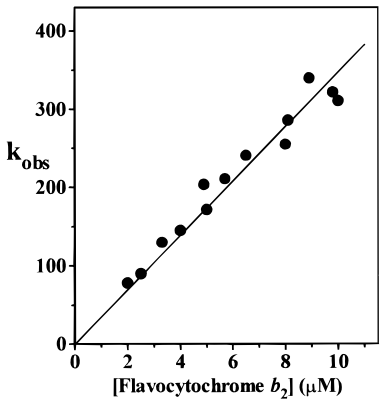

FIGURE 1: Determination of the second-order rate constant for cytochrome *c* reduction by pre-reduced wild-type flavocytochrome b_2 performed at 25 °C in 10 mM Tris−HCl buffer, pH 7.5, *I* 0.10, containing 10 mM L-lactate. k_{obs} is the pseudo-first-order rate constant for cytochrome *c* reduction derived from stopped-flow spectrophotometry at 416.5 nm (see Materials and Methods). The data are fitted by linear regression analysis. The gradient $k_2 = 34.8 \pm 0.9\ \mu M^{-1}\ s^{-1}$.

Scheme 1: Pre-Steady-State Reduction of Cytochrome *c* by Excess Flavocytochrome b_2 under Standard Conditions Follows a Single-Exponential Function[a]

(i) $F\!:\!H^{\bullet} + cyt\,c \underset{}{\overset{Fast}{\rightleftharpoons}} [F\!:\!H^{\bullet}\,cyt\,c] \xrightarrow{Slow} [F\!:\!H\ cyt\,c^{\bullet}]$

(ii) $F\!:\!H^{\bullet} + cyt\,c \overset{Slow}{\underset{}{\rightleftharpoons}} [F\!:\!H^{\bullet}\,cyt\,c] \xrightarrow{Fast} [F\!:\!H\ cyt\,c^{\bullet}]$

binding **electron transfer**

[a] This is only possible if one of the two schemes illustrated above is satisfied by the reaction mechanism. Therefore either (i) cytochrome *c* binding is fast and reversible and electron transfer slow by comparison, or (ii) cytochrome *c* association/ dissociation is slow and electron transfer is fast. F, flavin mononucleotide; H, b_2-heme; cytc, cytochrome *c*; ●, represents an electron thereby indicating redox state.

Reduction of wild-type flavocytochrome b_2-heme by L-lactate in the presence of zinc-substituted cytochrome *c* was monitored by stopped-flow spectrophotometry at 557 nm as described previously (Miles et al., 1992). The final concentrations were 5 μM flavocytochrome b_2, 10 mM L-lactate, and 0, 10, and 20 μM Zn-cytochrome *c*. Each trace shown in Figure 5 is an average of at least five different runs.

All data fitting was conducted by least-squares regression analysis using either Applied Photophysics software or Origin (Microcal).

RESULTS

Pre-Steady-State Reduction of Cytochrome c. Reduction of a substoichiometric amount of cytochrome *c* by reduced flavocytochrome b_2 was monitored by stopped-flow spectrophotometry as described in Materials and Methods. Figure 1 shows a plot of the observed rate constant against wild-type flavocytochrome b_2 concentration, which shows a linear dependency up to 10 μM, with the rate constant approaching 400 s^{-1}. Each of the generated traces fitted well to a single-exponential function (see Figure 3a), indicating that the reduction process is controlled by a single rate-determining step. The two possibilities for this mechanism are shown in Scheme 1. Either (i) complex formation is rapid and reversible, while electron transfer within the pre-formed complex is slow, or (ii) cytochrome *c* binding is slow while electron transfer is faster than both binding and dissociation. Model (i) would generally produce a hyperbolic curve saturating at the electron-transfer rate with $K_m = K_d$ for the bound complex. The linear region observed in Figure 1 would therefore have to occur at concentrations much less than the K_d for the bound complex. Model (ii) would generate a linear plot until the rate of binding approached the rate of electron transfer at which point single-exponential functions would no longer fit to the experimental traces. The second-order rate constant for cytochrome *c* reduction by wild-type flavocytochrome b_2 derived from Figure 1 is presented in Table 1 along with comparable values for the mutant enzymes E91K, F52A, and b_2-core (the separately expressed heme domain; Brunt et al., 1992). Neither point mutation has any effect on the rate of cytochrome *c* reduction, whereas the b_2-core mutant has a second-order rate constant less than half that of the wild-type value. Therefore residues E91 and F52 are unlikely to be important components of the cytochrome *c* binding site.

Table 1: Second-Order Rate Constants for the Reduction of Cytochrome *c* by Pre-Reduced Wild-Type and Mutant Flavocytochromes b_2[a]

	second-order rate constant ($\mu M^{-1}\ s^{-1}$)
wild-type	34.8 ± 0.9
E91K	37.7 ± 1.2
F52A	34.3 ± 1.4
b_2-core	16.2 ± 0.3

[a] All experiments were performed at 25 °C in 10 mM Tris-HCl buffer, pH 7.5, *I* 0.10 containing 10 mM L-lactate. The second-order rate constants were determined as described in Materials and Methods and as illustrated in Figure 1. Errors represent standard deviations from least-squares fit.

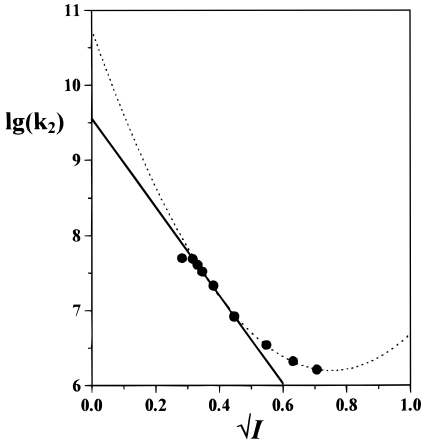

FIGURE 2: Log of the second-order rate constant (k_2) for cytochrome *c* reduction by wild-type flavocytochrome b_2 plotted against the square root of buffer ionic strength (10 mM Tris−HCl, pH 7.5 + NaCl + 1 mM L-lactate). (—) Fit to the Debye−Hückel equation ($\log k_2 = \log k_2^{\circ} + 2AZ_+Z_-\sqrt{I}$) gradient $(2AZ_+Z_-) = -5.9 \pm 0.1$ (NB/Fit restricted to five data points *I* 0.10−0.20); (---) Fit to parabolic curve ($\log k_2 = \log k_2^{\circ} + 2AZ_+Z_-\sqrt{I} - BI$) gradient at *I* 0 $(2AZ_+Z_-) = -12.1 \pm 0.5$. Errors represent standard deviations from a least-squares fit.

Variation of the Second-Order Rate Constant for Cytochrome c Reduction with Ionic Strength. The second-order rate constants for cytochrome *c* reduction by wild-type flavocytochrome b_2 at different ionic strengths are plotted in Figure 2. The straight line fit for $\log k_2$ versus $\sqrt{I}$ according to the Debye−Hückel equation has a gradient $2AZ_+Z_- = -5.9 \pm 0.1$, indicating a positive/negative

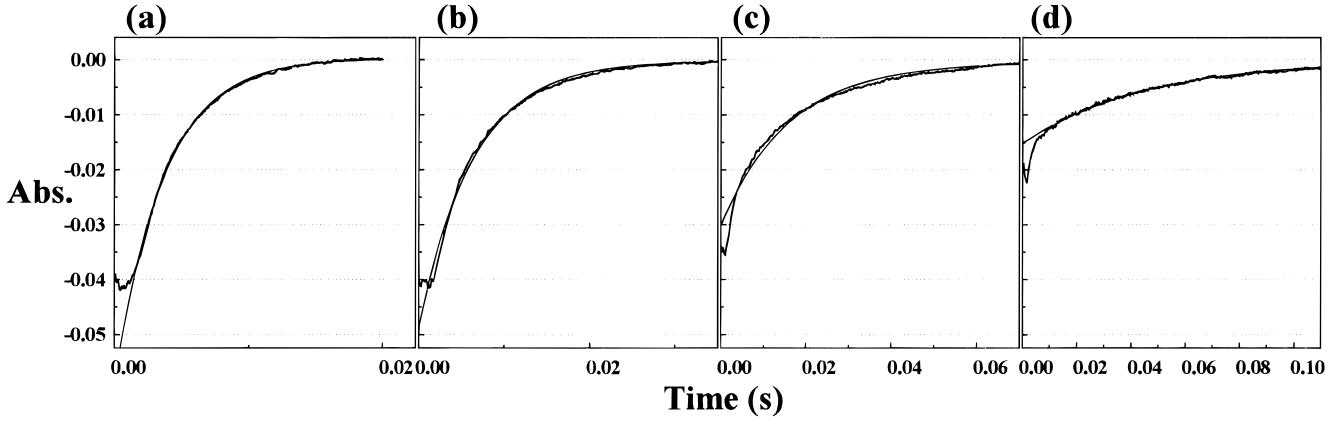

FIGURE 3: Stopped-flow traces showing pre-steady-state reduction of 1 μM cytochrome *c* by 8 μM reduced flavocytochrome b_2 at 25 °C, in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM L-lactate at four different ionic strengths (adjusted by addition of NaCl). Each trace is fitted by nonlinear least-squares regression analysis to a monophasic exponential function (shown) with rate constant *k* and amplitude *A*. (a) *I* 0.075, $k = 280\ s^{-1}$, $A = 0.060$; (b) *I* 0.050, $k = 171\ s^{-1}$, $A = 0.051$; (c) *I* 0.025, $k = 72\ s^{-1}$, $A = 0.031$; (d) *I* 0.010, $k = 24\ s^{-1}$, $A = 0.015$.

interaction at the cytochrome *c* binding site. However, it is clear from Figure 2 that the fit is far from satisfactory, deviating strongly at high ionic strength. This is not surprising in view of the well documented nonideal behavior exhibited during protein−protein interactions (Koppenol, 1980; Koppenol et al., 1978). Further, the ionic strength region studied (0.1−0.5) is well outside the range considered reasonable for an ideal solution (Robinson & Stokes, 1959). By introducing a term linearly dependent on ionic strength (with coefficient *B*) to the fitting function, a parabolic curve is generated. This fits better to the data and at $I = 0$ has a gradient of $2AZ_+Z_- = -12.1 \pm 0.5$, which again indicates a significant positive/negative interaction. The theoretical basis of this equation is also limited, as the coefficient *B* has no simple physical meaning (Perlmutter-Hayman, 1973). Its use in this case demonstrates the magnitude of uncertainty in the value of Z_+Z_- derived from Figure 2. Alternative more elaborate approaches use additional information to calculate the magnitude of charge−charge stabilization in a complex [e.g., Watkins et al. (1994)]. However, for the flavocytochrome b_2−cytochrome *c* complex the binding site location is unknown and such methods therefore inaccessible.

At lower ionic strength the stopped-flow method used to obtain second-order rate constants becomes unusable. The cytochrome *c* reduction traces no longer fit to single-exponential functions, suggesting that the reaction is now dependent on multiple rate-determining steps. Figure 3a−d shows four stopped-flow traces at different ionic strengths, each fitted to a single-exponential function. At lower ionic strength the amplitude of the observed reaction decreases and the apparent rate of reaction becomes slower, although the fit quality is poor. The decrease in amplitude indicates that a larger proportion of the reaction is occurring within the stopped-flow dead-time and is therefore happening too fast to be observed fully. This effect is to be expected if the trend of increasing second-order rate constant with decreasing ionic strength was continued as in Figure 2. The appearance of a slow phase indicates that not all the cytochrome *c* is reduced by this rapid step, but a proportion is delayed possibly by an inhibitory binding process. Such a deviation from pseudo-first-order behavior seriously limits the range of ionic strengths which can be used for the Debye−Hückel plot.

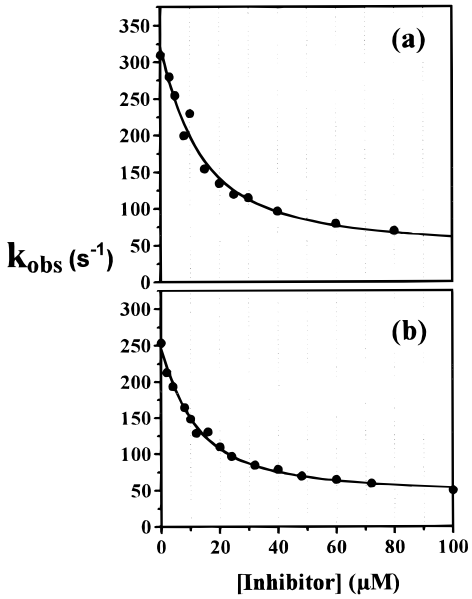

FIGURE 4: Rate constants observed (k_{obs}) for pre-steady-state reduction of ferricytochrome *c* (2 μM) by wild-type flavocytochrome b_2 are shown plotted against inhibitor concentration. (a) Inhibitor is ferrocytochrome *c* and concentration of flavocytochrome b_2 is 8.3 μM. (b) Inhibitor is zinc-substituted cytochrome *c* and concentration of flavocytochrome b_2 is 8.0 μM. Both data sets are fitted to the equation in Chart 1 by nonlinear least-squares regression analysis; (a) $K_i = 8.8 \pm 2.3$ μM (b) $K_i = 7.3 \pm 0.9$ μM.

Inhibition of Cytochrome c Reduction. Pre-steady-state cytochrome *c* reduction by wild-type flavocytochrome b_2 was inhibited by the addition of either zinc-substituted cytochrome *c* or ferrocytochrome *c* to the prereduced enzyme. The inhibition curves generated are plotted in Figure 4. The general similarity of the two data sets indicates that the use of the redox-inactive Zn−cytochrome *c* is unnecessary for this type of experiment. The electrode potential of cytochrome *c* (Loach, 1976) is some 270 mV more positive than that of the b_2-heme (White et al., 1993), and it is therefore unlikely that a significant reverse reaction would occur. Both systems appear to exhibit partial inhibition such that at high inhibitor concentration a significant rate constant is still observed (around 20% of the maximum value). This indicates that although the primary binding site is occupied rapidly by the inhibitor, cytochrome *c* reduction still occurs,

Chart 1[a]

$$k_{obs} = k_R E_T + \tfrac{1}{2}(k_2 - k_R)\left(E_T - I_T - K_i + \sqrt{(E_T - I_T - K_i)^2 + 4E_T K_i}\right)$$

k_2 Second-order rate constant at $I_T = 0$.

k_R Residual second-order rate constant (at $I_T = \infty$).

E_T Total enzyme concentration.

I_T Total inhibitor concentration.

K_i Dissociation constant for inhibitor at primary binding site.

[a] Cytochrome *c* reacts at a single primary binding site per flavocytochrome b_2 subunit with second-order rate constant $k_2 - k_R$. It also reacts at alternative positions whether the primary site is occupied or not. The cumulative effect of these electron-transfer encounters is accounted for by introducing a residual second-order rate constant k_R. The potential binding properties of these alternative electron-transfer sites are ignored for simplicity. Inhibitor binds to the primary binding site with dissociation constant K_i and prevents further reaction at this position. Since I_T is not a lot greater than E_T, it is considered inappropriate to use a classical Michaelis−Menten function for the fitting process.

Table 2: Inhibition Constants (K_i) and Residual Second-Order Rate Constants (k_R) Calculated for Inhibition by either Zinc-Substituted Cytochrome *c* or Ferrocytochrome *c* of the Pre-Steady-State Reduction of Ferricytochrome *c* by Both Wild-Type and Mutant (E91K) Flavocytochromes b_2[a]

enzyme	inhibitor	K_i (μM)	k_R ($\mu M^{-1}\ s^{-1}$)
wild-type	zinc-cytochrome *c*	7.3 ± 0.9	4.9 ± 0.6
wild-type	ferrocytochrome *c*	8.8 ± 2.3	4.5 ± 2.0
E91K	ferrocytochrome *c*	6.0 ± 2.0	7.5 ± 0.8

[a] Performed at 25 °C in 10 mM Tris-HCl buffer, pH 7.5 (*I* 0.10), containing 10 mM L-lactate. Rate constants generated by fitting stopped-flow data to monophasic exponential functions were plotted against inhibitor concentration, and fitted to the equation in Chart 1 by nonlinear regression analysis (as in Figure 4). Errors represent standard deviations from the least-squares fit.

albeit more slowly. K_i values for both curves in Figure 4 are presented in Table 2 along with the residual second-order rate constants (k_R). The model used to calculate these values assumes a single cytochrome *c* binding site per subunit of flavocytochrome b_2 and introduces a residual second-order rate constant k_R to account for the minimum rate constant observed. Both Zn−cytochrome *c* and ferrocytochrome *c* appear to cause partial inhibition with a dissociation constant of around 8 μM at the primary binding site. The additional data in Table 2 refer to the mutant E91K which shows no significantly different behavior with regard to inhibition by ferrocytochrome *c*, further supporting the view that this is not an important residue for binding cytochrome *c*.

Effect of Zinc-Substituted Cytochrome c Binding on b_2-Heme Reduction. Figure 5 shows three b_2-heme reduction traces for wild-type flavocytochrome b_2 generated by mixing with excess L-lactate in the presence of 0, 10, and 20 μM zinc-substituted cytochrome *c*. The Zn−cytochrome *c* is at high enough concentrations to ensure that a significant proportion of the binding sites are occupied (shown by the inhibition studies). The three traces overlay almost exactly, which indicates that there is no significant affect on either FMN to b_2-heme electron transfer or on the preceeding step, FMN reduction.

DISCUSSION

There have been several attempts to characterize the complexation between flavocytochrome b_2 and cytochrome *c*; however, the lack of correlation between these studies has made interpretation difficult. For example, stoichiometries reported vary between one cytochrome *c* per flavocytochrome b_2 tetramer to one per subunit (Tegoni et al., 1993). Previous studies on the flavocytochrome b_2 from *H. anomala* (60% sequence identity with the *S. cerevisiae* enzyme; Black et al., 1989) have led to the conclusion that the cytochrome *c* binding site involves both flavin and b_2-heme domains (Thomas et al., 1983a,b; Capeillère-Blandin & Albani, 1987). A similar conclusion can be drawn for the *S. cerevisiae* enzyme since the isolated heme domain (b_2-core) has a second-order rate constant for cytochrome *c* reduction which is less than half the value for the intact enzyme (Table 1). In addition, Capeillère-Blandin (1982) reported that the catalytically competent *H. anomala* flavocytochrome b_2−cytochrome *c* complex is stabilized by electrostatic attraction. Similar conclusions hold for the *S. cerevisiae* enzyme (see Figure 2 and Results). In line with the above results, Tegoni et al. (1993) proposed a model for the complex between flavocytochrome b_2 and cytochrome *c* dominated by electrostatic interactions and involving both heme and flavin domains. They also imposed a stoichiometry of one cytochrome *c* per subunit. We have constructed mutants of flavocytochrome b_2 to test this model. E91K reverses the charge of a flavocytochrome b_2 residue postulated to be involved in an electrostatic interaction critical to cytochrome *c* binding, and F52A removes a phenyl ring suggested to be a crucial part of the electron transfer pathway between the two hemes. If the hypothetical model were correct, both mutations would be expected to have a profound effect on the cytochrome *c* reductase ability of the enzyme. The results in Table 1 show that this is not the case.

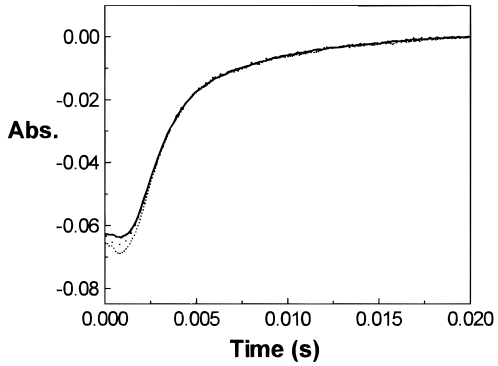

FIGURE 5: b_2-heme reduction traces for wild-type flavocytochrome b_2 (5 μM) on mixing with L-lactate (10 mM) in the presence of zinc-substituted cytochrome *c*: (—) 0 μM; (---) 10 μM; (•••) 20 μM. The traces were collected at 557 nm using stopped-flow spectrophotometry at 25 °C in 10 mM Tris−HCl buffer, pH 7.5, *I* 0.10.

Since the Tegoni model is unlikely to be correct, we have analyzed the flavocytochrome b_2−cytochrome *c* interaction in more detail. For wild-type flavocytochrome b_2, the rate constant for electron transfer from b_2-heme to cytochrome *c* within the pre-formed complex has been estimated by photochemical excitation to be 200 (± 80) s^{-1} at 25 °C (McLendon et al., 1987). For the *H. anomala* enzyme, the rate constant has been determined from stopped-flow/ionic strength experiments to be 380 s^{-1} at 5 °C (Capeillère-Blandin, 1982). In our experiments on the *S. cerevisiae* enzyme (at 25 °C), cytochrome *c* reduction occurs at rates beyond the reliable range of the stopped-flow technique and must be in excess of 1000 s^{-1}. While the second value is not inconsistent with this (in view of the lower temperature

used and different enzyme form), it seems unlikely that the electron-transfer rate constant could be as low as 200 s^{-1} as determined by photochemical excitation. At low ionic strength biphasic kinetic behavior is observed (Figure 3), the slow phase of which is probably caused by the inhibitory binding of cytochrome *c* to low activity sites on flavocytochrome b_2, as weaker electrostatic forces become more significant. At *I* 0.01 the amplitude is greatly diminished as the fast phase can no longer be resolved. Using the Debye−Hückel equation to calculate a rate constant at this ionic strength (based on Figure 2), we can estimate that for a 8 μM enzyme solution $k_{obs} = 7500$ s^{-1}. This would clearly not be observed in a stopped-flow experiment with a dead-time of 1 ms.

In order to resolve the confusion regarding cytochrome *c* binding to flavocytochrome b_2, kinetics can be used to discriminate for interactions at the catalytically active binding site. Vanderkooi et al. (1980) used iron free cytochrome *c* to inhibit the steady-state turnover of flavocytochrome b_2 and obtained a $K_i = 13$ μM. The catalytic cycle for flavocytochrome b_2 is discussed in Daff et al. (1996), and it is clear from the model presented that competitive inhibition would only occur at low cytochrome *c* concentrations. To demonstrate this, we conducted several steady-state assays in which redox-inactive zinc-substituted cytochrome *c* was used as an inhibitor. Zn−cytochrome *c* has been widely used in place of ferrocytochrome *c* to study binding interactions and electron transfer [e.g., Thomas et al. (1983a,b), McLendon et al. (1987) and Alleyne et al. (1992)] and has been shown to be structurally identical to the native protein (Anni et al., 1995). At high concentrations of ferricytochrome *c* (>100 μM) assays containing an equimolar amount of Zn−cytochrome *c* retained at least 90% of the rate observed in the absence of Zn−cytochrome *c*. Inhibition by Zn−cytochrome *c* was also followed in the pre-steady-state to ensure that the inhibition constants were as close as possible to dissociation constants. Figure 4 panels a and b plot observed rate constants against ferrocytochrome *c* and Zn−cytochrome *c* concentrations, respectively, for the pre-steady-state reduction of ferricytochrome *c*. In both cases the K_i was found to be around 8 μM based on the use of the equation in Chart 1. The fact that this equation fits the data suggests that the stoichiometry is one cytochrome *c* per flavocytochrome b_2 subunit, for the main catalytically active binding site [in agreement with Vanderkooi et al. (1980)]. However, the need to introduce a residual second-order rate constant indicates that this is not the sole site for b_2-heme to cytochrome *c* electron transfer, and that cytochrome *c* reduction occurs even when the main binding site is occupied. The equation in Chart 1 does not, however, take account of the number or nature of these alternative reaction sites and so is flawed in this respect. Nevertheless, if these reaction sites are numerous and binding to them is weak, the equation can be applied over a limited range. Pre-steady-state cytochrome *c* reduction is a single step reaction, which is dependent entirely on cytochrome *c* binding. Therefore, the K_i determined is likely to be equivalent to the actual dissociation constant for ferrocytochrome *c*. Since the protein−protein interaction is expected to be similar in the ferricytochrome *c*−flavocytochrome b_2 complex, as a first approximation the K_d for the catalytically active complex would be expected to be the same. In view of this, it seems unlikely that model (i), Scheme 1 could represent the pre-steady-state reaction. As explained in Results, this model requires that $K_d \gg 10$ μM. Model (ii), Scheme 1 can therefore be used to explain some of the effects already described. The rate constant for electron transfer has already been shown to be >1000 s^{-1}, which is consistent with this model. The rate constant for cytochrome *c* association would be equal to the second-order rate constant for cytochrome *c* reduction (i.e., 34.8 μM^{-1} s^{-1}). Finally, the dissociation rate must be less than the electron-transfer rate. By using the association rate constant and the inhibition constant, the dissociation rate constant can be estimated to be 280 s^{-1}. Although this value is significant when compared to the rate of enzyme turnover (207 s^{-1}) (Miles et al., 1992), the inhibition constant on which it is based is derived from partial inhibition. So although cytochrome *c* dissociation may be slow, the presence of alternative electron transfer sites means that at high concentration the turnover rate remains unaffected, whereas at low cytochrome *c* concentrations the binding affinity for cytochrome *c* remains high.

The possibility that inhibition by ferro-/Zn−cytochrome *c* in the steady-state may not be entirely competitive in nature was examined by monitoring flavocytochrome b_2 reduction by L-lactate in the presence of Zn−cytochrome *c*. Since b_2-heme reduction requires that both FMN reduction and FMN to b_2-heme intramolecular electron transfer take place, this was considered an appropriate experiment. Particular interest lies in the intramolecular electron transfer step which presumably depends on the relative orientation and position of the two domains. The traces displayed in Figure 5 overlay almost exactly suggesting either that the presence of bound Zn−cytochrome *c* has little affect on the enzyme's other catalytic processes or that the oxidized enzyme has a substantially lower Zn−cytochrome *c* binding affinity than the reduced form. Our current research is directed toward defining the precise location for cytochrome *c* reduction on flavocytochrome b_2.

ACKNOWLEDGMENT

We thank Prof. F. S. Mathews and Drs. F. Lederer, M. Tegoni, and C. Cambillau for helpful discussions.

REFERENCES

Alleyne, T. A., Wilson, M. T., Antonini, G., Malatesta, F., Vallone, B., Sarti, P., & Brunori, M. (1992) *Biochem. J. 287*, 951−956.
Anni, H., Vanderkooi, J. M., & Mayne, L. (1995) *Biochemistry 34*, 5744−5753.
Appleby, C. A., & Morton, R. K. (1954) *Nature 173*, 749−752.
Balme, A., Brunt, C. E., Pallister, R. L., Chapman, S. K., & Reid, G. A. (1995) *Biochem. J. 309*, 601−605.
Black, M. T., White, S. A., Reid, G. A., & Chapman, S. K. (1989) *Biochem. J. 258*, 255−259.
Brunt, C. E., Cox, M. C., Thurgood, A. G. P., Moore, G. R., Reid, G. A., & Chapman, S. K. (1992) *Biochem. J. 283*, 87−90.
Capeillère-Blandin, C. (1982) *Eur. J. Biochem. 128*, 533−542.
Capeillère-Blandin, C., & Albani, J. (1987) *Biochem. J. 245*, 159−165.
Capeillère-Blandin, C., Bray, R. C., Iwatsubo, M., & Labeyrie, F. (1975) *Eur. J. Biochem. 56*, 91−101.
Chapman, S. K., & Mount, A. R. (1995) *Nat. Prod. Rep. 12*, 93−100.
Chapman, S. K, Reid, G. A., Daff, S., Sharp, R. E., White, P. W., Manson, F. D. C., & Lederer, F. (1994) *Biochem. Soc. Trans. 22*, 713−718.
Daff, S., Ingledew, W. J., Reid, G. A., & Chapman, S. K. (1996) *Biochemistry* 35, 6345−6350.

Daum, G., Böhni, P. C., & Schatz, G. (1982) *J. Biol. Chem. 275*, 13028−13033.
Forestier, J-P., & Baudras, A. (1971) in *Flavins & Flavoproteins* (Kamin, H., Ed.) pp 599−605, University Park Press, Baltimore, MD.
Hazzard, J. T., McDonough, C. A., & Tollin, G. (1994) *Biochemistry 33*, 13445−13454.
Iwatsubo, M., Mével-Ninio, M., & Labeyrie, F. (1977) *Biochemistry 16*, 3558−3566.
Jacq, C., & Lederer, F. (1974) *Eur. J. Biochem. 41*, 311−320.
Koppenol, W. H. (1980) *Biophys. J. 29*, 493−508.
Koppenol, W. H., Vroonland, C. A. J., & Braams, R. (1978) *Biochim. Biophys. Acta 503*, 499−508.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Loach, P. A. (1976) in *Handbook of Biochemistry & Molecular Biology (Physical & Chemical Data Vol. 1)* (Fasman, G. D., ed.), 3rd ed., pp 122−130, CRC Press Inc., Cleveland, OH.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265−322.
McLendon, G., & Hake, R. (1992) *Chem. Rev. 92*, 481−490.
McLendon, G., Pardue, K., & Bak, P. (1987) *J. Am. Chem. Soc. 109*, 7540−7541.
Miles, C. S., Rouviere, N., Lederer, F., Mathews, F. S., Reid, G. A., Black, M. T., & Chapman, S. K. (1992) *Biochem. J. 285*, 187−192.
Moser, C. C., Page, C. C., Farid, R., & Dutton, P. L. (1996) *J. Bioenerg. Biomembr*. (in press).
Pajot, P., & Groudinsky, O. (1970) *Eur. J. Biochem. 12*, 158−164.
Perlmutter-Hayman, B. (1973) in *Progress in Reaction Kinetics* (Jennings, K. R., & Cundall, R. B., Ed.) Vol. 6, pp 239−267, Pergamon Press Oxford.
Pompon, D., Iwatsubo, M., & Lederer, F. (1980) *Eur. J. Biochem. 104*, 479−488.
Robinson, R. A., & Stokes, R. H. (1959) *Electrolyte Solutions*, 2nd ed., pp 230−231, Butterworth & Co. Ltd., London.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratoy Press, Cold Spring Harbor, NY.
Sharp, R. E., White, P. W., Chapman, S. K., & Reid, G. A. (1994) *Biochemistry 33*, 5115−5120.
Tegoni, M., White, S. A., Roussel, A., Mathews, F. S., & Cambillau, C. (1993) *Proteins 16*, 408−422, and references therein.
Thomas, M. A., Favoudon, V., & Pochon, F. (1983a) *Eur. J. Biochem. 135*, 569−576.
Thomas, M. A., Gervais, M., Favaudon, V., & Valat, P. (1983b) *Eur. J. Biochem. 135*, 577−581.
Vanderkooi, J. M., & Erecinska, M. (1975) *Eur. J. Biochem. 60*, 199−207.
Vanderkooi, J. M., Adar, F., & Erecinska, M. (1976) *Eur. J. Biochem. 64*, 381−387.
Vanderkooi, J. M., Glatz, P., Casadei, J., & Woodrow, G. V. (1980) *Eur. J. Biochem. 110*, 189−196.
Walker, M. C., & Tollin, G. (1991) *Biochemistry 30*, 5546−5555.
Watkins, J. A., Cusanovich, M. A., Meyer, T. E., & Tollin, G. (1994) *Protein Sci. 3*, 2104−2114.
White, P., Manson, F. D. C., Brunt, C. E., Chapman, S. K., & Reid, G. A. (1993) *Biochem. J, 291*, 89−94
Witt, H., Zickermann, V., & Ludwig, B. (1995) *Biochim. Biophys. Acta 1230*, 74−76.
Xia, Z.-X., & Mathews, F. S. (1990*) J. Mol. Biol. 212*, 837−863.

BI9522561