Mutation of the Heme-Binding Crevice of Flavocytochrome b_2 from *Saccharomyces cerevisiae*: Altered Heme Potential and Absence of Redox Cooperativity between Heme and FMN Centers†

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Received June 23, 1992; Revised Manuscript Received September 10, 1992

ABSTRACT: Kinetic and thermodynamic properties of yeast flavocytochrome b_2 (EC 1.1.2.3) are modified by the product pyruvate, which binds to the flavosemiquinone (FSQ) form of the prosthetic flavin and decreases the thermodynamic driving force for electron transfer from FSQ to heme. Pyruvate inhibits flavocytochrome b_2, but the catalytic competence of pyruvate-ligated FSQ in intramolecular electron transfer to heme is unclear; one kinetic study suggested pyruvate prevented this reaction [Tegoni, M, Janot J.-M., & Labeyrie, F. (1990) *Eur. J. Biochem. 190*, 329–342], while laser flash photolysis indicated pyruvate was essential [Walker, M. C., & Tollin, G. (1991) *Biochemistry 30*, 5546–5555]. To address this problem, wild-type (WT) and mutant (L36I) flavocytochromes b_2 have been expressed in *Escherichia coli*. Both forms incorporated heme and FMN prosthetic groups and were catalytically active. The mutation L36I was a conservative substitution within the heme-binding crevice and was designed to alter the midpoint potential (E_m) of the heme to alter the pyruvate-FSQ/heme equilibrium. Potentiometric titrations yielded E_m values (pH 7.0, 25 °C) of +8 and −28 mV for WT and L36I forms, respectively. The FMN midpoint potentials in the absence of pyruvate (−58 mV, $n = 2$) were identical within experimental error in WT and L36I species and were also identical (+5 mV, $n = 1$) in the presence of pyruvate. These results indicated the absence of redox cooperativity between FMN and heme. Turnover numbers with electron acceptors ferricyanide (311 and 304 s^{-1}, WT and L36I) and cytochrome *c* (211 and 192 s^{-1}, WT and L36I) were similar, indicating lack of rate limitation by FSQ to heme electron transfer. Pyruvate inhibition with cytochrome *c* as acceptor was competitive in WT and L36I forms and was inconsistent with inhibition by pyruvate ligation of FSQ.

Yeast flavocytochrome b_2 (L-lactate dehydrogenase, EC 1.1.2.3) catalyzes the oxidation of L-lactate to pyruvate, with the transfer of reducing equivalents to two cytochrome *c* molecules (Appleby & Morton, 1954). The protein is tetrameric with each identical subunit (57.5 kDa) containing one FMN[1] and one heme molecule (Pajot & Groundinsky, 1970; Appleby & Morton, 1954). Initially L-lactate reduces FMN, and reducing equivalents then pass sequentially to the b_5-type cytochrome which is the electron donor for cytochrome *c* reduction (Labeyrie et al., 1978; Capeillere-Blandin et al., 1980). The structure of the enzyme has been determined to a resolution of 2.4 Å (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990). Each subunit is comprised of distinct FMN-binding and cytochrome b_2 domains, and electron transfer occurs across the interface between them. In one subunit of the crystallographic unit cell, the heme and FMN rings are approximately coplanar, and the distance between the N5 atom of FMN and the nearest member of the heme porphyrin ring (C2A) is approximately 9.7 Å, apparently well situated for electron transfer. However, in the second subunit of the crystallographic unit cell, the cytochrome domain is not visible and a molecule of pyruvate is bound adjacent to the FMN, which is present as flavosemiquinone (FSQ).[1] The cytochrome domain is presumably not seen because it can adopt a number of alternative configurations within the crystal when pyruvate is bound. Recent NMR studies (Labeyrie et al., 1988) have suggested that the cytochrome domain is more mobile than the tetrameric core of FMN-binding domains.

The role of pyruvate in the catalytic cycle of flavocytochrome b_2 has been the focus of recent studies. Pyruvate binding selectively stabilizes the FSQ intermediate of FMN (Tegoni et al., 1986). This may lower the thermodynamic driving force for electron transfer from FSQ to cytochrome b_2 to close to 0 mV. Tegoni et al. (1990) analyzed inhibition of flavocytochrome b_2 from *Hansenula anomala* by pyruvate and concluded that pyruvate binding either severely decreased, or prevented, electron transfer between the FSQ and heme. Essentially the opposite conclusion was reached by Walker and Tollin (1991) using laser flash photolysis and the enzyme from *Saccharomyces cerevisiae*. No intramolecular electron transfer from FSQ to heme was observed unless pyruvate was present. Similar preliminary results were reported for the enzyme from *H. anomala*.

The small difference between the midpoint potentials for pyruvate-ligated FSQ and cytochrome b_2 suggested that the equilibrium constant for this intramolecular electron transfer should be sensitive to small changes in the heme midpoint potential. Consideration of the internal kinetics of flavocytochrome b_2 suggested that a heme E_m change should be reflected in the pyruvate inhibition kinetics of an altered

† Supported by Grant RO1 GM43911 from the National Institutes of Health (to C.J.K.).

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[1] Abbreviations: E_1, first one-electron midpoint potential of the FMN cofactor; E_2, second one-electron midpoint potential of the FMN cofactor; E_H, midpoint potential of the heme prosthetic group; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide (oxidized form); $FMNH_2$, two-electron reduced form of FMN; FSQ, one-electron reduced form of FMN; H_{ox} and H_{red}, oxidized and reduced heme, respectively; IPTG, isopropyl β-D-thiogalactopyranoside; L:CR, L-lactate:ferricytochrome *c* reductase activity; L:FC, L-lactate:ferricyanide reductase activity; PMSF, phenylmethanesulfonyl fluoride; WT, wild-type enzyme.

0006-2960/92/0431-11376$03.00/0

enzyme if pyruvate binding to FSQ was inhibitory. In addition, selective modification of the heme E_m would permit cooperative redox interactions between flavin and heme to be detected. Redox cooperativity has been suggested to explain the final electron distribution between heme and FSQ observed during laser flash photolysis studies, which was different to the distribution predicted from the known FMN and heme gross midpoint potentials (Walker & Tollin, 1991).

We have identified a conservative site-specific substitution among the residues that line the heme crevice which produces a 36-mV decrease in the heme E_m. The change is sufficiently large that electron transfer from FSQ to heme should become thermodynamically unfavorable. We examine the possibility of cooperativity between redox centers, and the steady-state kinetics of pyruvate inhibition of WT and mutant flavocytochrome b_2.

EXPERIMENTAL PROCEDURES

Enzyme Expression. The gene encoding *S. cerevisiae* flavocytochrome b_2 was isolated as a 1.9-kb *Spe*I fragment from plasmid YEp13-B2 (Guiard, 1985). The 5′ *Spe*I site was located in the presequence of the flavocytochrome b_2 precursor, 41 bp upstream of a fortuitous Shine–Dalgarno-like sequence (5′ACGAGC3′) and an initiation Met codon 11 bp downstream, which together direct expression of the mature protein in *Escherichia coli* (Black et al., 1989). The enzyme produced in *E. coli* lacks the first 5 N-terminal residues of the enzyme isolated from *S. cerevisiae*. The *Spe*I fragment was cloned into the *Sma*I site of expression plasmid pDS56 (Bujard et al., 1987), resulting in flavocytochrome b_2 expression plasmid pb2. Plasmid pb2 was transformed into several *E. coli* strains (HB101, SG13009, M15, DH5, TB1) and tested for flavocytochrome b_2 expression with and without *lac*-repressor-encoding plasmid pREP4 (Stuber et al., 1984). Optimum expression, determined by L-lactate:ferricyanide reductase activity (L:FC, described below) of crude cell lysates, was obtained in *E. coli* strain HB101 in the absence of plasmid pREP4, grown in Super medium containing 200 μg/mL ampicillin and 0.1 mM δ-aminolevulinic acid. Super medium contained (per liter) 25 g of bacto-tryptone, 15 g of bacto-yeast extract, and 5 g of NaCl. Cells were grown to a density (A_{600}) of 0.8 at 37 °C, induced with 1 mM IPTG, and grown for a further 8 h before harvesting.

Enzyme Purification. The purification scheme was similar to that of Black et al. (1989), and all steps were performed at 4 °C. Frozen pelleted cells were thawed and resuspended in 3 mL/g of cells, 10 mM Tris-Cl, 95 mM NaCl, and 10 mM EDTA, pH 7.5. Immediately before resuspension, 1 mM PMSF was added to the buffer. Lysozyme was added to a final concentration of 0.2 mg/mL, and after gentle stirring for 20 min, 4 mg of sodium deoxycholate was added per gram of *E. coli*. After 10 min, the solution was centrifuged at 39000*g* for 10 min to remove cell debris. Ammonium sulfate was added to 30% saturation with pH adjustment as necessary. The supernatant was collected following centrifugation at 39000*g* for 20 min. Ammonium sulfate was added to 70% saturation, and flavocytochrome b_2 was pelleted by centrifugation as above.

The pellet was resuspended in the minimum volume of 0.1 M sodium phosphate, 1 mM EDTA, and 1 mM D,L-lactate, pH 7.0, and dialyzed overnight against similar, Ar-purged, buffer. The sample was loaded onto a hydroxyapatite (Bio-Rad, Richmond, CA) column (30 × 1.5 cm) equilibrated with the same buffer and developed with a linear 500-mL ammonium sulfate gradient to a final concentration of 86 g/L. Fractions exhibiting an A_{269}/A_{423} ratio of less than 1 were pooled and precipitated with 70% saturated ammonium sulfate. Following dialysis, the sample was chromatographed over derivatized Sepharose, prepared, and used as described by Labeyrie et al. (1978). Enzyme samples containing L-lactate were frozen dropwise and stored in liquid N_2 until use. Immediately prior to use, enzyme samples were passed over a 25 × 1-cm G-25 column to remove lactate. Flavocytochrome b_2 concentration was determined using the Soret band of dithionite-reduced samples (ϵ_{423nm} = 183 mM^{-1} cm^{-1}; Pajot & Groudinsky, 1970).

Site-Specific Mutagenesis. A 0.3-kb *Bam*HI DNA fragment, isolated from a reverse-oriented clone otherwise similar to pb2, encoding residues 6–86 of the wild-type cytochrome b_2 domain, was subcloned into the *Bam*HI site of M13mp18 (Boehringer-Mannheim). Site-specific mutants were prepared by a gapped-duplex protocol (Boehringer-Mannheim) using the following primer, which was prepared using a Applied Biosystems Cyclone DNA synthesizer:

L36I: 5′TCGCGTTATGTCGTATA3′

Mutants were identified by single-stranded DNA sequencing and were subcloned back into pb2 as *Eco*RI/*Cla*I fragments. The entire heme-encoding regions of the final pb2-mutant plasmid was sequenced in both directions using custom primers (Operon Technologies, Alameda, CA) to ensure the absence of secondary mutations.

Potentiometric Measurements. Heme midpoint potentials were determined by potentiometric titrations in the presence of the following dye mediators to ensure equilibration (1.5 μM each): resorufin (–51 mV), methylene blue (+11 mV), 2,5-dihydroxybenzoquinone (–60 mV), thionin (+56 mV), dichlorophenolindophenol (+217 mV), indigotetrasulfonic acid (–46 mV), 2-hydroxy-1,4-naphthoquinone (–152 mV), indigocarmine (–125 mV), and anthroquinone-2-sulfonate (–225 mV). Electrochemical potentials were measured using a gold wire electrode and Ag/AgCl reference electrode (Microelectrodes Inc., Londonderry, NH). The reference electrode was calibrated relative to a saturated quinhydrone solution (Clark, 1960) after each experiment. Titrations were performed in a total volume of 1 mL in a stirred and thermostated 3-mL cuvette which was maintained anaerobic under a positive pressure of wetted Ar gas. Reduced methyl viologen (30 mM) was used as the reductant and was prepared by hydrogenation using a Pt–asbestos catalyst. The oxidant used was potassium ferricyanide (10 mM). Heme reduction was monitored at 423 and 555 nm, with 416 and 545 nm as the respective isosbestic points.

Flavin potentiometric titrations (19–25 μM flavocytochrome b_2 subunits) were performed using a similar procedure and the same dye mediators as for heme titrations. Flavin reduction at each potential was calculated from absorbance readings at 450, 545, and 557 nm essentially as described by Walker and Tollin (1991). The absorbance change at 450 nm due to FMN reduction was determined by correcting for the absorbance change due to heme reduction. Heme reduction was determined from the absorbance at 557 nm relative to the heme isosbestic point at 545 nm. The contribution of heme reduction to the absorbance change at 450 nm was assumed to be 48% (Capeillere-Blandin et al., 1975; Tegoni et al., 1986). Due to a slight increase in turbidity during titration, spectra were normalized at 545 nm (Tegoni et al., 1986).

Steady-State Kinetic Measurements. Initial-rate determinations were made at 25 °C using a Beckmann DU7500 diode-array spectrophotometer. The assay solution (2 mL)

Table I: Thermodynamic and Kinetic Properties of WT and L36I Flavocytochrome b_2[a]

	wild type	L36I
$E_H(H_{ox}/H_{red})$(mV)	+8 ± 6	−28 ± 7
$E_{(FMN/FMNH_2)}$ (mV)[b] − pyruvate	−58 ± 9	−55 ± 10
$E_{(FMN/FSQ)}$ (mV)[c] + pyruvate	+5 ± 7	+1 ± 8
k_{cat} (L:CR) (s^{-1})[d]	211 ± 22 (3)	194 ● 17 (3)
k_{cat} (L:FC) (s^{-1})[e]	311 ± 30 (3)	304 ± 35 (2)
K_m L-lactate (L:FC) (mM)	0.46 ± 0.03 (3)	0.43 ± 0.06 (3)
K_m L-lactate (L:CR) (mM)	0.14 ± 0.03 (2)	0.20 ± 0.04 (2)
K_i pyruvate (L:CR) (mM)[f]	3.9	5.1

[a] All determinations were made at 25 °C. [b] Values were derived from data presented in Figure 3 for the two-electron reduction of FMN. [b] Values were derived from data presented in Figure 3 for the two-electron reduction of FMN. [c] Values were derived from data presented in Figure 3 for the one-electron reduction of FMN (E_1). [d] Steady-state turnover numbers are expressed as mol of cytochrome *c* reduced per mole of flavocytochrome b_2 monomer. [e] Steady-state turnover numbers are expressed as mol of ferricyanide reduced per mole of flavocytochrome b_2 monomer. [f] Experimental uncertainties are not quoted, due to the nonlinearity of the corresponding inhibition plots (see text).

contained sodium potassium phosphate (100 mM) and 1 mM EDTA, together with L-lactate (Sigma), pyruvate (Sigma), horse heart cytochrome *c* (type VI, Sigma), and ferricyanide (1 mM) as appropriate. L-Lactate and pyruvate stock solutions were 190 and 240 mM, respectively, in order to match the ionic strength of the assay medium. Ferricyanide reduction was monitored at 420 nm (ϵ_{420nm} = 1.04 mM^{-1} cm^{-1}), and reduction of ferricytochrome *c* was monitored at 550 nm ($\Delta\epsilon_{550nm}$ = 19.1 mM^{-1} cm^{-1}). Reactions were started by addition of an appropriate aliquot of diluted enzyme using a Hamilton syringe. Initial rates were accurately determined from the initial linear portion of the recorded time courses. Assays were performed in duplicate, and mean rates are expressed as μmol of reducing equivalents/(min·nmol of flavocytochrome b_2 monomer).

RESULTS

Flavocytochrome b_2 Expression. Plasmid pb2, when transformed into *E. coli* HB101, resulted in high-level expression of wild-type and L36I flavocytochrome b_2. Plasmid pb2 contains promoter/operator N25OPSN25OP29 which is repressed by *lac* and induced by IPTG. As first reported by Black et al. (1989), a fortuitous Shine–Dalgarno sequence, located 11 bp upstream of the Met6 codon of the mature enzyme expressed in yeast, is sufficient to direct translation in *E. coli*. The bacterially-expressed enzyme therefore lacks the first 5 amino acids of the yeast enzyme, but appears kinetically identical (Black et al., 1989). Optimum host strains and growth conditions (see Experimental Procedures) resulted in expression of 12–18 mg of flavocytochrome b_2/L of cell culture for both wild-type (WT) and mutant L36I. The absorption spectra from 350 to 650 nm for wild-type and L36I forms were identical. Heme Soret peak wavelengths for oxidized and dithionite-reduced samples were identical to previously published values (Labeyrie et al., 1978), and the characteristic spectra of oxidized FMN were present, underlying the heme spectra in both WT and L36I species (Figure 2), suggesting that both heme and FMN were correctly bound to both enzyme forms expressed in *E. coli*. Optimum L-lactate: ferricyanide reductase activities (L:FC)[1] were 311 and 304 s^{-1}, and optimum L-lactate:cytochrome *c* reductase activities (L:CR)[1] were 211 and 192 s^{-1}, for WT and L36I, respectively (Table I). The WT values (determined at 25 °C) were close to previously published values (Labeyrie et al., 1978) and were consistent with full incorporation of FMN and heme into the apoenzymes.

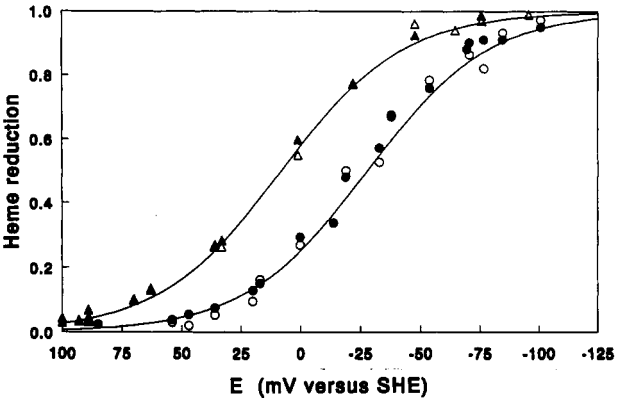

FIGURE 1: Potentiometric titration of WT and L36I flavocytochrome b_2 heme. Potentiometric titrations of 2 μM WT (triangles) and L36I (circles) flavocytochrome b_2 subunits were performed in the presence of dye mediators in 0.1 M sodium potassium phosphate and 0.1 mM EDTA, pH 7.0. Heme reduction was monitored using the wavelength pairs 423–416 nm (closed symbols) and 555–545 nm (open symbols). The data points were collected in both oxidative and reductive directions. The curves represent theoretical n = 1 Nernstian processes with E_m values of +8 mV (WT) and −28 mV (L36I).

Heme Potentials. Potentiometric titrations of the heme prosthetic group were performed to measure the effect of substitution L36I on the heme E_m. Anaerobic samples of flavocytochrome b_2 (2–3 μM monomers) were titrated in the presence of dye mediators with small aliquots of reduced methyl viologen or ferricyanide. Heme reduction was measured using the wavelength pairs 423–416 nm (Soret band) and 555–545 nm (α-band) and plotted as a function of applied potential (Figure 1). The data at both wavelength pairs were consistent with an n = 1 reversible reduction with a midpoint potential of +8 mV, in close agreement with previous reports for both the cleaved (S_x) *S. cerevisiae* enzyme (Capeillere-Blandin et al., 1975) and the intact (S_I) enzyme (Walker & Tollin, 1991). Results from titration of L36I heme under identical conditions are also shown in Figure 1. The data were also consistent with a simple n = 1 reversible reduction, but the midpoint potential was −28 mV. The mutation L36I, which is a conservative substitution within the rear of the heme-binding crevice (see Discussion), therefore produced a 36-mV decrease in the heme midpoint potential.

FMN Potentials. To determine whether the mutation of the heme-binding domain L36I also altered the thermodynamic properties of the FMN cofactor, further potentiometric titrations were performed. Although the mutation L36I was located in the heme domain, cooperativity between heme and FMN redox states has been suggested (Walker & Tollin, 1991). Under certain circumstances, cooperative interactions between cofactors may become apparent in mutagenesis experiments as a shift in the E_m of one cofactor when the second E_m is perturbed (see Discussion). Visible spectra obtained during potentiometrically controlled reduction of WT and L36I flavocytochrome b_2, obtained in the presence of minimal redox mediator concentrations, are presented in Figure 2. In the absence of pyruvate, reduction of WT heme occurred at significantly higher potentials than the bleaching of FMN absorption at 450 nm. Consequently, the heme is almost completely reduced throughout the potential range where flavin bleaching is seen (Figure 2A). Spectra obtained during titration of L36I in the absence of pyruvate (Figure 2B) showed less separation of heme and FMN reductions, as expected from the lower heme potential. Similar titrations were repeated in the presence of 10 mM pyruvate. The visible spectra (Figure 2C,D) clearly demonstrate the previously

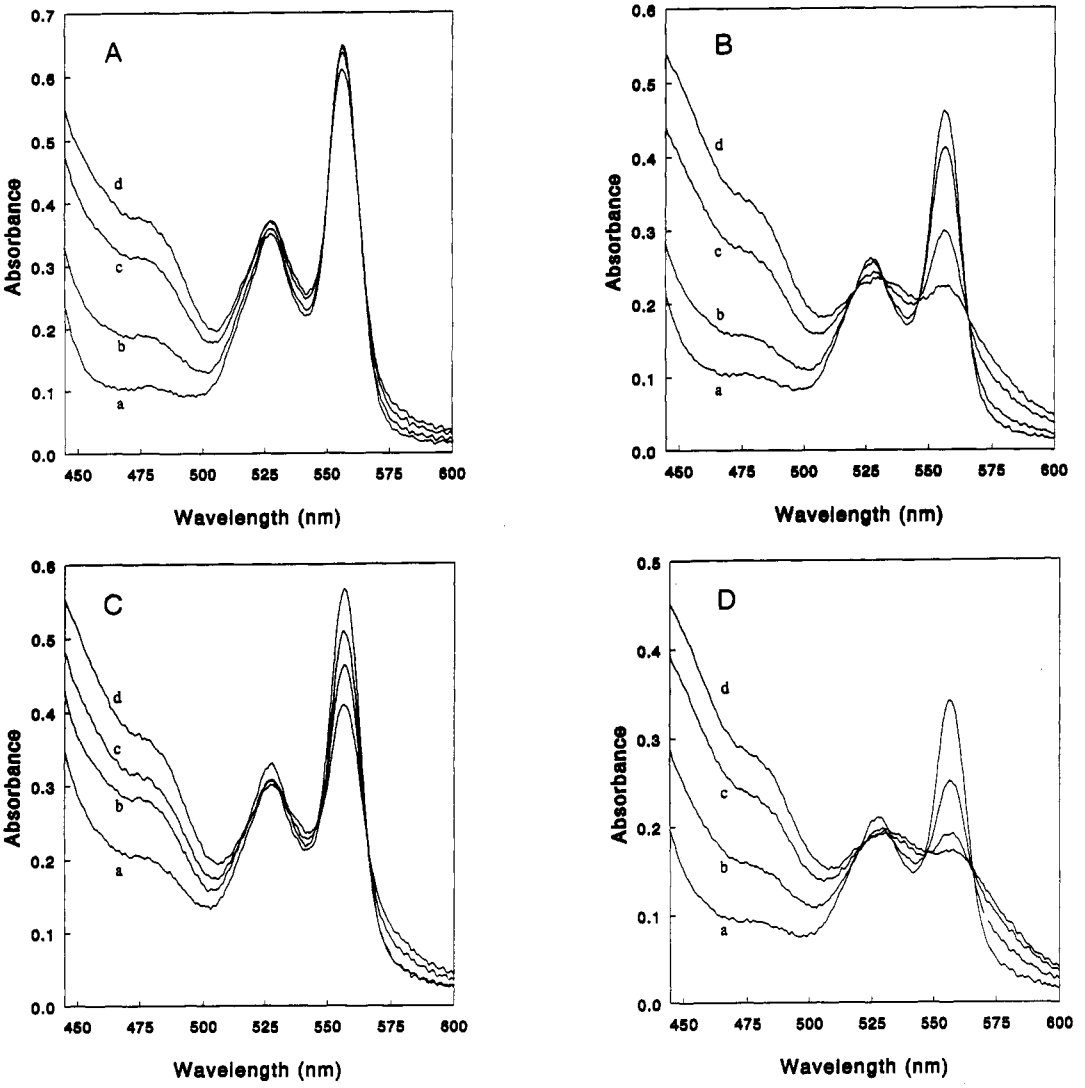

FIGURE 2: Visible potentiometric spectra of heme and FMN reduction of WT and L36I flavocytochrome b_2 in the presence and absence of pyruvate. Flavocytochrome b_2 was suspended in 0.1 M sodium potassium phosphate and 0.1 mM EDTA, pH 7.0, in the presence of minimal dye mediators under anaerobic conditions, and the potential adjusted by addition of reductant (methyl viologen) or oxidant (ferricyanide). Spectra were measured following redox equilibration at the indicated potentials. (A) WT flavocytochrome b_2 (25 μM subunits) in the absence of pyruvate: a, −94 mV; b, −66 mV; c, −47 mV; d, −32 mV. (B) L36I flavocytochrome b_2 (22 μM subunits) in the absence of pyruvate: a, −87 mV; b, −63 mV; c, −43 mV; d, −27 mV. (C) WT flavocytochrome b_2 (19 μM subunits) in the presence of 10 mM pyruvate: a, −45 mV; b, −17 mV; c, +25 mV; d, +65 mV. (D) L36I flavocytochrome b_2 (16 μM subunits) in the presence of 10 mM pyruvate: a, −52 mV; b, −15 mV; c, +21 mV; d, +52 mV.

reported stabilization of a reduced form of FMN (Tegoni et al., 1986), since in the WT heme reduction and FMN reduction occurred concurrently at potentials significantly higher than those of Figure 2A, while in L36I, FMN reduction preceded heme reduction (Figure 2D).

The absorption decrease at 450 nm attributable to FMN reduction was determined by correcting spectra for the known heme contribution (see Experimental Procedures). The extent of FMN reduction is plotted as a function of potential in Figure 3. The behavior of WT flavocytochrome b_2 FMN was consistent with a reversible reduction with a midpoint potential of −58 mV. The data were well fitted by a line with slope of 30 mV (least-squares fitting yielded 28 mV), indicating little stabilization of the one-electron-reduced FSQ. Instead, the results were consistent with two-electron reduction:

$$FMN + 2e^- \rightleftharpoons FMNH_2$$

It should be noted that the protonation state of the reduced FMN cannot be inferred from these data. Within experimental error, the results obtained for L36I were identical, also yielding an n value of close to 2 and a midpoint potential of −55 mV. The FMN redox behavior was therefore indistinguishable from that of the WT enzyme in the absence of pyruvate. Upper and lower bounds for the two one-electron reductions E_1^1 and E_2^1 can be derived from the observation that the data of Figure 3 were not consistent with accumulation of more than 10% FSQ during the titration (Elema, 1933). These bounds were therefore $E_1 < -85$ mV and $E_2 > -25$ mV.

In the presence of saturating (10 mM) pyruvate, the FMN spectral changes yielded midpoint potentials of +5 and +1 mV for WT and L36I flavocytochrome b_2, respectively. The n values in both cases were close to 1 (least-squares fitting yielded $n = 0.97$ and 1.05 for WT and L36I, respectively), consistent with selective stabilization of the FSQ intermediate by pyruvate, as previously reported (Tegoni et al., 1986). The relevant reduction was therefore

$$FMN + 1e^- \rightleftharpoons FSQ$$

The midpoint potential for the second reduction E_2 cannot be directly derived from these data, but making the approximation

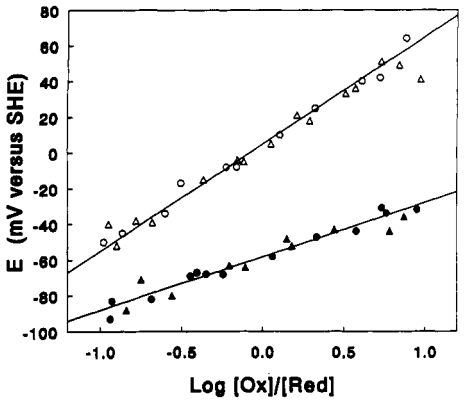

FIGURE 3: Potentiometric titration of the flavin of WT and L36I flavocytochrome b_2 in the presence and absence of pyruvate. Potentiometric titrations were performed in the presence of dye mediators as described in the legend to Figure 2 and in Experimental Procedures. Titrations were performed in the absence (closed symbols) and presence (open symbols) of 10 mM pyruvate, and using WT (circles) and L36I (triangles) flavocytochrome b_2. The degree of FMN reduction was determined as described above (Experimental Procedures) and is plotted according to the Nernst equation. The lines correspond to reversible Nernstian processes: (solid symbols) $E_m = -58$ mV, $n = 2$; (open symbols) $E_m = +5$ mV, $n = 1$.

that pyruvate binding affects only the stability of FSQ, approximate values of −110 mV can be derived for E_2 of both WT and L36I enzymes. The reported E_2 value for *H. anomala* flavocytochrome b_2 from EPR measurements was −133 mV (Tegoni et al., 1986). Walker and Tollin (1991) estimated a value for $E_2 < -55$ mV for the *S. cerevisiae* S_I enzyme, based on the n value for the FMN titration which was close to 1.

The potentiometric data for heme and FMN cofactors for the WT enzyme were similar to those reported by Walker and Tollin (1991). The WT and L36I potentiometric results are presented in Table I.

Pyruvate Inhibition. Two recent studies have reached essentially opposite conclusions regarding the kinetic consequences of pyruvate binding to flavocytochrome b_2. Pyruvate binds preferentially to the FSQ intermediate of FMN, and since the equilibrium between this species and reduced heme may depend directly on the difference in their microscopic midpoint potentials, it was of interest to see how the altered heme midpoint potential of L36I affected the kinetics of pyruvate inhibition. Initial rate determinations for L:CR activities of WT and L36I enzymes were made in the presence of fixed pyruvate concentrations (0–15 mM). The effect of pyruvate on L:FC activity was not examined due to the complexities of ferricyanide reduction, which can accept reducing equivalents at both heme and FMN centers. The data for WT and L36I enzymes were obtained under strictly identical conditions; the same stock solutions were used and the experiments were performed in parallel, and efforts were made to maintain a constant ionic strength. The patterns of pyruvate inhibition of WT and L36I enzymes were similar. In both cases the inhibition patterns were mixed competitive/noncompetitive, but the points of intersection of the double-reciprocal plots (not shown) were close to the $1/v$ axes, indicating almost competitive inhibition with only a slight effect on V_m. Indeed, at higher pyruvate concentrations (>5 mM), the lines of the double-reciprocal plots tended to curve downward near the $1/v$ axis, approaching a common intercept. Competitive inhibition of L:FC activity by pyruvate has been reported previously (Lederer, 1978; Walker & Tollin, 1991), though, in the case of the *S. cerevisiae* enzyme, inhibition of neither L:FC nor L:CR has been thoroughly investigated. In contrast, the enzyme from *H. anomala* exhibited noncompetitive kinetics for this activity (Tegoni et al., 1990). Secondary plots (not shown) yielded K_I estimates of 3.9 and 5.1 mM, for WT and L36I, respectively (Table I). These values were derived assuming that the mode of inhibition was primarily competitive.

DISCUSSION

Heme Environment. The mutation L36I is a conservative substitution (isomerization) of a residue located within the heme-binding crevice of cytochrome b_2. The position of L36 relative to the heme ring is depicted in Figure 4 and is one of 10 nonpolar residues which contact the heme (Xia & Mathews, 1990). Residue L36 is located within the six-stranded mixed β-sheet which forms the base of the heme-binding pocket. Heme atom CMC (Brookhaven nomenclature; Bernstein et al., 1977) is located 3.5 Å from one of the terminal side-chain methyl groups (CD2). This residue was selected for mutation since it has been proposed that the polarity of the nonpolar heme environment is a significant determinant of midpoint potential in cytochromes (Kassner, 1973), and because the site is relatively distant from the FMN domain and the domain interface. Other substitutions were also made, but with less effect on E_m (L36V, V70A, V70I; data not shown). Some observations regarding L36I can be made using the WT coordinates, on the tentative assumption that this mutation does not significantly perturb the structure. In the WT structure, a terminal methyl group (CD2) makes van der Waals contact with heme CMC and also the side chain of V27. The side chain of L36 is surrounded by residues C25, V27, Y34, D35, F39, and I75. In addition, there is a cavity surrounding heme atom CHC adjacent to L36 CD2. Modeling of the substitution L36I suggested that a terminal methyl group could approach heme CMC as closely as in L36 without significant strain, while still maintaining preferred χ values (McGregor et al., 1987). In contrast, the approximately methyl group-sized cavity surrounding CHC could not be occupied by I36 CD1 without either assigning unfavorable (eclipsed) χ values or making larger structural changes. According to Kassner (1973), the midpoint potentials of cytochromes are substantially more positive than those of model heme–ligand complexes primarily because of destabilization of Fe(III) relative to Fe(II) within the nonpolar interior of proteins. The prediction of this mechanism is that a decrease in the polarity of the local heme environment, or an increase in the thickness of the nonpolar layer shielding the heme, would tend to increase the heme E_m. Other factors, such as the existence of solvent channels to the heme, were also considered important (Kassner, 1973). In the present case, the E_m change of −36 mV due to the mutation is significant within the context of the redox equilibrium between heme and FSQ (below), but is in the opposite direction to that predicted by this theory. A number of scales have been proposed to quantify hydrophobicity of amino acid side chains, and depending on the methods (e.g., solvent partitioning or empirical determinations from known protein structures) used in their preparation, they rank leucine and isoleucine differently. Either isoleucine is more hydrophobic than leucine (Janin, 1979; Kyte & Doolittle, 1982; Rose et al., 1985), or the two are comparable (Woolfenden et al., 1981). An increase in E_m would therefore have been predicted for L36I, and an explanation of the E_m shift, possibly through indirect effects on the coordination of Fe by histine (Moore & Williams, 1977), will require a more detailed molecular explanation from determination of the mutant structure.

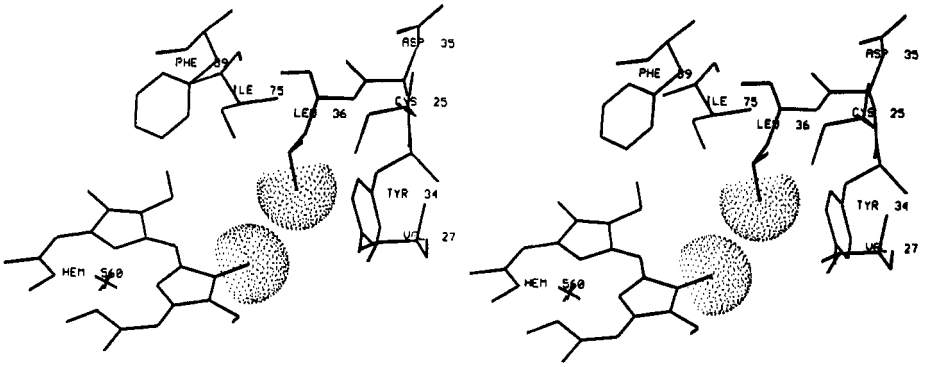

FIGURE 4: Position of L36 within the heme-binding crevice. The relative positions of heme CMC atom (Brookhaven nomenclature, Bernstein et al., 1977) and the side chain of leucine 36 are shown.

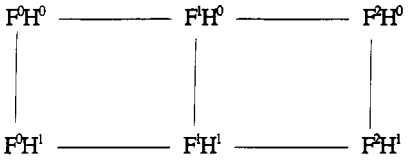

FIGURE 5: Detection of redox cooperativity between heme and FMN prosthetic groups. Each one-electron reduction possible within a monomer of flavocytochrome b_2 is shown. Superscripts denote the number of reducing equivalents relative to the fully oxidized form. F, flavin; H, heme.

Cooperative Redox Interactions. The redox titration results presented above suggest that cooperative redox interactions between the heme and FMN prosthetic groups are either small or absent within the enzyme forms populated during potentiometric titration. There remains the possibility that cooperativity could occur between metastable states found on the time scale of flash photolysis experiments. Walker and Tollin (1991) have raised this possibility as one explanation of why the amplitude of heme reduction due to intramolecular electron transfer from FSQ to heme during laser flash photolysis experiments in the presence of pyruvate was less than that predicted from heme and flavin midpoint potentials. Because the mutation L36I decreases the separation between FMN and heme E_m values in the absence of pyruvate, it is possible to evaluate redox cooperativity among the equilibrium states. A scheme allowing for redox cooperativity is shown in Figure 5. The six possible electron transfers within a subunit of flavocytochrome b_2 are shown. Redox cooperativity would imply that heme and flavin E_m values were coupled (i.e., the three vertical redox transitions of Figure 7 were unequal). For each of the three cycles present in Figure 5, the microscopic E_m values must sum to zero. The difference between the two heme microscopic E_m values in each cycle will therefore be reflected in compensating changes in flavin potentials. If the heme and FMN potentials are well separated, cooperativity will not be apparent even if possible, since only three of the six species of Figure 5 will be substantially populated during the titration. Between the two conditions (with and without pyruvate), and including data for WT and L36I, all species would be expected to be significantly populated during potentiometric titration. The failure of the altered heme E_m of L36I to change flavin potentials in the absence or presence of pyruvate therefore suggests that cooperativity is weak or absent.

Kinetic Effects of Mutation. The equilibrium distribution of reducing equivalents between pyruvate-ligated FSQ and heme flavocytochrome b_2 may be significantly different in L36I than WT. In the absence of cooperativity (so that microscopic and macroscopic E_m values are equal), the relevant E_m values translate into equilibrium constants of 1.12 and 0.28 for WT and L36I, respectively. That the mutation had no effect on optimal L:FC and L:CR activites (Table I) indicates a lack of rate limitation at this intramolecular electron-transfer step, consistent with earlier studies assigning the rate-limiting step to abstraction of the α-proton of L-lactate (Lederer, 1974). The rate constant for heme reduction by FSQ has been measured as 520 s^{-1} by laser flash photolysis (0.1 M phosphate; Walker & Tollin, 1991) in the presence of saturating (5 mM) pyruvate. Studies of the effect of thermodynamic driving force on rate constants for electron transfer from reduced flavins to cytochromes (Meyer et al., 1983) suggest that, at driving forces <100 mV, $\partial \log k/\partial \Delta E_m \approx 8.2 \times 10^{-3}$ mV^{-1}. This predicts a value of 260 s^{-1} for the corresponding intramolecular electron transfer in L36I. Measurement of this rate constant is currently underway.

Pyruvate Inhibition. On the basis of the analysis of Tegoni et al. (1990), we predicted that changing the equilibrium between pyruvate-ligated FSQ and heme would result in enhanced inhibition of L:CR activity by pyruvate. The reasoning for this prediction was that the lower E_m of L36I compared to WT would result in an increase in FSQ. This should occur under both equilibrium and nonequilibrium conditions and result in a lowered K_I value. This did not occur, since the differences between pyruvate inhibition patterns of WT and L36I were within experimental error. The patterns of inhibition were not simple, since double-reciprocal plots obtained at high pyruvate concentrations were not linear. To a first approximation, inhibition was competitive, which was in contrast to pyruvate inhibition of L:CR of flavocytochrome b_2 from *H. anomala*, which was noncompetitive (Tegoni et al., 1990). The noncompetitive kinetics were explained as a mixture of competitive inhibition by pyruvate binding to oxidized FMN and uncompetitive inhibition due to formation of a dead-end complex between pyruvate and FSQ (Tegoni et al., 1990). The decreased ability of the dead-end complex to transfer reducing equivalents to heme in the presence of pyruvate was supported by previous temperature-jump measurements (Tegoni et al., 1984), but was in contrast to the findings of Walker and Tollin (1991) where intramolecular electron transfer occurred only in the presence of pyruvate. In the present case, inhibition of *S. cerevisiae* L:CR activity by pyruvate can, to a first approximation, be explained most simply as competitive inhibition through binding to oxidized FMN. A full description of inhibition is likely to be complex due to the large number of enzyme species involved (Tegoni et al., 1990). To reconcile the failure of L36I to exhibit different inhibition kinetics to WT enzyme with the observations of Walker and Tollin (1991), one can speculate that pyruvate does not inhibit by binding FSQ in the case of the *S. cerevisiae* enzyme. If pyruvate is

required for intramolecular electron transfer from FSQ to heme, the catalytic competence of the enzyme in the absence of added pyruvate suggests that the pyruvate formed at the active site does not dissociate until after FSQ is oxidized by heme, though more study would be necessary to determine this.

ACKNOWLEDGMENT

We thank Dr. F. S. Mathews for kindly providing the coordinates of flavocytochrome b_2 prior to their deposition in the Brookhaven Protein Data Bank, Dr. B. Guiard (CNRS, Gif-sur-Yvette, France) for the gene encoding flavocytochrome b_2 from *S. cerevisiae*, and Dr. D. Stuber (Hoffmann-La Roche, Basel, Switzerland) for providing the bacterial expression vector pDS56. Molecular modeling was performed at the San Diego Supercomputer Center, Advanced Scientific Visualization Laboratory.

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Registry No. FMN, 146-17-8; flavocytochrome b_2, 9078-32-4; heme, 14875-96-8; ferricyanide, 13408-62-3; cytochrome *c*, 9007-43-6; pyruvate, 127-17-3; L-lactic acid, 79-33-4; leucine, 61-90-5; isoleucine, 73-32-5.