Functional Properties of the Histidine−Aspartate Ion Pair of Flavocytochrome b_2 (L-Lactate Dehydrogenase): Substitution of Asp282 with Asparagine†

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ABSTRACT: The FMN prosthetic group of flavocytochrome b_2 or L-lactate dehydrogenase oxidizes lactate to pyruvate. The reducing equivalents are then transferred one by one, intramolecularly, to heme b_2 and then to external acceptors. Substrate oxidation is thought to begin with abstraction of the substrate α-hydrogen as a proton by an enzyme base. It has been proposed that this role is played by His373, which lies close to the flavin in the crystal structure and interacts with Asp282. It has also been shown before, using hydrogen exchange measurements, that the pK_a of His373 is substantially increased in the wild-type reduced enzyme compared to that in the oxidized state. We report here the enzymatic properties of the D282N mutant flavocytochrome b_2. Steady-state rate measurements with [2-^{1}H]lactate and [2-^{2}H]-lactate indicate that, as predicted, the Michaelis complex stability is hardly affected, whereas the transition state for proton abstraction increases in energy by 2.8 kcal/mol. Steady-state inhibition studies were conducted with a number of active-site ligands: sulfite, D-lactate, pyruvate, and oxalate. Binding was found to be most affected for oxalate, but kinetic patterns indicated oxalate and pyruvate were still capable of binding to the enzyme both at the oxidized and semiquinone stages, whereas inhibition by excess substrate, due to lactate binding at the semiquinone stage, was lost. Finally, analysis of the intermolecular hydrogen transfer catalyzed by the enzyme between [2-^{3}H]lactate and fluoropyruvate indicated that the substitution with asparagine facilitates exchange of the histidine-bound proton and hence induces a decrease in the pK_a value of H373 in the reduced enzyme of about 1.4 pH units. Nevertheless, the rate constant value for exchange with the solvent of the enzyme-bound substrate α-proton indicates that H373 is still protonated in the reduced mutant enzyme at neutral pH. Thus, the D282N mutation destabilizes the transition state for proton abstraction and decreases the pK_a of H373 in the reduced enzyme but is insufficient to bring it back to a normal value.

Flavocytochrome b_2 oxidizes L-lactate to pyruvate. The reaction is catalyzed by its FMN prosthetic group, which then reduces heme b_2 in the same subunit in two successive one-electron transfer steps. Cytochrome *c* is the physiological external oxidant and can be replaced by artificial electron acceptors (Lederer, 1991). The enzyme belongs to a family of FMN-dependent α-hydroxy-acid-oxidizing enzymes, which can be separated into two categories according to the nature of the flavin oxidative half-reaction. The dehydrogenases−oxidases make use of oxygen, with formation of H_2O_2, whereas the dehydrogenases−electron transferases need monoelectronic acceptors.

Numerous mechanistic studies, carried out mainly with lactate monooxygenase from *Mycobacterium smegmatis* (formerly called lactate oxidase, EC 1.13.12.4) (Ghisla & Massey, 1991) and flavocytochrome b_2 (Lederer, 1991), support the idea that the substrate dehydrogenation reaction proceeds by initial formation of a carbanion which then yields the electrons to the flavin (Ghisla, 1982). Amino acid sequence comparisons, as well as comparison between the active-site crystal structures of flavocytochrome b_2 and glycolate oxidase, suggested a similar mechanism for all family members (Xia & Mathews, 1990; Lindqvist & Brändén, 1989; Lindqvist et al., 1991). The three-dimensional structure of flavocytochrome b_2 showed the presence of pyruvate, the reaction product, bound at the active site (Xia & Mathews, 1990); this suggested a possible substrate binding mode and enabled Lederer and Mathews (1987) to propose a role for active-site side chains in catalysis on the basis of the carbanion mechanism. The first proposed reaction step is presented in Figure 1. The catalytic base would be H373, which in the resting oxidized enzyme forms

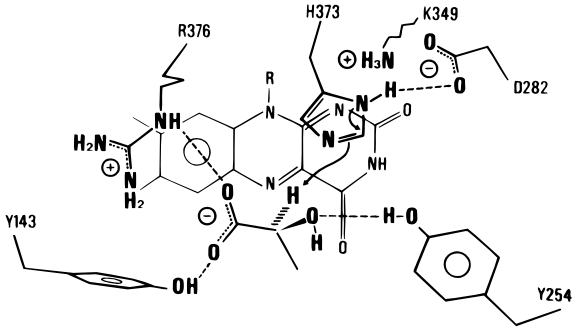

FIGURE 1: Proposed Michaelis complex and first catalytic step for the flavocytochrome b_2-catalyzed lactate oxidation. The lactate binding mode is deduced from that of the pyruvate molecule observed in the three-dimensional structure (Lederer & Mathews, 1987; Xia & Mathews, 1990).

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Table 1: Steady-State Kinetic Parameters and Deuterium Isotope Effect with Ferricyanide as Acceptor for D282N Mutant Flavocytochrome b_2[a]

	K_m (mM)			k_{cat} (s^{-1})		k_{cat}/K_m (10^3 M^{-1} s^{-1})			
enzyme	L-[^{1}H]lactate	L-[^{2}H]lactate	ferricyanide	L-[^{1}H]lactate	L-[^{2}H]lactate	L-[^{1}H]lactate	L-[^{2}H]lactate	^DV	$^DV/K$
wild-type[b]	0.49 ± 0.10	0.50 ± 0.20	≤ 0.1[c]	270 ± 30	65 ± 22	551 ± 174	130 ± 96	4.1 ± 1.8	4.2 ± 4.4
D282N	0.73 ± 0.05	0.72 ± 0.06	≤ 0.01	3.9 ± 0.1	0.66 ± 0.03	5.3 ± 0.5	0.92 ± 0.12	5.9 ± 0.4	5.8 ± 1.3

[a] k_{cat} is expressed as mol of substrate oxidized s^{-1} (mol of subunit)$^{-1}$. The data for the D282N enzyme are the average of three independent experiments for [^{1}H]lactate, two for [^{2}H]lactate, and two for ferricyanide. For experiments at varying lactate concentrations, 1 mM ferricyanide was used; for experiments at variable ferricyanide concentrations, 20 mM lactate was used. Error values on ratios (k_{cat}/K_m, ^DV, and $^DV/K$) were calculated from the sum of relative errors on values in the numerator and denominator. [b] Dubois et al. (1990). [c] Rouvière-Fourmy (1992).

a hydrogen bond with D282. As the histidine becomes protonated, a favorable electrostatic interaction should develop between the two side chains. It has been shown before that this ion pair is stably established in reduced flavocytochrome b_2, due to an elevation of the histidine pK_a upon flavin reduction (Urban & Lederer, 1985; Lederer, 1992). In the oxidized enzyme at neutral pH the histidine is normally unprotonated. Stopped-flow studies of flavin reduction indicated catalysis to depend on a group with pK_a of 6.0 in the free enzyme and 5.3 in the ES complex (Suzuki & Ogura, 1970). These pK_a values can perhaps be ascribed to His373.

Mechanistic proposals have been, in recent years, analyzed by site-directed mutagenesis. The active-site H → Q mutations for lactate monooxygenase (Müh et al., 1994) and flavocytochrome b_2 (Gaume et al., 1995) showed that the histidine indeed plays a critical part in catalysis. We present here the properties of the D282N mutant flavocytochrome b_2. We have analyzed the effects of the mutation on the catalysis of substrate dehydrogenation, on the binding of a number of inhibitory ligands, as well as on the ionization state of H373 in the reduced enzyme. This is the first report about the effects of a mutation at this position in the family of α-hydroxy-acid-oxidizing enzymes [for a review, see Lederer et al. (1996)]. The D282N mutant enzyme has been found before to be synthesized in *Escherichia coli* as a mixture of flavin-free and holo-enzyme, owing probably to an alteration of the folding process in the bacterium (Gondry et al., 1995); but the two forms can be separated by chromatography, so the present work could be carried out with an essentially homogeneous enzyme.

MATERIALS AND METHODS

Enzyme. The D282N protein was expressed in *E. coli* MM294 cells grown in LB medium (Black et al., 1989). The purification was carried out as described before; the holoenzyme was separated from flavin-free enzyme by chromatography on a Blue-Trisacryl column; alternatively, the late fractions from the hydroxyapatite column, which contained a stoichiometric amount of FMN, were pooled (Gondry et al., 1995). After ammonium sulfate precipitation and dissolution of the enzyme pellet, the flavin/heme ratio was 0.8 to 0.9, due to some prosthetic group dissociation at high salt. The enzyme concentration was determined using the heme extinction coefficients ($\epsilon^{ox}_{413} = 129.5$ mM^{-1} cm^{-1}; $\epsilon^{red}_{423} = 183$ mM^{-1} cm^{-1}). The FMN/heme ratio was determined by measuring flavin fluorescence after release from the protein in 6 M guanidinium chloride (Gondry et al., 1995). Activities were expressed relative to flavin.

Steady-State Kinetics. Enzyme assays were carried out with a Uvikon 930 spectrophotometer, at 30 °C in 0.1 M Na^+/K^+ phosphate buffer, 1 mM EDTA, pH 7. Standard activity assays were usually carried out in the presence of 20 mM L-lactate and 2 mM ferricyanide ($\epsilon^{red\text{-}ox}_{420} = 1.04$ mM^{-1} cm^{-1}). When cytochrome *c* was used as acceptor, the reaction was monitored at 550 nm ($\epsilon^{red\text{-}ox}_{550} = 20.5$ mM^{-1} cm^{-1}). K_m and k_{cat} values were extracted from experimental data using a least squares non-linear regression program for fitting to the Michaelis−Menten equation. Inhibition constants were calculated using a linear regression analysis for fitting to the Dixon equation (Dixon, 1953). When Dixon plots indicated simple competitive inhibition, a confirmation was obtained using the graphical analysis proposed by Cornish-Bowden (1974).

Kinetics of the Reverse Reaction with Fluoropyruvate. The rate of fluoropyruvate reduction was monitored as described before (Urban et al., 1983). Briefly, DL-2-hydroxy-3-butynoate was used as reducing substrate; 2-keto-3-butynoate was trapped by an excess of glutathione, yielding an adduct with $\epsilon_{315} = 10.6$ mM^{-1} cm^{-1} (Pompon & Lederer, 1982). Reactions were carried out under argon, at 30 °C, in 0.1 M Na^+/K^+ phosphate buffer in the presence of 700 μM DL-2-hydroxy-3-butynoate, 5.5 mM reduced glutathione, 10 μM enzyme, and varying fluoropyruvate concentrations.

Intermolecular Transhydrogenation Experiments. They were carried out as described before (Urban & Lederer, 1985; Balme & Lederer, 1994). The reaction mixture contained 34.5 μM enzyme, 20 mM DL-[2-^{3}H]lactate (0.25 μCi/μmol), and varying fluoropyruvate concentrations. After 45 min at 30 °C, the mixture was acidified and products were separated on an AG1-X8 column (18 × 1 cm, OH^- form). It was first washed with 60 mL of H_2O, in order to collect all the tritium appearing in the solvent; a linear gradient was then applied between 250 mL of H_2O and 250 mL of 2 M formic acid. The effluent fractions were analyzed for hydroxy acids, keto acids, and radioactivity (LKB Wallac 1410 scintillator) as described (Urban et al., 1983).

RESULTS AND DISCUSSION

Steady-State Kinetic Parameters and Isotope Effect for the D282N Mutant Flavocytochrome b_2. Table 1 presents a comparison of wild-type and mutant enzymes parameters using L-lactate as substrate and ferricyanide as acceptor. The substrate K_m value is slightly increased, whereas k_{cat} decreases by a factor of 70. The deuterium isotope effect is at least as high as that of the wild-type enzyme, if no higher. It is known from previous work that α-proton abstraction is the rate-limiting step for wild-type enzyme flavin reduction: ^Dk values for flavin reduction as determined in stopped-flow experiments lie between 7 and 8; nevertheless, in the steady-state, ^DV values for ferricyanide reduction are 4−5 (Pompon

Table 2: Steady-State Kinetic Parameters with Cytochrome *c* as Acceptor for D282N Mutant Flavocytochrome b_2[a]

	cytochrome *c*		lactate	
enzyme	K_m (μM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
wild-type[b]	87 ± 15	155 ± 15	0.29 ± 0.05	155 ± 15
D282N	3.9 ± 0.1	4.9 ± 0.1	0.44 ± 0.04	4.1 ± 0.2

[a] The experiments at varying cytochrome *c* concentrations were carried out in the presence of 20 mM L-lactate. The kinetics at varying lactate concentrations were performed in the presence of 450 μM cytochrome *c* for wild-type enzyme and 45 μM cytochrome *c* for the D282N enzyme (respectively 84% and 92% of saturation). k_{cat} is expressed as mol of substrate oxidized s^{-1} (mol of subunit)$^{-1}$. The data for the D282N protein are the average of two independent experiments. [b] Rouvière-Fourmy (1992).

et al., 1980; Miles et al., 1992; Rouvière-Fourmy et al., 1994), showing that steps of the catalytic cycle after flavin reduction introduce a partial rate limitation. Table 1 thus suggests that the mutation essentially affects the rate-limiting step. Furthermore, from a consideration of the isotope effect values and of the equation $[^{D}V - 1]/[^{D}(V/K) - 1] = K_m/K_s$ (Northrop, 1975; Klinman & Mathews, 1985), it can be observed that K_m values for both wild-type and D282N enzymes are identical to K_s values. Therefore, the mutation has a negligible effect on Michaelis complex formation but destabilizes the transition state for proton abstraction by 2.8 kcal/mol.

The apparent K_m for ferricyanide, which has a low value and is therefore difficult to measure accurately [for a review, see Lederer (1991)], did not appear to undergo a significant change (or at least a significant increase) (Table 1). In contrast, when cytochrome *c* was used as acceptor, a more than 20-fold decrease in its K_m value was noted (Table 2). This is the expected result of the reduction in the rate of a slow step preceding cytochrome *c* binding and reduction and cannot be taken as necessarily indicating a better cytochrome *c* affinity. Within error, the k_{cat} values obtained in the various experiments with cytochrome *c* and ferricyanide were identical. Thus, results show that, for the D282N mutant enzyme, the α-proton abstraction step is entirely rate-limiting in the steady-state, whereas for the wild-type enzyme, the steps that follow flavin reduction introduce a partial rate limitation; in particular, for cytochrome *c* reduction, k_{cat} values are lower than those for ferricyanide reduction (Lederer, 1991; Miles et al., 1992; Rouvière-Fourmy, 1992). It follows that the flavin reduction rate for the D282N mutant protein should be identical to the steady-state rate of acceptor reduction. Stopped-flow methods cannot be used to verify this point nor to assess possible effects of the mutation on the flavin to heme electron transfer rate. This is because, at low reduction rates, the slow electron transfer between protomers, which takes place in the absence of acceptor (stopped-flow conditions), reoxidizes the flavin in competition with its reduction by lactate. Thus, at low deuterolactate concentrations, the flavin reduction rate was found to be lower than the rate of overall heme reduction by lactate, even though FMN is reduced before heme (Pompon et al., 1980; Pompon, 1980). A similar phenomenon was observed even at saturating lactate concentrations for the Y254F mutant flavocytochrome b_2 (A. Balme, S. K. Chapman, and F. Lederer, unpublished experiments), the k_{cat} value for which is similar to that of the D282N mutant enzyme (Dubois et al., 1990).

Binding of Inhibitors. In order to probe further the consequences of the mutation, we analyzed its effects on the binding of several inhibitory ligands. Assuming no important enzyme structure change (see conclusion), any modification would be expected to arise from the destabilization of the protonated state of H373. This could lead to altered affinity with or without a change in ligand binding mode. The lack of effect of the mutation on kinetic constants could actually also arise from a change in binding mode with the establishment of compensatory interactions; this case is, however, kinetically indistinguishable from the absence of interactions with the mutated residue. Recent crystallographic studies of the Y143F mutant flavocytochrome b_2 suggested the possibility of binding mode alterations, but kinetic constants were not reported (Tegoni et al., 1995).

Sulfite behaves as a competitive inhibitor of lactate by forming a rapidly reversible covalent bond with the flavin at position N5 (Lederer, 1978). The crystal structure of the wild-type enzyme−sulfite complex has been described (Tegoni & Mathews, 1988; Tegoni & Cambillau, 1994). It showed a close distance between one of the sulfite oxygens and the Nϵ position of H373, suggesting that the latter becomes protonated upon sulfite addition. It would appear from Table 3 that the loss of the electrostatic interaction between the H373 imidazolium ion and the D282 carboxylate entailed by the D to N mutation affects only slightly the stability of the flavin−sulfite complex. The negative charges of the bound sulfite may provide some stabilization to the histidine acidic form. The D to N mutation had an even smaller effect, if any, on the stability of the D-lactate−enzyme complex (Table 3), whereas the H373Q mutant enzyme shows a 12-fold increase in the D-lactate K_i value (Gaume et al., 1995). These results obtained with two different mutant forms may perhaps be interpreted as indicating the formation of a hydrogen bond between the inhibitor hydroxyl and the unprotonated Nϵ position of H373 in the oxidized enzyme.

The discussion of the inhibition exerted by lactate, pyruvate, and oxalate requires that one considers the complexity of the kinetic scheme describing the steady-state functioning of flavocytochrome b_2 (Scheme 1). When cytochrome *c* is the acceptor, it first binds to the enzyme and accepts an electron from heme b_2 in intermediates **3** and **5**. Ferricyanide, in contrast, is believed to react in a second-order fashion (Iwatsubo et al., 1977). Various pieces of evidence [reviewed in Lederer (1991)] suggest that this acceptor can be reduced both by heme b_2, as is cytochrome *c*, and by Fl_{sq}.[1] In that case, species **3** goes over directly to species **5**, and species **4** to **1**.

L-Lactate has been shown to inhibit the WT enzyme at high concentration, with $K_i^{app} = 150$ mM (27 °C) (Somlo & Slonimski, 1966). Inhibition by excess substrate is generally considered to arise from substrate binding to an enzyme form different from the one with which it reacts. Indeed, a combination of steady-state and stopped-flow studies recently indicated that inhibition by lactate arises from its binding to species **3** (and possibly **4**), preventing electron transfer to heme b_2 and ferricyanide (Mayer et al., 1996). For the D282N mutant enzyme, the inhibition by excess substrate

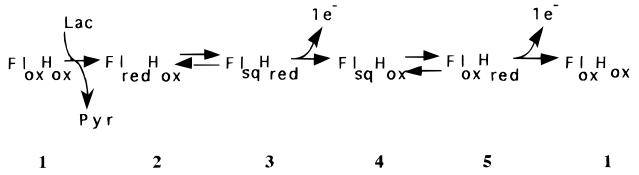
[1] Abbreviations: Fl_{ox}, oxidized flavin; Fl_{sq}, flavin semiquinone; Fl_{red}, reduced flavin; H_{ox} and H_{red}, oxidized and reduced heme; WT, wild-type.

Table 3: Inhibition of Wild-Type and D282N Flavocytochromes b_2 by Various Ligands

	wild-type enzyme		D282N enzyme[e]	
inhibitor	inhibition type	K_i (mM)	inhibition type	K_i (mM)
sulfite	competitive[a]	$(1.4 \pm 0.5) \times 10^{-3}$ [a]	competitive	$(3.5 \pm 0.4) \times 10^{-3}$
D-lactate	competitive[b]	1.4 ± 0.5[b]	competitive	2.4 ± 0.1
L-lactate	excess substrate[b]	150[b]	none[c]	
pyruvate	competitive[a,b]	3.0[a,b]	competitive	5.8 ± 0.1
	noncompetitive[a]	30[a]	noncompetitive	25.7 ± 0.3
oxalate	mixed	0.3−0.5[d]	competitive	3.6 ± 0.1
			noncompetitive	27.6 ± 0.7

[a] Lederer (1978). [b] Somlo and Slonimski (1966). [c] Tested up to 500 mM lactate. [d] Secondary plots of slope values taken from Figure 4 of Lederer (1978) yielded a value of 0.3 mM for the oxalate K_i. Difference spectral titrations reported in the same paper gave $K_d = 0.5$ mM for the E_{ox}•oxalate complex. [e] The results for the D282N enzyme are the average of two experiments with sulfite, D-lactate, and L-lactate and three experiments with oxalate and pyruvate. Ferricyanide (2 mM) was used as electron acceptor, at varying inhibitor concentrations in the presence of several fixed lactate concentrations (see, for example, Figure 2).

Scheme 1



is not observed any more (Table 3), at least in the concentration range studied. The wild-type enzyme crystal structure indicates H373 to be protonated when the FMN is in the one-electron reduced state (Xia & Mathews, 1990; see also below). It can then be suggested that the weak-affinity inhibitory mode of lactate at the flavin semiquinone stage requires a strongly stabilized His−Asp ion pair.

Pyruvate shows a complex inhibition pattern with the wild-type enzyme. For the flavocytochrome b_2 from *H. anomala*, pyruvate was found to be a noncompetitive inhibitor at low concentrations and an uncompetitive inhibitor at high concentrations (Tegoni et al., 1990). The authors gave a molecular explanation for the phenomenon by showing that pyruvate has a higher affinity for the enzyme at the semiquinone stage than at the oxidized or reduced stages. Furthermore, it was shown that pyruvate binding induced an 85 mV upward shift of the Fl_{ox}/Fl_{sq} couple (Tegoni et al., 1986). As a consequence, the pyruvate-complexed flavin semiquinone was incapable of transferring electrons to acceptors, heme b_2 or ferricyanide (Tegoni et al., 1990; Janot et al., 1990). Tegoni et al. (1990) considered binding of pyruvate to reduced enzyme not to play any role in the kinetic inhibition pattern, even though, in the absence of monoelectronic acceptors, the product can act as a reverse substrate for the reduced enzyme (Urban et al., 1983). Although pyruvate K_m values were determined for the reverse reaction, the actual K_d value of the E_{red}•pyruvate complex is not known for either of the two homologous enzymes.

For the *Saccharomyces cerevisiae* WT enzyme, Hinkson and Mahler (1963) already suggested that pyruvate exhibited its inhibitory action at two different stages of the reaction cycle. More recently, Dixon plots for pyruvate inhibition gave two slopes at each substrate concentration, indicating competitive inhibition at low pyruvate concentrations ($K_i = 3$ mM) and noncompetitive inhibition at higher concentrations ($K_i' = 30$ mM) [Table 3 and Lederer (1978)]. It would appear that, although the steady-state kinetic inhibition pattern is not exactly identical to that observed for the homologue from *Hansenula anomala*, pyruvate inhibition also arises from both competitive binding at the active site of oxidized enzyme and higher affinity inhibitory binding at the semiquinone stage. Indeed, Walker and Tollin (1991) showed the midpoint potential of the Fl_{ox}/Fl_{sq} couple to be shifted upward upon pyruvate binding, as it is for the *H. anomala* enzyme. The authors interpreted the results of their flash photolysis study as indicating that pyruvate binding at the semiquinone stage favored flavin to heme electron transfer. This conclusion is in contradiction with the results obtained with the *H. anomala* enzyme; it also does not fit with our steady-state results [Lederer (1978) and this work], which only indicate inhibition; it also stands in contrast with the interpretation of subsequent results by the same group (Hazzard et al., 1994), namely, that pyruvate binding to species **2** (Scheme 1), where the flavin is fully reduced, would on the contrary inhibit flavin to heme electron transfer. In the following discussion, we shall adhere to the idea that ligand binding at the active site when the FMN is in the fully-reduced or half-reduced state prevents electron transfer from flavin to acceptors.

The crystal structure of the *S. cerevisiae* enzyme provides the basis for understanding the higher pyruvate affinity for the semiquinone state than for the oxidized form (Xia & Mathews, 1990): pyruvate is observed to engage into an ion pair with R376 as well as a hydrogen bond with Y143 on the carboxylate side (Figure 1); most importantly, its carbonyl oxygen lies within hydrogen bonding distance of both Y254 and H373 Nϵ; the latter is therefore protonated whereas it is not in the oxidized enzyme. If we now consider the results obtained with the D282N mutant enzyme, then the substitution with asparagine does not appear to modify substantially the inhibition pattern: Dixon plots still indicate competitive and noncompetitive inhibition according to the pyruvate concentration range (not shown, but see Figure 3C for an example of the outlook). The derived kinetic constant values are very similar to those measured for the WT enzyme (Table 3). It may be reasonable to consider the competitive K_i value as similar to if not identical with the K_d value for the E_{ox}•pyruvate complex, in view of the rate-limiting nature of the first step in the reaction sequence, in particular for the mutant enzyme. If this is correct, the result is not surprising. Indeed, if pyruvate binds to E_{ox} in the same orientation as observed in the crystal structure for the semiquinone stage, then it would not be able to form a hydrogen bond to H373, since the latter is in its basic form at that point (hence the lower affinity of pyruvate for the oxidized enzyme). Therefore, the mutation would not be expected to have an important effect on the binding of ligands that either do not

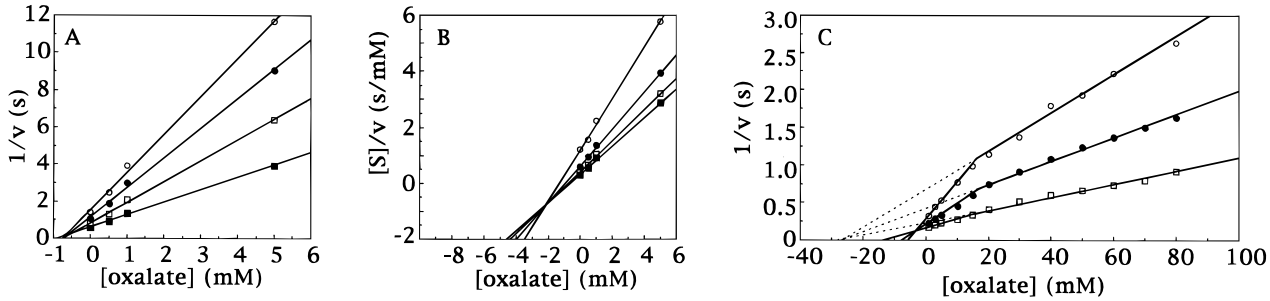


FIGURE 2: Inhibition by oxalate. (A,B) WT enzyme. The data are taken from the double-reciprocal plot in Lederer (1978) and replotted as shown: L-lactate concentrations (mM), 0.25 (○); 0.33 (●); 0.5 (□); and 1 (■). (C) D282N mutant flavocytochrome b_2: lactate concentrations (mM), 0.52 (○); 1 (●); and 2 (■).

require a protonated H373 or do not bind to this residue at all, when the enzyme is in the oxidized state. In contrast, the Y254F mutation was found to significantly decrease pyruvate binding to E_{ox} (M. Gondry, J. Dubois, and F. Lederer, to be published). As far as the noncompetitive part of inhibition by pyruvate is concerned, it is at present impossible to give a quantitative interpretation to the lack of change in the parameter value in the absence of a more detailed kinetic analysis. The fact that the inhibition kinetic pattern is again complex at least indicates that pyruvate still has affinity for the semiquinone state of the D282N mutant enzyme. This suggests that a strong stabilization of the H373 protonated state is not essential for pyruvate binding.

The interaction of oxalate with flavocytochrome b_2 has been studied for both forms of the wild-type *S. cerevisiae* enzyme (intact and proteolytically nicked or Morton form) and for the *H. anomala* enzyme, by a variety of methods. In the steady state, mixed inhibition was observed (lines for different inhibitor concentrations intersecting in the upper quadrant left of the ordinate, in the double-reciprocal plot) (Blazy et al., 1976; Lederer, 1978). Replots of slope values versus inhibitor concentration yielded $K_i = 0.3$ mM for intact enzyme and $K'_1 = 2$ mM, while flavin spectral perturbations induced by oxalate led to titrate a K_d value of 0.5 mM (Table 3). Rapid kinetic methods indicated a two-step binding to the *H. anomala* enzyme (Blazy, 1982), similarly to observations made on lactate monooxygenase (Ghisla & Massey, 1975). With the D282N mutant enzyme, a Dixon plot indicated, as for pyruvate, that inhibition was competitive at low oxalate concentrations and noncompetitive at higher ones (Figure 2C, Table 3), in contrast with the mixed inhibition observed for the wild-type enzyme using the double-reciprocal plot (Lederer, 1978). In order to avoid any ambiguity, the data of the 1978 paper were used for constructing Dixon plots as well as the $[S]/v = f([I])$ plot proposed by Cornish-Bowden (1974) (Figure 2A,B). The combination of the two plots confirms the mixed nature of the wild-type enzyme inhibition by oxalate.

Thus, between the wild-type and the mutant enzymes, there is a clear change in the kinetic pattern of inhibition by oxalate. Does this imply a change in the molecular basis of the inhibition by this compound? This is less than certain. From the combined data, it appears legitimate to assume that oxalate binds to the oxidized form, thereby competing with the substrate. Table 3 indicates a 10-fold increase in the competitive K_i value as a consequence of the mutation. This suggests a decreased oxalate affinity for the oxidized enzyme. It has been shown for lactate monooxygenase that the second step of oxalate binding implies uptake of a proton by a group

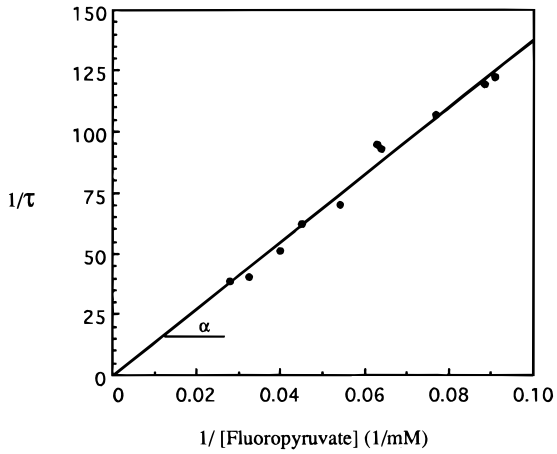


FIGURE 3: Dependence of τ (fraction of intermolecular tritium transfer) on fluoropyruvate concentration. Experimental details are described in Materials and Methods.

which undergoes a pK_a shift from 4.7 in the unliganded enzyme to 9.8 in the oxalate complex. This group was suggested to be the active-site base (Ghisla & Massey, 1977). In view of the similarities in primary structure and function between lactate monooxygenase and flavocytochrome b_2, it seems reasonable to assume that oxalate binding would induce protonation of H373 and its homologue H290 in the monooxygenase. The lesser stabilization of the imidazolium form of H373 induced in the oxidized D282N enzyme, compared to the wild-type oxidized form, could account for the affinity drop because of a weaker electrostatic interaction between the negatively charged ligand and the less acidic imidazolium ion. One may then wonder why this effect is felt less strongly for sulfite than for oxalate binding (Table 3). There is a plausible interpretation of these differences. Indeed, one of us has argued that when the flavin N1 position is negatively charged, it contributes, together with D282, to the stabilization of the H373 acidic form (Lederer, 1992). Results described below support this contention. The flavin N1 position is negatively charged at the semiquinone and reduced stages as well as in the flavin−sulfite adduct. Therefore, the destabilizing effects of the D282N mutation should be more strongly felt when protonation of the histidine is required at the Fl_{ox} stage, when the flavin is neutral. The result of the mutation is a 10-fold increase in the oxalate K_i (K_d) value.

As to the other aspects of the inhibition by oxalate, it is likely that they arise from binding to the semiquinone and possibly the reduced enzyme forms. The Dixon plot for oxalate (Figure 2C) shows exactly the same pattern as the one for pyruvate and, strikingly enough, very similar values

for the deduced inhibition parameters (Table 3). This suggests that, at least in the mutant enzyme, pyruvate and oxalate inhibit in the same way, by binding at the Fl_{ox} and Fl_{sq} stages with similar affinities. Therefore the differences observed for oxalate between the WT and the D282N mutant enzymes may have as origin a drop in the affinity of the ligand for all redox states to which it binds (oxidized, semiquinone, and possibly reduced), combined with the changes in substrate kinetic parameters. More detailed investigations are required to fully elucidate the phenomenon. As explained above, a stopped-flow analysis is precluded in the slow mutant cases. Analysis of inhibition patterns using wild-type and mutant flavodehydrogenase domains might provide interesting information.

Influence of the D282N Mutation on the pK_a of H373. It has been demonstrated before that the active-site base undergoes an upward pK_a shift upon enzyme reduction. The structural factors responsible for the stabilization of the protonated histidine were discussed by Lederer (1992); among them was obviously the electrostatic interaction with D282. It was thus of interest to evaluate the effect of the D to N mutation on the histidine pK_a.

The basic observation concerning the H373 protonation state was that the enzyme-bound substrate α-proton was sticky: in the absence of a monoelectronic acceptor, it could be transferred to a keto acid acting as a reverse substrate, while a certain proportion, depending on reaction conditions, was also lost to the solvent (Urban & Lederer, 1985). Monitoring the amount of intermolecular tritium transfer from [2-^{3}H]lactate as a function of the keto acid acceptor concentration offers a means to determine the first-order rate constant for exchange with the solvent of the enzyme-bound α-proton, k_e^H, according to eq 1:

$$k_e^H = \alpha[k_{cat}/K_m] \quad (1)$$

where k_{cat} and K_m are the kinetic parameters for keto acid reduction by reduced enzyme and α is given by eq 2:

$$1/\tau = 1 + \alpha[1/(\text{keto acid})] \quad (2)$$

where τ is the fractional amount of isotope transferred from [2-^{3}H]lactate to the keto acid substrate (Urban & Lederer, 1985); α is also equal to the keto acid concentration that gives rise to 50% transfer (Rose et al., 1974; Balme & Lederer, 1994). If the nature of the solvent species which catalyzes proton exchange is known (buffer ion or water), then the second-order rate constant for exchange can be calculated and fed into the Eigen equation for estimating the catalytic base pK_a (Eigen, 1964). In our case, the instability of the enzyme at low buffer concentrations has thus far made it difficult to estimate the buffer sensitivity of the α-proton exchange with the solvent. Nevertheless, assuming everything else to remain constant, it is possible to calculate a pK_a shift between the wild-type enzyme and a mutant form using first-order rate constants for exchange, according to eq 3:

$$\Delta pK_a(\text{mutant}-\text{WT}) = \log[k_e^H(\text{WT})/k_e^H(\text{mutant})] \quad (3)$$

We first determined k_{cat} and K_m values for reduction of fluoropyruvate by the reduced D282N mutant enzyme. Results are compared in Table 4 to those obtained previously for the wild-type and the Y254F enzymes. The specific activity of the latter for lactate oxidation is rather similar to that of the D282N mutant form (6 s^{-1} versus 4 s^{-1}) compared to that of the WT (Table 1). A low k_{cat} value for the reverse reaction would therefore be expected for the two mutant enzymes, unless they had undergone important flavin redox potential changes.

Table 4: Steady-State Kinetic Parameters for the Reverse Reaction with Fluoropyruvate[a]

enzyme	K_m (mM)	k_{cat} (s^{-1})
wild-type[b]	8.9 ± 3.2	2.8 ± 0.7
Y254F[b]	3.9 ± 1.0	0.20 ± 0.02
D282N	4.8 ± 0.2	0.22 ± 0.02

[a] The data were obtained as described under Methods. They are the average of three independent experiments. [b] Balme and Lederer (1994).

Table 5: Determination of the Rate Constant for Exchange with the Solvent of the Protein-Bound Substrate α-Proton

enzyme	slope α (mM)[b]	k^H (s^{-1})[c]	ΔpK_a (pH units)[d]
wild-type[a]	10.6 ± 1.6	2.5 ± 0.6[e]	−
Y254F mutant[a]	161 ± 8	8.2 ± 2.8	−0.5
D282N mutant	1379 ± 20	63 ± 4	−1.4

[a] Balme and Lederer (1994). [b] Derived from plots such as that of Figure 2. [c] Calculated using eq 1. [d] Calculated using eq 3. [e] Average of values obtained for intact and Morton wild-type enzymes and the recombinant flavodehydrogenase domain (Balme & Lederer, 1994).

Next, the fraction of intermolecular tritium transfer from [2-^{3}H]lactate at varying fluoropyruvate concentrations was determined as described in Materials and Methods. Results are presented in Figure 3 under a double-reciprocal form. The line intersects the ordinate at $1/\tau = 0.95$, in good agreement with the theoretical value of 1 (eq 2). The slope value, α (Table 5), is more than 2 orders of magnitude larger than that for the wild-type protein and nearly 10-fold larger than that of the Y254F enzyme. It was clear during the experiments that exchange with the solvent must be much more rapid than that for the two other enzymes. Indeed, the τ values determined at the extremes of the fluoropyruvate concentration range were 0.8% and 2.6% compared to about 40% and 70% for the wild-type enzyme and 6% and 18% for the tyrosine mutant protein over the same concentration range. In principle, one could determine higher experimental transfer levels by going to even higher fluoropyruvate concentrations. But there is a limit to this, owing to the fact that fluoropyruvate labels the oxidized enzyme and inactivates it (Urban & Lederer, 1988).

With the values given in Tables 4 and 5, eq 1 yields a first-order rate constant for the proton exchange of 63 s^{-1}, and eq 3 yields a ΔpK_a^{app} of −1.4 pH units, a figure that gives an approximate measure of the weakening of the electrostatic interaction induced in the reduced enzyme by the D282N mutation (about 2 kcal/mol). Nevertheless, if we assumed the effects of the mutations at Y254 and D282 to be additive, then the proton exchange rate would still be no more than about 200 s^{-1}; in other words, it would still be very much slower than that for a freely accessible histidine in the buffer used for these experiments (10^6−10^7 s^{-1}). Therefore, the protein structure provides additional stabilizing elements. It is most likely that a hydrogen bond still exists between N282 and H373 (see below), but its effect should be less than that of the lost electrostatic interaction and thus cannot account for a difference of 4−5 orders of magnitude.

It has been suggested before, upon detailed inspection of the crystal structure, that the negative charge on the reduced flavin N1 position contributes in a major way to the pK_a increase experienced by H373 upon enzyme reduction (Lederer, 1992). The present results support but do not prove this suggestion. Further studies aimed at destabilizing the negatively charged state of the reduced flavin (for example, removing the positive charge of K349) should contribute to shed light on the matter. It must, however, be noted that, with the 25-fold increase of k_e^H induced by the mutation, we have been working rather close to the limits of detection of intermolecular hydrogen transfer. Therefore, for any increase in k_e^H value that is significantly larger than the one observed in this paper, another quantification method would probably have to be devised.

The present work brings no direct information as to the possible effects of the D282N mutation on the electron transfer properties of the Fl_{red} and Fl_{sq} states. The semiquinone is an intermediate in intramolecular electron transfer during the catalytic cycle. Owing to the reciprocal stabilization effects which are postulated to exist between the Fl_{red} and Fl_{sq} N1 anions and the protonated imidazolium of H373 (Lederer, 1992), one may wonder if the latter's lesser stabilization in the mutant enzyme may influence electron transfer rates. Since the first step of the catalytic cycle, α-proton abstraction, is entirely rate limiting in the mutant enzyme, heme reduction rates must still be quite rapid compared to that of this first step. Furthermore, there is a strong suspicion that, in the WT enzyme, flavin reoxidation and heme reduction may be limited by heme-binding domain movements; in other words, they could be conformationally gated (Miles et al., 1992; Lederer, 1994). In this case, no alteration in the interactions between the domains would be expected in the mutant enzyme, since the H373-D282 pair does not lie at the interface.

CONCLUSION

In the flavocytochrome b_2 active site, one of the D282 carboxylate oxygens accepts a hydrogen bond from H373 Nδ; the other oxygen is hydrogen bonded to WAT 609; in turn, the latter interacts with S371 Oγ (Xia & Mathews, 1990). Identical interactions are formed by the active-site aspartate, D157, in glycolate oxidase (Lindqvist & Brändén, 1989; Lindqvist et al., 1991). Replacement of the second carboxylate oxygen with the asparagine amido nitrogen should not appreciably disturb the hydrogen bond network; in particular the proton of neutral H373 could still reside at Nδ rather than Nϵ. Several observations support these hypotheses. Firstly, the D282N mutant flavocytochrome b_2 undergoes a decrease in k_{cat} value of less than 100-fold; one would expect a much larger drop if Nϵ were protonated instead of Nδ, because the histidine could not act as a base toward the substrate. In comparison, the D102N mutant of rat trypsin, where the polarity of the H bond network was reversed and Nϵ carried the proton instead of Nδ, was only 10^{-4}-fold as active as the wild-type enzyme (Craik et al., 1987; Sprang et al., 1987). Secondly, the similarity of K_m and K_i values for L- and D-lactate and sulfite, between wild-type and mutant enzymes, are indicative of a substantial structural conservation. Consequently, the modified properties of the mutant enzyme are in all likehood essentially due to the different electrostatic influence of Asn compared to Asp. The very modest effect of the mutation on substrate affinity and the sizable decrease in stabilization of the transition state for α-proton abstraction (2.8 kcal/mol) are in agreement with mechanistic predictions (Lederer & Mathews, 1987; Lederer, 1991). Furthermore, active-site mutant enzymes offer means of obtaining information on possible binding modes for inhibitory ligands. The results given in Table 3 indicate that the mutation and the resulting greater difficulty of protonating H373 have little consequence for the binding of most ligands, except for lactate at the semiquinone stage and for oxalate at several if not all redox stages. A rationalization of the observed effects is presented in the preceding section. Finally, the value of the rate constant for exchange with the solvent of the protein-bound substrate α-proton indicates that the electrostatic field of D282 is insufficient to account for the whole extent of stabilization of the H373 acidic state in the reduced enzyme. This adds support to the suggestion that the anionic charge of the reduced flavin is a major contributor to the phenomenon (Lederer, 1992).

In what precedes, we have been analyzing the properties of the D282N mutant enzyme in terms of the carbanion mechanism. It has recently been argued that a hydride transfer mechanism was more likely (Stenberg et al., 1995), in view of the lack of effect of the Y254F mutation on apparent substrate affinity (Figure 1) (Stenberg et al., 1995; Dubois et al., 1990). It was suggested that H373 would rather remove the substrate hydroxyl proton, thus facilitating direct hydride transfer to the flavin N5 position. We believe the present results strengthen the case for the carbanion mechanism. Firstly, a similar D→N mutation of the catalytic His−Asp pair in NADH-linked lactate dehydrogenase resulted in a drop of the k_{cat}/K_m value by a factor of about 200 for lactate oxidation and of about 2000 for pyruvate reduction (Clarke et al., 1988). This result showed that stabilization of the protonated histidine was more important for reduction than for oxidation. In our case, the k_{cat}/K_m value decreased about 100-fold for lactate oxidation and less than 10-fold for fluorolactate reduction. The comparison does not speak in favor of an identical mechanism. Secondly and more importantly, we find it hard to explain how the altered interaction between H373 and N282 could induce a pK_a decrease (as monitored by the proton exchange rate) at the reduced flavin N5 position, since the substrate α-hydrogen would be transferred to and from that position in a hydride transfer reaction. In the WT enzyme, the electrostatic field of the imidazolium ion would be expected to confer some stabilization, if anything, to the N5 anion formed transiently during proton exchange with the solvent and hence to facilitate exchange; the mutation would then be expected to make the proton more sticky, whereas the opposite is observed.

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