Molecular Interpretation of Inhibition by Excess Substrate in Flavocytochrome b_2: A Study with Wild-Type and Y143F Mutant Enzymes†

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ABSTRACT: The crystal structure of flavocytochrome b_2 (L-lactate dehydrogenase) from *Saccharomyces cerevisiae* suggests that Tyr143 plays a dual role at the active site: it contributes to substrate binding and, most importantly, makes a hydrogen bond to a heme propionate, which could facilitate communication between the domains. Previous work on the Y143F mutant enzyme provided support for these hypotheses [Miles, C. S., Rouvière-Fourmy, N., Lederer, F., Mathews, F. S., Reid, G. A., Black, M. T., & Chapman, S. K. (1992) *Biochem. J. 285*, 187−192; Rouvière-Fourmy, N., Capeillère-Blandin, C., & Lederer, F. (1994) *Biochemistry 33*, 798−806]. In the course of kinetic comparisons between the wild-type (WT) enzyme and the Y143F mutant protein, we observed for the latter signs of inhibition by excess substrate at much lower concentrations than observed for the former. A detailed investigation of the phenomenon has shown that, for the wild-type and Y143F forms, lactate at high concentrations inhibits both cytochrome *c* and ferricyanide reduction. In these cases, inhibition appears to be a specific effect, since acetate at identical concentrations exerts an inhibitory effect that is markedly weaker than that of lactate. In the pre-steady-state, in the absence of acceptor, flavin and heme reduction are unaffected by high substrate concentrations in the WT enzyme case. For the Y143F mutant, flavin reduction is similarly unaffected, but heme reduction is inhibited to nearly the same extent by high lactate and acetate concentrations. In this case, inhibition can probably be ascribed to ionic strength effects. The combination of stopped-flow and steady-state results suggests that lactate binds with weak affinity at the active site when the flavin is in the semiquinone state, preventing electron transfer to heme b_2 and hence to acceptors. This phenomenon is analogous to the inhibition exerted by pyruvate when bound to the enzyme at the semiquinone stage [Tegoni, M., Janot, J. M., & Labeyrie, F. (1990) *Eur. J. Biochem. 190*, 329−342]. We suggest that the substrate carboxylate and the heme propionate of the mobile heme-binding domain compete for the Tyr143 hydroxyl group, hence for approach to the flavin. In the Y143F mutant enzyme, in which the interdomain interaction is impaired, competition would play in favor of the substrate, resulting in the inhibition at lower lactate concentrations than observed for the wild-type enzyme.

Flavocytochrome b_2 (EC 1.1.2.3), a tetrameric flavohemoprotein, catalyzes the oxidation of lactate to pyruvate at the expense of cytochrome *c* in the intermembrane space of mitochondria. The prosthetic group FMN oxidizes the substrate and transfers the reducing equivalents intramolecularly, one by one, to heme b_2, thus forming an intermediate flavosemiquinone (Fl_{sq}) of the anionic or red type (Capeillère-Blandin et al., 1975; Lederer, 1991). Heme b_2 is the exclusive donor to cytochrome *c* (Ogura & Nakamura, 1966; Forestier & Baudras, 1971; Iwatsubo et al., 1977; Capeillère-Blandin et al., 1980). In vitro, other electron acceptors can be used; ferricyanide in particular can reportedly be reduced by heme b_2 or FMN in its fully and/or half-reduced states, depending on the enzyme form [Forestier & Baudras, 1971; Iwatsubo et al., 1977; for a review, see Lederer (1991)].

The three-dimensional structure of *Saccharomyces cerevisiae* flavocytochrome b_2 has been refined to 2.4 Å resolution (Xia & Mathews, 1990). The two subunits of the asymmetric unit are not equivalent. S1 shows two folding units: the heme-binding domain (residues 1−99), which belongs to the family of b_5-like cytochromes (Lederer, 1994), and the flavin-binding domain or flavodehydrogenase (residues 100−511). The latter is a member of a family of FMN-dependent α-hydroxy acid-oxidizing enzymes, which present the $\beta_8\alpha_8$ barrel fold (Lê & Lederer, 1991; Lindqvist et al., 1991). In subunit S1, a heme propionate points toward the flavin and forms a hydrogen bond with an active site residue, Tyr143, as well as indirect hydrogen bonds to the flavin through water molecules (Figure 1A). The prosthetic groups planes are parallel, and the shortest edge to edge distance is 9.6 Å. In subunit S2, the heme-binding domain cannot be located, which indicates positional mobility. A piece of extraneous electron density close to the flavin was identified as pyruvate, the reaction product (Figure 1B). A correlation with solution studies on flavocytochrome b_2 from *Hansenula anomala* suggested that the S2 flavin in the *S. cerevisiae*

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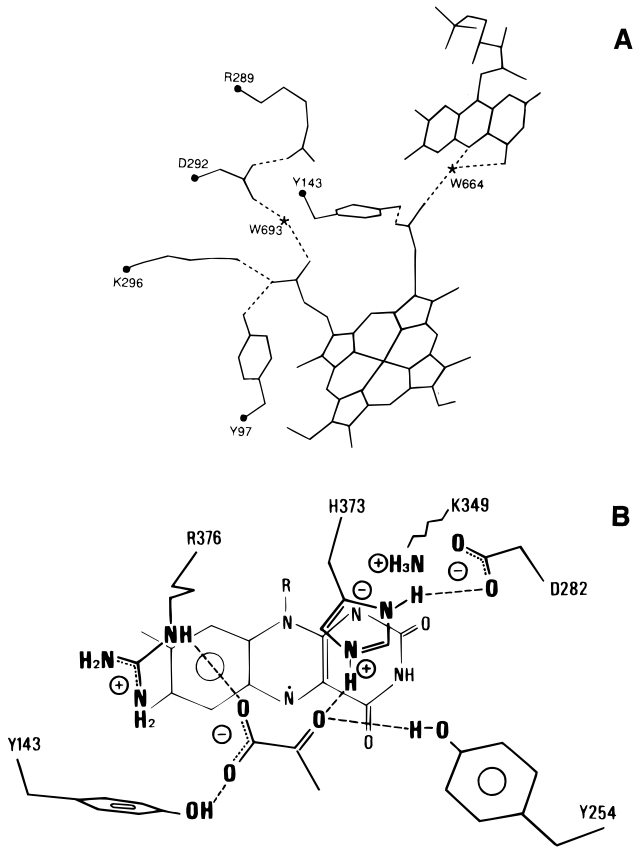


FIGURE 1: Structure of the flavocytochrome b_2 active sites: (A) subunit S1, (B) subunit S2 (after Xia and Mathews, 1990).

enzyme crystal structure was in the semiquinone state, since pyruvate has a high affinity for the enzyme with the flavin in this redox state (Tegoni et al., 1986; Xia & Mathews, 1990). The S1 flavin should be in the $2e^-$-reduced state, because lactate was still present in the mother liquors. The structure of the recombinant flavocytochrome b_2 purified from *Escherichia coli* presents the same general features (Tegoni & Cambillau, 1994).

The pyruvate orientation in subunit S2 (Xia & Mathews, 1990) suggested a binding mode for the substrate, lactate, as well as a role for active-site side chains in catalysis, on the basis of the generally accepted carbanion mechanism (Lederer & Mathews, 1987). In particular, it was proposed that Y143 forms a hydrogen bond with a substrate carboxylate oxygen during catalysis.

The location of this tyrosine in the active site as well as at the domain interface and its interaction with a heme propionate in S1 made Y143 an interesting target for site-directed mutagenesis. The Y143F mutant enzyme was submitted to a detailed kinetic analysis in Tris/HCl buffer (I = 0.1 M), pH 7.5, 25 °C (Miles et al., 1992). Flavin and heme reduction as well as ferricyanide and cytochrome *c* reduction were studied by stopped-flow and steady-state methods, respectively. The complete set of kinetic parameters, combined with deuterium isotope effects, clearly showed that the main effect of the mutation was to decrease the rate of flavin to heme electron transfer; as a consequence, cytochrome *c* reduction was also considerably slowed down and ferricyanide acted as an alternate electron acceptor for Fl_{red} instead of heme b_2. The effect of the mutation on flavin reduction was more modest, consisting essentially in a small substrate K_m increase. An intriguing decrease of the deuterium isotope effect on flavin reduction was also observed (Miles et al., 1992). We carried out an independent study in 0.1 M phosphate buffer (I = 0.22 M) pH 7, at 5 °C, which yielded somewhat different relative rate constants values [Rouvière-Fourmy et al. (1994) and this work]. We have published those aspects of that study which dealt with the effect of the mutation on the catalysis of substrate dehydrogenation. We qualitatively confirmed the results of Miles et al. (1992); in addition, we showed that the intrinsic isotope effect on flavin reduction was indeed lowered by the mutation. Nevertheless, it was clear that the postulated hydrogen bond between Y143 and the substrate carboxylate was less stabilizing for the transition state than for the Michaelis complex. This ruled out Y143 as an electrophilic catalyst in lactate dehydrogenation, an interesting hypothesis proposed by Ghisla and Massey (1991). As a consequence of the greater destabilization of the Michaelis complex than of the transition state, the Y143F enzyme exhibited a 2-fold higher flavin reduction rate than the wild-type enzyme [Rouvière-Fourmy et al., (1994) and Table 1].

In our first paper (Rouvière-Fourmy et al., 1994), we presented fast-kinetic and steady-state studies that yielded kinetic parameters for flavin and cytochrome *c* reduction. We now present a continuation of that study, i.e., results dealing with heme and ferricyanide reduction by the Y143F mutant enzyme. As we observed, early on, an inhibition of the Y143F enzyme heme reduction at lactate concentrations above 5 mM, we were reminded of an observation by Somlo and Slonimski (1966); these authors had shown an inhibition by excess substrate on wild-type enzyme-catalyzed ferricyanide reduction at 27 °C, with $K_{i'}$ = 130 mM, and on cytochrome *c* reduction. Therefore, we carried out rate measurements up to 500 mM L-lactate as well as at optimal lactate concentrations in the presence of high acetate concentrations, in order to distinguish between inhibition by excess substrate and by ionic strength. Our results enable us to propose a molecular explanation for the inhibition by excess substrate observed under steady-state conditions; furthermore, they shed an interesting light on the consequences of the Y143F mutation.

MATERIALS AND METHODS

Chemicals. L-[2-^{2}H]Lactate was enzymatically synthesized as described by Pompon et al. (1980).

Enzymes. *S. cerevisiae* recombinant wild-type and mutant enzymes were purified from *E. coli*, desalted, and stored as described before (Dubois et al., 1990; Rouvière-Fourmy et al., 1994). Enzyme concentrations were expressed as heme concentrations [ϵ^{ox}_{413} = 129 mM^{-1} cm^{-1}; ϵ^{red}_{423} = 183 mM^{-1} cm^{-1} (Labeyrie et al., 1978)]. Unless stated otherwise, the buffer used throughout was 0.1 M Na^+/K^+ phosphate buffer, 1 mM EDTA, pH 7 (I = 0.22 M).

Stopped-Flow Studies. Two different instruments were used in the course of this work: a Gibson-Durrum apparatus modified as described by Capeillère-Blandin (1991) (2.2 ms dead time and 1.9 cm optical path length) and a SFM-3 Biologic instrument (Grenoble, France), a motor-driven, three-syringe apparatus (2 ms dead time and 1 cm optical path length); the kinetic traces were acquired, saved, and analyzed using the BIO-KINE software application. The temperature was controlled at 5.0 ± 0.5 °C. Conditions for flavin reduction were described previously (Rouvière-Fourmy

Table 1: Stopped-Flow Kinetic Parameters and Deuterium Isotope Effects for Heme Reduction in Wild-Type and Y143F Enzymes at 5 °C. Comparison with Flavin Parameters[a]

		flavin reduction				heme reduction			
enzyme	substrate	k_{red}^F (s^{-1})	K_m (mM)	k_{red}^F/K_m ($mM^{-1}\,s^{-1}$)	$^Dk_{red}^F$	k_{red}^H (s^{-1})	K_m (mM)	k_{red}^H/K_m ($mM^{-1}\,s^{-1}$)	$^Dk_{red}^H$
				Phosphate, 5 °C[b]					
WT	[2-^{1}H]lactate	144 ± 4	0.89 ± 0.07	162 ± 17	7.2 ±0.9	108 ± 5	0.54 ± 0.07	200 ± 35	4.7 ± 0.4
	[2-^{2}H]lactate					23 ± 1	0.57 ± 0.07	40 ± 7	
Y143F	[2-^{1}H]lactate	310 ± 19	2.55 ± 0.26	121 ± 20	4.4 ± 0.5	49 ± 3	0.40 ± 0.04	122 ± 20	2.0 ± 0.3
	[2-^{2}H]lactate					25 ± 2	0.61 ± 0.08	41 ± 9	
				Tris/HCl, 25 °C[c]					
WT	[2-^{1}H]lactate	604 ± 60	0.84 ± 0.20	719 ± 243	8.1 ± 1.4	445 ± 50	0.53 ± 0.05	840 ± 174	6.3 ± 1.2
Y143F	[2-^{1}H]lactate	735 ± 80	2.81 ± 0.30	261 ± 56	4.3 ± 0.8	21 ± 2	0.19 ± 0.02	110 ± 22	1.6 ± 0.5

[a] k_{red}^F and k_{red}^F are the rate constants for flavin and heme reduction, respectively, extrapolated to infinite substrate concentrations. The error values given for $k_{red}/K_{m\ and}\ ^Dk_{red}$ are calculated from the sum of the relative errors on values in the numerator and denominator. [b] The values for flavin reduction are taken from Rouvière-Fourmy et al. (1994); those for heme reduction were determined in this work. Buffer: 0.1 M Na^+/K^+ phosphate, 1 mM EDTA, pH 7, $I = 0.22$ M. [c] The values for flavin and heme reduction are taken from Miles et al. (1992). Buffer: 0.1 M Tris/HCl, pH 7.4, adjusted with NaCl to $I = 0.1$ M (25 °C).

et al., 1994). Heme reduction was monitored at 557 nm. As described before, reduction traces were biphasic, but the slow phase is catalytically irrelevant (Suzuki & Ogura, 1970a; Capeillère-Blandin et al., 1975; Pompon et al., 1980). Only rapid phase rates will be reported here. For each substrate or salt concentration, reduction was monitored over two separate time ranges (0.2 s and 1 s) for better precision at early times. The files were then merged, and experimental traces were fitted to an equation for two exponential terms using a non-linear regression program based on a least-squares criterion. The observed rate constants were obtained from the average of two to six traces.

Steady-State Kinetics. Experiments were carried out at 5 °C with a Uvikon 930 spectrophotometer. As enzyme concentrations were low (between 10 and 100 nM), 10 μM FMN was added to assay cuvettes in order to stabilize the enzyme, and sometimes 50 μg/mL bovine serum albumin when ferricyanide was the acceptor. These additions did not alter experimental rates but provided a somewhat longer linear response. For horse heart cytochrome *c* (Sigma type VI), the concentration was calculated using $\Delta\epsilon_{red-ox}$ (550 nm) = 21 $mM^{-1}\,cm^{-1}$, and for ferricyanide it was calculated using $\Delta\epsilon_{red-ox}$ (420 nm) = 1.04 $mM^{-1}\,cm^{-1}$. All measurements, except those with 1 mM ferricyanide, were carried out in 0.2 cm path length cuvettes. Data analysis was carried out in several ways. For k_{cat} and K_m values, experimental points from several independent determinations were pooled and fitted to a Michaelis−Menten equation with a least-squares nonlinear regression program. For the determination of substrate inhibition constants, the data were directly fitted to the general equation

$$v/[E] = k_{cat}\,[S]/(K_m + S + S^2/K_i) \quad (1)$$

Ionic strength effects were analyzed according to the simplified Debye−Hückel equation:

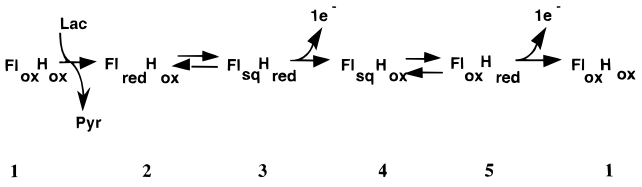
$$\log k = \log k_o + 2Z_A Z_B a\sqrt{I} \quad (2)$$

using a least-squares linear regression program.

RESULTS

The sequence of events occurring in the catalytic cycle of the wild-type enzyme is depicted in Scheme 1, which applies

Scheme 1



when cytochrome *c* is the electron acceptor (Fl, flavin; H, heme b_2). When ferricyanide is used instead, it is believed it can also be reduced by Fl_{sq} of species 3 and 4, but not by Fl_{red} (species 2) (Iwatsubo et al., 1977; Lederer, 1991; Capeillère-Blandin, 1995). Flavin reduction by the substrate can be followed in the stopped-flow spectrophotometer (step 1 → 2), as well as heme reduction (steps 1 → 3), using specific wavelengths (Capeillère-Blandin et al., 1975; Pompon et al., 1980).

Stopped-Flow Study of Heme Reduction for Wild-Type and Y143F Flavocytochromes b_2. Kinetic parameters for flavin and heme reduction were obtained by varying the concentration of L-[2-^{1}H]lactate and L-[2-^{2}H]lactate. In the range from 0.05 to 25 mM substrate, the observed rate constant for overall heme reduction by lactate obeyed a simple Michaelis−Menten equation for the WT enzyme. In contrast, for the Y143F mutant enzyme, velocities started decreasing above 3 mM L-lactate (Figure 2). The deduced kinetic parameters are presented in Table 1 together with our previously published values for flavin reduction (Rouvière-Fourmy et al., 1994), as well as corresponding parameters obtained in Tris/HCl buffer, pH 7.5, 25 °C (Miles et al., 1992) in order to facilitate comparison. Our values for the wild-type recombinant enzyme are, within experimental error, in agreement with those determined by Pompon et al. (1980) for the enzyme purified from *S. cerevisiae*, using the same reaction conditions. The comparison between the mutant and the wild-type enzymes confirms the observation of Miles et al. (1992) under different conditions; in the mutant protein, the low k^H_{red} and kinetic isotope effects values compared to the corresponding parameters for flavin reduction reflect the most important kinetic consequence of the mutation: a lowered rate of heme reduction by flavin.

Steady-State Study of Ferricyanide Reduction by Wild-Type and Y143F Flavocytochromes b_2. Table 2 presents the steady-state kinetic parameters obtained at 5 °C for ferri-

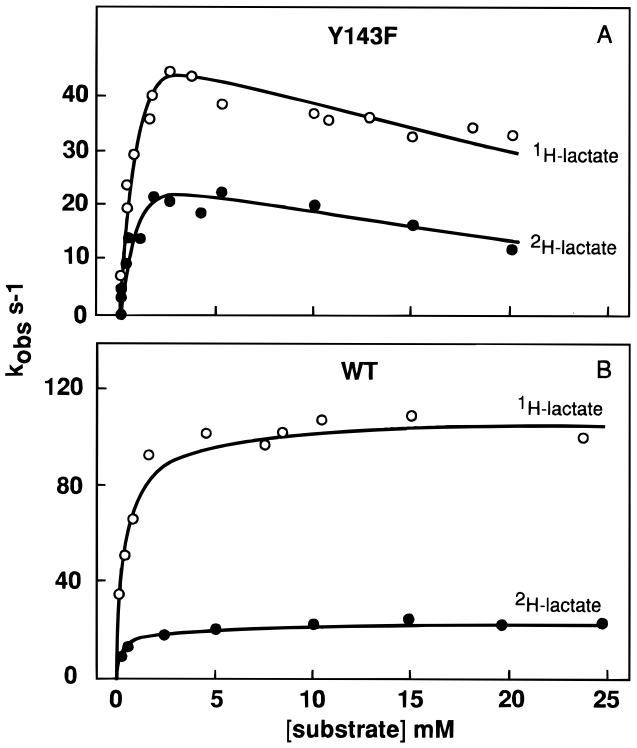


FIGURE 2: Dependence of global heme reduction rates on lactate concentration, at 5 °C. For experimental conditions, see Materials and Methods.

cyanide reduction by wild-type and Y143F mutant enzymes, at varying concentrations of lactate and L-[2-^{2}H]lactate concentrations. They are compared with values for cytochrome *c* reduction. The latter have been determined before (Rouvière-Fourmy et al., 1994), and the results obtained during the present work with the Y143F enzyme do not differ significantly from the published ones. For the wild-type enzyme, however, we found a significantly lower k_{cat} value (61 s^{-1} instead of 98 s^{-1}), although K_m (lactate) and ^DV values were consistent with the published ones. Three different enzyme preparations and several commercial cytochrome *c* lots gave similar lower k_{cat} values. The origin of this discrepancy is unclear. The revised figures are given in Table 2. As discussed before, the substrate K_m increase determined with ferricyanide as acceptor reflects one of the expected consequences of the mutation on substrate binding (Miles et al., 1992; Rouvière-Fourmy et al., 1994). Let us recall that, using the isotope effects on flavin reduction, our previous study yielded actual K_d values, which were 0.45 and 2.1 mM for the wild-type and mutant enzymes, respectively (Rouvière-Fourmy et al., 1994).

With respect to rates, as already observed by Miles et al. (1992) under different experimental conditions, the Y143F enzyme turnover rate in the presence of ferricyanide (120 s^{-1}) and the deuterium isotope effect on this rate ($^DV = 4.3$) (Table 2) are distinctly larger than those for the overall heme reduction as determined in rapid kinetic studies (49 s^{-1} and $^DV = 2.0$) (Table 1). It follows that ferricyanide must receive electrons directly from Fl_{red} and from the ensuing Fl_{sq}. Thus, ferricyanide reduction can entirely bypass the heme in the mutant enzyme. It is however clear that electron transfer to the artificial acceptor from either Fl_{red} or Fl_{sq} or from both is not very fast as compared to flavin reduction by lactate (310 s^{-1}).

The idea that flavin acts as the direct ferricyanide reductant is supported by the increased K_m value of this acceptor (Table 3). For the wild-type enzyme, the ferricyanide half-saturation value is difficult to determine accurately because it is very low. Values ranging between 5 and 180 μM are reported in the literature [for review, see Lederer (1991)]. Under our conditions, as well as in Tris buffer (Miles et al., 1992), 1 mM ferricyanide appears to be saturating. Nevertheless, saturation kinetics with this acceptor do not necessarily indicate it has affinity for flavocytochrome b_2; indeed, it has been shown to react with the enzyme in a second order fashion (Iwatsubo et al., 1977). These authors showed an increase of the ferricyanide K_m value from 5 μM for the cleaved form of flavocytochrome b_2 to 0.5 mM for the corresponding heme-free enzyme. Their analysis indicated that this could be ascribed to the fact that heme b_2 in the holoenzyme oxidizes $2e^-$ -reduced flavin to the semiquinone faster than ferricyanide can. In agreement with the analysis of Iwatsubo et al. (1977), higher K_m values for ferricyanide were obtained with the recombinant flavodehydrogenase (Balme & Lederer, 1994; Balme et al., 1995). By analogy, an increased ferricyanide K_m value for the Y143F enzyme is another indication that Fl_{red} can be oxidized directly by the artificial acceptor, as already discussed by Miles et al. (1992).

As for cytochrome *c*, its reduction rate is slower than that of heme (Table2); this suggests an additional slow step in the steady-state as compared to pre-steady-state. Knowing that electron transfer from heme b_2 to cytochrome *c* is fast (Capeillère-Blandin, 1982; Daff et al., 1996), this step may be reasonably identified as the transfer of the second electron to heme by Fl_{sq} (Scheme 1, step 4 → 5), since the stopped-flow experiments indicate the mutation to have appreciably slowed down transfer of the first electron from fully reduced flavin to heme.

All these results lead to conclusions that are in qualitative agreement with those drawn by Miles et al. (1992) after their study in Tris buffer at $I = 0.1$ M at 25 °C. For a better quantitative comparison with the results of these authors, we also present steady-state kinetic parameters at 30 °C, in the same 0.1 M phosphate buffer ($I = 0.22$ M) that we used at 5 °C as well as in 43 mM phosphate buffer pH 7 ($I = 0.1$ M) (Table 4). The pH difference between these buffers and the Tris buffer used by Miles et al. (1992) should not be of much consequence, because the pH dependence profile shows a rather broad maximum between pH 7 and pH 8 (Suzuki & Ogura, 1970b). Altogether, it would appear, for lactate oxidation by ferricyanide, that the various buffer conditions at the higher temperatures do not much affect rates, lactate K_m values, or isotope effects for either enzyme. Values for the physiological acceptor cytochrome *c*, however, present interesting differences between WT and mutant enzymes under the various conditions. For the WT form, these rates show little variation with buffer composition, taking into account the temperature difference; in contrast, for Y143F flavocytochrome b_2, reaction conditions do appear to make a difference: the drop in cytochrome *c* reduction rate between the wild-type and the mutant form is 3-fold in 0.1 M phosphate, 6-fold in 43 mM phosphate, and 9-fold in Tris. Looking back at the stopped-flow values of Table 1, a similar trend can be observed for heme reduction, although the comparison is made more ambiguous by the large temperature difference. Interestingly enough, with the mutant

Table 2: Steady-State Kinetic Parameters and Deuterium Isotope Effects for Ferricyanide Reduction at 5 °C by Wild-Type and Y143F Flavocytochrome b_2. Comparison with Cytochrome *c* Parameters[a]

		ferricyanide reduction				cytochrome *c* reduction			
enzyme	substrate	k_{cat}^{Fecya} (s^{-1})	K_m (mM)	k_{cat}^{Fecya}/K_m (mM^{-1} s^{-1})	^DV	k_{cat}^{c} (s^{-1})	K_m (mM)	k_{cat}^{c}/K_m (mM^{-1} s^{-1})	^DV
WT	[2-^{1}H]lactate	101 ± 1	0.62 ± 0.01	163 ± 4	4.0 ± 0.2	61 ± 4	0.24 ± 0.01	254 ± 27	3.6 ± 0.4
	[2-^{2}H]lactate	25 ± 1	0.42 ± 0.01	59 ± 4		17 ± 1	0.52 ± 0.04	33 ± 5	
Y143F[b]	[2-^{1}H]lactate	120 ± 1	3.4 ± 0.1	35 ± 2	4.3 ± 0.2	20 ± 4	0.37 ± 0.16	54 ± 34	1.5 ± 0.2
	[2-^{2}H]lactate	28 ± 1	5.6 ± 0.1	5.0 ± 0.3					

[a] Buffer: 0.1 M Na^+/K^+ phosphate, 1 mM EDTA, pH 7. k_{cat}^{Fecya} and k_{cat}^{c} are the rate constants for ferricyanide and cytochrome *c* reduction, respectively, extrapolated to infinite substrate concentrations. The units are moles of substrate oxidized per second per mole of enzyme. The error values given for k_{cat}/K_m and ^DV are calculated from the sum of the relative errors on values in the numerator and denominator. The ferricyanide concentration was 1 mM for experiments with the WT enzyme (94% saturation, see Table 4) and 13 mM for the Y143F mutant enzyme (95% saturation). The cytochrome *c* concentration was 450 μM for the WT enzyme and 1 mM for the mutant one (91% and 88% saturation, respectively). [b] The values for cytochrome *c* reduction are taken from Rouvière-Fourmy et al. (1994).

Table 3: K_m Values of Acceptors[a]

	reaction conditions				acceptor	
temp (°C)	pH	buffer	ionic strength (M)	enzyme	ferricyanide (mM)	cytochrome *c* (μM)
5	7.0	0.1 M phosphate	0.22	WT	0.07 ± 0.02	45 ± 4
				Y143F	0.61 ± 0.01	131 ± 7
		0.1 M phosphate + acetate[b]		WT	<0.1	355 ± 28
				Y143F	2.50 ± 0.1	61 ± 4
30	7.0	0.1 M phosphate	0.22	WT	<0.1[c]	131 ± 2
				Y143F	1.5 ± 0.4	121 ± 2
30	7.0	0.043 M phosphate	0.1	WT	<0.1[c]	17.9 ± 0.1
				Y143F	0.85 ± 0.17	9.0 ± 1.8
25[d]	7.5	0.01 M Tris	0.1	WT	≪0.1	10 ± 1
				Y143F	3.3 ± 0.6	1.5 ± 0.2

[a] Lactate concentration was 10 mM for all the determinations of the present work. [b] Acetate concentration was 500 mM for ferricyanide for both enzymes and for cytochrome *c* with the WT enzyme and 50 mM for cytochrome *c* with the mutant enzyme. [c] See Lederer (1991) for details. [d] Taken from Miles et al. (1992).

Table 4: Steady-State Kinetic Parameters and Deuterium Isotope Effects for Lactate Oxidation at 30 °C by Wild-Type and Y143F Flavocytochromes b_2[a]

		acceptor: ferricyanide				acceptor: cytochrome *c*			
enzyme	substrate	k_{cat}^{Fecya} (s^{-1})	K_m (mM)	k_{cat}^{Fecya}/K_m (mM^{-1} s^{-1})	^DV	k_{cat}^{c} (s^{-1})	K_m (mM)	k_{cat}^{c}/K_m (mM^{-1} s^{-1})	^DV
				Phosphate, I = 0.22 M, pH 7[b]					
WT	[2-^{1}H]lactate	270 ± 30	0.49 ± 0.10	551 ± 174	4.2 ± 1.9	155 ± 15	0.29 ± 0.05	534 ± 144	3.0 ± 0.3
	[2-^{2}H]lactate					51 ± 1	0.53 ± 0.06	96 ± 13	
Y143F	[2-^{1}H]lactate	382 ± 1	1.4 ± 0.1	227 ± 3	2.7 ± 0.1	61 ± 7	0.34 ± 0.22	179 ± 60	1.8 ± 0.4
	[2-^{2}H]lactate	140 ± 1	2.5 ± 0.1	55 ± 1		34 ± 4	0.66 ± 0.33	51 ± 31	
				Phosphate, I = 0.1 M, pH 7[c]					
WT	[2-^{1}H]lactate	218 ± 9	0.30 ± 0.11	727 ± 296	4.6 ± 0.4	165 ± 1	0.57 ± 0.01	289 ± 6	3.5 ± 0.1
	[2-^{2}H]lactate	47 ± 2	0.61 ± 0.16	77 ± 11		47 ± 1	0.95 ± 0.02	49 ± 2	
Y143F	[2-^{1}H]lactate	272 ± 15	1.3 ± 0.3	209 ± 60	2.3 ± 0.3	28 ± 2	0.17 ± 0.13	162 ± 134	1.3 ± 0.2
	[2-^{2}H]lactate	116 ± 7	2.2 ± 0.4	53 ± 13		22 ± 2	0.92 ± 0.34	24 ± 11	
				Tris, I = 0.1 M, pH 7.5, 25 °C[d]					
WT	[2-^{1}H]lactate	200 ± 5	0.49 ± 0.05	408 ± 52	4.7 ± 0.4	103 ± 5	0.24 ± 0.04	429 ± 92	3.0 ± 0.6
Y143F	[2-^{1}H]lactate	200 ± 15	2.9 ± 0.2	69 ± 11	2.0 ± 0.5	11 ± 1	0.73 ± 0.03	15 ± 2	1.7 ± 0.5

[a] k_{cat}^{Fecya} and k_{cat}^{c} are the rate constants for ferricyanide and cytochrome *c* reduction, respectively. Activity units are moles of substrate oxidized per second per mole of enzyme. For error values calculations, see the legend of Table 1. Acceptor concentrations were the following: ferricyanide for WT enzyme: 1 mM (>91% saturation under all conditions; see Table 3); ferricyanide for Y143F enzyme: 13 mM (*i.e.*, 90% and 94% saturation in phosphate at I = 0.22 and 0.1 M, respectively); cytochrome *c* for both enzymes: 450 μM (*i.e.*, for the WT enzyme, 84% and 96% saturation in phosphate buffer at I = 0.22 and 0.1 M, respectively; for the Y143F mutant enzyme, 79% and 99% saturation, in phosphate at I = 0.22 and 0.1 M, respectively, see Table 3). [b] Buffer molarity = 100 mM. For the ferricyanide reduction by WT enzyme, results are taken from Dubois et al. (1990). [c] Buffer molarity = 43 mM. [d] 10 mM Tris buffer adjusted to I = 0.1 M with NaCl. Results are taken from Miles et al. (1992).

enzyme, turnover with cytochrome *c* is faster at the higher phosphate concentration (Table 4), a result that is unexpected in view of the known sensitivity to ionic strength of the cytochrome *c* interactions with its redox partners. This sensitivity rather appears to be reflected in K_m values for both enzymes (Table 3). Whatever the case, taken together, all the results indicate that, for the mutant enzyme, the main rate-limiting step for cytochrome c reduction is always flavin to heme electron transfer.

Effects of High Substrate Concentrations. In view of the inhibition observed above 3−5 mM lactate on heme reduction (Scheme 1, steps 1 → 3) for the mutant enzyme (Figure

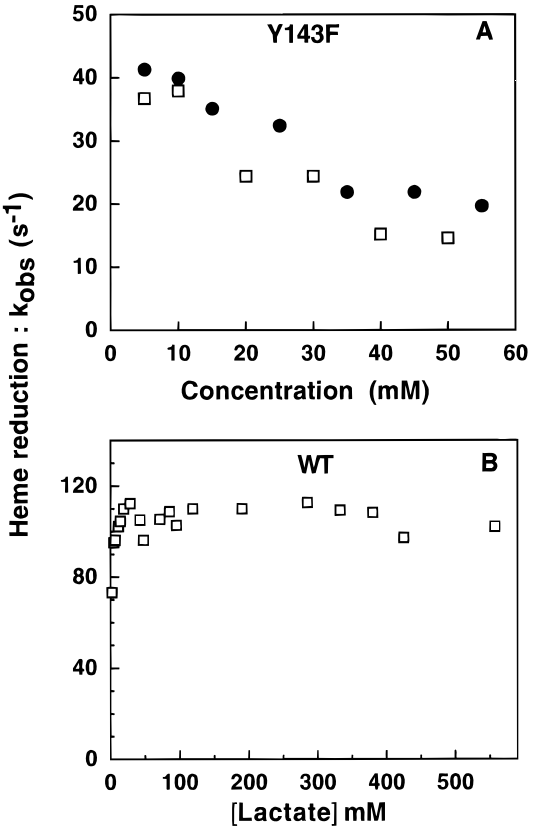


FIGURE 3: Influence of high lactate and acetate concentrations on stopped-flow heme reduction. All experiments were carried out in 0.1 M phosphate buffer, 1 mM EDTA, pH 7, at 5 °C. (A) Y143F mutant flavocytochrome b_2, comparison between high lactate (□) and high acetate concentrations in the presence of 5 mM lactate (●). (B) WT enzyme heme reduction at high lactate concentrations.

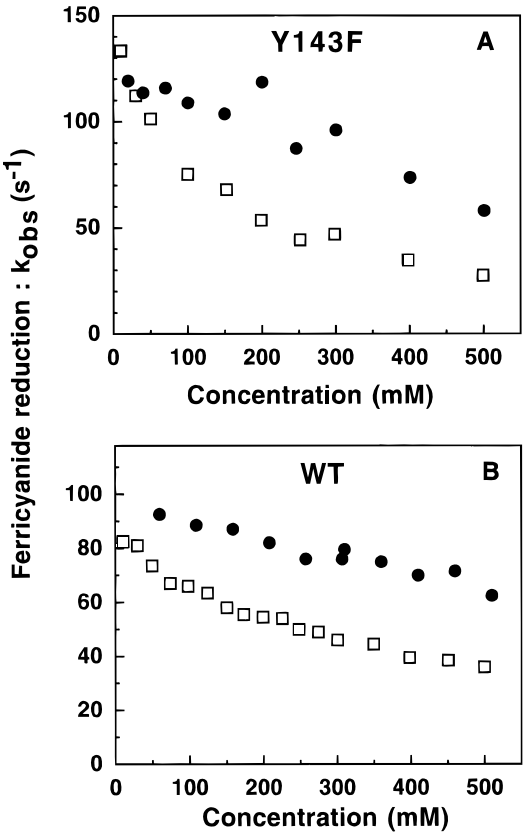


FIGURE 4: Steady-state rates of ferricyanide reduction at high lactate and acetate concentrations: (□) lactate, (●) acetate + 30 mM lactate (A) or 10 mM lactate (B). All experiments were carried out in 0.1 M phosphate buffer, 1 mM EDTA, pH 7, at 5 °C. (A) Y143F mutant flavocytochrome b_2 (13 mM ferricyanide). (B) WT enzyme (1 mM ferricyanide).

2), we decided to carry out a detailed study of the phenomenon, hoping it would bring interesting information concerning Y143F flavocytochrome b_2. Indeed, Somlo and Slonimski (1966) described an inhibition by excess substrate for ferricyanide reduction by the wild-type enzyme; a Dixon plot yielded a K_i of 130 mM at 27 °C (67 mM phosphate buffer, pH 7.2), whereas acetate at high concentrations had hardly any influence.

We also observed inhibition at 30 °C, but we only carried out an extensive study at 5 °C in view of the impossibility of obtaining reliable fast-kinetics data at the higher temperature. We determined rates at increasing lactate concentrations, up to 500 mM. The effect of similar acetate concentrations at optimal or near optimal lactate concentrations was studied in parallel. Figure 3 shows representative stopped-flow results for heme reduction in the two enzymes. For the Y143F mutant protein, lactate above 5 mM inhibits the process, as already indicated in Figure 2. But acetate appears to also exert a strong inhibitory effect. In several experiments, the difference between the two curves was found to be either non-existent or small. In contrast, heme reduction for the WT enzyme was insensitive to high lactate concentration up to 500 mM (Figure 3B); acetate was therefore not tested. In this case, it thus appears that the WT heme reduction is not subject to substrate inhibition, and the effect observed with the Y143F enzyme can rather be ascribed to a salt effect. Our previously published observations for flavin reduction [Figure 2 in Rouvière-Fourmy et al. (1994)] showed normal hyperbolic curves up to 25 and 40 mM lactate for the Y143F and the WT enzymes, respectively. Taken together with the present results for heme reduction (Figure 3), these observations indicate a lack of substrate inhibition for flavin reduction in both cases.

In the steady-state, ferricyanide reduction was sensitive to both high lactate and acetate concentrations, but differentially so (Figure 4). For the WT enzyme, control experiments indicated that, at 500 mM acetate, the lactate and ferricyanide concentrations used were still saturating. Therefore, the inhibition by acetate was not due to a salt effect on K_m values. For the Y143F mutant, the lactate and ferricyanide K_m values were altered in 500 mM acetate compared to no acetate; they were found to be 6.4 (± 1.0) mM as compared to 3.4 mM (Table 2) for substrate and 2.5 mM as compared to 0.6 mM for the acceptor (Table 3). The data of Figure 4A for acetate were thus acquired at 30 mM lactate, a reasonable compromise between substrate inhibition at low acetate and insufficient substrate saturation at high acetate concentrations. When inhibition by acetate was determined at 10 mM lactate, the data points were very close to those obtained with lactate only (not shown).

For the steady-state reduction of cytochrome *c*, the influence of high acetate concentrations on acceptor half-saturation values was also checked. In the WT enzyme case, the cytochrome *c* K_m value was raised from 45 (no acetate) to 355 μM (500 mM acetate). Figure 5B shows the results of inhibition studies at 450 μM (open symbols) and at 1 mM (filled symbols) cytochrome *c*. Inhibition by acetate was slightly less at the higher acceptor concentration, but substrate inhibition was not affected. For the mutant enzyme, the

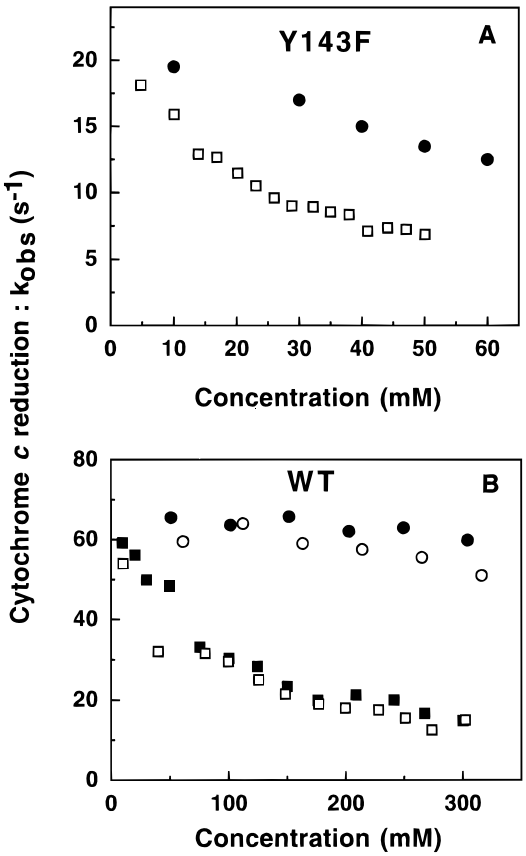


FIGURE 5: Steady-state rates of cytochrome *c* reduction at high lactate and acetate concentrations: (□ and ■) lactate, (○ and ●) acetate. Experiments were carried out in 0.1 M phosphate buffer, 1mM EDTA, pH 7, at 5 °C. (A) Y143F mutant flavocytochrome b_2 (450 μM cytochrome *c* and 10 mM lactate for acetate inhibition). (B) WT enzyme; open symbols: 450 μM cytochrome *c*; filled symbols: 1 mM cytochrome *c*.

cytochrome *c* K_m value was found to be lower in 50 mM acetate (61 μM) than without acetate (131 μM, Table 3). Here again then, the inhibition exerted by acetate (Figure 5A) could not have arisen from insufficient acceptor saturation. The overall conclusion from the data of Figures 4 and 5 is that the reduction of both acceptors is differentially affected by lactate and acetate.

In order to better understand the significance of these results, we tested the idea that acetate could be a specific inhibitor, since propionate has been shown to be a competitive inhibitor with K_i = 28 mM in the WT enzyme case (Genet & Lederer, 1990). For this enzyme, the acetate data of Figure 4B fitted a straight line in the Dixon representation for competitive inhibition (not shown). Nevertheless, when acetate inhibition was monitored at other lactate concentrations (0.5, 0.75, and 1 mM) than that used for the experiment of Figure 4B (10 mM), with ferricyanide as acceptor, the results did not fit the pattern expected for competitive, uncompetitive, or mixed inhibition. In contrast, the data for acetate gave satisfactory fits to the Debye−Hückel equation, both for the wild-type enzyme and for the mutant enzyme (Table 5).

Finally, the data for inhibition by high lactate concentrations were analyzed by direct fitting to eq 1 for inhibition by excess substrate. An example of fitting is shown in Figure 6, and all the derived K_i' values are presented in Table 6. It can be observed that, for each enzyme, a higher lactate concentration is required for 50% inhibition of ferricyanide

Table 5: Slopes of Debye−Hückel Plots

conditions	WT	Y143F
heme reduction		
acetate		-5.63 ± 0.92 ($n = 3$)
lactate		-8.06 ± 1.00 ($n = 3$)
ferricyanide reduction[a]		
acetate	-0.36 ± 0.08 ($n = 2$)	-1.00 ± 0.15 ($n = 4$)
cytochrome *c* reduction		
450 μM + acetate	-0.36 ± 0.21 ($n = 1$)	-1.36 ± 0.09 ($n = 3$)
1 mM + acetate	-0.17 ± 0.09 ($n = 1$)	

[a] The ferricyanide concentration was taken into account in the calculation of ionic strength values.

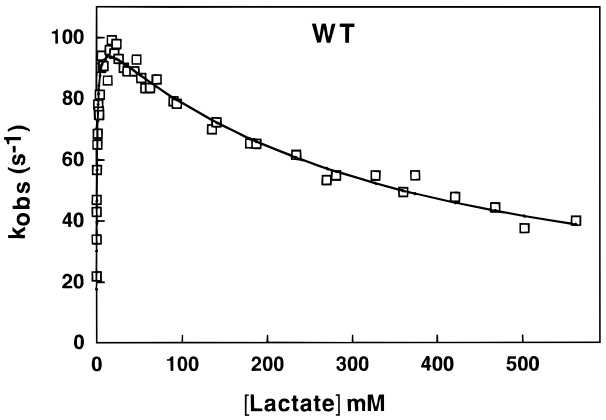


FIGURE 6: Inhibition of ferricyanide reduction by excess substrate for the wild-type enzyme: fitting of experimental data to eq 1.

Table 6: Kinetic Constants (K_i') for Inhibition by Excess Substrate at 5 °C

acceptor	WT enzyme (mM)	Y143F enzyme (mM)
ferricyanide	347 ± 3 ($n = 4$)	75 ± 18 ($n = 4$)
cytochrome *c*		
450 μM	87 ± 16 ($n = 1$)	25 ± 1 ($n = 4$)
1 mM	73 ± 3 ($n = 1$)	

than of cytochrome *c* reduction and that half-inhibitory values are lower for the mutant form than for the WT one.

DISCUSSION

The new data presented above together with previously published ones provide a complete set of experimental kinetic parameters describing the catalytic cycle of Y143F mutant flavocytochrome b_2 and enable a full comparison with the WT enzyme in 0.1 M phosphate buffer at 5 °C. The steady-state data at 30 °C, moreover, permit a comparison with other mutant flavocytochromes b_2 studied under the same conditions as well as a better comparison with data obtained in Tris/HCl buffer, pH 7.5, at 25 °C (Miles et al., 1992). The main conclusions concerning the effect of the Y143F mutation appear to be, independently of buffer and temperature effects, a somewhat decreased substrate affinity and a decrease in the rate of electron transfer from flavin to heme; the latter effect results in lower cytochrome *c* reduction rates and furthermore in direct reduction of ferricyanide by two-electron reduced flavin. Our data strengthen the conclusions presented previously (Miles et al., 1992; Rouvière-Fourmy et al., 1994). It is useful to keep them in mind for the discussion of the other aspects of this work. In particular, previous work makes it appear likely that the impairment of interdomain electron transfer by the Y143F mutation is a consequence of a less facile recognition between the domains,

of a docking problem, more than from an alteration of the electron pathway by the suppression of the phenolic group (Miles et al., 1992). The effects of mutations that alter the hinge region connecting the domains lead to similar conclusions (Sharp et al., 1994, 1996). The crystal structure of the Y143F mutant flavocytochrome b_2 has been solved at 2.9 Å resolution (Tegoni et al., 1995). The structure of the individual domains appeared identical to those of the natural and recombinant wild-type enzymes, except for the absence of the phenolic hydroxyl group. However, the contacts between the domains appeared looser than in the wild-type structure, a feature that was taken as supporting the conclusions of the solution studies and the concept of a dynamic interdomain interface. A possibly interesting new feature disclosed by the present study is the specific sensitivity to buffer nature of the flavin-to-heme electron transfer rate and of the cytochrome *c* reduction rate (Tables 1 and 4). This will be the object of further investigations. In the light of recent results showing that transfer of the second electron from flavin to heme is much slower than the first transfer in Tris/HCl (Daff *et al*., 1996), our observations suggest that the second transfer could be a particularly sensitive step.

Inhibition by Excess Substrate. The results described above indicate a stronger inhibition by lactate than acetate on steady-state reduction of the two different acceptors, for the two enzyme forms. We shall assume in the following discussion that this presumably specific inhibition arises from a binding of lactate, with weak affinity, to one or several of the redox species other than fully oxidized enzyme (Scheme 1). As discussed below, an analysis of the data appears to provide a molecular explanation of the phenomenon.

For the WT enzyme, the stopped flow studies indicated no inhibitory effect of lactate on flavin reduction (step 1 → 2, Scheme 1) or on heme reduction (steps 1 → 3). Inhibition was only observed in the steady-state, which includes the later steps of the catalytic cycle. Since there is no obvious reason to suppose that lactate could have a specific binding site on the heme domain of flavocytochrome b_2, we propose that lactate inhibits electron transfer to acceptors by binding at the active site when the flavin is in the semiquinone state (species 3 and/or 4). Binding to species 4 in particular would prevent further heme reduction and completion of the catalytic cycle in the presence of cytochrome *c* as acceptor. EPR combined with freeze quench techniques could possibly be used to monitor semiquinone stabilization upon lactate binding. An analogy can be found in the inhibition exerted by pyruvate (Tegoni et al., 1984, 1986, 1990). With the *Hansenula anomala* flavocytochrome b_2, these authors showed that pyruvate has a higher affinity for the enzyme when the flavin is in the Fl_{sq} than in the Fl_{ox} or Fl_{red} states; its binding raises the Fl_{sq}/Fl_{ox} mid-point potential above that of the heme, and electron transfer to heme b_2 and consequently to both external acceptors is inhibited. Walker and Tollin (1991, 1992) and Kay and Lippay (1992) confirmed the thermodynamic change imposed by pyruvate binding to the semiquinone state, for both the *H. anomala* and the *S. cerevisiae* enzymes. Furthermore, Daff et al. (1996) showed in a stopped-flow study of the *S. cerevisiae* enzyme that pyruvate inhibits pre-steady-state flavin reoxidation by cytochrome *c*, in agreement with the effects observed for the *H. anomala* enzyme.

The flavocytochrome b_2 crystal structure (Xia & Mathews, 1990) gives a picture of pyruvate bound at the active site with flavin in the semiquinone state (Figure 1B). This structure suggested a mode for lactate binding at the oxidized enzyme active site (Lederer & Mathews, 1987; Dubois et al., 1990). It is easily conceived that lactate could be bound in a rather similar fashion when the flavin is one-electron reduced. It is probable that lactate binding to the $2e^-$-reduced active site, a possibility that is excluded by the present results, would be difficult because of a steric conflict between the substrate tetrahedral C2 atom and the proton on the reduced FMN N5 atom (Rüterjans et al., 1996).

The K_i' values listed in Table 6 are clearly not those of lactate binding constants; they are only half-saturation concentrations for the phenomenon under observation. We want to suggest a rationale for their being lower for the mutant enzyme. It is based on the likely hypothesis that, in solution, the heme domain is mobile (see the introduction), and on the further hypothesis that at each catalytic cycle the domain must somehow move away from the flavodehydrogenase and then dock to it again for electron transfer between the prosthetic groups [for a discussion, see Miles et al. (1992) and Lederer (1994)]. In the crystal structure, the Y143 phenol group is involved in substrate binding in one subunit, in binding to a heme propionate carboxyl group in the other subunit (Figure 1) (Xia & Mathews, 1990). Previous studies of the Y143F mutant enzyme provided support for a role of the phenol group both in Michaelis complex stabilization and in electron transfer to the heme; the latter role has been suggested to be due to a contribution to the docking of the two domains (Miles et al., 1992; Rouvière-Fourmy et al., 1994). Nevertheless, since the phenol group carries only one proton, it seems clear that it cannot bind at the same time to its two carboxylate partners. We thus want to propose that there exists a competition between ligands and the heme-binding domain for approach to Y143 and the flavin; the competition would necessarily increase the substrate concentration required for observing inhibition, relative to the true binding constant value.

As the Y143F mutation leads to an increase in the substrate K_d value from 0.5 to 2.2 mM (Rouvière-Fourmy et al., 1994), loss of the phenol group should also lower the affinity of lactate for the semiquinone state. Nevertheless, inhibition of cytochrome *c* reduction is observed at a lower substrate concentration than in the WT enzyme ($K_i' = 25$ mM versus 80 mM, Table 6). We suggest that the mutation affects the heme domain docking more than lactate binding. In other words, we propose that the more efficient lactate inhibition of cytochrome *c* reduction by the Y143F mutant enzyme is due to a less effective competition by the heme domain.

The different K_i' values for reduction of ferricyanide and cytochrome *c* require a different rationalization. As observed in Tables 2 and 4 and previously described (Lederer, 1991; Miles et al., 1992), ferricyanide reduction by the WT enzyme is faster than cytochrome *c* reduction. This has always been taken as evidence that ferricyanide can be reduced in part by Fl_{sq} (species 3 and 4, Scheme 1) rather than only by the heme, as is cytochrome *c* (Forestier & Baudras, 1971; Iwatsubo et al., 1977). The different K_i' values for reduction of the two acceptors is another indication that the electron flow from lactate may not follow an identical kinetic pathway for the two acceptors. For the Y143F mutant enzyme, ferricyanide reduction appears to bypass the heme, as discussed above. In this case as well, the K_i' value is higher than that for inhibition of cytochrome *c*, the reduction of

which goes via heme b_2. It would thus appear that lactate binding at the flavin semiquinone stage also inhibits transfer to the small reagent ferricyanide. This could arise from a physical block to electron transfer and/or from an electrostatic repulsion between the carboxylate group and the negatively charged ferricyanide.

The inhibition by excess lactate, first detected by Somlo and Slonimski (1966), is not observed for the Morton form of flavocytochrome b_2 (a proteolytically nicked enzyme with lowered k_{cat} and higher substrate K_m). It is not observed either in the Y254L and D282N mutant flavocytochromes b_2 (Gondry, 1994). In these cases, it is possible that lactate affinity for the semiquinone state is decreased. In contrast, Sharp et al. (1996) described mutations leading to more efficient substrate inhibition. In this series of mutations, the hinge region between the two domains was gradually shortened; the authors observed a progressive decrease of the flavin-to-heme electron transfer rate together with a concomitant decrease in K_i' values for cytochrome *c* reduction (from 173 mM for the wild-type enzyme to 14 mM for the slowest mutant, which was slower than the Y143F protein). These results can possibly receive the same interpretation as our own: the efficiency of substrate inhibition might be correlated with decreased competition by the heme-binding domain, since the mutations were devised to disrupt the functional interface between the domains.

Inhibition by High Salt Concentrations. Concentrated acetate inhibits the reduction of both acceptors by the WT enzyme in the steady-state, but only weakly as compared to lactate (Figures 4B and 5B). For the Y143F enzyme, inhibition by acetate is observed already for heme reduction, and in this case lactate appears to be hardly more inhibitory (Figure 3 and Table 5). These inhibitions are therefore exerted at the level of the first electron transfer from flavin to heme (step 2 → 3, Scheme 1). At first sight it seems difficult to imagine that acetate could have a specific effect similar to that of lactate, simply because it has a carboxylate group. Moreover, separate experiments indicated that acetate did not behave as a specific competitive, uncompetitive, or mixed type inhibitor. Finally, a few exploratory experiments showed that chloride also inhibited acceptors reduction as well as heme reduction for the Y143F enzyme. Therefore, we ascribe the inhibitory effects observed with acetate and with lactate on heme reduction for the Y143F mutant enzyme essentially to a medium effect, in all likelihood to an ionic strength effect. The data gave linear Debye−Hückel plots. Their slope values are given in Table 5. Their physical meaning cannot be interpreted directly due to the complexity of the kinetic scheme and the high experimental ionic strengths. Nevertheless, the figures are a means of describing the phenomenon in quantitative terms, and they probably bear a relationship to the sensitivity of the various steps to ionic strength.

The weak inhibition by acetate observed with the WT enzyme in the steady-state obviously affects one or several of the steps occurring after the formation of species 3 (Scheme 1). In the absence of further data, it is impossible to decide which. But it is interesting to consider the situation with the Y143F mutant enzyme. With the latter, ferricyanide is directly reduced by the flavin; it follows that electron transfer from Fl_{red} to both acceptors, heme b_2 and ferricyanide, is sensitive to ionic strength, whereas heme b_2 reduction in the WT enzyme is not. We cannot exclude at present that this difference is a direct consequence of the mutation. Nevertheless, it could also be an indirect one, namely, the result of the uncoupling between the domains.

CONCLUSION

We interpret our combined stopped-flow and steady-state data on WT and Y143F flavocytochromes b_2 as indicating that the inhibition by excess substrate observed in the steady-state arises from binding of lactate at the active site when the flavin is under the Fl_{sq} redox state; the bound ligand appears to prevent transfer of the Fl_{sq} electron to heme b_2 and ferricyanide, with ensuing inhibition of external acceptor reduction. In addition, different inhibitory effects of ionic strength were observed for the WT and the mutant enzymes. In particular, electron transfer from Fl_{red} to heme in the Y143F mutant enzyme is inhibited at high salt concentrations, whereas it is not in the wild-type enzyme. These differences could be a direct consequence of the mutation, or an indirect one caused by the uncoupling between the domains that results from the Tyr → Phe substitution. Studies on the isolated corresponding flavodehydrogenase domains should contribute to distinguishing between the two possibilities.

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