Role of Tyrosine 143 in Lactate Dehydrogenation by Flavocytochrome b_2. Primary Kinetic Isotope Effect Studies with a Phenylalanine Mutant†

Nathalie Rouvière-Fourmy,‡ Chantal Capeillère-Blandin,§ and Florence Lederer*

Centre National de la Recherche Scientifique, URA 1461, Hôpital Necker, 161 rue de Sèvres, 75743 Paris Cédex 15, France

Received July 30, 1993; Revised Manuscript Received October 28, 1993®

ABSTRACT: Flavocytochrome b_2 catalyzes the oxidation of lactate at the expense of cytochrome *c*. After flavin (FMN) reduction by the substrate, reducing equivalents are transferred one by one to heme b_2, and from there on to cytochrome *c*. The crystal structure of the enzyme is known at 2.4-Å resolution, and specific roles in catalysis have been assigned to active side chains. Tyr143 in particular, located at the interface between the flavodehydrogenase moiety and the heme-binding domain, was thought to take part in substrate binding, as well as to orient the heme-binding domain for efficient electron transfer. A first study of the properties of a Tyr143Phe mutant showed that the major effect of the mutation was to decrease the rate of electron transfer from flavin to heme [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]. In the present paper, we focus on the effect of the mutation on catalysis of lactate dehydrogenation. We report the deuterium kinetic isotope effects on flavin reduction as measured with stopped-flow methods and on cytochrome *c* reduction in the steady-state using L-[2-^{2}H]lactate. For the wild-type enzyme, isotope effects on FMN reduction, $^D(k^F_{red})$ and $^D(k^F_{red})/K_m$), were 7.2 ± 0.9 and 4.2 ± 1.3, respectively, and for the Y143F mutant values of 4.4 ± 0.5 and 3.9 ± 1.1 were obtained. Calculations, from deuterium isotope effects, of substrate K_d values, combined with knowledge of k_{cat}/K_m values, lead to the conclusion that Tyr143 does stabilize the Michaelis complex by hydrogen bonding to a substrate carboxylate, as was postulated; but the mutation does not destabilize the transition state more than the Michaelis complex. It is concluded that Tyr143 does not play the role of an acid or an electrophilic catalyst which would stabilize the carbanion-like transition state formed in the initial step of the reaction. Tritium isotope effects were also determined using DL-[2-^{3}H]lactate and yielded $^TV/K$ figures of 15.8 ± 1.7 and 11.3 ± 0.5 for the wild-type and the Y143F mutant. Analysis of the results supports the idea that the ^DV effects determined for FMN reduction are intrinsic isotope effects values and therefore that the mutation induces a change in the structure of the transition state.

Flavocytochrome b_2 (L-lactate cytochrome *c* oxidoreductase, EC 1.1.2.3) catalyzes the oxidation of lactate to pyruvate. In the intermembrane space of yeast mitochondria, this reaction is coupled to cytochrome *c* reduction. The enzyme is a homotetramer of 240 000 Da; each one of its subunits carries two cofactors, FMN and protoheme IX (Lederer, 1991a).

The three-dimensional structure of flavocytochrome b_2 from *Saccharomyces cerevisiae* has been refined to 2.4-Å resolution (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990). The monomer is folded into two distinct domains: the heme-binding domain, comprised of the first 100 residues, and the flavodehydrogenase domain. Furthermore the asymmetric unit contains two distinguishable subunits. One of them (S1) has both domains visible; the cofactors are approximately coplanar, and the closest distance between the heme edge and FMN N5 is 9.6 Å. A heme propionate points toward the flavin; its carboxylate oxygens receive hydrogen bonds respectively from the phenol group of Tyr143 and from a fixed water molecule, which is itself hydrogen bonded to atoms N5 and O4 of FMN. In this subunit, both cofactors are presumably reduced. On the other hand, in subunit S2, only the flavodehydrogenase domain is visible. The other domain is not seen, due to positional disorder. On the *si* face of the flavin, a piece of extraneous electron density is ascribed to pyruvate (the reaction product) complexed at the active site, where the cofactor is thought to be in the semiquinone state (Xia & Mathews, 1990).

Roles in catalysis of lactate dehydrogenation have been ascribed to active-site side chains on the basis of the crystal structure, of previous mechanistic studies in solution which had pointed to a carbanion mechanism, as well as of molecular modeling studies (Lederer & Mathews, 1987; Dubois et al., 1990; Lederer, 1991a,b). These are summarized in Figure 1. Briefly, the Michaelis complex is proposed to be stabilized by an electrostatic interaction between substrate carboxylate and Arg376, as well as by three hydrogen bonds to Arg376, Tyr143, and Tyr254. His373 is the general base which generates the carbanion. Tyr254, initially thought to act as a general base in the catalysis of electron transfer to the flavin (Lederer & Mathews, 1987), now rather appears to be particularly important for transition state stabilization during carbanion formation (Dubois et al., 1990). In addition, Ghisla and Massey (1991) suggested that Tyr143 could act as an electrophilic catalyst and provide stabilization to the carbanion-like transition state. On the other hand, the exact mechanism of flavin reduction by the carbanion, through either intermediate covalent adduct formation or two single electron-transfers in quick succession, is still open to debate (Ghisla,

† This work was supported by a grant from the CNRS (Interface Chimie-Biologie).

* Address correspondence to this author at URA CNRS 1461, Clinique Néphrologique 161, rue de Sèvres, Hôpital Necker, 75743 Paris Cédex 15, France. Telefax: (33-1) 43 06 23 88.

‡ Present address: Institut National de la Santé et de la Recherche Médicale, U 33, 80 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cédex, France.

§ Present address: Centre National de la Recherche Scientifique, URA 400, 45 rue des Saints Pères, 75006 Paris.

® Abstract published in *Advance ACS Abstracts*, December 15, 1993.

0006-2960/94/0433-798$04.50/0

1984; Ghisla & Massey, 1989, 1991; Bruice, 1980; Lederer, 1991b).

In the wild-type enzyme, the essentially rate-limiting step was shown to be α-proton abstraction from the substrate (Lederer, 1974; Pompon et al., 1980). After flavin reduction, electron transfer to heme b_2 takes place in two monoelectronic steps, with the intermediate formation of an anionic flavin semiquinone (Hiromi & Sturtevant, 1965; Ogura & Nakamura, 1966; Capeillère-Blandin et al., 1975). The crystal structure of the enzyme shows that the medium between the two cofactors consists essentially of the solvent, and that their mutual distance appears favorable for electron tunneling. Nevertheless, the location of Tyr143 appears intriguing, at the interface between the two functional domains, with its aromatic ring more or less perpendicular to the cofactors' planes (but not interposed between them) and with its capacity to hydrogen bond either to the substrate carboxylate or to a heme propionate in the crystal structure. It was suggested that, besides its probable function in catalysis of substrate dehydrogenation, Tyr143 could perhaps play a role in electron transfer to heme.

The importance of Tyr143 in the flavocytochrome b_2 function has been probed by site-directed mutagenesis. Miles et al. (1992) carried out a study of the properties of a Tyr143Phe mutant, using steady-state and stopped-flow studies combined with kinetic isotope effect determinations. The mutation was found to induce an important reduction of the flavin to heme electron transfer rate but to have less marked effects on lactate dehydrogenation proper, with, however, an intriguing decrease of the kinetic isotope effect on flavin reduction. We report here part of a study carried out under somewhat different conditions, the results of which illuminate the effects of Tyr143Phe mutation on substrate interactions at the active site and provide a better definition of the Tyr143 role in catalysis of cofactor reduction by lactate.

MATERIALS AND METHODS

Chemicals. L-[2-^{2}H]Lactate was synthesized enzymatically as described by Pompon et al. (1980). DL-[2-^{3}H]lactate was synthesized by chemical reduction of pyruvate by Na[^{3}H]-BH_4, as follows. To 60 μmol of pyruvate in 0.9 mL of 0.1 M Na^+/K^+ phosphate buffer and 1 mM EDTA, pH 8.2 was added 0.45 μmol of Na[^{3}H]BH_4 (60 Ci/mmol, CEA-Saclay) in 0.1 mL of 1 M NaOH. After 20 min at room temperature, 680 μmol of solid unlabeled $NaBH_4$ was added in 3–4 portions. The reaction was stopped with 100 μL of concentrated HCl after another 30 min. The released tritium gas was evaporated under a nitrogen stream before applying the reaction mixture to a column of AG1-X8 (OH^- form; 0.9 × 17 cm). The column was washed with water to remove tritiated water before elution with a 400-mL linear gradient of formic acid (0–1.2 M). The fractions containing DL-[2-^{3}H]lactate were concentrated to a small volume by rotary evaporation and submitted to a second chromatography under identical conditions. The pooled fractions were concentrated to a volume of about 1 mL and neutralized with solid $NaHCO_3$. The specific radioactivity of the final product was 388 mCi/mmol; severe radiolysis took place when the sample was stored at −80 °C, so that eventually samples were diluted about 11-fold with unlabeled L-lactate and stored in liquid nitrogen.

Enzymes. Both wild-type and the Y143F mutant flavocytochrome b_2 were prepared from transformed *Escherichia coli* (Black et al., 1989; Miles et al., 1992). Cells were grown and the enzyme extracted and purified as described in Dubois et al. (1990). Enzyme stocks were stored reduced under argon

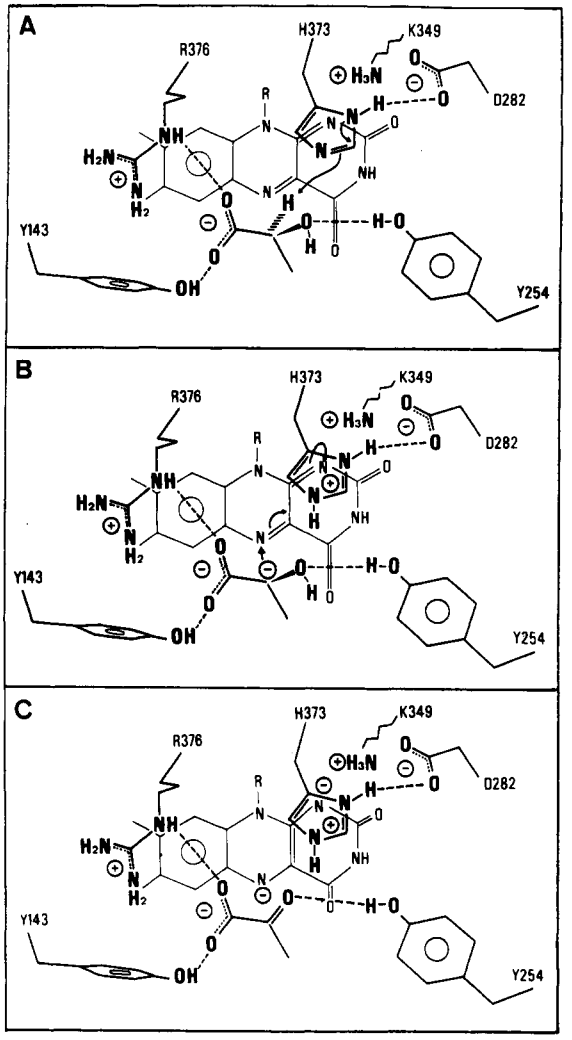

FIGURE 1: Proposed mechanism for lactate dehydrogenation by flavocytochrome b_2 [from Lederer (1992)]. (A) Michaelis complex; (B) carbanion stage; (C) Fl_{red}·product complex. For details, see the introduction. In panel B, the arrow between the substrate carbanion and FMN N5 is only meant to indicate that electrons leave the substrate and are eventually found on the cofactor. For a discussion of the possible mechanism for this step, see Lederer (1991b) and the introduction.

at 4 °C as 70% ammonium sulfate precipitates in standard buffer (0.1 M Na^+/K^+ phosphate buffer, 1 mM EDTA, pH 7.0) containing 20 mM DL-lactate. For the experiments, the precipitates were redissolved in a small volume of lactate-free buffer and either dialyzed overnight or filtered on a Sephadex G-25 column (2 × 12 cm) in standard buffer. The resulting fully oxidized enzyme could be stored at −80 °C for several months without any activity loss. Flavocytochrome b_2 concentration was expressed relative to one heme [ϵ^{ox}_{413}) = 129 mM^{-1} cm^{-1}; ϵ^{red}_{423} = 183 mM^{-1} cm^{-1} (Labeyrie et al., 1978)]. Enzyme activities were routinely checked at 30 °C in the presence of 20 mM L-lactate, using as acceptor 1 mM ferricyanide for the wild-type protein and 13 mM ferricyanide in 2-mm pathway cuvettes for the mutant ($\Delta\epsilon_{420}$ = 1.04 mM^{-1} cm^{-1}). Average values were 223 ± 27 s^{-1} (n = 9) and 300 ± 48 s^{-1} (n = 22) for wild-type and mutant enzymes, respectively (units are moles of substrate reduced per second and per heme).

Stopped-Flow Studies. Stopped-flow studies were carried out with a Gibson-Durrum apparatus modified as described by Capeillère-Blandin (1991), with a 2.2-ms dead time and an optical pathlength of 1.9 cm. The temperature was controlled at 5.0 ± 0.5 °C. Flavin was monitored at 438.3

nm, which corresponds to a heme isosbestic point. This wavelength was regularly verified by calibrating the monochromator with oxidized and reduced cytochrome b_2 core, as described in Capeillère-Blandin et al. (1975). For each substrate concentration, at least two traces were recorded at each time sweep. Data analysis was carried out with an Apple II computer. Experimental traces were fitted to an equation for two exponential terms by application of a nonlinear iterative regression program based on a least-squares criterion, as previously described (Capeillère-Blandin, 1991). Finally, the observed rate constants were extracted from analysis of four to six traces.

Steady-State Studies. Steady-state studies were carried out at 5 °C with a Uvikon 930 spectrophotometer, in standard buffer. The concentration of the acceptor cytochrome *c* was 450 μM, and cuvettes of 0.2-cm pathlength were used. The reaction was monitored at 550 nm, using $\epsilon_{red\text{-}ox} = 20\ 500\ M^{-1}\ cm^{-1}$. The $K_{m,app}$ values for cytochrome *c* were found to be 87 ± 15 μM and 8.5 ± 3.4 μM (30 °C) for the wild-type and the mutant enzymes, respectively. Thus, a 450 μM concentration was 84% saturating for the wild-type protein and 98% for Y143F. Higher concentrations could not be used without running the risk of exceeding the linearity range of the spectrophotometer.

Experiments with [2-^{3}H]Lactate. The reaction was carried out in 5 mL of standard buffer at 5 °C. At time zero, the reaction mixture contained 2.2 μM enzyme (wild-type or mutant), 8 mM ferricyanide, and 66.8 mM L-lactate in DL-[2-^{3}H]lactate (2.25 μCi/μmol). When all the ferricyanide had been consumed, as attested by disappearance of the yellow color, a 2-mL aliquot was withdrawn, and the reaction mixture acidified with 0.1 volume of concentrated HCl. To the rest of the sample was added another concentrated ferricyanide aliquot so as to bring back its concentration to 8 mM. When this second aliquot had been consumed, a 1-mL aliquot of the sample was acidified, and the rest was again supplemented with ferricyanide, etc. The volume of the successive aliquots was calculated so that they should contain more or less the same amount of pyruvate each time, and this was essentially determined by the total amount of ferricyanide consumed up to that time. With this procedure, the degree of substrate conversion increased from one aliquot to the next. These samples were kept frozen at −80 °C until they were chromatographed on an AG1-X8 column as described by Urban and Lederer (1985), in order to separate tritiated water, residual tritiated lactate, and nonradioactive pyruvate. L-Lactate was titrated with flavocytochrome b_2 in the presence of excess ferricyanide, pyruvate with beef heart lactate dehydrogenase and excess NADH, as described (Urban & Lederer, 1985). Radioactive fractions were counted in an LKB Wallac 1410 scintillation counter. For each aliquot, $^TV/K$ was determined according to

$$^TV/K = \frac{\ln(1-f)}{\ln[1-f(SA_{(f)}/SA_0)]}$$

where *f* is the fractional conversion to product, $SA_{(f)}$ is the specific activity of the product at fractional conversion *f* (in our case total dpm in water/pyruvate formed), and SA_0 the initial lactate specific activity (Melander, 1960; Cleland, 1977). The radioactivity found in water was corrected for the radioactivity obtained in the same fraction upon chromatography of an identical amount of DL-[2-^{3}H]lactate under identical conditions.

RESULTS

The first published comparison between wild-type and Y143F mutant flavocytochrome b_2 reported, among other things, a detailed stopped-flow analysis of flavin and heme reduction by L-[2-^{1}H]lactate and L-[2-^{2}H]lactate (Miles et al., 1992). Thus a $^D(k^F_{red})$ of 8.1 ± 1.4 was determined for the flavin reduction of the wild-type recombinant enzyme in 10 mM Tris-HCl, pH 7.5 (I = 0.1 M), at 25 °C. This value was in satisfactory agreement with the value of 8.0 ± 1.0 obtained by Pompon et al. (1980) using the enzyme purified from yeast at 5 °C in 0.1 M phosphate buffer and 1 mM EDTA, pH 7.0 (I = 0.22 M). For the mutant, the $^D(k^F_{red})$ value was found to be 4.3 ± 0.8. This result was intriguing since flavin reduction is thought to involve at least two steps, namely, abstraction of substrate α-proton and then electron transfer from carbanion to flavin (with or without intermediate covalent adduct formation) (Figure 1). The lowering of the isotope effect between WT and mutant could arise from either of two causes: a lowering of the rate of the electron transfer step so that it would become partially rate-limiting or a change in the intrinsic isotope effect on proton abstraction. Furthermore, initial studies with the mutant appeared to indicate that it worked faster in phosphate buffer (Miles et al., 1991). Therefore, we decided to carry out a stopped-flow study in phosphate buffer, which would be complemented by a determination of the discrimination against tritium during the oxidation of [2-^{3}H]lactate, so as to hopefully be able to calculate the intrinsic isotope effect on proton abstraction. In view of the high reaction rates observed, we had to work at 5 °C.

Stopped-Flow Experiments. Flavin reduction was monitored at 438.3 nm as described under Materials and Methods, using a range of L-[2-^{1}H]lactate and L-[2-^{2}H]lactate concentrations from 0.1 mM to between 20 and 40 mM. The stopped-flow traces were generally biphasic, as observed by previous investigators (Suzuki & Ogura, 1970a; Capeillère-Blandin et al., 1975; Pompon et al., 1980; Miles et al., 1992). Under these conditions, the rate constants for the fast and slow phases differ by a factor of 20–30. It has been pointed out before that the second phase is too slow to have kinetic relevance during turnover (Capeillère-Blandin et al., 1975). Indeed, in the presence of acceptors, each subunit functions as a two-electron shuttle; but in absence of acceptors full reduction requires a total of three electrons per subunit, or six lactate molecules per tetramer, and not four. In the absence of acceptor, after each subunit has received two reducing equivalents from a lactate molecule, a slow intramolecular electron reshuffling occurs which leads to the final oxidation of two more substrate molecules by two reoxidized flavins per tetramer. It is the combination of these events which gives rise to the two phases (Capeillère-Blandin et al., 1975; Pompon et al., 1980; Pompon, 1980).

Figure 2 gives the variation of the first phase rate constant (k^F) as a function of lactate and deuterolactate concentration for both wild-type and mutant enzymes. In both cases, typical hyperbolic saturation curves were obtained. The best fits for parameters k^F_{red} and K_m are given in Table 1. The values obtained under the same conditions with the wild-type enzyme purified from yeast are also recalled in Table 1, as well as those obtained with the recombinant enzyme under the different experimental conditions mentioned above (Miles et al., 1992).

The overall agreement between the values obtained with the wild-type enzyme from *S. cerevisiae* and the recombinant one should first be noted. Secondly, when one compares the

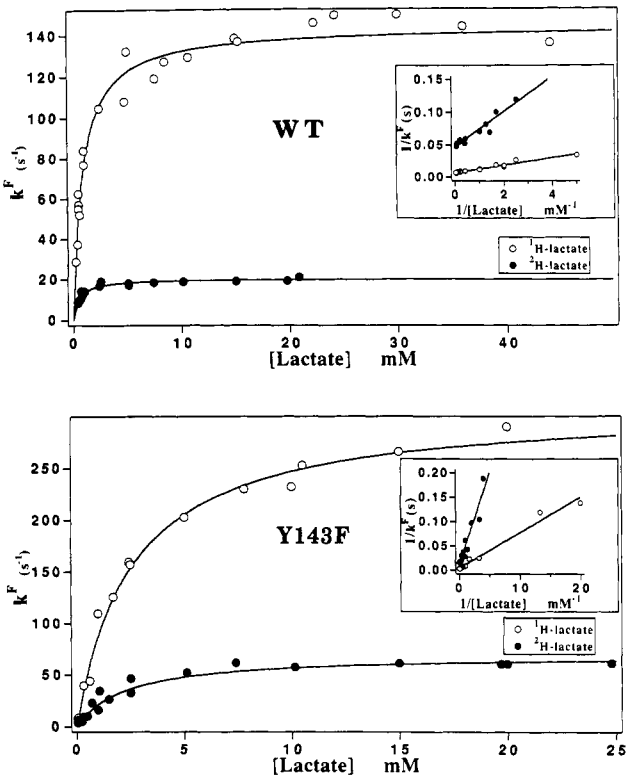

FIGURE 2: Flavin reduction rate as a function of substrate concentration (fast phase), for L-[2-^{1}H]lactate and L-[2-^{2}H]lactate. The enzyme concentration was on the order of 10 μM in 0.1 M phosphate buffer and 1 mM EDTA, pH 7.0, at 5 °C. The experimental points are the average of four to six determinations. The results of two independent series of measurements were merged in order to obtain the profile shown. The solid curves were calculated by fitting the data to a hyperbolic function with a nonlinear iterative regression program. The insets show the same data as double-reciprocal plots.

present results with those of Miles et al. (1992), although absolute rate values cannot be directly compared between experiments at 5 and 25 °C, it is clear that neither the temperature nor the buffer composition nor a slight pH difference seem to affect the K_m values for lactate or the $^D(k^F_{red})$ values. Therefore, the studies in phosphate at 5 °C and in Tris-HCl/NaCl at 25 °C both lead to the observation that the mutation induces a 3-fold increase in K_m and roughly a halving of the isotope effect on k^F_{red}. But an obvious difference in relative k^F_{red} values for wild-type and mutant enzymes appears between the conditions used by Miles et al. (1992) and our conditions: in 0.1 M phosphate buffer, pH 7.0, at 5 °C, the mutant enzyme at lactate saturation has its flavin reduced about twice as fast as the wild-type one, so that on the whole the catalytic efficiency of the Y143F mutant, with respect to flavin reduction, is hardly different from that of the wild-type enzyme.

Steady-State Experiments with Cytochrome c as Acceptor. Table 2 reports the results of steady-state studies carried out with cytochrome *c* as electron acceptor. Under these conditions, after initial flavin reduction, further electron transfer steps take place: $Fl_{red} \rightarrow$ heme b_2^{3+}, $Fl_{sq} \rightarrow$ heme b_2^{3+}, and heme $b_2^{2+} \rightarrow$ cytochrome c^{3+}. Cytochrome *c* is known to be reduced only by heme b_2, not by FMN (Ogura & Nakamura, 1966; Capeillère-Blandin et al., 1980). The overall conclusion drawn from our results is similar to that of Miles et al. (1992) (figures reproduced in Table 2): the Y143F mutant is less efficient than the wild-type enzyme for cytochrome *c* reduction. This is reflected in a near insensitivity of cytochrome *c* reduction to deuterium substitution in the substrate. Miles et al. (1992) showed that this consequence of the mutation arises from an alteration of the flavin to heme electron transfer rate which appears to become the rate-limiting step in cytochrome *c* reduction in the mutant. Again, as for the stopped-flow results (Table 1), differences in relative values can be observed between the wild-type and the mutant enzymes when one compares the results obtained in Tris buffer at 25 °C and in phosphate buffer at 5 °C. It would, however, be premature to make any comment at this stage, in view of the complexity of the kinetic mechanism of cytochrome *c* reduction by flavocytochrome b_2.

Tritium Kinetic Isotope Effects. DL-[2-^{3}H]lactate was used as a substrate with ferricyanide as electron acceptor; the choice of this electron acceptor, rather than of cytochrome *c*, will be justified under Discussion. Discrimination against tritium was determined for various degrees of conversion of substrate to pyruvate, as described under Materials and Methods. The results are presented in Table 3. It can be seen that the values of $^TV/K$ do not appear to depend on the degree of conversion up to at least 50% conversion, while on the other hand they are significantly different between the wild-type and the mutant enzymes.

DISCUSSION

As mentioned in the introduction, the first paper about the properties of mutant Y143F showed that the main effect of the mutation was to reduce the rate of flavin to heme electron transfer (Miles et al., 1992). Our aim, upon undertaking the experiments reported here, was to obtain more detailed information on the role of the phenolic hydroxyl in the catalysis of lactate dehydrogenation which leads to flavin reduction. While the K_m increase in the mutant was expected in view of the loss of the putative hydrogen bond to a substrate carboxylate oxygen, the drop in $^D(k^F_{red})$ raised questions, as detailed below.

Scheme for Flavin Reduction. As shown in Scheme 1, flavin reduction by lactate involves in kinetic terms at least two chemical steps: α-proton abstraction (step 2) followed by electron transfer (step 3) (see also Figure 1). Electron transfer itself is not a single chemical step either (see the introduction) but for kinetic purposes can be considered as such since it has been impossible to show the existence of intermediates. The $^D(k^F_{red})$ value determined under all experimental conditions for WT flavin reduction ($^D(k^F_{red})$ = 7–8, Table 1) suggests that for this enzyme the first chemical step, namely, the isotope-sensitive step, is fully rate-determining for flavin reduction. We cannot of course rigorously exclude that this value would rather be a low one in a case of proton tunneling with partial rate limitation by the electron transfer step(s) (Klinman, 1978). Indeed, a number of cases of hydrogen tunneling in enzymes has been demonstrated recently, with or even without noticeably large intrinsic isotope effects (Cha et al., 1989; Grant et al., 1989; Bahnson et al., 1993). If, however, we consider the flavocytochrome b_2 case in the most straightforward manner, k^F_{red} should then reflect the proton abstraction rate constant (k_2); k_3 would have to be much larger and could actually be several orders of magnitude higher than k^F_{red}. Thus, in the mutant, the lower $^D(k^F_{red})$ value could originate from two alternative phenomena. A first, perhaps unlikely, hypothesis would propose an even higher proton removal rate than experimental flavin reduction rate combined with a perhaps precipitous drop in the electron transfer rate, so that this step would become partially rate-limiting in the mutant. The second hypothesis would propose a change in the intrinsic isotope effect on the C_α–H bond rupture step induced by the

Table 1: Stopped-Flow Kinetic Parameters and Deuterium Isotope Effects for Flavin Reduction in Wild-Type and Y143F Flavocytochrome b_2[a]

	L-[2-^{1}H]lactate			L-[2-^{2}H]lactate				
	k^F_{red} (s^{-1})	K_m (mM)	k^F_{red}/K_m (mM^{-1} s^{-1})	k^F_{red} (s^{-1})	K_m (mM)	k^F_{red}/K_m (mM^{-1} s^{-1})	$^D(k^F_{red})$	$^D(k^F_{red}/K_m)$
phosphate, 5 °C, (this work)[b]								
WT (*E. coli*)	144 ± 4	0.89 ± 0.07	162 ± 17	20 ± 2	0.52 ± 0.08	39 ± 8	7.2 ± 0.9	4.2 ± 1.3
Y143F	310 ± 19	2.55 ± 0.26	121 ± 20	70 ± 3	2.26 ± 0.18	31 ± 4	4.4 ± 0.5	3.9 ± 1.1
phosphate, 5 °C, (Pompon et al., 1980)[b,d]								
WT (*S. cerevisiae*)	163 ± 18	0.69 ± 0.15	236 ± 77	20.8 ± 1.5	0.13 ± 0.50	160 ± 73	7.8 ± 1.4	1.5 ± 1.2
Tris-HCl, 25 °C, (Miles et al., 1992)[c]								
WT (*E. coli*)	604 ± 60	0.84 ± 0.20	719 ± 243	75 ± 5	1.33 ± 0.28	56 ± 15	8.1 ± 1.4	12.8 ± 7.7
Y143F	735 ± 80	2.8 ± 0.3	261 ± 56	171 ± 10	3.97 ± 0.40	46 ± 7	4.3 ± 0.8	5.7 ± 2.1

[a] k^F_{red} is the rate constant for the fast phase of flavin reduction at saturating substrate concentration, obtained as described in the legend to Figure 2. The errors given for k^F_{red}/K_m, $^D(k^F_{red})$, and $^D(k^F_{red}/K_m)$ are calculated from the sum of the relative errors on values in the numerator and denominator. $^D(k^F_{red}/K_m)$ values can also be obtained by taking the ratio of the slopes V_{max}/K_m of a Lineweaver–Burk representation of the data. For the experiments described in this work, this would give values of 4.5 ± 1.6 instead of 4.2 ± 1.3 for WT and 5.1 ± 1.5 instead of 3.9 ± 1.1 for the Y143F mutant.[b] 0.1 M Na^+/K^+ phosphate buffer, EDTA 1 mM, pH 7.0 (I = 0.22 M) at 5 °C. [c] 10 mM Tris-HCl, pH 7.5, adjusted to I = 0.1 with NaCl, at 25 °C.[d] The values given here are slightly different from the published ones (Pompon et al., 1980) because they were recalculated from the experimental rate constants given in the paper, using the same nonlinear least-squares regression method as used in the present work.

Table 2: Steady-State Kinetic Parameters and Deuterium Isotope Effects for Cytochrome c as Acceptor[a]

	L-[2-^{1}H]lactate			L-[2-^{2}H]lactate				
	k^c_{cat} (s^{-1})	K_m (mM)	k^c_{cat}/K_m (mM^{-1} s^{-1})	k^c_{cat} (s^{-1})	K_m (mM)	k^c_{cat}/K_m (mM^{-1} s^{-1})	^DV	$^DV/K$
phosphate, 5 °C, (this work)[b]								
WT	98 ± 6	0.36 ± 0.02	272 ± 32	26 ± 1	0.38 ± 0.02	68 ± 7	3.8 ± 0.1	4.0 ± 0.8
Y143F	20 ± 4	0.37 ± 0.16	54 ± 34	14 ± 1	0.63 ± 0.30	22 ± 12	1.5 ± 0.2	2.4 ± 2.8
Tris-HCl, 25 °C, (Miles et al., 1992)[c]								
WT	103 ± 5	0.24 ± 0.04	429 ± 92	35 ± 5	0.48 ± 0.10	73 ± 26	3.0 ± 0.6	5.9 ± 3.4
Y143F	11 ± 1	0.23 ± 0.03	48 ± 10	6.5 ± 1.0	0.73 ± 0.03	8.9 ± 1.7	1.7 ± 0.5	5.4 ± 2.1

[a] k^c_{cat}, the rate constant for cytochrome c reduction at saturating lactate concentration, was obtained in the present study by fitting experimental points to the Michaelis–Menten equation with a nonlinear iterative regression program; it is expressed in moles of substrate reduced per second per mole of enzyme. The errors given for k^c_{cat}/K_m, ^DV, and $^DV/K$ are calculated from the sum of the relative errors on values in the numerator and denominator. $^DV/K$ values can also be obtained by taking the ratio of the slopes V_{max}/K_m of a Lineweaver–Burk representation of the data. For the experiments described in this work, this would give values of 5.0 ± 1.0 instead of 4.0 ± 0.8 for WT and 5.3 ± 6.0 instead of 2.4 ± 2.8 for the Y143F mutant. [b] 0.1 M Na^+/K^+ phosphate buffer and 1 mM EDTA, pH 7.0 (I = 0.22 M), at 5 °C. Results are the average of two independent series of experiments. [c] 10 mM Tris-HCl, pH 7.5, adjusted to I = 0.1 with NaCl, at 25 °C.

Table 3: Discrimination against Tritium in the Conversion of [2-^{3}H]Lactate to Pyruvate[a]

WT		Y143F	
f(%)	$^TV/K$	f(%)	$^TV/K$
8.2	17.6	7.3	12.2
14.8	15.8	12.3	10.6
21.4	15.6	19.2	11.3
27.5	11.8	25.7	10.6
46	18.1	36.5	11.9
	M = 15.8 ± 1.7		M = 11.3 ± 0.5

[a] The results were obtained as described under Materials and Methods.

Scheme 1: Kinetic Scheme for Flavin Reduction in Flavocytochrome b_2[a]

$$E{\cdot}Fl_{ox}{\cdot}H_{ox} + LH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}Fl_{ox}{\cdot}H_{ox}{\cdot}LH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}Fl_{ox}{\cdot}H_{ox}{\cdot}L^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$$

$$E{\cdot}Fl_{red}{\cdot}H_{ox}{\cdot}P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E{\cdot}Fl_{red}{\cdot}H_{ox} + P \longrightarrow \longrightarrow$$

[a] LH represents lactate, L^- is its α-carbanion, and P is pyruvate. The multiple arrows after step 4 symbolize the succession of monoelectronic transfer steps between cofactors which lead to complete enzyme reduction in the stopped-flow experiments (Capeillère-Blandin et al., 1975; Pompon et al., 1980). With the wild-type and the mutant enzymes, the fast phase essentially reflects steps 1–4 (see the text for more comments).

mutation, so that k^F_{red} would still reflect the rate of proton abstraction. In this case, its higher value in the mutant (Table 1) would have to be explained.

In Scheme 1, k_{-2} and k_{-3} can be considered as negligible on several grounds, even though flavocytochrome b_2 was shown to catalyze transhydrogenation reactions between hydroxy acids and ketoacids (Urban et al., 1983). First, the redox potential of the lactate/pyruvate couple (–190 mV) is much below that reported for the Fl_{red}/Fl_{ox} couple in flavocytochrome b_2 (–50 mV; Capeillère-Blandin et al., 1975), so that lactate oxidation is favored. Secondly, a k_{cat} of 0.08 s^{-1} (30 °C) was measured for the reduction of pyruvate by reduced flavocytochrome b_2 under transhydrogenation conditions (Urban et al., 1983). By analogy with bromopyruvate, for the reduction of which a strong deuterium isotope effect was measured (Urban & Lederer, 1985), this value should be dominated, in large part if not entirely, by k_{-2}. Taking into account the temperature difference between the measurements, it can be calculated that k_{-2} should be at least several thousand-fold smaller than k^F_{red}. Thirdly, the double-reciprocal plots of the stopped-flow data (Figure 1, insets) are found to be linear. According to Strickland et al., (1975) this is in agreement with Scheme 2, where k_{-2} is zero. If we assume, as is most likely in our case, that the observed flavin reduction rate is

Scheme 2

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP$$

Table 4: Calculation of K_d Values for Lactate from Kinetic Deuterium Isotope Effects[a]

	WT	Y143F
K_d (from steady-state data) (mM)[b]	0.38 ± 0.11	1.04 ± 1.35
K_d (from stopped-flow data) (mM)[b]	0.46 ± 0.23	2.10 ± 1.05
K_d (from stopped-flow K_m) (mM)[c]	0.46 ± 0.34	2.18 ± 1.21
k_{+1} (from stopped-flow K_m) (mM^{-1} s^{-1})[d]	335 ± 121	829 ± 232
k_{-1} (from stopped-flow K_m) (s^{-1})[d]	154 ± 55	1805 ● 505

[a] The error values are calculated from the sum of the relative errors on values of the parameters which appear in the equations used for calculating the relevant constants. [b] Values calculated using the equation $({}^{D}V - 1)/({}^{D}V/K - 1) = K_m/K_d$ (see Discussion). [c] Value calculated as k_{-1}/k_{+1}. [d] Values calculated using the equations $K_m^H = (k_{-1} + k_{red}^{F,H})/k_{+1}$ and $K_m^D = (k_{-1} + k_{red}^{F,D})/k_{+1}$ (see Discussion).

dominated by the rate of carbanion formation, Scheme 1 essentially reduces to Scheme 2. All this reasoning holds for the wild-type enzyme. For the Y143F mutant, it is not known whether the flavin redox potential is altered. If it is, one would expect it to be higher than in the wild-type enzyme, since flavin to heme electron transfer has been noticeably slowed down in the mutant (Miles et al., 1992). Thus pyruvate reduction should be even less favored in the mutant than in the wild-type enzyme. This reasoning, combined with the linearity of the reciprocal plots of stopped-flow data, leads us to conclude that k_{-2} and k_{-3} can also be neglected in the Y143F case. These considerations will be helpful for the discussion of isotope effects. They also justify the use of the more convenient ferricyanide as electron acceptor for the tritium isotope effect experiments, since V/K isotope effects are insensitive to steps occurring after the first irreversible step following the isotope-sensitive step. In our case, then, the nature of the electron acceptor is irrelevant (Scheme 1).

Calculation of the Lactate Dissociation Constant. Several pieces of useful information can be extracted from the experimental data presented above. First, we can calculate K_d values for lactate using the isotope effect values reported in Table 1, according to the following equation (Northrop, 1975; Klinman & Mathews, 1985):

$$\frac{{}^{D}V - 1}{{}^{D}V/K - 1} = \frac{K_m}{K_d}$$

This equation was proposed for steady-state data, and it makes no assumptions as to the values of the intrinsic isotope effect. In Table 4 we compare the value obtained with this equation using the steady-state results of Table 2, to those derived in the same way from the stopped-flow data of Table I. Furthermore, we carried out another calculation, assuming this time that the stopped-flow ${}^{D}(k_{red}^{F})$ values for both wild-type and mutant enzymes are intrinsic values. With this hypothesis, $k_{red}^{F} = k_{+2}$ and we notice that the K_m values for [2-^{1}H]lactate and [2-^{2}H]lactate are different (Table 1). As already pointed out by Pompon et al. (1980), this suggests that k_{+2} is not negligible compared to k_{-1}. We can thus write

$$K_m^H = \frac{k_{-1} + k_2^H}{k_1} \quad \text{and} \quad K_m^D = \frac{k_{-1} + k_2^D}{k_1}$$

since deuterium substitution is not expected to have any effect on substrate on- and off-rate constants (Jencks, 1969). The system of two equations in two unknowns can be solved, yielding k_{-1} and k_{+1} values, from which K_d is obtained. The results of this calculation are also presented in Table 4. It can be seen that, for the wild-type enzyme, the three figures for the calculated K_d are in excellent agreement. For the Y143F mutant, the K_d from steady-state data is fraught with a larger error and thus cannot be considered as significantly different from the other two values, which are pratically identical. Therefore, this series of calculations, which lead to the same conclusion by independant routes, strongly support the idea that in both wild-type and mutant enzymes the measured ${}^{D}(k_{red}^{F})$ is an intrinsic isotope effect and that it is substantially lower in the Y143F enzyme. Furthermore, the figures calculated for the substrate on- and off-rate constants make sense insofar as removal of the Tyr143 hydroxyl group makes binding about 3-fold faster but dissociation more than 10-fold easier.

Kinetic Tritium Isotope Effect. In principle, flavocytochrome b_2 should constitute a favorable case for calculating intrinsic isotope effects on rates using Northrop's equation (Northrop, 1975, 1982):

$$\frac{{}^{D}V/K - 1}{{}^{T}V/K - 1} = \frac{{}^{D}k - 1}{{}^{D}k^{1.44} - 1}$$

where ${}^{D}k$ is the intrinsic isotope effect on k. This is because, in the general expression for kinetic isotope effects,

$${}^{DT}V/K = \frac{{}^{DT}k + C_f + C_r K_{eq}}{1 + C_f + C_r}$$

C_r for Scheme 1 is k_{-2}/k_3 and can be neglected, since $k_{-2} << k_3$ as discussed above. In practice, it has been pointed out repeatedly that Northrop's equation can only be used in cases where the experimental error is less than 3% (Albery & Knowles, 1977; Northrop, 1982; Miller & Klinman, 1982). It is clear that our experimental errors are higher than this, in particular those on deuterium isotope effects. In order to have a satisfactory precision, ${}^{D}V/K$ determinations would have to be carried out with a different method, such as competitive measurements using doubly labeled substrate [see, for example, Grant and Klinman (1989)]. Nevertheless, the tritium isotope effects reported here provide interesting information.

It is clear that the ${}^{T}V/K$ values in Table 3 are different between wild-type and mutant enzymes: using the Swain–Schaad equation (Swain et al., 1958), intrinsic ${}^{D}V/K$ values can be calculated and compared to the experimental ones. For the wild-type enzyme, the figure is 6.8 ± 0.5 compared to the experimental value of 4.2 ± 1.3. For the mutant, the calculation yields 5.4 ± 0.2 compared to 3.9 ± 1.1. Thus, it would appear that in this latter case, within experimental error, both calculated and experimental ${}^{D}V/K$ [i.e., ${}^{D}(k_{red}^{F}/K_m)$] values are very similar to, if not identical with, the ${}^{D}(k_{red}^{F})$ value of 4.4 ± 0.5. This comparison strengthens the idea that the experimental ${}^{D}(k_{red}^{F})$ for the mutant is an intrinsic kinetic isotope effect. For the wild-type protein, on the other hand, there is a clear breakdown of the Swain–Schaad relationship. This can be understood if the commitment factor C_f is not negligible. With Scheme 1, $C_f = k_{+2}/k_{-1}$. The k_{-1} values of Table 4 and the assumption that $k_{red}^{F} = k_{+2}$ lead to $C_f = 0.93$ and 0.17 for the wild-type and the mutant enzymes, respectively. Large commitment factors result in a decrease of ${}^{D}V/K$ values relative to ${}^{D}V$ values. If we use the commitment factors estimated from the deuterium isotope effect studies to calculate ${}^{T}k = {}^{D}k^{1.44}$ from

$${}^{T}V/K - 1 = \frac{{}^{D}k^{1.44} - 1}{1 + C_f}$$

the results are $^{D}k = 10.5 \pm 3.2$ and 5.9 ± 1.0 for the wild-type and the mutant enzymes, respectively. These figures are in fair agreement with the experimental $^{D}(k^{F}_{red})$ values and, more importantly, again lead to the conclusion that the substitution of Tyr143 with Phe leads to a lowering of the intrinsic isotope effect on the rate of C_α–H bond cleavage. We consider that the best values of the intrinsic isotope effects are those given by the ratio of k^{F}_{red} values (Table 1).

Effect of the Mutation on Catalysis of Lactate Dehydrogenation. With the knowledge of K_d, k_{+2}, and ^{D}k, it becomes possible to analyze the effect of the mutation on catalysis of lactate dehydrogenation by flavocytochrome b_2. Several interesting observations can be made. First, Table 4 indicates that in the mutant the dissociation constant for lactate is 4–5-fold higher than in the wild-type enzyme. The figures lead to $\Delta\Delta G^{S}_{app} = -0.86$ kcal/mol. This value corresponds to the difference in energy between the Y143F and wild-type Michaelis complexes. This figure falls within the range of 0.5–1.8 kcal/mol proposed for ΔG^{S}_{app} and ΔG^{S}_{bind} in cases when a hydrogen bond between uncharged groups is removed (Fersht, 1988). This is not necessarily the energy of the hydrogen bond between Tyr143 and the substrate carboxylate since there could still exist a weakly polar interaction between a carboxylate oxygen and the F143 aromatic ring (Burley & Petsko, 1988); such an interaction could itself amount to up to 1 kcal/mol (Thomas et al., 1982). Alternatively, a water molecule could hydrogen bond to the substrate carboxylate and be oriented by a weakly polar interaction with the edge of the phenylalanine ring. Whatever the case, the results are in favor of the existence of the postulated hydrogen bond between Tyr143 and the substrate carboxylate in the Michaelis complex (Lederer & Mathews, 1987; see Figure 1).

The second interesting piece of information pertains to the alteration in activation energy induced by the mutation. The comparison between k^{F}_{red}/K_m values (Table 1) leads to a $\Delta\Delta G^{\ddagger}$ of 0.16 kcal/mol. Thus, it would appear that the Y143 hydroxyl group plays a lesser role in transition state stabilization than in Michaelis complex stabilization under our experimental conditions. One may even wonder at first sight whether the bond exists at all in the transition state. Yet it is still present, otherwise one would not expect a change in intrinsic deuterium isotope effect such as the one observed [a conformational change at the mutant active site is ruled out by preliminary crystallographic results (Tegoni & Cambillau, 1993)]. The absolute value of an intrinsic isotope effect depends on a number of factors, in particular the degree of bond rupture and of symmetry in the transition state (Jencks, 1969; Klinman, 1978). With a $^{D}(k^{F}_{red})$ of 7–8, the lactate α-proton is expected to be equally shared in the transition state between lactate and His373 Nϵ. With a lower isotope effect, the degree of C_α–H bond rupture could be either smaller or higher, and at present we cannot choose between these alternatives. It seems clear, at least, that with little change in energy level the transition state in the mutant has an altered structure and probably does not occur at the same point along the reaction coordinate as for the wild-type. This is an interesting, if ill-understood, difference between the two enzymes disclosed by our isotope effect study.

A third interesting point, in view of the observations presented above, is that it can now be easily explained why k^{F}_{red} is faster in the mutant than in the wild type, a phenomenon which was at first sight surprising. It now turns out to be a clear-cut example of how to maximize k^{F}_{red} by a better stabilization of the transition state than of the Michaelis complex. In the mutant, the transition state is less destabilized than the Michaelis complex; therefore, the energy difference between the two states is smaller and k_{cat} [k^{F}_{red}] is larger than for the wild-type protein (Figure 3).

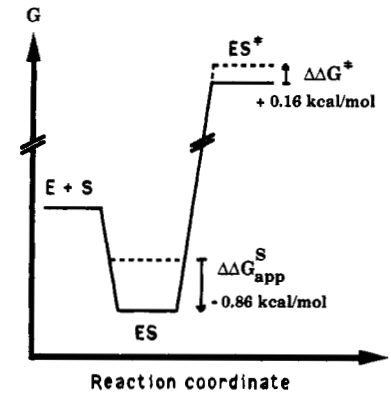

FIGURE 3: Free energy diagram for carbanion formation. $\Delta\Delta G^{S}_{app}$ was calculated using the equation $\Delta\Delta G^{S}_{app} = RT \ln(K_d^{WT}/K_d^{Y143F})$, where the K_d values are taken from Table 4 (stopped-flow data). $\Delta\Delta G^{\ddagger}$ was calculated using $\Delta\Delta G^{\ddagger} = RT \ln[(k^{F}_{red}/K_m)^{WT}/(k^{F}_{red}/K_m)^{Y143F}]$ (Table 1). The values on the diagram correspond to the studies reported in the present paper (phosphate buffer, pH 7, $I = 0.22$ M, 5 °C). The kinetic constants reported by Miles et al. (1992) (see also Table 1) for Tris buffer, pH 7.5, $I = 0.1$ M, 25 °C, yield $\Delta\Delta G^{S}_{app} = -0.6$ kcal/mol and $\Delta\Delta G^{\ddagger} = 0.6$ kcal/mol. The dashed line refers to the mutant.

Finally, when analyzed in the same way as the results reported in this paper, the data obtained by Miles et al. (1992) (recalled in Table 1) yield K_d values of 1.4 and 4 mM for the wild-type and the mutant enzyme, respectively. In Tris buffer, pH 7.5 at 25 °C, $I = 0.1$ M, it would thus appear that both the Michaelis complex and the transition state are destabilized by the same amount (0.6 kcal/mol), which is in agreement with the very similar k^{F}_{red} values reported for the two enzymes (Figure 3). On the other hand, the difference between the $^{D}(k^{F}_{red})$ values for wild-type and mutant enzymes in Tris buffer at 25°C is similar to what was found in the present study (using phosphate buffer at 5°C). Therefore, in spite of small differences in kinetic parameters, due to changes in buffer composition and/or temperature (probably more than to pH changes, see Suzuki & Ogura, 1970b), the general conclusion from the two studies of the Y143F mutant (Miles et al., 1992; this work) is that removal of the Tyr143 hydroxyl group does not destabilize the transition state more than the Michaelis complex and shifts the transition state along the reaction coordinate.

Does Tyr143 Play the Role of an Electrophilic or Acid Catalyst? It is generally considered by organic chemists that formation of a carbanion adjacent to a carboxylate group is a highly unfavorable reaction. The problem of forming such an intermediate in enzymatic reactions has been recently discussed in detail (Gerlt et al., 1991; Gerlt & Gassman, 1992). It was proposed that electrophilic catalysis is a major contributor to the stabilization of the charged transition state; a metal ion, a hydrogen bond or even full transfer of a proton to an enolate or a carboxylate were felt necessary to lower the pK_a of the carbon-bonded hydrogen and hence make the ΔpK_a between this hydrogen and the active-site general base compatible with observed reaction rates. In the case of flavocytochrome b_2, the proximity of Arg376 to the substrate carboxylate indicates the existence of an electrostatic interaction between the two groups which must stabilize the negative charge on the carboxylate oxygen throughout the reaction. It was, however, also conceivable that Tyr143 could provide additional stabilization, in the transition state in particular,

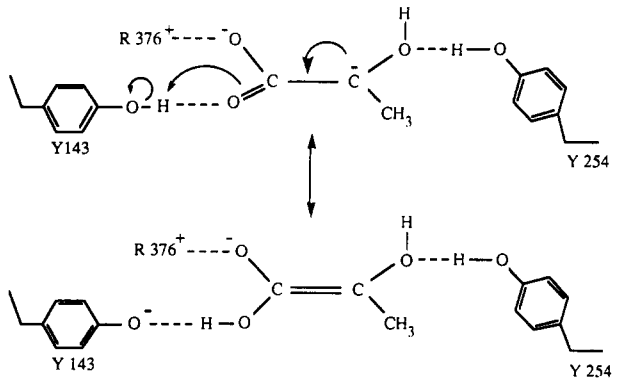

FIGURE 4: Extreme resonance forms of the putative carbanion (Ghisla & Massey, 1991).

by partial or even total transfer of the phenolic proton to an enolized carboxylate (Figure 4). Such a possibility was indeed suggested for α-hydroxy acid-oxidizing flavoenzymes on the basis of the active-site structure of flavocytochrome b_2 and the conservation of active-site residues among family members (Ghisla & Massey, 1991; Lê & Lederer, 1991).

Site-directed mutagenesis, when the putative electrophilic catalyst is not a metal ion but a side chain, constitutes a good method for probing the validity of some hypotheses. Indeed, the literature offers several illustrative examples in this respect. In citrate synthase, where His274 is believed to protonate the enolate of acetyl-CoA in the transition state, the mutation His274 → Gly lowers k_{cat} by about 10^3-fold, with little change in K_m (Alter et al., 1990). In triose phosphate isomerase, His95 is believed to protonate the enediolate intermediate (Lodi & Knowles, 1991); the substitution of Gln for His95 leads again to little change in K_m, while the forward isomerization rate is decreased 140-fold and the reverse rate 380-fold (Nickbarg et al., 1988). For 3-oxo-Δ^5-steroid isomerase, where Tyr14 is thought to act as an electrophile rather than as an acid catalyst, the Tyr14Phe mutant shows an even more spectacular decrease in efficiency: with a less than 3-fold increase in K_m, the mutant is 10^5-fold less active than the wild-type enzyme (Kuliopoulos et al., 1989; Zeng et al., 1992). In a different reaction type, electrophilic catalysis provided by amide side chains (together with backbone >NH groups) during peptide bond hydrolysis by serine and cysteine proteases contributes rate accelerations of one to several hundred-fold (Bryan et al., 1986; Ménard et al., 1991). Thus in all these cases, electrophilic or acid catalysis appears to stabilize the transition state by at least 3 kcal/mol when not much more.

With these examples in mind, the modest destabilization of the transition state introduced by the Tyr143Phe mutation makes it clear that Tyr143 does not act as an electrophilic catalyst. Whatever supplementary stabilization is provided by the enzyme to the carbanion, or carbanion-like transition state, must be ascribed to Arg373 and the rest of the active-site structure. In view of the slowness of carbanion protonation in the reverse reaction (Urban & Lederer, 1985) and of the affinity of ethane nitronate for the enzyme active site (Genet & Lederer, 1990), it could perhaps be believed that some delocalization of the carbanion charge occurs in the transition state (Kresge, 1975). Other phenomena, however, may be at work, like geometrical factors (Scheiner & Hillenbrand, 1985) or solvent effects. Furthermore, there is a fundamental difference between flavocytochrome b_2 and related flavoproteins, on the one hand, and enzymes such as those mentioned above, on the other hand. For isomerases as well as racemases [see, for example, Neidhart et al. (1991)], the carbanion electrons are used for reprotonation at another position or with a different stereochemistry. For enzymes such as citrate synthase or ribulose bisphosphate carboxylase oxygenase, they are used for carbon–carbon bond formation. But, in the case of flavocytochrome b_2 and related enzymes, electrons have to leave the substrate in the course of the oxidation–reduction reaction. Therefore it does not seem reasonable to expect an important delocalization of the carbanion charge. Since charge delocalization is one of the factors considered to be important in lowering the pK_a of the carbon acid and hence to favor proton abstraction, what could be the driving force in the flavocytochrome b_2 case? We would like to suggest that it is the pK_a raise undergone by the active-site base in the reduced enzyme (Urban & Lederer, 1985; Lederer, 1992). The relative orientation of the cofactor and of the His373 imidazole ring induced one of us to compare the two aromatic structures to the plates of a biological capacitor (Lederer, 1992): the positive charge acquired by the imidazole plate upon proton abstraction from substrate would be stabilized by interaction not only with Asp282 but also with the negative charge appearing at N1 on the flavin plate upon transfer of the reducing equivalents from the carbanion (Figure 1).

In conclusion, the comparison of reaction rates and kinetic isotope effects between wild-type flavocytochrome b_2 and mutant Y143F has led to a better definition of the role of the Tyr143 phenolic group: it forms a hydrogen bond with the substrate carboxylate in the Michaelis complex, thus stabilizing it. No electrophilic catalysis is provided by Tyr143, since the hydrogen bond does not appear to be stronger in the transition state. But it is still present at that stage, since its absence appears to shift the position of the transition state for C_α–H bond breaking along the reaction coordinate.

ACKNOWLEDGMENT

We are indebted to Drs. F. Labeyrie and M. Tegoni for their interest in the initial phases of this work and to A. Balme for fruitful discussions concerning the tritium isotope effects.

REFERENCES

Albery, W. J., & Knowles, J. P. (1977) *J. Am. Chem. Soc. 99*, 637–638.
Alter, G. M., Casazza, J. P., Zhi,W., Nemeth, P., Srere, P. A., & Evans, C. T. (1990) *Biochemistry 29*, 7557–7563.
Bahnson, B. J., Park, D. H., Kim, K., Plapp, B. V., & Klinman, J. P. (1993) *Biochemistry 32*, 5503–5507.
Black, M. T., White, S. A., Reid, G. A., & Chapman, S. K. (1989) *Biochem. J. 258*, 255–259.
Bruice, T. C. (1980) *Acc. Chem. Res. 13*, 256–262.
Bryan, P., Pantoliano, M. W., Quill, S. G., Hsiao, H.-Y., & Poulos, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3743–3745.
Burley, S. K., & Petsko, G. A. (1988) *Adv. Protein Chem. 39*, 125–189.
Capeillère-Blandin, C. (1991) *Biochem. J. 274*, 207–217.
Capeillère-Blandin, C., Bray, R. C., Iwatsubo, M., & Labeyrie, F. (1975) *Eur. J. Biochem. 54*, 549–566.
Capeillère-Blandin, C., Iwatsubo, M., Testylier, G., & Labeyrie, F. (1980) in *Flavins & Flavoproteins* (Yagi, K., & Yamamoto, T., Eds.) pp 617–630, Japan Scientific Societies Press, Tokyo.
Cha, Y., Murray, C. J., & Klinman, J. P. (1989) *Science 243*, 1325–1330.
Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273–387.
Dubois, J., Chapman, S. K., Mathews, F. S., Reid, G. A., & Lederer, F. (1990) *Biochemistry 29*, 6393–6400.
Fersht, A. (1988) *Biochemistry 27*, 1577–1580.
Genet, R., & Lederer, F. (1990) *Biochem. J. 266*, 301–304.

Gerlt, J. A., & Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928–5934.
Gerlt, J. A., Kozarich, J. W., Kenyon, G. L., & Gassman, P. G. (1991) *J. Am. Chem. Soc. 113*, 9667–9669.
Ghisla, S. (1982) in *Flavins & Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 133–143, Elsevier North Holland, Amsterdam.
Ghisla, S., & Massey, V. (1980) *J. Biol. Chem. 225*, 5688–5696.
Ghisla, S., & Massey, V. (1989) *Eur. J. Biochem. 181*, 1–17.
Ghisla, S., & Massey, V. (1991) in *Flavins & Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 132–130, Walter De Gruyter & Co., Berlin.
Grant, K. L., & Klinman, J. P. (1989) *Biochemistry 28*, 6597–6605.
Hiromi, K., & Sturtevant, J. M. (1965) *J. Biol. Chem. 240*, 4662–4668.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.
Klinman, J. P. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 46*, 415–494.
Klinman, J. P., & Matthews, R. G. (1985) *J. Am. Chem. Soc. 107*, 1058–1060.
Kresge, A. J. (1975) *Acc. Chem. Res. 8*, 354–360.
Kuliopoulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 28*, 149–159.
Labeyrie, F., Baudras, A., & Lederer, F. (1978) *Methods Enzymol. 53*, 238–256.
Lê, K. H. D., & Lederer, F. (1991) *J. Biol. Chem. 266*, 20877–20881.
Lederer, F. (1974) *Eur. J. Biochem. 46*, 393–399.
Lederer, F. (1991a) in *Chemistry and Biochemistry of Flavoenzymes*, pp 154–242, CRC Press, Boca Raton, FL.
Lederer, F. (1991b) in *Flavins & Flavoproteins 1990* (Curti, B. Ronchi, S., & Zanetti, G., Eds.) pp 773–782, Walter De Gruyter & Co., Berlin.
Lederer, F. (1992) *Protein Sci. 1*, 540–548.
Lederer, F., & Mathews, F. S. (1987) in *Flavins & Flavoproteins* (Edmonson, D. E., & McCormick, D. B., Eds.) pp 133–143, Walter De Gruyter & Co., Berlin.
Lodi, P. J., & Knowles, J. R. (1991) *Biochemistry 30*, 6948–6956.
Mathews, F. S., & Xia, Z.-X. (1987) in *Flavins & Flavoproteins* (Edmonson, D. E., & McCormick, D. B., Eds.) pp 123–132, Walter De Gruyter & Co., Berlin.
Melander, L. (1960) *Isotope Effects on Reaction Rates*, The Ronald Press Company, New York.
Ménard, R., Carrière, J., Laflamme, P., Plouffe, C., Khouri, H. E., Vernet, T., Tessier, D. C., Thomas, D. Y., & Storer, A. C. (1991) *Biochemistry 30*, 8924–8928.
Miles, C. S., Chapman, S. K., Reid, G. A., White, S. A., Mathews, F. S., Rouvière, N., & Lederer, F. (1991) in *Flavins & Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 783–786, Walter De Gruyter & Co., Berlin.
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.
Miller, S. M., & Klinman, J. P. (1982) *Methods Enzymol. 87*, 711–732.
Neidhart, D. J., Howell, P. L., Petsko, G. A., Powers, V. M., Kenyon, G. L., & Gerlt, J. A. (1991) *Biochemistry 30*, 9264–9273.
Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5946–5960.
Northrop, D. B. (1975) *Biochemistry 14*, 2644–2651.
Northrop, D. B. (1982) *Methods Enzymol. 87*, 607–625.
Ogura, Y., & Nakamura, T. (1966) *J. Biochem. (Tokyo) 60*, 77–86.
Pompon, D. (1980) *Eur. J. Biochem. 106*, 151–159.
Pompon, D., Iwatsubo, M., & Lederer, F. (1980) *Eur. J. Biochem. 104*, 479–488.
Reid, G. A., White, S. A., Black, M. T., Lederer, F., Mathews, F. S., & Chapman, S. K. (1988) *Eur. J. Biochem. 178*, 329–333.
Scheiner, S., & Hillenbrand, E. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2741–2745.
Suzuki, H., & Ogura, Y. (1970a) *J. Biochem. (Tokyo) 67*, 277–289.
Suzuki, H., & Ogura, Y. (1970b) *J. Biochem. (Tokyo) 67*, 291–295.
Swain, C. G., Stevers, E. C., Reuwer, J. F., & Schaad, C. J., (1958) *J. Am. Chem. Soc. 80*, 5885–5893.
Tegoni, M., & Cambillau, C. (1993) in *Flavins and Flavoproteins* (Yagi, K., Ed.) Walter De Gruyter, Berlin.
Thomas, K. A., Smith, G. M., Thomas, T. B., & Feldman, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4843–4847.
Urban, P., & Lederer, F. (1985) *J. Biol. Chem. 260*, 11115–11122.
Urban, P., Alliel, P. M., & Lederer, F. (1983) *Eur. J. Biochem. 134*, 275–281.
Xia, Z.-X., & Mathews, F. S. (1990) *J. Mol. Biol. 212*, 837–863.
Xia, Z.-X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F., & Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2629–2633.
Zeng, B., Bounds, P. L., Steiner, R. F., & Pollack, R. M. (1992) *Biochemistry 31*, 1521–1528.