Temperature-Jump and Potentiometric Studies on Recombinant Wild Type and Y143F and Y254F Mutants of *Saccharomyces cerevisiae* Flavocytochrome b_2: Role of the Driving Force in Intramolecular Electron Transfer Kinetics[†,‡]

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ABSTRACT: The kinetics of intramolecular electron transfer between flavin and heme in *Saccharomyces cerevisiae* flavocytochrome b_2 were investigated by performing potentiometric titrations and temperature-jump experiments on the recombinant wild type and Y143F and Y254F mutants. The midpoint potential of heme was determined by monitoring redox titrations spectrophotometrically, and that of semiquinone flavin/reduced flavin (Fsq/Fred) and oxidized flavin (Fox)/Fsq couples by electron paramagnetic resonance experiments at room temperature. The effects of pyruvate on the kinetic and thermodynamic parameters were also investigated. At room temperature, pH 7.0 and I = 0.1 M, the redox potential of the Fsq/Fred, Fox/Fsq, and oxidized heme/reduced heme (Hox/Hred) couples were −135, −45, and −3 mV, respectively, in the wild-type form. Although neither the mutations nor excess pyruvate did appreciably modify the potential of the heme or that of the Fsq/Fred couple, they led to variable positive shifts in the potential of the Fox/Fsq couple, thus modulating the driving force that characterizes the reduction of heme by the semiquinone in the −42 to +88 mV range. The relaxation rates measured at 16 °C in temperature-jump experiments were independent of the protein concentrations, with absorbance changes corresponding to the reduction of the heme. Two relaxation processes were clearly resolved in wild-type flavocytochrome b_2 ($1/\tau_1 = 1500\ s^{-1}$, $1/\tau_2 = 200 \pm 50\ s^{-1}$) and were assigned to the reactions whereby the heme is reduced by Fred and Fsq, respectively. The rate of the latter reaction was determined in the whole series of proteins. Its variation as a function of the driving force is well described by the expression obtained from electron-transfer theories, which provides evidence that the intramolecular electron transfer is not controlled by the dynamics of the protein.

In aerobic yeast, L-lactate can transfer electrons to cytochrome *c* via the catalytic action of flavocytochrome b_2 (EC 1.1.2.3) (*1*, *2*). Purified, this enzyme was found to be a tetramer consisting of four identical subunits, each of which had a mass of 58 kDa, and carried a flavin mononucleotide (FMN)[1] and a *b*-type heme. The FMN and the b_2 heme each catalyze distinct reactions, namely, the dehydrogenation of L-lactate and the reduction of cytochrome *c*, respectively. The link between these two half-reactions is the intramolecular electron transfer from the FMN to the b_2 heme. This functional separation is clearly visible in the three-dimensional structure (*3*, *4*): a $\beta_8\alpha_8$ flavin domain, homologous to that present in spinach glycolate oxidase (*5*), and a cytochrome b_2 domain homologous to that observed in cytochrome b_5 (*3*, *6*, *7*), are connected to each other by a hinge. Presumably to optimize the interaction between the flavoprotein and the cytochrome, the two genes have become fused in the course of evolution and now code for a single polypeptide chain (*7*, *8*). The hinge region is known to play a crucial role in intramolecular electron transfer (*9*−*11*). The position of the prosthetic groups in the protein appears to favor an efficient electron transfer: the propionates of the b_2 heme extrude from the heme pocket toward the FMN, and the two prosthetic groups are approximately coplanar and lie 9.7 Å apart edge-to-edge (Figure 1A) (*3*). Analysis of the flavin site structure (Figure 1B) suggests a possible mechanism for the oxidation of L-lactate and the intramolecular electron transfer. In particular, a putative role in the catalysis of some of the residues at the active site has been hypothesized (*12*, *13*).

Five main steps are required for the two electrons of L-lactate to reach cytochrome *c*: the oxidation of L-lactate into pyruvate, along with the reduction of the FMN to flavin hydroquinone (Fred); the electron transfer from Fred to the b_2 heme, accompanied by the formation of reduced heme

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[1] Abbreviations: FMN, flavin mononucleotide; Fox, oxidized flavin; Fsq, semiquinone flavin; Fred, reduced flavin; Hox, oxidized heme; Hred, reduced heme; Y143F, mutant Tyr143→Phe; Y254F, mutant Tyr254→Phe; EPR, electron paramagnetic resonance.

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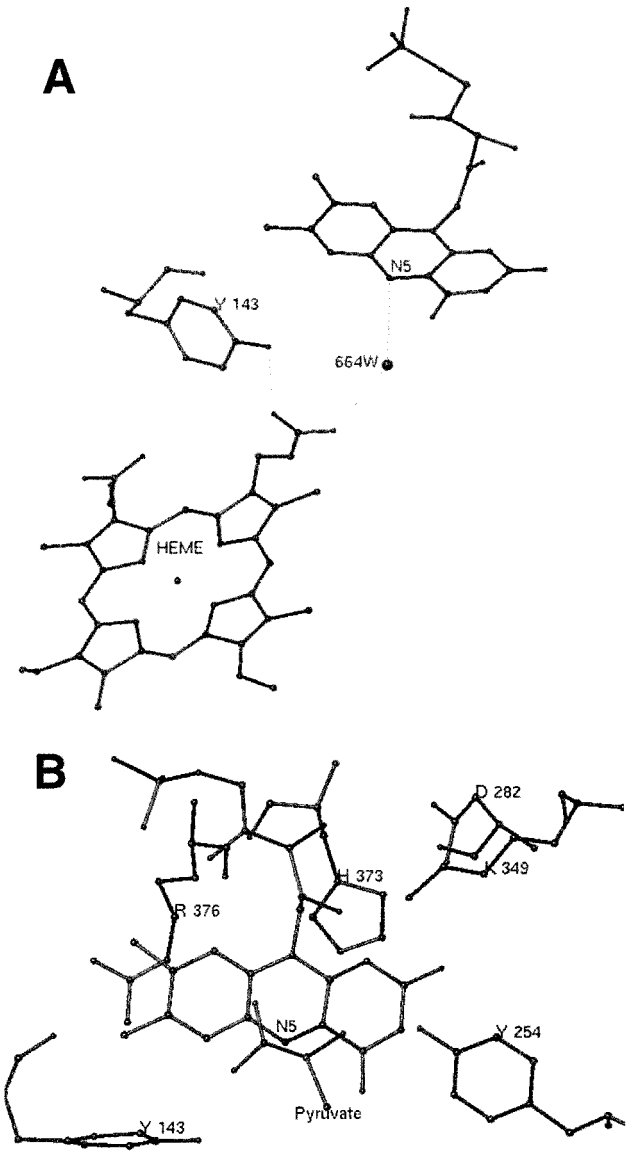
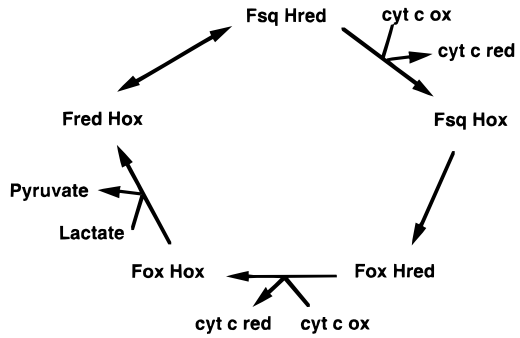


FIGURE 1: (A) Solid (ball-and-stick) diagram of the interaction between FMN and heme in flavocytochrome b_2. One of the propionates of the heme interacts with Tyr143 and with FMN via a water molecule (664W). (B) Solid (ball-and-stick) diagram of the FMN site in the three-dimensional structure of flavocytochrome b_2. Pyruvate is in front of flavin N5.

(Hred) and flavin semiquinone (Fsq); the reduction of the first molecule of cytochrome *c* by the reduced b_2 heme; the electron transfer from Fsq to the b_2 heme, which involves the formation of a second reduced heme (H_{red}) and oxidized flavin (F_{ox}); and again the reduction of cytochrome *c* by the reduced b_2 heme (Scheme 1).

The reactions of both the *Saccharomyces cerevisiae* and the *Hansenula anomala* flavocytochrome b_2 prosthetic groups with the physiological electron donor (L-lactate) and acceptor (cytochrome *c*) have been characterized on the basis of detailed rapid-mixing experiments (*14*−*20*). Analysis of electron transfer between FMN and heme is more complicated, however. In fact, the abstraction of Cα-hydrogen from L-lactate completely limits the turnover of the *S. cerevisiae* enzyme (*21*) and partly limits that in *H. anomala* flavocytochrome b_2 (*18*). In the first enzyme, the stopped-flow time courses of the reduction of the FMN and the heme are almost synchronous, which makes it difficult to accurately estimate

Scheme 1: Elementary Steps in the Kinetic Mechanism of Electron Transfer from Lactate to Cytochrome *c*, Catalyzed by Flavocytochrome b_2[a]



[a] In the intermediate states of the enzyme, the FMN is in the oxidized, totally reduced, or 1 electron−reduced state (Fox, Fred, and Fsq, respectively), and the heme is in the oxidized or reduced state (Hox and Hred, respectively). Pyruvate and reduced cytochrome *c* are the reaction products.

the electron-transfer step from Fsq to the b_2 heme (*10*, *14*, *17*, *18*). This rate has been estimated for *H. anomala* flavocytochrome b_2, however, since a lag is observed between the traces of the FMN and the heme reduction (*18*). The intramolecular electron-transfer reactions show a high degree of equilibrium, the redox potentials of the FMN and the b_2 heme being quite similar to each other (*14*, *22*). This has made it possible to characterize the intramolecular electron transfers in the *H. anomala* flavocytochrome b_2 by using the temperature-jump procedure (*22*, *23*). More recently, these reactions were studied by flash-photolysis (*24*−*26*). Contrary to what our results on *H. anomala* flavocytochrome b_2 seem to indicate, the latter data were interpreted in terms of an electron-transfer reaction controlled by a conformational change in the protein induced by the binding of pyruvate (*24*−*26*). The electron transfer from Fsq to heme in *S. cerevisiae* enzyme has been characterized in the presence of cytochrome *c* by rapid-mixing experiments (*27*), but no evidence of "conformational gating" induced by pyruvate was detected in that study (*27*).

Cloning of the gene (*28*−*31*) and expression of the *S. cerevisiae* protein in *Escherichia coli* (*32*) have made it possible to produce the recombinant protein and construct specifically designed site-directed mutants for probing the reaction mechanism. The steady-state parameters of the recombinant protein are very similar to those of the wild-type enzyme (*33*, *34*). If differences between the experimental conditions are taken into account, the rate of reduction of the recombinant protein by L-lactate is in reasonable agreement with that of the wild-type enzyme (*17*, *34*). The redox potentials of both prosthetic groups have also been found to be similar to those of the wild-type enzyme (*14*, *24*, *35*).

Some mutants of the residues at the flavin site have been expressed and characterized, particularly, Tyr143Phe (Y143F) and Tyr254Phe (Y254F). The residue Tyr143 belongs to the FMN domain and is located at the interface with the b_2 heme domain, roughly between FMN and heme (Figure 1B). It is the only residue at the active site that forms a strong hydrogen bond with the heme, via propionate A (*3*, *4*). The particular location of this residue suggests that it may be involved in electron transfer between the prosthetic groups, where it may either contribute to the superexchange pathway

or form one of the fundamental interactions required for the approach between the FMN and heme domains. Replacing Tyr143 by Phe143 has been used to test the role of the hydroxyl side chain in the wild-type enzyme. The apparent rate constant of the electron transfer to the heme has been studied by stopped-flow and found to be 20 times lower in the mutant Y143F and limiting the turnover of the enzyme in the presence of cytochrome *c* (*34*). One possible explanation for this kinetic behavior, as suggested by X-ray crystallography, is that the number of interactions between the FMN and heme domains may be severely reduced in this mutant, which may decrease the number of efficient approaches (*36*). However, other explanations for this kinetic difference should be considered, since the thermodynamic parameters of the two flavin couples may be affected by the mutation and the superexchange pathway may change according to the chemical nature of the side chain.

The residue Tyr254 is hydrogen bonded to the C−O of the product, pyruvate (Figure 1B) (*3*, *4*). This residue was initially thought to act as a general base in the first steps of L-lactate oxidation (*12*). The replacement of Tyr254 by Phe254 was therefore expected to completely abolish the specific activity. Instead, however, steady-state kinetic data have shown that the mutant is still active, although its activity is very low (*37*). X-ray data also argue against the general base hypothesis, having shown that the product is differently oriented in the active site, that it does not interact with Phe254, and that it is hydrogen-bonded to His373 (*38*). Tyr254 is now thought to stabilize the transition state intermediate, as required for the carbanion formation to occur (*13*).

Here, we describe a series of temperature-jump experiments that were performed to characterize the electron-transfer kinetics between FMN and the b_2 heme in the recombinant flavocytochrome b_2 of *S. cerevisiae*. In the same enzyme, we measured the two potentials of the flavin at room temperature by electron paramagnetic resonance (EPR)−monitored potentiometric titration and determined the potential of the heme by spectrophotometrically monitored titration. Three proteins were studied: the wild type flavocytochrome b_2 and the two site-directed mutants Y143F and Y254F. The experiments were carried out under conditions similar to those used in previous thermodynamic and kinetic studies. The effects of pyruvate, which was already known to affect the thermodynamic and kinetic parameters of *H. anomala* (*22*, *23*) and *S. cerevisiae* flavocytochrome b_2 (*24*, *26*), were analyzed. Our results are consistent with the idea that the intramolecular electron transfer kinetics may be mainly determined in these proteins by the driving force of the reaction.

MATERIALS AND METHODS

Cell Growth and Protein Purification. Plasmid-bearing *E. coli* cells were grown at 37 °C in Luria broth supplemented with ampicillin at 100 μg/mL; cell lysis in the presence of lysozyme and the first purification steps were carried out as described by Black et al. (*32*) with minor modifications. The chromatographic steps were as described by Labeyrie et al. (*39*). At the end of the purification procedure, the enzyme was precipitated at 70% saturation of ammonium sulfate, in the presence of 50 mM D,L-lactate and 100 mM Na/K_2 phosphate buffer at pH 7.0, and is stored at 4 °C. Under these conditions, the enzymes kept their standard molar activity for several weeks.

The L-lactate oxidase−ferricyanide reductase activity was assayed as described by Labeyrie et al. (*39*); the L-lactate and ferricyanide concentrations in the assay mixture varied between 10 and 25 mM and between 1 and 13 mM, respectively, depending on the K_M of the specific mutant with these substrates. The standard molar activity, expressed throughout this paper as moles of mono-electronic acceptor reduced per mole of monomeric enzyme per second, was comparable with the results reported in the literature (*33*, *34*, *40*) for the mutants. The purified protein had an $A_{280\ nm}/A_{423\ nm}$ (reduced) ratio = 0.5 and showed a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The protein concentration was determined by using $\epsilon_{423\ nm}$ = 183 mM $^{-1}$cm^{-1} for the dithionite-reduced enzyme and $\epsilon_{413\ nm}$= 129.5 mM $^{-1}$cm^{-1} for the oxidized enzyme (*41*). The value $\epsilon_{557\ nm}$ taken for the reduced minus oxidized forms was 21.7 mM $^{-1}$cm^{-1} (*22*). Before each experiment, the suitable amount of precipitated protein was resolubilized and filtered on Sephadex G25 that had been preequilibrated in 100 mM Na/K_2 phosphate buffer, pH 7.0, at 4 °C.

Redox Titrations. The enzyme obtained after the molecular filtration procedure was first concentrated to about 1 mM by centrifugation at 4 °C on a Centricon 100. All the redox titrations were carried out in degassed 100 mM phosphate buffer, pH 7.0. The potentials were adjusted with small amounts of a 1.0 M L-lactate solution, in the presence of the following mediators: phenazine methosulfate ($E_{m,7}$ = +80 mV), methylene blue ($E_{m,7}$ = +11 mV), resorufin ($E_{m,7}$ = −51 mV), and naphthoquinone ($E_{m,7}$ = −137 mV). When necessary, small amounts of 20 mM dithionite solution were added at the end of the titration to achieve complete reduction. The reversibility of each titration was checked by reoxidizing the reduced solution with aliquots of partially reduced enzyme. Redox potentials were measured with a combined Pt− Ag/AgCl/KCl (3 M) electrode. All the values given in the text are expressed with respect to the standard hydrogen electrode. To determine the b_2 heme midpoint potential $E_{m,H}$, we used an enzyme concentration of 15 mM and deduced the percentage of reduced heme from absorbance measurements at 557 nm. To determine the midpoint potentials $E_{m,F1}$ and $E_{m,F2}$, corresponding to the F_{sq}/F_{red} and F_{ox}/F_{sq} couples, respectively, we used an enzyme concentration of 500 μM and deduced the amount of Fsq from X-band EPR experiments carried out on a Bruker ESP300 spectrometer equipped with the ESP1620 data-processing unit. In the first series of titrations, 30 μL of the equilibrated solution was transferred anaerobically into a calibrated capillary tube (i.d. 1.2 mm), which was sealed and probed at room temperature by EPR. In the second series of titrations, a sample containing 150 μL of the solution was transferred into a calibrated EPR tube, which was then rapidly frozen and cooled at 100 K with an Air Products gas-flow system. The semiquinone radical signal was recorded with a 0.5 mT peak-to-peak modulation amplitude and a nonsaturating power equal to 0.5 mW at room temperature and 10 μW at 100 K. In all the proteins, the peak-to-peak line width of the signal was equal to 1.5 ± 0.1 mT, a value characteristic of a red semiquinone, as previously observed in the case

of the *S. cerevisiae* (*14*) and *H. anomala* (*22*) enzymes. The maximum amount of Fsq present during the titration was determined by comparing the integrated intensity corresponding to the peak in the titration curve with that obtained at the same temperature for a control solution of $CuSO_4$.

The b_2 heme absorbance variations were fitted to a simple Nernst equation. Variations in the amplitude of the semiquinone radical signal as a function of the potential of the solution were fitted to theoretical curves calculated from the following equation:

fraction (Fsq) $= 1/(1+ Q_2 + Q_1^{-1})$, where $Q_1 = \exp q\ (E - E_{m,F1})/k_BT$, $Q_2 = \exp q\ (E - E_{m,F2})/k_BT$, and q is the elementary electric charge. The equilibrium constants K_1 and K_2, corresponding to reactions Fred + Hox ↔ Fsq + Hred and Fsq + Hox ↔ Fox + Hred, respectively, were calculated as: $\ln K_1 = q(E_{m,H} - E_{m,F1})/k_BT$, $\ln K_2 = q(E_{m,H} - E_{m,F2})/k_BT$, where $E_{m,H}$, $E_{m,F1}$ and $E_{m,F2}$ are the midpoint potentials of the couples Hox/Hred, Fsq/Fred and Fox/Fsq, respectively. For the dismutation reaction, 2 Fsq ↔ Fox + Fred, the equilibrium constant $K_{dism} = [Fox][Fred]/[Fsq]^2$ was calculated by taking the K_2/K_1 ratio.

Temperature-Jump Experiments. Temperature-jump experiments were carried out with flavocytochrome b_2 samples at a starting temperature of 11 °C, using an instrument manufactured by Messenlagen Gesellshaft (Göttingen, FRG). The final temperature reached was 16 °C. The oxidized enzyme samples obtained after molecular filtration were diluted (final concentration 10−63 μM) in degassed 100 mM phosphate buffer (pH 7.0), anaerobically transferred to the temperature-jump cell (volume 7 mL), and reduced with a stoichiometric amount of L-lactate. A slow oxygen leak into the cell gradually led to a very slow oxidation of the enzyme ($t_{1/2} \approx 1$ h at 11 °C), which made it possible to monitor the relaxation kinetics as a function of the percentage of Hred. The slow oxidation kinetics did not interfere with the electron-transfer processes to be monitored, since the redox equilibria between heme and flavin are known to be reached within a much shorter time. The absorption spectra recorded during the experiments in the temperature-jump cell fitted to an OLIS modified Cary-14 spectrophotometer were used to directly monitor the heme reduction levels. The courses of relaxation time are given later (see Figure 4) as differences between the measured absorbance and that at time zero (t_0). All the relaxation time courses were fitted by using the nonlinear regression fitting program Igor Pro (Wavemetrics, Inc. U.S.A.).

Effects of Pyruvate. In the *H. anomala* enzyme, a complex pattern of inhibition kinetics was observed under steady-state conditions, with two inhibition constants (K_I) of ∼1 and 5 mM (*42*). These constants are not directly related to the dissociation constants of pyruvate from Fox, Fsq, and Fred, which had been roughly estimated to be 10, 0.2, and 15−20 mM, respectively (*22*, *42*). In the case of *S. cerevisiae* enzyme, a complex pattern of inhibition was determined by Lederer (*43*) with two inhibition constants of 3 and 33 mM, respectively. In agreement with these results, the kinetic inhibition of pyruvate in the recombinant *S. cerevisiae* enzyme, mutantsY143F and Y254F, were also found to be complex, with K_I values in the same range as those of the wild-type enzyme.

In addition, for the recombinant *S. cerevisiae* enzyme, the stabilization of Fsq by pyruvate was investigated by Walker and Tollin (*24*). The effects of pyruvate on the kinetic behavior and the potentiometric titrations were reported to be practically complete at 5 mM. Therefore, this concentration was used in the flash-photolysis and potentiometric experiments (*24*). With this same enzyme, pyruvate was recently found to be able to inhibit the flavin oxidation under pre-steady-state conditions (*27*). Under those particular experimental conditions, the K_I was 40 mM, a value that agrees with neither the results of Tollin and co-workers (*24*, *26*) nor those obtained here (which are both measured for the recombinant enzyme), but is in line with the value of 33 mM obtained on the enzyme purified from *S. cerevisiae* (*43*).

In the experiments described here for the recombinant *S. cerevisiae* enzyme, the concentration of pyruvate was chosen on the basis of the results by Tollin and co-workers for this enzyme (*24*, *26*), the similarities observed in the kinetic inhibition data between *H. anomala* and *S. cerevisiae* enzymes (reported above), and some preliminary results for temperature-jump experiments on the wild type and mutant enzymes, which basically confirmed the results of Tollin and co-workers. In these experiences, the effects of 1 mM pyruvate on the kinetic pattern were only slightly enhanced by increasing the pyruvate concentration 10-fold. These results seem to confirm that the dissociation constant of pyruvate to Fsq may be an order of magnitude less than that determined by inhibition kinetics, as previously reported for recombinant *S. cerevisiae* (*24*) and as found for the *H. anomala* enzyme (*42*). The concentration of 10 mM was thus presumed to be saturating or quasi-saturating and was used in subsequent kinetic and potentiometric experiments.

RESULTS

Redox Titration. The percentage oxidation of the b_2 heme was plotted as a function of the potential of the solution in Figure 2A for both the recombinant wild-type enzyme and the Y143F and Y254F mutants. Allowing for the experimental error, the three proteins gave identical variations, which are well described by a Nernst curve centered at −3 ± 5 mV (Figure 2A). Variations in the amplitude of the EPR signal for Fsq as a function of the potential of the solution are also shown for the recombinant wild-type enzyme and the two mutated proteins (Figure 2A). Fitting these variations to the equation in *Materials and Methods* yielded the values of $E_{m,F1}$ and $E_{m,F2}$ given in Table 1. From these values, we deduced that the maximum percentage of Fsq likely to be observed during the titration was 68%, 94%, and 92% with the wild-type protein and the Y143F and Y254F mutants, respectively. Comparing these numbers with those obtained directly by spin quantitation of the corresponding EPR signals recorded at room temperature showed that the maximum Fsq content given by these two independent methods agreed within 15%, which confirms the consistency of our analysis.

High concentrations of pyruvate, the product of the reaction with L-lactate, have been reported to strongly stabilize the semiquinone form of FMN in the *H. anomala* enzyme (*22*). To compare the sensitivity of the redox behavior of the FMN with respect to pyruvate in the two enzymes, we carried out redox titrations in the presence of 10 mM pyruvate with the recombinant wild-type form and

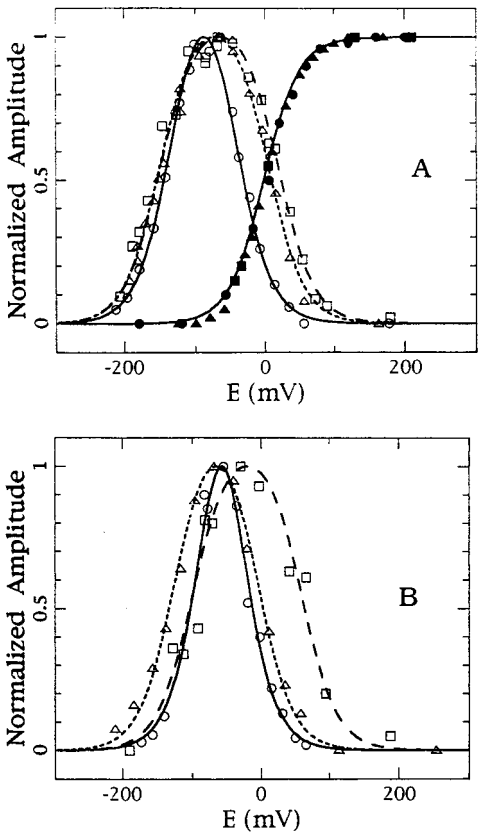


FIGURE 2: Potentiometric titration of the FMN and heme groups of the wild-type and mutated forms of *S. cerevisiae* flavocytochrome b_2. Filled symbols: normalized variations in the heme absorbance at 557 nm. Open symbols: normalized amplitude of the FMN semiquinone EPR signal recorded at (A) room temperature and (B)-100 K. Circles: wild-type protein; rectangles: Y143F mutant; triangles: Y254F mutant. The theoretical curves were calculated as explained in *Materials and Methods*, using the midpoint potentials given in Table 1.

Table 1: Midpoint Potentials of the Flavin and Heme Groups of Recombinant *S. cerevisiae* Flavocytochrome b_2

Recombinant b_2	$E_{m,F}$ (mV) F_{ox}/F_{red}	$E_{m,F1}$(mV) F_{sq}/F_{red}	E_{m,F_2}(mV) F_{ox}/F_{sq}	$E_{m,H}$(mV) H_{ox}/H_{red}	T
wild type	−90	−135 ± 5	−45 ± 5	−3 ± 5	27 °C
wild type + pyruvate	−38	−125 ± 5	+50 ± 5	−3 ± 5	24 °C
Y143F	−68	−155 ± 5	+20 ± 5	−3 ± 5	22 °C
Y143F + pyruvate	−33	−150 ± 5	+85 ± 5	−3 ± 5	25 °C
Y254F	−73	−150 ± 5	+5 ± 5	−3 ± 5	22 °C
Y254F + pyruvate	−35	−150 ± 5	+80 ± 5	−3 ± 5	24 °C
wild type	−55	−70 ± 10	−40 ± 10	-	100 K
Y143F	−20	−100 ± 10	+60 ± 10	-	100 K
Y254F	−65	−125 ± 10	−5 ± 10	-	100 K

the Y143F and Y254F mutants of *S. cerevisiae* flavocytochrome b_2. The results are shown in Figure 3 and the corresponding E_m values are given in Table 1. At room temperature, the relevant E_m values in the intramolecular electron transfer were $E_{m,F1} = -135$ mV, $E_{m,F2} = -45$ mV, and $E_{m,H} = -3$ mV with the wild-type protein (Table 1). In the presence of pyruvate the first potential increased by only 10 mV, whereas the second potential was shifted 95 mV and became positive. As expected, the redox potential of the heme was not affected by the presence of pyruvate (Table 1).

Upon replacing Tyr143 or Tyr254 with Phe, the potential of the Fsq/Fred couple varied slightly and became more

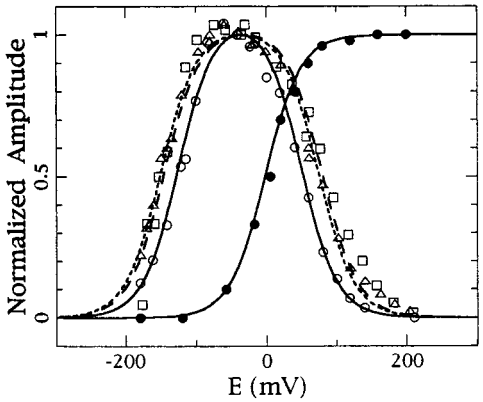


FIGURE 3: Potentiometric titration of the FMN and heme groups of the wild-type and mutated forms of *S. cerevisiae* flavocytochrome b_2 in the presence of 10 mM pyruvate. Symbols as in Figure 2. The FMN semiquinone EPR spectrum was recorded at room temperature. Identical results were obtained for the heme with all three proteins, so only the data for the wild-type protein are shown here. The theoretical curves were calculated as explained in *Materials and Methods*, using the midpoint potentials given in Table 1.

Table 2: Equilibrium Constants of the Two Electron-Transfer Reactions in Recombinant *S. cerevisiae* Flavocytochrome b_2

Recombinant b_2	K_1 F_{sq}/F_{red}	K_2 F_{ox}/F_{sq}	$1/K_{dism}$ K_1/K_2
wild type	188	5.3	35
wild type + pyruvate	127	0.12	1060
Y143F	417	0.40	1040
Y143F + pyruvate	342	0.03	11400
Y254F	342	0.73	470
Y254F + pyruvate	342	0.037	9240

negative, whereas the second potential increased considerably (Table 1). With both mutants, the presence of pyruvate had no effects, within the experimental error limits, on the potential of the Fsq/Fred couple, but the Fox/Fsq potential was 65 and 75 mV more positive with Y143F and Y254F, respectively. The redox potential of the heme was not affected by the mutations (Table 1). In all three recombinant proteins, therefore, both the Tyr→Phe mutation and the presence of pyruvate affected the redox potential of the Fox/Fsq couple, making this redox potential notably more positive. This can be seen from the equilibrium constants K_1 and K_2, calculated from the experimental redox potentials (see *Materials and Methods*), and shown in Table 2: K_2 varied by 13−45-fold upon the addition of pyruvate, whereas K_1 was only slightly if at all affected by the presence of the product. Likewise, K_2 decreased 7−13-fold at the mutation of Tyr to Phe, whereas K_1 increased only 1.5−2-fold. The differences between the dismutation constants therefore mainly reflected the changes in K_2.

In a previous study, the midpoint potentials of the FMN group in *S. cerevisiae* flavocytochrome b_2 were determined by performing low-temperature EPR-monitored redox titrations experiments (*14*). To compare the results of that study with our own data, we carried out a redox titration experiment in which the concentration of the semiquinone radical was monitored by recording its EPR signal at 100 K. The titration curves obtained are shown in Figure 2B and the corresponding midpoint potentials in Table 1.

Relaxation Kinetics. Wild-type flavocytochrome b_2. The temperature-jump experiment with partially reduced wild-

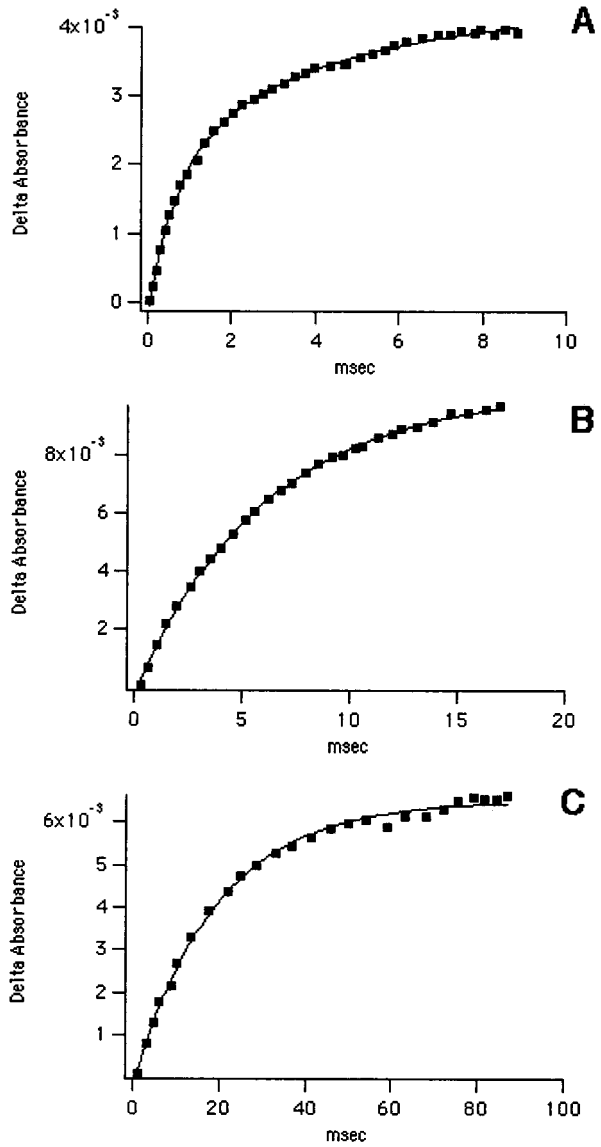


FIGURE 4: Time course of the relaxation obtained by performing temperature jump experiments on recombinant flavocytochrome b_2. (A) relaxation with wild-type protein, monitored at 557 nm, protein concentration 52 μM; (B) Y143F mutant, at 423 nm, protein concentration of 16 μM; (C) Y254F mutant, at 557 nm, protein concentration 58 μM. Experiments were carried out in phosphate buffer, 100 mM, pH 7.0. The ΔA calculated as differences from the absorbance at $t = 0$. Continuous lines give the fit obtained with Igor Pro (see *Materials and Methods*) with the rate constants values listed in Table 3.

Table 3: Relaxation Times and Rate Constants of the Two Electron-Transfer Reactions in Recombinant *S. cerevisiae* Flavocytochrome b_2

	(s^{-1})				
Recombinant b_2	$1/\tau_1$	$k_{1/k-1}$	$1/\tau_2$	k_2/k_{-2}	$1/\tau_3$
wild type	1500 ± 300	1490/10	200 ± 50	170/30	5
wild type + pyruvate	900−1000	942/8	510 ± 50	60/450	
Y143F			170 ± 30	50/120	
Y143F + pyruvate			250 ± 20	10/240	5−10
Y254F			70 ± 15	27/43	
Y254F + pyruvate			345	13/330	13

type flavocytochrome b_2, clearly demonstrated at least two relaxation processes (Figure 4A and Table 3): a very fast process ($1/\tau_1 = 1500 \pm 300$ s^{-1}), which was clearly visible only at high heme reduction percentages (70−100%), and a much slower one ($1/\tau_2 = 200 \pm 50$ s^{-1}), which was observed at low reduction percentages. The second relaxation was followed by a much slower process ($1/\tau_3 = 5$ s^{-1}) with a small amplitude (5−10% of the total ΔA), which was not further analyzed. Since the mean reciprocal relaxation times given in Table 3 did not depend on the observation wavelength or on the enzyme concentration (33−52 μM), we concluded that the electron transfer observed was an intramolecular process.

The direction of the absorbance change at the wavelengths where heme was the only absorbing chromophore showed that the proportion of the reduced heme increased after the temperature jump. In principle, two different forms of the flavin, the hydroquinone (Fred) and the semiquinone (Fsq), may act as electron donors to the heme in the following reactions:

$$\text{Fred} + \text{Hox} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{Fsq} + \text{Hred} \quad (1)$$

$$\text{Fsq} + \text{Hox} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \text{Fox} + \text{Hred} \quad (2)$$

These two reactions were monitored at the wavelengths at which the flavin absorbs much more than the heme (from 460 to 520 nm), and characteristic difference spectra were obtained, at least in the *H. anomala* enzyme (*23*, *44*), where an unambiguous assignment of the relaxations to one or the other reaction was possible. Since no difference was observed between the optical spectra of the *S. cerevisiae* and *H. anomala* enzymes, and since the semiquinone radical is of the same type, i.e., a red one in both cases, as demonstrated by EPR spectroscopy (*14*, *22*, and this study), we can reasonably assume that the static and kinetic difference spectra in reactions 1 and 2 are likely to be identical or very similar in both proteins. In the wild-type recombinant *S. cerevisiae* flavocytochrome b_2, however, the difference in absorbance (ΔA) of the two relaxation processes was very small or absent at these critical wavelengths, possibly because the enthalpy variation ΔH of reactions 1 and 2 was much smaller than those observed with *H. anomala*. Accordingly, we could not completely characterize the two processes on the basis of kinetic difference spectroscopy. However, the optical variation associated with the fast relaxation was negative at 460 nm and positive at 500 nm, whereas the sign of the second relaxation was negative at 500 nm.

The probability of observing reactions 1 and 2 depends on the relative concentrations of the possible electron donors at a certain level of reduction. At very high reduction levels in the system, Fred is the more abundant flavin species; when the reduction level decreases, Fred becomes rare and the Fsq concentration increases, tends to a maximum, and then decreases. At low reduction levels, the flavin is mainly in the oxidized state. On the basis of the ΔA sign at 500 and 460 nm and the amplitude distribution of the two phases, and by analogy with the relaxation rates observed in the *H. anomala* enzyme ($1/\tau_1 = 2500$ s^{-1}, $1/\tau_2 = 190$ s^{-1} at pH 6.0), we confidently assign the rapid relaxation process to reaction 1 and the second process to reaction 2.

In the case of a reversible intramolecular process, the relaxation time can be simply expressed in terms of the two kinetic constants by $1/\tau = k_+ + k_-$. Since an equilibrium

constant of 190 was obtained in the case of reaction 1 (Table 2), the electron-transfer rates were $k_1 = 1490\ s^{-1}$ and $k_{-1} = 10\ s^{-1}$ (Table 3). The value of $(k_1 + k_{-1})$ was identical to that deduced from stopped-flow experiments carried out on the same enzyme at 25 °C and pH 7.5 (*10*). The equilibrium constant of 5.3 for reaction 2 (Table 2) yielded electron-transfer rates of $k_2 = 170\ s^{-1}$ and $k_{-2} = 30\ s^{-1}$ (Table 3). The k_2 value is in good agreement with the $120\ s^{-1}$ value determined on the same enzyme by performing stopped-flow experiments at 25 °C (*27*) as well as with that measured by the temperature-jump method on *H. anomala* flavocytochrome b_2 (*23*).

The effects of pyruvate (10 mM) on the relaxation have mainly been studied at low heme reduction levels, where Fsq is the predominant reductant species. As previously noted for the *H. anomala* protein, the process was monophasic, with a relaxation time $1/\tau = 510 \pm 50\ s^{-1}$ (Table 3); the relaxation amplitude increased slightly. Pyruvate significantly affected the equilibrium constant of reaction 2 ($K_2 = 0.12$, Table 2), stabilizing the semiquinone intermediate which gave the following values for the individual rate constants in the presence of pyruvate: $k_2 = 60\ s^{-1}$ and $k_{-2} = 450\ s^{-1}$ (Table 3). This compares to $k_2 = 100\ s^{-1}$ at 10 mM pyruvate with the recombinant *S. cerevisiae* enzyme (*27*) and with $k_2 = 140\ s^{-1}$ and $k_{-2} = 500\ s^{-1}$ for the *H. anomala* enzyme (*44*). In a few cases, at high enzyme concentrations and high levels of heme reduction, a fast phase was detected. Our tentative estimation of this relaxation rate was $1/\tau = 900-1000\ s^{-1}$.

Mutant Y143F. The presence of the Y143F mutation strongly affected the relaxation kinetics. The rapid relaxation phase that occurs with the wild-type enzyme (which we assigned to reaction 1) was absent in this mutant enzyme, whereas a relaxation rate similar to $1/\tau_2$ ($1/\tau = 170 \pm 30\ s^{-1}$) was observed at all heme reduction levels (3−95%) (Figure 4B and Table 3). This relaxation did not depend on the protein concentration (16−65 μM), which is consistent with the occurrence of an intramolecular process. In this mutant, the kinetic difference spectrum was characterized in the flavin region between 500 and 460 nm, and on this basis the relaxation observed is unambiguously assigned to reaction 2. The following individual rate constants were calculated from the relaxation rate and the equilibrium constant ($K_2 = 0.40$, Table 2): $k_2 = 50\ s^{-1}$ and $k_{-2} = 120\ s^{-1}$; hence, k_2 decreased and k_{-2} increased with respect to the wild-type enzyme values (Table 3).

Adding pyruvate had little effect on the relaxation rate, but a slow phase ($k = 5-10\ s^{-1}$) with a significant amplitude (30−70% of the total) independent of protein concentration developed (Table 3). Given the equilibrium constant $K_2 = 0.03$ recorded in the presence of pyruvate, the microscopic rate constants were $k_2 = 10\ s^{-1}$ and $k_{-2} = 240\ s^{-1}$ (Table 3).

Mutant Y254F. The presence of the Y254F mutation also strongly affected the relaxation kinetics. The rapid relaxation assigned in the wild-type enzyme to reaction 1 was no longer visible, even at high heme reduction levels. Only one slow relaxation ($1/\tau = 70\ s^{-1}$), which did not depend on the protein concentration (36−58 μM), was observed (Figure 4C and Table 3). The difference spectrum of this transition was characteristic of reaction 2. The dependence of relaxation amplitude was also consistent with the assignment of the relaxation to reaction 2, since upon starting with the completely reduced enzyme, the amplitude increased toward a maximum, peaked between 40 and 60% heme reduction, and then decreased. The absolute amplitude of the relaxation was greater than that observed in the wild-type enzyme, possibly because this mutant has a larger ΔH value. The relaxation constant was slower here than that observed with either the wild-type or the Y143F enzyme. Given the equilibrium constant of 0.73 determined for reaction 2 (Table 2), we obtained $k_2 = 27\ s^{-1}$ and $k_{-2} = 43\ s^{-1}$ (Table 3).

The relaxation time course was significantly affected by pyruvate in the case of mutant Y254F, becoming faster and biphasic ($1/\tau_2 = 345\ s^{-1}$ and $1/\tau_3 = 13\ s^{-1}$); the slow phase covered only 20% or less of the total amplitude (Table 3). The difference spectrum of the main phase indicates that we were still looking at reaction 2. Focusing on the preponderant fast phase, we calculated the following rate constants from the relaxation constant and the equilibrium constant of reaction 2 in the presence of pyruvate ($K_2 = 0.037$, Table 2): $k_2 = 13\ s^{-1}$ and $k_{-2} = 330\ s^{-1}$ (Table 3).

DISCUSSION

Redox Potential Determinations. Let us first discuss the results of the potentiometric experiments. All the experimental data satisfactorily fitted the Nernst curves calculated on the assumption that the heme and the flavin titrate independently from one another (Figure 2A, B); this shows that the redox behavior of the system is not complicated by any significant cooperative or anticooperative redox interactions. With the recombinant protein, the E_m values of the couples Fsq/Fred, Fox/Fsq and Hox/Hred are −135, −45, and −3 mV, respectively (Table 1). These values can be compared with several determinations previously published in the literature. Since the redox potential of cytochromes can depend on the ionic strength and temperature (*45*−*47*), meaningful comparisons can be carried out only between data obtained under identical conditions. Allowing for experimental errors and temperature effect, the potential of -3 ± 5 mV at 24 °C and $I = 0.1$ M is thus reasonably in agreement with the values determined previously (*14*, *24*, *35*) and is slightly more positive than that of the *H. anomala* enzyme (*18*, *22*). As expected, the redox potential of the heme was not affected by the presence of pyruvate in our experiments. Nor did this value undergo any changes as the result of the mutation of the Tyr143 and Tyr254 residues belonging to the flavin domain.

Based on the E_m values of the Fsq/Fred and Fox/Fsq couples, the potential characterizing the bielectronic exchange between Fox and Fred was evaluated to be -90 ± 10 mV, much more negative than those previously published: −52 mV (*14*), −64 mV (*24*), and −58 mV (*35*). The latter values, however, were deduced from difference spectrophotometry measurements, which can lead to large experimental errors in the case of flavocytochrome b_2, where the FMN absorbance is much less than that of the heme and where the FMN cofactor is relatively labile. The $E_{m,F1}$ and $E_{m,F2}$ values determined by performing EPR-monitored potentiometric titrations experiments seem to be more reliable, especially when the Fsq content is directly measured by spin quantitation. This procedure has been used in a previous study on the *S. cerevisiae* enzyme, in which the EPR signal of the

flavin radical was examined at cryogenic temperature, giving $E_{m,F1} = -63$ mV and $E_{m,F2} = -50$ mV (*14*). Since these values differ appreciably from those we measured at room temperature, we carried out another series of titrations in which the EPR signal was recorded at 100 K (see *Materials and Methods*). Here we obtained $E_{m,F1} = -70$ mV and $E_{m,F2} = -40$ mV (Figure 2B), in good agreement with the results of Capeillère-Blandin et al. (*14*). In this system, therefore, the thermodynamic equilibrium is conspicuously modified during the freezing of the samples, so that the concentration of the semiquinone radical deduced from low-temperature EPR experiments does not reflect the redox equilibrium reached at room temperature. Similar problems have been previously encountered in other systems (*48*−*50*).

Previous EPR-monitored redox titration experiments carried out at room temperature on *H. anomala* flavocytochrome b_2 showed that the semiquinone form of the flavin was stabilized by an excess of pyruvate, the reaction product. This stabilization was due both to a decrease in $E_{m,F1}$ and to an increase in $E_{m,F2}$: $E_{m,F1} = -62$ mV and $E_{m,F2} = -45$ mV without pyruvate *(22)*, and $E_{m,F1} = -130$ mV, $E_{m,F2} = +65$ mV in the presence of pyruvate (*22*, *42*). In the *S. cerevisiae* enzyme, however, the stabilization of the semiquinone form induced by excess pyruvate was related only to an increase in the $E_{m,F2}$ of 95 mV, whereas the $E_{m,F1}$ remained practically unchanged (Figure 3 and Table 1); accordingly, the potential corresponding to the bielectronic exchange was shifted positively. A similar effect has been observed in other studies on *S. cerevisiae* flavocytochrome b_2 (*24*, *35*). In fact, in the *S. cerevisiae* enzyme, the potential $E_{m,F2}$ corresponding to the F_{ox}/F_{sq} redox couple shifts conspicuously in the positive direction in response to both an excess of pyruvate and the Tyr→Phe mutations, and the shifts are roughly additive (Table 1); in contrast, the potential $E_{m,F1}$ of the F_{sq}/F_{red} redox couple appears to be almost insensitive to changes in the flavin environment (Table 1).

Relaxation Kinetics. Two kinetic processes ($1/\tau_1 = 1500$ s^{-1}, $1/\tau_2 = 200$ s^{-1}) were observed here in the wild-type recombinant *S. cerevisiae* flavocytochrome b_2 (Table 3) and were assigned to the electron transfer from Fred and Fsq to Hox, respectively. At 16 °C, the microscopic rate constants calculated on the basis of the equilibrium constant were $k_1/k_{-1} = 1490$ $s^{-1}/10$ s^{-1} and $k_2/k_{-2} = 170$ $s^{-1}/30$ s^{-1}. Taking into account the effects of temperature, the k_1 value is in general agreement with the rate constant value of 500 s^{-1} at 5 °C, which was used to simulate the whole reduction reaction in the *S. cerevisiae* wild-type enzyme (*17*). The k_1 value was also in good agreement with the value of $k_1 = 1500 \pm 500$ s^{-1} used to simulate the complete reduction of the recombinant protein by L-lactate at 25 °C (*10*) and with the experimental value of 1900 s^{-1} at 24 °C (*26*). The k_{-1} values of 270 ± 90 s^{-1} and 120 s^{-1} reported previously (*10*, *17*) differ somewhat from the value obtained here, probably because a different equilibrium constant was used in the calculations for reaction 1. The rate constants of this reaction in the *H. anomala* enzyme were estimated to be 1500 $s^{-1}/1000$ s^{-1} (*44*). Reaction 1 observed here in the *S. cerevisiae* enzyme is quasi-irreversible ($k_1/k_{-1} = 1490$ $s^{-1}/10$ s^{-1}), which is in striking contrast to the results for *H. anomala*, where the same reaction has a real character of equilibrium ($k_1/k_{-1} = 1500$ $s^{-1}/1000$ s^{-1} (*44*). This difference between the two enzymes, previously observed in the reaction of FMN reduction by L-lactate (*14*, *17*), can now be said to extend to the Fred to Hox transfer process.

The value of the second equilibrium constants determined here for the recombinant enzyme were 170 $s^{-1}/30$ s^{-1} at 16 °C and differ significantly from the $k_2/k_{-2} = 500$ $s^{-1}/75-100$ s^{-1} at 5 °C estimated by Pompon et al. (*17*) or the 80 $s^{-1}/120$ s^{-1} at 24 °C reported by Capeillère-Blandin et al. (*14*). These differences may be attributed to the different forms of the enzymes used, rather than to the different techniques, since a k_2 of 120 s^{-1} was indeed obtained for the recombinant enzyme by Daff et al. (*27*); using stopped-flow, a value in good agreement with our results. In any case, the intrinsic discrepancy of the previous two results (*14*, *17*), as we take into account the experimental temperatures, makes it hard to draw any satisfactory conclusions. Reaction 2 was not visible in the flash photolysis experiments without pyruvate (*24*, *25*); the explanation given by the latter authors was that conformational gating may have taken place in the presence of the reaction product, which was always present under our experimental conditions in the temperature-jump cell. More stringent comparisons with the flash-photolysis results would in fact have been possible if the "stoichiometric pyruvate" conditions had been tested with that technique. A qualitative dependence of the kinetic pattern on the pyruvate concentration (1−10 mM) is mentioned by Walker and Tollin (*24*), which is in agreement with our results but differs noticeably from those reported by Daff et al. (*27*). This discrepancy may be due to the differences between the flash-photolysis and temperature-jump experimental conditions on the one hand and the stopped-flow conditions on the other hand: Fsq and pyruvate have a time of preequilibration in the former case, whereas any interactions in the latter case should be completed in the mixing time.

The rates determined have been compared with various *S. cerevisiae* enzyme turnover numbers published in the literature: 124 s^{-1} at 5 °C and 400 s^{-1} at 25 °C with ferricyanide as acceptor (*17*, *34*), and 207 s^{-1} in the presence of cytochrome *c* at 25 °C (*34*). Note that all these figures are of the same order of magnitude, particularly if one compares our 170 s^{-1} at 16 °C with the turnover of 207 s^{-1} at 25 °C. It is therefore tempting to suggest that the electron transfer from Fsq to Hox, which is mandatory for the transfer to cytochrome *c*, may contribute to limiting the k_{cat}. The idea that this step may determine the k_{cat} was previously proposed by Daff et al. (*27*).

A single kinetic process was observed here in mutants Y143F and Y254F ($1/\tau_2 = 170$ s^{-1} and 74 s^{-1}, respectively) and was attributed to the transfer from Fsq to Hox. Spectral characterization was performed for the mutant Y254F reactions because the amplitude of the relaxation was larger in this species, whereas the data for the Y143F mutant were interpreted mainly on the basis of the distribution of the time course as a function of the reduction level in the system. The ΔH associated with the relaxation of Y143F was small, even smaller than that with the wild-type enzyme. The mutation of Tyr143 and Tyr254 to Phe significantly affected the redox potential of the Fsq/Fox couple and made the equilibrium very unfavorable for the formation of oxidized flavin. The values of the microscopic constants calculated from the equilibrium constants were $k_2/k_{-2} = 50$ $s^{-1}/120$ s^{-1}

and 27 s^{-1}/43 s^{-1} for the Y143F and the Y254F mutant, respectively, at 16 °C.

Reaction 2 was investigated by performing steady-state kinetic and stopped-flow experiments on mutant Y143F: The k_{app} of heme reduction was 21 s^{-1} at 25 °C [in Tris buffer (*34*)], and the turnover number with cytochrome *c* as acceptor was 20 s^{-1} at 5 °C [in phosphate buffer (*51*)]. Our k_2 value is in general agreement with the figures reported above; moreover, if one allows for the effects of the temperature, our rate constant is in very good agreement with the turnover value obtained under the buffer conditions most similar to ours. The electron transfer from Fsq to Hox appears to be the rate-determining step in the Y143F mutant, as previously suggested (*10*, *34*).

The Y254F mutant enzyme has previously been characterized only on the basis of the steady-state kinetics; the turnover number for ferricyanide reduction was determined to be 12 s^{-1} at 30 °C (*37*). The value of 27 s^{-1} we obtained for k_2 at 16 °C, which was clearly attributable to the Fsq−Hox electron exchange, is of the same order of magnitude as the turnover value. The fact that K_1 becomes roughly 2 times larger in this mutant than in the wild-type species suggests that the intramolecular electron transfer from Fred to Hox may be faster than in the wild type. In addition, the large decrease in K_2 strongly suggests the presence of conditions that are unfavorable for electron transfer from the Fsq and are a possible limitation at this step. In fact, since no pre-steady-state data are available, it is not easy to compare these rate constants. All that can be stated is that, in view of the turnover reported so far, the electron transfer from Fsq at 27 s^{-1} might be rate limiting.

The fast relaxation process observed was attributed here to the electron transfer from Fred to Hox in the wild-type enzyme. No relaxation time corresponding to this equilibrium was detected in either the Y143F or the Y254F mutant. Indeed, the doubling of the first equilibrium constant observed by performing redox determinations in the mutants may suggest that the rate from Fred to Hox increases greatly, e.g., >3000 s^{-1}, which thus would make it difficult to detect. Moreover, the very small overall ΔA observed in these experiments, particularly in the Y143F mutant, makes the relaxation process even more difficult to characterize.

Dynamic or Thermodynamic Control of the Rate Constants? Several independent arguments based on NMR (*61*) and crystallographic studies (*3*, *4*, *38*), as well as other results (*45*, *52*), indicate that in flavocytochrome b_2, the cytochrome domain is mobile relative to the flavin domain. Since the intramolecular electron-transfer process can be expected to take place only when the two domains form an appropriate complex in which the respective arrangement of the redox centers is favorable, this conformational mobility may affect the electron-transfer kinetics. Besides, the idea that a dynamic control of the intramolecular electron transfer may be at work in flavocytochrome b_2 has been put forward before (*11*, *36*). If we denote as k_{et} the electron-transfer rate constant and as k_c the rate of formation and dissociation of the competent complex, a dynamic control can be expected to occur when k_c is similar to or less than $k_{et.}$ "Conformationally gated" electron transfers of this kind have been studied theoretically (*53*) and have also been identified in biological systems (*54*). In particular, the kinetics of electron transfer in *S. cerevisiae* from flavocytochrome b_2 to cytochrome *c*

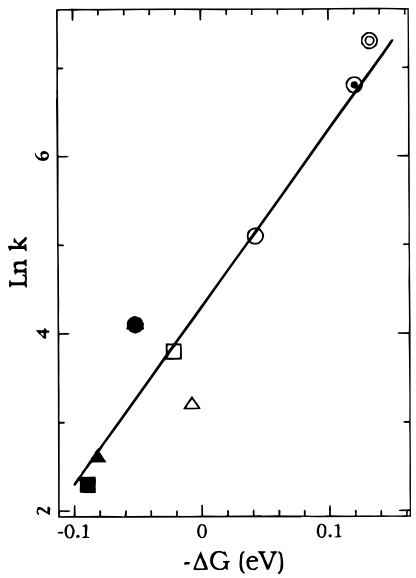


FIGURE 5: Plot of k_1 and k_2 values versus the driving force ΔG, from the data given in Tables 1 and 3. For k_2, the following symbols are used: circles, wild-type protein; rectangles, Y143F mutant; triangles, Y254F mutant; filled and empty symbols correspond to experiments with and without pyruvate. For k_1, the same symbols are surrounded by a circle. The solid line gives the best fit between the data and a line with the slope $1/2k_BT = 20$ eV^{-1}.

is practically independent of the driving force, which suggests that the electron transfer is strongly gated in this system such that $k_c \ll k_{et}$ (*55*). In the other limiting situation, k_c is much larger than k_{et}, so no conformational dynamics are involved in the kinetics, and the measured rate constant corresponds to k_{et}. Analysis of the data in Tables 1 and 3 provides useful information as to which situation prevails in *S. cerevisiae* flavocytochrome b_2.

We have plotted in Figure 5 the values of the rate constant k_2 given in Table 3 as a function of the driving force ΔG, which is equal to $F(E_{m,F_2} - E_{m,H})$ in reaction 2 (see *Relaxation Kinetics*). Note first that the rate constant increases by 17-fold when $-\Delta G$ varies from −88 to +42 meV. It is especially interesting to compare these variations with those predicted by electron-transfer theories, i.e., that the expression of the electron-transfer rate constant k_{et} in the high-temperature limit is given by (*56*):

$$k_{et} = (4\pi^2/h)\,[1/(4\,\lambda\,k_BT)^{1/2}]\,(T_{ab})^2 \exp(-(\Delta G + \lambda)^2/4\,\lambda\,k_BT) \quad (3)$$

where h and k_B are Planck and Boltzmann constants, respectively, λ is the reorganization energy, ΔG the driving force and T_{ab} the electronic factor. Although several experiments have yielded a consistent value of 0.5 eV for the reorganization energy of a low-spin heme (*57*, *58*), little is known about the contribution of a flavin group. In a recent study on a electron transfer in a modified form of *S. cerevisiae* iso-1-cytochrome *c*, in which a flavin group was covalently linked to cysteine 39, a large flavin contribution value of 0.7 eV was obtained, which was attributed to the high exposure of the flavin group to the solvent (*59*). Because a smaller value is therefore to be expected in the case of flavocytochrome b_2, in which the FMN moiety is buried inside the flavodehydrogenase domain, in what follows we will assume that the λ value of the whole protein

falls within the 0.5−1 eV range. Since ΔG is much smaller than λ in flavocytochrome b_2, expression 3 can be simplified so that the dependence of k_{et} on ΔG becomes

$$\ln k_{et} \approx A - \Delta G/(2\ k_B T) \qquad (4)$$

where $A = \ln[(4\pi^2/h)(1/(4\ \lambda\ k_B T)^{1/2})\ T_{ab}^2] - \lambda/4k_B T$ does not depend on ΔG. From expression 4, a linear dependence of $\ln k_{et}$ as a function of $-\Delta G$ is expected, with a slope equal to $1/(2\ k_B T)$. The data given in Figure 5 fit a line with a slope $1/(2\ k_B T) \approx 20\ eV^{-1}$ at 16 °C, the temperature of the temperature-jump study, with an intercept A = 4.3. Moreover, the k_1 values in Table 3, corresponding to the reduction of the heme by Fred, when plotted as a function of the driving force of reaction 1, $\Delta G = F(E_{m,F1} - E_{m,H})$, also fit the same line well (Figure 5). By using the full expression 3, we checked that the deviation from the linear approximation is negligible for λ values >0.5 eV. From the value of A, it can be deduced that T_{ab} ranges between 5×10^{-3} and 8×10^{-2} cm^{-1} if λ ranges between 0.5 and 1 eV. The magnitude of T_{ab} in *S. cerevisiae* flavocytochrome b_2 is therefore similar to that of $T_{ab} = 4 \times 10^{-2}$ cm^{-1} measured in the electron transfer between the flavin group and the low-spin heme in the modified form of *S. cerevisiae* iso-1-cytochrome *c* (*59*). When the redox state of the enzyme is such that the flavin is in the semiquinone form and the heme is oxidized, both centers are paramagnetic, and intercenter magnetic interactions can be expected to occur. This redox state is the initial state in the electron transfer corresponding to k_2, so that the antiferromagnetic contribution J of the exchange interaction is related to the electronic factor T_{ab} by $J = (T_{ab})^2/U$, where U is equal to the reorganization energy λ when $|\Delta G| \ll \lambda$ (*55*). Given the previously quoted values of T_{ab} and λ, J can be predicted to be in the 3×10^{-9} to 8×10^{-7} cm^{-1} range. Such small values are consistent with the fact that no effects attributable to the exchange interactions have been observed on the EPR spectrum (*60*). The fact that no dipolar effects are observed either has been previously discussed elsewhere (*52*).

From the above discussion, we conclude that the magnitude of the rate constant and its variation with ΔG (given in Table 3) are accurately described by theoretical expression 3. This strongly suggests that these rate constants are true electron-transfer rate constants and that the electron transfer is not controlled by the protein dynamics in the flavocytochrome b_2 variants studied here, not even in the wild-type enzyme. This conclusion could be strengthened by investigating the temperature dependence of the electron-transfer rate. According to expression 3, the activation energy E_a is equal to $\lambda/4 + \Delta H/2$ at low ΔG values (*57*). If the enthalpic contribution is neglected, this expression predicts a moderate activation energy E_a equal to 12 and 24 kJ mol^{-1} for λ = 0.5 and 1 eV, respectively. The changes in the protein brought about by the binding of pyruvate and the mutations Y143F and Y254F may of course affect the electronic factor T_{ab}, which might explain the scattering of the data observed in Figure 5. Nevertheless, we believe that the overall consistency of the available data emerging from our analysis argues in favor of the idea that the electron transfer rate is thermodynamically controlled in this series of proteins.

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