Substitution of Tyr254 with Phe at the Active Site of Flavocytochrome b_2: Consequences on Catalysis of Lactate Dehydrogenation†

Joëlle Dubois,‡ Stephen K. Chapman,§ F. Scott Mathews,‖ Graeme A. Reid,⊥ and Florence Lederer*,‡

INSERM U 25, CNRS UA 122, Hôpital Necker, 161 Rue de Sèvres, 75015 Paris, France, Department of Chemistry and Department of Microbiology (School of Agriculture), University of Edinburgh, West Mains Road, Edinburgh EH9 3JG, U.K., and Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received December 6, 1989; Revised Manuscript Received February 16, 1990

ABSTRACT: A role for Tyr254 in L-lactate dehydrogenation catalyzed by flavocytochrome b_2 has recently been proposed on the basis of the known active-site structure and of studies that had suggested a mechanism involving the initial formation of a lactate carbanion [Lederer, F., & Mathews, F. S. (1987) in *Flavins and Flavoproteins*, Proceedings of the Ninth International Symposium, Atlanta, GA, 1987 (Edmondson, D. E., & McCormick, D. B., Eds.) pp 133–142, Walter de Gruyter, Berlin]. This role is now examined after replacement of Tyr254 with phenylalanine. The k_{cat} is decreased about 40-fold, K_m for lactate appears unchanged, and the mainly rate-limiting step is still α-hydrogen abstraction, as judged from the steady-state deuterium isotope effect. Modeling studies with lactate introduced into the active site indicate two possible substrate conformations with different hydrogen-bonding partners for the substrate hydroxyl. If the hydrogen bond is formed with Tyr254, as was initially postulated, the mechanism must involve removal by His373 of the C2 hydrogen, with carbanion formation. If, in the absence of the Tyr254 phenol group, the hydrogen bond is formed with His373 N3, the substrate is positioned in such a way that the reaction must proceed by hydride transfer. Therefore the mechanism of the Y254F enzyme was investigated so as to distinguish between the two mechanistic possibilities. 2-Hydroxy-3-butynoate behaves with the mutant as a suicide reagent, as with the wild-type enzyme. Similarly, the mutant protein also catalyzes the reduction and the dehydrohalogenation of bromopyruvate under transhydrogenation conditions. It also catalyzes the intermolecular hydrogen transfer between [2-^{2}H]- and [2-^{3}H]lactates and bromopyruvate already observed with the wild-type protein. Although the quantitative aspects of these two reactions are somewhat different between wild-type and mutant enzymes, the results show the latter still uses a carbanion mechanism. It can be concluded that the Tyr254 hydroxyl group takes part in Michaelis complex formation as well as transition-state stabilization, as predicted, but that its role as an active-site base is not essential for electron transfer.

Flavocytochrome b_2 is a tetrameric protein found in the intermembrane space of yeast mitochondria. It catalyzes the oxidation of lactate to pyruvate at the expense of a number of monoelectronic acceptors, cytochrome *c* being the physiological one. The three-dimensional structure of the enzyme has been solved and refined to 2.4-Å resolution (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990). Its gene has been cloned in two laboratories (Guiard, 1985; Reid et al., 1988). Numerous studies in solution have provided clues as to the chemical mechanism of the lactate dehydrogenation reaction (Capeillère-Blandin et al., 1975; Pompon & Lederer, 1985; Pompon et al., 1980; Urban & Lederer, 1984, 1985; Urban et al., 1983; Tegoni et al., 1986). It is thought to involve, as a first and mainly rate-limiting step, abstraction of the substrate α-proton by an enzyme base, followed by electron transfer to FMN. Similar mechanisms are thought to be operative in a number of flavooxidases (Ghisla, 1982; Lederer, 1984; Urban & Lederer, 1985; Urban et al., 1988).

The enzyme crystal structure showed pyruvate liganded to the active site with the flavin in the semiquinone state (Mathews & Xia, 1987; Lederer, 1990). With this model it became possible to assign specific roles in catalysis to the active-site residues (Lederer & Mathews, 1987; Lederer, 1990). The predicted substrate binding modes and the possible catalytic functions of a number of side chains are presented in Figure 1. In particular, Tyr254 was predicted to hydrogen bond to the substrate O2 at all stages of the reaction and to facilitate electron departure to the flavin by deprotonating the substrate hydroxyl. The proposed series of events can now be studied by using site-directed mutagenesis (Reid et al., 1988; Black et al., 1989). This paper deals with a number of properties of the Y254F mutant, a preliminary characterization of which has been published (Reid et al., 1988).

Experimental Procedures

Enzymes. Wild-type flavocytochrome b_2 (intact form) was prepared from commercial bakers' yeast according to Labeyrie et al. (1978). The Y254F mutant was prepared either from *Saccharomyces cerevisiae* GR20 transformed with the appropriate plasmid, as described (Reid et al., 1988), or from transformed *Escherichia coli* (Black et al., 1989). In the first case, purification was carried out as described for the wild type except that further chromatography after the hydroxyapatite step was not necessary. In the second case, plasmid-bearing cells were grown at 37 °C in Luria broth supplemented with ampicillin (100 μg/mL). Frozen cells were resuspended (10 mL/g) in 0.1 M Tris-HCl buffer, pH 7.5, in the presence of 1 mM EDTA and 20 mM D,L-lactate at 4 °C. The suspension was incubated at 4 °C for 2 h in the presence of 0.1 mg/mL lysozyme and 5 μg/mL DNase I and RNase A. The lysate was centrifuged at 39000*g* for 10 min, and the supernatant was purified by precipitation between 30 and 70% ammonium sulfate saturation, followed by chromatography on hydroxy-

† This work was supported by grants from the Ministère de la Recherche et de la Technologie (87C080), the SERC, the Nuffield Foundation, the USPHS (GM20530), and NATO (174187).

‡ Hôpital Necker.
§ Department of Chemistry, University of Edinburgh.
‖ Washington University School of Medicine.
⊥ Department of Microbiology, University of Edinburgh.

0006-2960/90/0429-6393$02.50/0

Table I: Steady-State Kinetic Parameters and Deuterium Isotope Effect for Wild-Type and Y254F Mutant Flavocytochrome b_2[a]

	K_m (mM)			k_{cat} (s^{-1})			k_{cat}/K_m [M^{-1} s^{-1} ($\times 10^{-5}$)]			
enzyme	[^{1}H]Lac	[^{2}H]Lac	Phe-Lac	[^{1}H]Lac	[^{2}H]Lac	Phe-Lac	[^{1}H]Lac	[^{2}H]Lac	Phe-Lac	^DV(Lac)
WT	0.49 ± 0.10	0.50 ± 0.23	0.40 ± 0.02[b]	270 ± 30	65 ± 22	16.5 ± 3[b]	5.51	1.3	0.41	4.5 ± 1.1
Y254F	0.35 ± 0.07	0.25 ± 0.11	0.084 ± 0.001	6.1 ± 0.25	1.12 ± 0.045	0.29 ± 0.03	0.17	0.045	0.034	5.4 ± 0.4

[a] [^{1}H]Lac, L-[^{1}H]lactate; [^{2}H]Lac, L-[^{2}H]lactate; Phe-Lac, L-phenyllactate; WT, wild type. Figures are the average of three experiments for lactate and two experiments for phenyllactate. k_{cat} is expressed as mol of substrate reduced s^{-1} (mol of subunit)$^{-1}$. [b] N. Rouvière, personal communication.

apatite as described by Labeyrie et al. (1978). Phenylmethanesulfonyl fluoride (0.5 mM) was present throughout the purification.

Enzyme stocks were stored reduced under nitrogen at 4 °C as 70% ammonium sulfate precipitates in standard buffer (0.1 M sodium/potassium phosphate buffer, 1 mM EDTA, pH 7.0) containing 20 mM D,L-lactate. Working solutions were prepared daily as described by Mulet and Lederer (1977). Enzyme concentrations were expressed relative to one heme (ϵ^{ox}_{413} = 129.5 mM^{-1} cm^{-1}; ϵ^{red}_{423} = 183 mM^{-1} cm^{-1}).

Chemicals. D,L-2-Hydroxy-3-butynoic acid was synthesized by Dr. D. Pompon according to Glattfeld and Hoehn (1935) and purified as described by Pompon and Lederer (1982). D,L-[2-^{3}H]Lactic acid and 3-bromo[2-^{14}C]pyruvic acid were kindly given by Dr. P. Urban after being synthesized according to Alliel et al. (1980). Sodium L-[2-^{2}H]lactate was prepared as described by Pompon et al. (1980).

Methods. Enzymatic assays were carried out at 30 °C with a Cary 118CX or a Uvikon 930 spectrophotometer, in standard buffer containing 1 mM potassium ferricyanide and the required concentrations of substrates or inhibitors (10 mM L-lactate for the standard assay). The amount of substrate oxidized was calculated from the amount of ferricyanide consumed by using $\Delta\epsilon_{420}$ = 1.04 mM^{-1} cm^{-1}.

Dehydrohalogenation and transhydrogenation reactions were carried out anaerobically as described by Urban et al. (1983). Products were separated after acidification on an AG 1-X8 column (Bio-Rad) (16 × 0.9 cm, OH^- form). After the sample was applied, the column was first washed with 25 mL of water and then eluted with a 500-mL gradient from 0 to 2 M formic acid. Hydroxy acids were titrated with flavocytochrome b_2 in the presence of 1 mM ferricyanide, keto acids were titrated with beef heart lactate dehydrogenase (Boehringer) in the presence of 200 μM NADH, and bromopyruvate was titrated by reaction with 2-nitro-5-thiobenzoate dianion (Yun & Suelter, 1977). For the kinetic deuterium isotope effect experiments in the reverse direction, aliquots of the incubation mixture were withdrawn with a Hamilton syringe every 30 s for monitoring bromopyruvate concentration and every minute for total ketoacid concentration. The reaction was stopped by addition of an equal volume of 0.2 M HCl.

Modeling studies were carried out with an Evans & Sutherland PS300 or PS390 molecular graphics system, using the software MANOSK (Cherfils et al., 1988).

Results and Discussion

Steady-State Kinetic Parameters and Deuterium Isotope Effect. Table I presents the results of steady-state rate measurements with L-lactate, L-[^{2}H]lactate, and L-phenyllactate. The values obtained with the normal substrate are in good agreement with those determined before under somewhat different experimental conditions (Reid et al., 1988; Black et al., 1989); they confirm the lack of effect of the mutation on K_m and the 40- to 50-fold lowering of k_{cat}. L-Phenyllactate, quoted as a substrate by Armstrong (1965) but for which no quantitative data exist in the literature, also shows a lowering of k_{cat} but a 5-fold drop in K_m. Furthermore, within

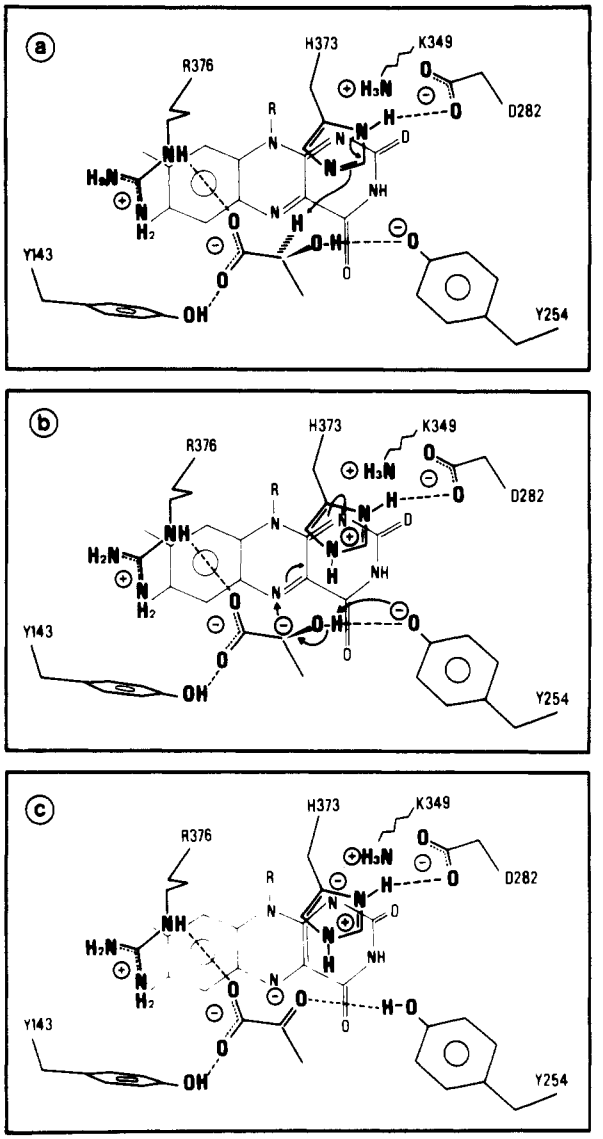


FIGURE 1: Proposed mechanism for lactate dehydrogenation by flavocytochrome b_2 (Lederer, 1990; reproduced with permission from CRC Press, Inc.) (a) Michaelis complex; (b) carbanion stage; (c) final E_{red}·product complex. Tyr254 was postulated to be ionized in (a) and (b) because of its proposed function as a general base in the transition from (b) to (c). The picture in (b) implies flavin reduction through two monoelectronic transfers in quick succession. No experimental evidence exists concerning this point, and the intermediate formation of a covalent bond between substrate C2 and flavin N5 is not ruled out at present. In (c), Fl_{red} is pictured as its N1–N5 dianion because there does not appear to be enough space around N5 to accommodate a protonating solvent species before pyruvate has left the active site. This proposed mechanism is discussed in part by Lederer and Mathews (1987) and more fully by Lederer (1990).

experimental error, the deuterium isotope effect on lactate oxidation is the same for wild-type and mutant enzymes [previously published values for the wild-type enzyme are 5 at 30 °C (Lederer, 1974) and 3.8 ± 0.8 at 4 °C (Pompon et al., 1980)]. For the wild-type enzyme, stopped-flow experiments indicated a deuterium isotope effect of 8 on flavin re-

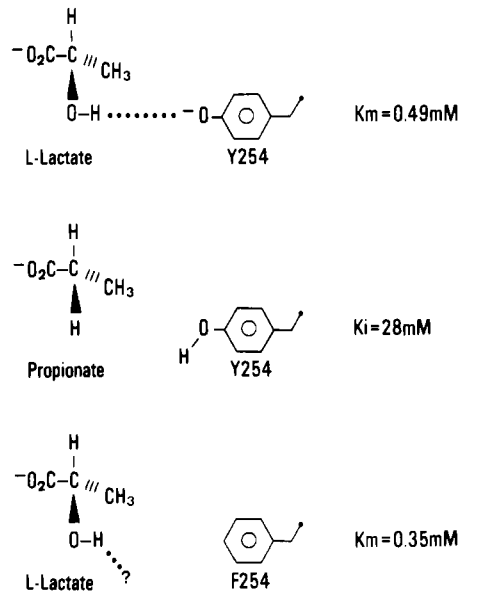


FIGURE 2: Three hydrogen-bonding situations. The propionate K_i value is taken from Genet and Lederer (1990). It can be seen that, compared to the postulated hydrogen bond between lactate and Tyr254 (top), the symmetrical removal of the substrate hydroxyl group (middle) or of the enzyme phenol group (bottom) does not lead to a symmetrical change in affinity. It should be concluded that in Y254F flavocytochrome b_2 the lactate hydroxyl forms a hydrogen bond with a group on the protein.

duction, showing that α-hydrogen abstraction is the mainly rate-limiting step in the overall transfer from lactate to ferricyanide (Pompon et al., 1980). Pending similar rapid kinetic studies with the mutant enzyme, one can thus conclude that it has the same mainly or totally rate-limiting step as the wild-type protein.

The K_m for lactate of the *Saccharomyces* flavocytochrome b_2 is known to be close to K_s (Hinkson & Mahler, 1963; Blazy et al., 1976; Mulet & Lederer, 1977; Pompon et al., 1980); it can therefore be used as a good indicator of the substrate affinity. The fact that it is not altered in the mutant protein would seem to imply that, contrary to the proposal of Figure 1, Tyr254 is not involved in Michaelis complex formation. However, the comparison of lactate K_m values for wild-type and mutant enzymes with the propionate K_i value of 28 mM (Genet & Lederer, 1990) (Figure 2) suggests that the substrate hydroxyl group in the mutant enzyme must be hydrogen bonded to some group on the protein.

Modeling Studies. The active site was inspected on a molecular graphics system, and it appeared that the only potential partner, other than Tyr254, for a hydrogen bond with the substrate was His373. Pyruvate in the active site was replaced with L-lactate and modeling studies were carried out without introducing any alteration in the protein, assuming implicitly that the mutation had not entailed any gross structural change in the active-site topography. A crystallographic study of the mutant is planned in order to check this point.

The coordinates of the lactate carboxylate as well as those of C2 were kept identical with those of the corresponding pyruvate atoms in the crystal structure, and the C2 substituents were rotated around the C1–C_2 axis. Two sterically possible geometries were found which were particularly interesting in terms of catalysis (Lederer, 1990). They are shown in Figure 3. In Figure 3A, the substrate α-hydrogen points toward His373 N3, the C3 methyl group is oriented toward the flavin but below its plane, and the hydroxyl group points away from the flavin; it is closer to the Tyr254 phenol group than to His373 N3. The distance for the former (3.8 Å) is, however, too long for a hydrogen bond. Further modeling is required to optimize the interaction; nevertheless, Figure 3A presents an interesting approximation of an active-site configuration poised for a carbanion mechanism. On the other hand, by rotating the C2 substituents clockwise, one can reach the substrate conformation shown in Figure 3B, with the substrate hydroxyl forming a good hydrogen bond to His373 N3 and the α-hydrogen pointing directly toward flavin N5. In this case, His373 would play in the mutant the same general base role with respect to the substrate hydroxyl as has been ascribed to the active-site histidine in nicotinamide-dependent lactate and malate dehydrogenases (Birktoft & Banaszak, 1983), and hydride transfer to flavin N5 would ensue.

Since solution studies provided strong evidence in favor of a carbanion mechanism for the wild-type protein (Urban & Lederer, 1985), the modeling studies suggested that, in the mutant, the substrate might have changed its hydrogen-bonding partner, with His373 replacing Tyr254. While providing a similar stabilization for the Michaelis complex, this change would lead to a switch from a carbanion mechanism to a hydride transfer reaction. This hypothesis was all the more appealing since previous studies had suggested that the wild-type enzyme, when reconstituted with 5-carba-5-deazaflavin, could perhaps work by hydride transfer (Pompon & Lederer, 1979; Ghisla & Massey, 1986). Consequently, the mutant enzyme mechanism was probed with the same kind of experiments that had shown a carbanion mechanism for the wild-type protein.

Reaction with 2-Hydroxy-3-butynoate. This reagent was first used with lactate oxidase and was found to act as a suicide substrate by forming a covalent adduct with the flavin, the structure of which has been established (Ghisla et al., 1976; Schonbrunn et al., 1976). Adduct formation was explained by attack at the flavin C4a position of an allenic carbanion arising from an initially formed, catalytically competent acetylenic carbanion; however, Michael addition of reduced flavin on the acetylenic keto acid, the normal oxidation product, would have led to the same product and this pathway, although less likely, could not be excluded. Thus, this kind of experiment, however suggestive, cannot be considered as an absolute proof of a carbanion mechanism.

2-Hydroxy-3-butynoate has also been found to inactivate wild-type flavocytochrome b_2 by formation of an adduct which was shown to be identical with that obtained with lactate oxidase (Lederer, 1974; Pompon & Lederer, 1985). When the Y254F enzyme (15 μM) was incubated with 3–6 mM D,L-2-hydroxy-3-butynoate in standard buffer containing 1 mM ferricyanide, at 30 °C, the rate of acceptor reduction rapidly decreased to zero, indicating enzyme inactivation. After addition of fresh enzyme, ferricyanide consumption resumed and then ceased again. When this was repeated, a linear relationship was found between the number of inactivation events and the number of turnovers (i.e., the amount of ferricyanide consumed each time) (not shown). In other words, inactivation appeared to be independent from product accumulation, as observed for the wild-type enzyme. The partition ratio between catalytic and inactivating events was found to be 680, compared to 3200 for the wild-type protein (Pompon & Lederer, 1985). These figures suggest that the inactivation rate is less affected by the mutation than is the turnover rate. There may be several explanations for this, but it seems premature to present speculations. The flavin absorption spectra of mutant and wild-type inactivated enzymes presented the same modifications with respect to control

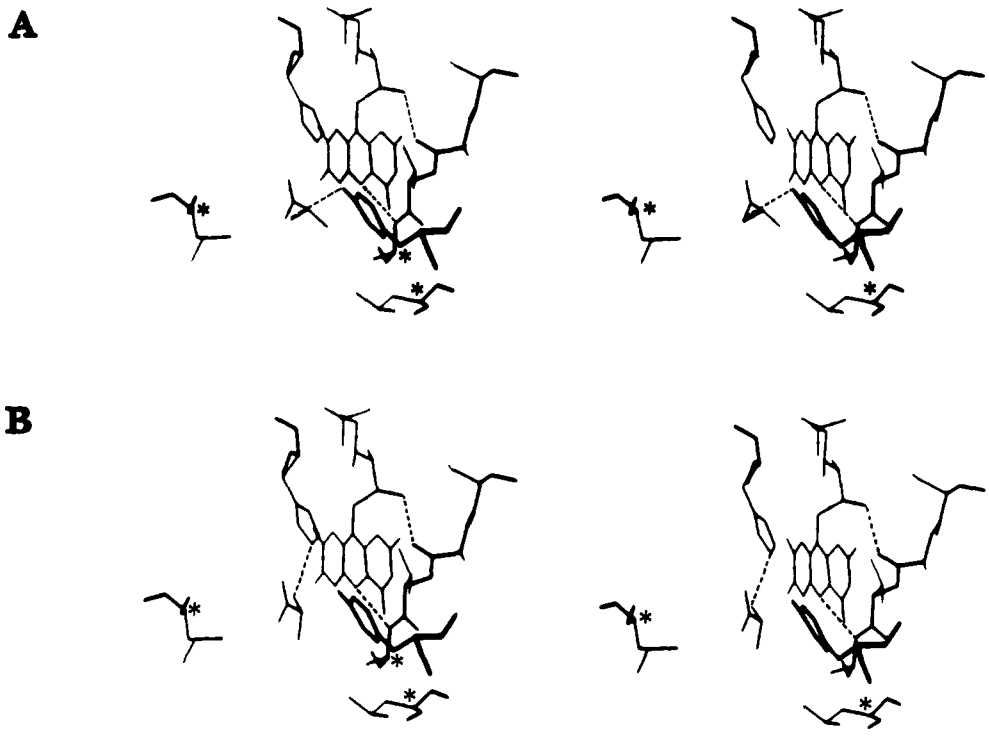
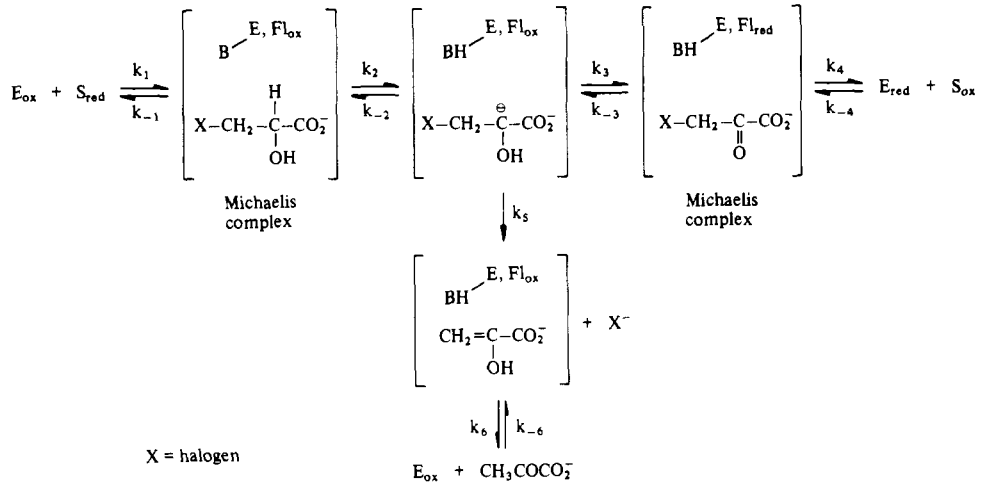


FIGURE 3: Modeling of L-lactate at the active site (Lederer, 1990; reproduced with permission from CRC Press, Inc.). (A) Proposed lactate conformation for a carbanion mechanism. The torsion angle between the planes defined by substrate atoms O1–C1–C2 and C1–C2–O2 is 105° (this angle is 0° when O1 and O2 are eclipsed). The substrate coordinates for the carboxylate atoms and C2 are the same as those of pyruvate in the crystal structure. Besides His373 and Tyr254, several side chains are shown that do not appear in Figure 1. Leu230 (bottom right) and Leu286 (extreme left) are the closest side chains to the lactate C2 substituents together with Ala198. The peptide chain from Thr195 to Ala198, running from top to bottom, is shown behind the flavin (on the right). The Cα atoms of Ala198, Leu230, and Leu283 are starred. Dashed lines represent hydrogen bonds between Ala196 amide oxygen and a ribityl phosphate oxygen and between Ala198 amide nitrogen and flavin N5 (Mathews & Xia, 1987; Lederer & Mathews, 1987) and the putative hydrogen bond between substrate O2 and Tyr254 phenol oxygen. (B) Proposed lactate conformation in the case of a hydride transfer reaction. The torsion angle as defined in A is 40°. The putative hydrogen bond between substrate O2 and N3 of His373 is shown with a dashed line.

Scheme I: Proposed Reaction Scheme for a Halogeno Substrate[a]



[a] X = Br or Cl.

protein, indicating that the same adduct was formed (not shown). All these results showed that the mutation had not led to any qualitative change in the reaction between enzyme and 2-hydroxy-3-butynoate.

The Dehydrohalogenation Reaction. Halide ion elimination from β-halogeno substrates was proposed as a diagnostic test for a carbanion mechanism in flavoenzyme-catalyzed dehydrogenation of α-hydroxy and α-amino acids; its occurrence, however, constituted strongly supportive evidence but not absolute proof (Ghisla, 1982). With flavocytochrome b_2, dehydrohalogenation was more easily observed under transhydrogenation conditions, using lactate and bromo- or chloropyruvates as substrates, than under forward reaction conditions (lactate and ferricyanide) (Urban & Lederer, 1984). This was due to a more favorable partition between carbanion protonation and halide ion elimination (reverse reaction) than between carbanion oxidation and elimination (forward reaction) (Scheme I).

We therefore analyzed the products of a transhydrogenation reaction between lactate and [2-^{14}C]bromopyruvate, catalyzed by the Y254F mutant. The resulting incubation mixture was analyzed in two ways. First, the final keto acid concentration

Table II: Intermolecular Tritium Transfer between [2-^{3}H]Lactate and Halogenopyruvates[a]

halogeno-pyruvate substrate	initial concn (mM)	enzyme form	sp radioact of L-[2-^{3}H]lactate ($\times10^{-3}$ cpm μmol^{-1})	radioact ($\times10^{-3}$ cpm) H_2O	halogeno-lactate	pyruvate	product formed (μmol) halogeno-lactate	pyruvate	sp radioact ($\times10^{-3}$ cpm μmol^{-1}) H_2O	halogeno-lactate	pyruvate	fraction transferred[b] (τ)
FPyr[c]	20	WT	276	116	294.1		16.6	0	0.002	17.7		0.72
FPyr[d]	20	Y254F	477	190	20.6		2.5	0	0.003	8.2		0.10
BrPyr[e]	7.5	WT	276	244	267.8	28.2	15	5.4[f]	0.004	17.9	5.2[f]	0.55
BrPyr[g]	7.5	Y254F	350	214	12.3	3.3	2	3.1[f]	0.003	6.1	1.1[f]	0.07

[a] All incubations were carried out at 30 °C in an initial volume of 1 mL, with an initial L-lactate concentration of about 25 mM; for these experiments, the radioactive D,L-[2-^{3}H]lactate was diluted between 50- and 100-fold in L-lactate. [b] The amount of transfer, τ, is the ratio of the sum of radioactivity found in the halogenohydroxy acid and pyruvate to the total radioactivity transferred from [^{3}H]lactate (found in water and the two reaction products). [c] Enzyme concentration was 6.4 μM. The reaction was stopped after 20 min by freezing. [d] Enzyme concentration was 15 μM. The reaction was stopped after 30 min by addition of 20 μL of concentrated HCl at 0 °C. [e] The initial enzyme concentration was 6.4 μM. The following anaerobic additions were made: 7.5 μmol of bromopyruvate and 1.1 nmol of enzyme after 9 min; 8.5 μmol of lactate, 15.5 μmol of bromopyruvate, and 1.1 nmol of enzyme after 18 min; 1.1 nmol of enzyme after 24 min. The reaction was stopped by freezing after 30 min (final volume 1.31 mL). [f] This figure takes into account only the pyruvate produced from bromopyruvate by halide ion elimination. [g] The initial enzyme concentration was 35 μM. The following anaerobic additions were made: 3.9 nmol of enzyme after 5 and 10 min; 7.5 μmol each of lactate and bromopyruvate and 3.9 nmol of enzyme after 15 min; 3.9 nmol of enzyme after 20 and 25 min. The reaction was quenched by addition of 20 μL of concentrated HCl at 0 °C after 30 min (final volume 1.33 mL).

was compared to the initial one: it should not change when no dehydrohalogenation takes place, but it should increase in the case of an elimination reaction (Scheme I; Urban & Lederer, 1984). Second, the reaction mixture was fractionated so as to detect the possible presence of [2-^{14}C]pyruvate, the product of Br^- elimination from [2-^{14}C]bromopyruvate. In view of the low catalytic rate of the mutant protein, the reaction conditions (Figure 4) were chosen so as to favor the detection of even very low amounts of elimination. The results (Figure 4) showed unambiguously that all the radioactive bromopyruvate had been transformed into two compounds: bromolactate (2.2 μmol) and pyruvate (3.4 μmol of [2-^{14}C]-pyruvate out of 8.6 μmol of total keto acid present at the end of the reaction). The calculated amount of [2-^{14}C]pyruvate corresponded well to the increase of 3.2 μmol of total keto acids during the incubation, a figure independently determined before chromatography.

Since a control experiment carried out under identical conditions but without enzyme showed no formation of [2-^{14}C]pyruvate, the experiment demonstrated that the mutant enzyme, as the wild type, catalyzes the dehydrohalogenation reaction.

Intermolecular Hydrogen Transfer. (A) Tritium Transfer. As discussed in a previous paper, the best evidence in favor of a carbanion mechanism comes from the study of the intermolecular hydrogen transfer catalyzed by flavocytochrome b_2, using either [2-^{3}H]lactate or [2-^{2}H]lactate and a halogenopyruvate (Urban & Lederer, 1985).

With the 2-^{3}H substrate, the wild-type enzyme has been previously shown to transfer tritium both to the C2 position of halogenopyruvates in the reduction reaction and to the C3 position of the pyruvate formed in the elimination reaction (Scheme II). It was also shown that exchange with solvent of the protein-bound substrate α-proton only occurred when the active site was free (after product dissociation and before reverse substrate binding), so that the amount of tritium transfer increased with increasing starting halogeno keto acid concentration (see Schemes I and II) (Urban & Lederer, 1985).

All these intermolecular hydrogen transfer experiments had been carried out with the so-called Morton form, or cleaved form, of flavocytochrome b_2. This species is obtained when no precautions are taken against proteolysis during purification and has been previously shown to have undergone selective proteolytic cleavage in an area lying between positions 305 and 314 in the sequence (Ghrir & Lederer, 1981). This form has

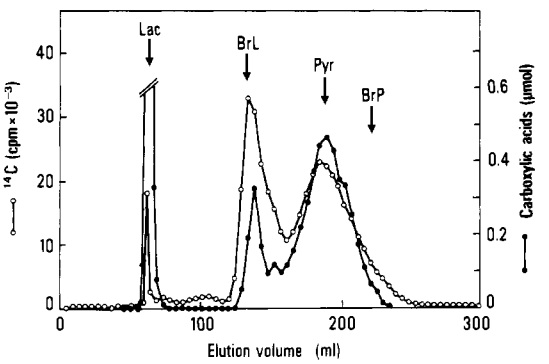
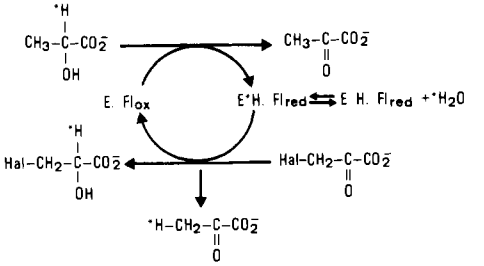


FIGURE 4: Reaction between reduced Y254F flavocytochrome b_2 and bromo[2-^{14}C]pyruvate: separation of reactants and reaction products. Initial conditions were 25.5 mM L-lactate, 3.2 mM bromo[2-^{14}C]-pyruvate (132×10^3 cpm μmol^{-1}), and 28.5 μM Y254F enzyme in 1 mL of standard buffer at 30 °C. The following anaerobic additions were subsequently made: 4 nmol of enzyme after 5 and 10 min; 8.5 μmol of L-lactate, 3.2 μmol of bromo[2-^{14}C]pyruvate, and 4 nmol of enzyme after 15 min; 8.1 nmol of enzyme after 20 and 25 min; 4 nmol of enzyme after 30 min. The reaction was stopped by addition of 20 μL of concentrated HCl after 35 min of incubation. Lac, lactate; BrL, bromolactate; Pyr, pyruvate; BrP, bromopyruvate. The reaction mixture was fractionated on AG 1-X8 as described under Methods.

Scheme II: Intermolecular Hydrogen Transfer between Labeled Lactate and a Halogenopyruvate[a]



[a] Hal = Br or Cl.

2–2.5-fold lower specific activity than the intact form to which we are comparing the mutant enzyme, as well as a usually lower affinity for active-site ligands (Somlo & Slonimski, 1966; Jacq & Lederer, 1972). With respect to the intermolecular hydrogen transfer experiments, a number of quantitative aspects now also appear to differ between wild-type intact and Morton forms, as will be seen below.

Table II shows the results of comparative experiments carried out with both mutant and wild-type enzymes (intact

forms), looking at the transfer from L-[2-^{3}H]lactate to fluoropyruvate, a substrate which does not undergo halide ion elimination, and to bromopyruvate, which does. A first interesting point emerges from the results of the reaction with fluoropyruvate. The mutant protein also catalyzes tritium transfer to this reverse substrate, but a striking difference is observed in the amount of transfer, τ, catalyzed by the wild-type and the mutant enzymes. It cannot be excluded that these differences arise from different saturation levels in fluoropyruvate during the experiments, since no data are yet available concerning the K_m values of fluoropyruvate for the intact wild-type enzyme and for the mutated one [for Morton enzyme, the expected value of transfer at 20 mM fluoropyruvate (K_m = 7.2 mM) is 0.3 (Urban et al., 1983; Urban & Lederer, 1985)]. If the fluoropyruvate K_m values are not too different for the two enzymes examined in the present work—since their lactate K_m values are similar—the observed disparity between the amounts of transfer may mean that, in the mutant, exchange with the solvent of the protein-bound substrate α-hydrogen is faster than in the wild type. This point deserves further investigation.

The amount of transfer catalyzed by the two enzymes in the bromopyruvate case cannot be compared in a similar fashion because of the experimental protocol adopted, which involved multiple additions (see Table II) [a compromise between the necessity of accumulating enough turnovers and that of avoiding high bromopyruvate concentrations, which lead to an inactivating enzyme alkylation (Mulet & Lederer, 1977)]. Nevertheless, the additional information given by the experiments with bromopyruvate constitutes the second point of interest in Table II: the specific radioactivity is substantially lower for pyruvate than for bromolactate, with both enzymes. A similar observation had been made with Morton enzyme, with proper controls to verify that this result was not due to nonenzymatic exchange of labile pyruvate protons (Urban & Lederer, 1985). This fact had been ascribed to partial dissociation of enolpyruvate from the enzyme before isomerization (Scheme I). In the case of a hydride transfer reaction a similar isotope discrimination factor would have been expected for protonation at C2 and at C3.

(B) Deuterium Isotope Effect on Bromopyruvate Transformation. It has been previously shown that, under transhydrogenation conditions, the use of [2-^{2}H]lactate enables the determination of an isotope effect on the reverse reaction, confirming that the slow step in the reverse direction is carbanion protonation (Urban & Lederer, 1985). After extrapolation to infinite bromopyruvate concentration, a ^DV of 4.4 was determined for the disappearance of bromopyruvate. For the dehydrohalogenation reaction, no similar extrapolation could be carried out because of experimental difficulties due to the slowness of the reaction. But at concentrations where bromopyruvate was transformed with an overall isotope effect of about 3, pyruvate was found to appear with a ^DV of 0.5. This was taken as a strong piece of evidence in favor of a branched pathway such as the one shown in Scheme I, where an intermediate partitions between an isotope-sensitive and an isotope-insensitive pathway.

We investigated the situation with the mutant enzyme. The experiments were difficult ones because no reliable determination could be carried out below 3 mM bromopyruvate, owing in part to the faster exchange catalyzed by the mutant enzyme under the experimental conditions used; on the other hand, above 15 mM, enzyme inactivation was too fast. Nevertheless, a concentration-dependence study of bromopyruvate disappearance in the presence of saturating L-[^{1}H]lactate and L-[^{2}H]lactate yielded the results of Figure 5: a ^DV of 1.96 ± 0.08 was obtained by extrapolation to infinite bromopyruvate concentration. In so doing, a K_m of 18 ± 2 mM and a k_{cat} of 0.94 ± 0.1 s^{-1} were determined for global bromopyruvate transformation. For the isotope effect on pyruvate formation, the values obtained are given in Table III: they show no reliable deviation from 1.

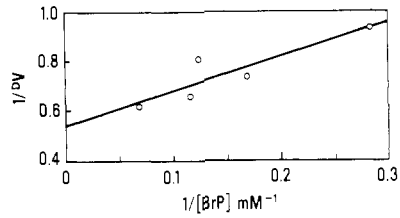


FIGURE 5: Substrate concentration dependence of the deuterium isotope effect on bromopyruvate disappearance catalyzed by reduced Y254F flavocytochrome b_2. At each initial concentration of bromopyruvate, its disappearance was monitored as described under Methods. Initial rates were linear for at least 4 min. Other reactants were 23.7 mM L-[2-^{1}H]lactate or 23.5 mM L-[2-^{2}H]lactate and 40 μM enzyme.

Table III: Deuterium Isotope Effect on Mutant-Catalyzed Elimination from Bromopyruvate[a]

expt	isotope transferred	bromopyruvate concn (mM)	k_{obs} (pyruvate formation) (s^{-1})	$^Dk_{obs}$
1	^{1}H	9.3 ± 0.4	0.215 ± 0.020	1.00 ± 0.30
	^{2}H		0.215 ± 0.045	
2	^{1}H	4.25 ± 0.5	0.046 ± 0.004	0.81 ± 0.13
	^{2}H		0.057 ± 0.004	
3	^{1}H	3.85 ± 0.25	0.073 ± 0.007	0.76 ± 0.30
	^{2}H		0.096 ± 0.029	

[a] The experiments were carried out as described under Methods, with 24 mM L-[2-^{1}H]lactate or L-[2-^{2}H]lactate and 25–50 μM Y254F enzyme in standard buffer at 30 °C.

These results can be qualitatively understood in the following way. If halide ion elimination is the rate-determining step on the pathway to pyruvate formation via enolpyruvate, as it is with Morton enzyme (Urban & Lederer, 1984), then in the case of exclusive occurrence of the elimination pathway, bromopyruvate disappearance and pyruvate formation rates would be equal and subject to an isotope effect of 1. When reduction and elimination compete for the same intermediate (Scheme I), the isotope effect on pyruvate formation will decrease below 1; its decrease will be a function of the value of the isotope effect on bromopyruvate disappearance. In turn, the latter will be a function both of the degree of enzyme saturation in bromopyruvate for a given experiment (i.e., of the degree of enzyme deuteration) and of the partition ratio between carbanion reduction and elimination. While for thermodynamic reasons reduction must be distinctly slower in the Y254F enzyme than in the wild-type protein, the elimination rate is not expected to vary as much, if at all. In other words, the partition between reduction and elimination is expected to be more favorable to elimination with the mutant protein. All these reasons appear sufficient to qualitatively explain the low extrapolated value of $^Dk_{cat}$ for bromopyruvate disappearance and the absence of reliable deviation from 1 of the isotope effect on pyruvate formation.

Finally, a reasoning ad absurdum confirms the idea that the mutant enzyme, as the wild-type one, uses a carbanion mechanism. In the case of a hydride transfer reaction, the results of the tritium transfer experiments would imply a higher discrimination against tritium for isotope transfer to substrate C3 than to C2. In turn this would imply a higher deuterium isotope effect on pyruvate formation than on bromolactate

formation; hence one would expect the former effect to be at least as high if not higher than the composite isotope effect determined for bromopyruvate disappearance, which clearly is not the case. We conclude from all this that the results obtained rule out a hydride transfer reaction.

CONCLUSION

The absence of K_m change in the Y254F mutant seemed at first sight incompatible with the postulated existence of a hydrogen bond between the substrate hydroxyl function and Tyr254 phenol group. The experiments described above rule out an alternative hydrogen bond between the substrate and His373 N3. It follows that new interactions between the substrate and the protein must provide in the mutant enzyme an energetic compensation for the loss of the substrate–Tyr254 hydrogen bond. The following suggestion can be made concerning the nature of the compensation. Thomas et al. (1982), after examining a number of protein crystal structures, found a preferred interaction between oxygen atoms and the edge of phenylalanine aromatic rings. Similar observations were also made by Gould et al. (1985), and a number of other examples are summarized in a recent review (Burley & Petsko, 1988). The origin of these preferred interactions is presumably a weak electrostatic attraction between the negatively polarized oxygen atoms and the positively polarized aromatic ring edge. Quantum mechanical calculations using formamide and benzene as a model showed a stabilization energy of up to 2 kcal/mol in favorable cases (Thomas et al., 1982). Therefore, it appears possible that at the mutant flavocytochrome b_2 active site the substrate reorients itself so that its hydroxyl group can make a favorable interaction with the Phe254 side chain, which may itself also shift its position. This reorientation would lead to incorrect positioning of the α-hydrogen with respect to His373. One finds support in favor of this idea by considering the case of L-phenyllactate (Table I). The k_{cat}/K_m values indicate that the transition-state energy for the reaction between wild-type enzyme and phenyllactate is higher than that for the reaction with lactate. But the transition-state destabilization induced by the mutation is more important for lactate than for phenyllactate (2.1 versus 1.5 kcal/mol, respectively). It looks as though steric hindrance caused by the phenyllactate aromatic ring already introduces in the wild-type transition state a deviation from the optimal geometry for carbanion formation. The distortion increases in the mutant, as concluded from the k_{cat}/K_m values, but this time the phenyllactate K_m decreases. This suggests that in the absence of the hydrogen bond between the hydroxyl groups the substrate and Phe254 are free to adopt a conformation which will optimize the interaction between the aromatic rings in the Michaelis complex. Thus, the detailed examination of the kinetic parameters shown in Table I finally also gives evidence in favor of the existence of a hydrogen bond between the substrate and the Tyr254 phenol group in the Michaelis complex, even though the absence of lactate K_m alteration in the mutant enzyme could have been taken at first sight as evidence against the existence of this hydrogen bond.

The present work was undertaken with the aim of checking the function of Tyr254 in the catalysis of lactate dehydrogenation by flavocytochrome b_2. The results described above confirm that Tyr254 plays a role in both Michaelis complex formation and transition-state stabilization (Lederer & Mathews, 1987). Its main function appears to be that of freezing the substrate alcohol function in a conformation such that the α-hydrogen is well oriented for abstraction by His373 N3. Furthermore, it is clear that, in the absence of the phenol group, electron transfer to the flavin still takes place, so that the function of general base predicted for Tyr254 does not appear essential; in the case of the Y254F enzyme, at least, it seems that the substrate hydroxyl proton can be removed directly by a solvent species.

The work described in this paper, based on a three-dimensional structure, constitutes the first step toward the elucidation of the role of side chains in catalysis of a flavin-dependent α-hydroxy acid dehydrogenation reaction. It will be interesting to see to what extent the conclusions will also be applicable to the other enzymes catalyzing this reaction, which are known to be homologous to flavocytochrome b_2 but which belong to the oxidase class: glycolate oxidase (Volokita & Somerville, 1987), long-chain hydroxy acid oxidase (Urban et al., 1988), and lactate oxidase from *Mycobacterium smegmatis* (Giegel et al., 1990).

ACKNOWLEDGMENTS

Two of us (J.D. and F.L.) thank Dr. J. P. Mornon for access to his graphics system, as well as Drs. M. C. Vaney, N. Colloc'h, and C. Gaboriaud for help and advice in the use of the software. We are indebted to D. Mazella for competent secretarial help.

REFERENCES

Alliel, P. M., Mulet C. & Lederer, F. (1980) *Eur. J. Biochem. 105*, 343–351.
Armstrong, J. McD. (1965) *Ann. N.Y. Acad. Sci. 119*, 877–887.
Birktoft, J. J., & Banaszak, L. J. (1983) *J. Biol. Chem. 258*, 472–482.
Black, M. T., White, S. A., Reid, G. A., & Chapman, S. K. (1989) *Biochem. J. 258*, 255–259.
Blazy, B., Thusius, D., & Baudras, A. (1976) *Biochemistry 15*, 257–261.
Burley, S. K., & Petsko, G. A. (1988) *Adv. Protein Chem. 39*, 125–189.
Capeillère-Blandin, C., Bray, R. C., Iwatsubo, M., & Labeyrie, F. (1975) *Eur. J. Biochem. 54*, 549–566.
Cherfils, J., Vaney, M. C., Morize, I., Surcourf, E., Colloc'h, N., & Mornon, J. P. (1988) *J. Mol. Graphics 6*, 155–160.
Genet, R., & Lederer, F. (1990) *Biochem. J. 266*, 301–304.
Ghisla, S. (1982) in *Flavins and Flavoproteins*, Proceedings of the Seventh International Symposium, Ann Arbor, MI, 1981 (Massey, V., & Williams, C. H., Eds.) pp 133–142, Elsevier, Amsterdam.
Ghisla, S., & Massey, V. (1986) *Biochem. J. 239*, 1–12.
Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H., & Walsh, C. T. (1976) *Biochemistry 15*, 1791–1797.
Ghrir, R., & Lederer, F. (1981) *Eur. J. Biochem. 120*, 279–287.
Giegel, D. A., Williams, C. H., Jr., & Massey, V. (1990) *J. Biol. Chem. 265*, 6626–6632.
Glattfeld, J. W. E., & Hoehn, R. E. (1935) *J. Am. Chem. Soc. 57*, 1405–1407.
Gould, R. O., Gray, A. M., Taylor, P., & Walkinshaw, M. D. (1985) *J. Am. Chem. Soc. 107*, 5921–5927.
Guiard, B. (1985) *EMBO J. 4*, 3265–3272.
Hinkson, J. W., & Mahler, H. R. (1963) *Biochemistry 2*, 216–220.
Jacq, C., & Lederer, F. (1972) *Eur. J. Biochem. 25*, 41–48.
Jacq, C., & Lederer, F. (1974) *Eur. J. Biochem. 41*, 311–320.
Labeyrie, F., Baudras, A., & Lederer, F. (1978) *Methods Enzymol. 53*, 238–256.
Lederer, F. (1974) *Eur. J. Biochem. 46*, 393–399.
Lederer, F. (1984) in *Flavins and Flavoproteins*, Proceedings of the Eighth International Symposium, Brighton, U.K.,

1984 (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 513-526, Walter de Gruyter, Berlin.
Lederer, F. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) CRC Press, Inc., Boca Raton, FL (in press).
Lederer, F., & Mathews, F. S. (1987) in *Flavins and Flavoproteins*, Proceedings of the Ninth International Symposium, Atlanta, GA, 1987 (Edmondson, D. E., & McCormick, D. B., Eds.) pp 133-142, Walter de Gruyter, Berlin.
Mathews, F. S., & Xia, Z. X. (1987) in *Flavins and Flavoproteins*, Proceedings of the Ninth International Symposium, Atlanta, GA, 1987 (Edmondson, D. E., & McCormick, D. B., Eds.) pp 123-132, Walter de Gruyter, Berlin.
Mulet, C., & Lederer, F. (1977) *Eur. J. Biochem. 73*, 443-447.
Pompon, D., & Lederer, F. (1979) *Eur. J. Biochem. 96*, 571-579.
Pompon, D., & Lederer, F. (1982) *Eur. J. Biochem. 129*, 143-147.
Pompon, D., & Lederer, F. (1985) *Eur. J. Biochem. 148*, 145-154.
Pompon, D., Iwatsubo, M., & Lederer, F. (1980) *Eur. J. Biochem. 104*, 479-488.
Reid, G. A., White, S., Black, M. T., Lederer, F., Mathews, F. S., & Chapman, S. K. (1988) *Eur. J. Biochem. 178*, 329-333.
Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H., & Massey, V. (1976) *Biochemistry 15*, 1798-1807.
Somlo, M., & Slonimski, P. (1966) *Bull. Soc. Chim. Biol. 48*, 1221-1249.
Tegoni, M., Janot, J. M., & Labeyrie, F. (1986) *Eur. J. Biochem. 155*, 491-503.
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. (1984) *Eur. J. Biochem. 144*, 345-351.
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.
Urban, P., Chirat, I., & Lederer, F. (1988) *Biochemistry 27*, 7365-7371.
Volokita, M., & Somerville, C. R. (1987) *J. Biol. Chem. 262*, 15825-15828.
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.
Yun, S. L., & Suelter, C. H. (1978) *Anal. Biochem. 85*, 437-441.

Buffer Enhancement of Proton Transfer in Catalysis by Human Carbonic Anhydrase III†

Chingkuang Tu,‡ Shanthi R. Paranawithana,‡ David A. Jewell,§ Susan M. Tanhauser,§ Philip V. LoGrasso,‡ George C. Wynns,‡ Philip J. Laipis,§ and David N. Silverman*,‡

Department of Pharmacology and Therapeutics and Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, Florida 32610

Received November 8, 1989; Revised Manuscript Received March 8, 1990

ABSTRACT: Among the isozymes of carbonic anhydrase, isozyme III is the least efficient in the catalysis of the hydration of CO_2 and was previously thought to be unaffected by proton transfer from buffers to the active site. We report that buffers of small size, especially imidazole, increase the rate of catalysis by human carbonic anhydrase III (HCA III) of (1) ^{18}O exchange between HCO_3^- and water measured by membrane-inlet mass spectrometry and (2) the dehydration of HCO_3^- measured by stopped-flow spectrophotometry. Imidazole enhanced the rate of release of ^{18}O-labeled water from the active site of wild-type carbonic anhydrase III and caused a much greater enhancement, up to 20-fold, for the K64H, R67H, and R67N mutants of this isozyme. Imidazole had no effect on the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium. Steady-state measurements showed that the addition of imidazole resulted in increases in the turnover number (k_{cat}) for the hydration of CO_2 catalyzed by HCA III and for the dehydration of HCO_3^- catalyzed by R67N HCA III. These results are consistent with the transfer of a proton from the imidazolium cation to the zinc-bound hydroxide at the active site, a step required to regenerate the active form of enzyme in the catalytic cycle. Like isozyme II of carbonic anhydrase, isozyme III can be enhanced in catalytic rate by the presence of small molecule buffers in solution.

Human carbonic anhydrase III (HCA III)[1] is found predominantly in skeletal muscle. It has a maximal turnover number in the hydration of CO_2 near 1×10^4 s^{-1}, about 100-fold less than that of red cell carbonic anhydrase II. There is a significant body of evidence suggesting that, like isozyme II, catalysis by HCA III of CO_2 hydration and HCO_3^- dehydration occurs in two separate and distinct steps (Silverman & Lindskog, 1988). The first step is the conversion of HCO_3^-

† This work was supported by a grant from the National Institutes of Health (GM 25154).

* Address correspondence to this author at Box J-267 Health Center, Department of Pharmacology, University of Florida, Gainesville, FL 32610.
‡ Department of Pharmacology and Therapeutics.
§ Department of Biochemistry and Molecular Biology.

[1] Abbreviations: HCA III, human carbonic anhydrase III; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

0006-2960/90/0429-6400$02.50/0