Probing the Relative Timing of Hydrogen Abstraction Steps in the Flavocytochrome b_2 Reaction with Primary and Solvent Deuterium Isotope Effects and Mutant Enzymes†

Pablo Sobrado,‡ S. Colette Daubner,‡ and Paul F. Fitzpatrick*,‡,§

Departments of Biochemistry and Biophysics and of Chemistry, Texas A&M University, College Station, Texas 77843-2128

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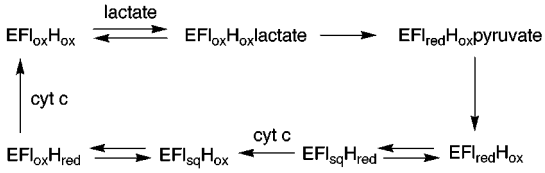
ABSTRACT: Flavocytochrome b_2 catalyzes the oxidation of lactate to pyruvate. Primary deuterium and solvent kinetic isotope effects have been used to determine the relative timing of cleavage of the lactate O−H and C−H bonds by the wild-type enzyme, a mutant protein lacking the heme domain, and the D282N enzyme. The $^{D}V_{max}$ and $^{D}(V/K_{lactate})$ values are both 3.0 with the wild-type enzyme at pH 7.5 and 25 °C, increasing to about 3.6 with the flavin domain and increasing further to about 4.5 with the D282N enzyme. Under these conditions, the $^{D20}V_{max}$ values are 1.38, 1.18, and 0.98 for the wild-type enzyme, the flavin domain, and the D282N enzyme, respectively; the $^{D20}(V/K_{lactate})$ values are 0.9, 0.44, and 1.0, respectively. The $^{D}k_{red}$ value is 5.4 for the wild-type enzyme and 3.5 for the flavin domain, whereas the solvent isotope effect on this kinetic parameter is 1.0 for both enzymes. The V_{max} values for the wild-type enzyme and the flavin domain are 32 and 15% limited by viscosity, respectively. In contrast, the $V/K_{lactate}$ value for the flavin domain increases about 2-fold at moderate concentrations of glycerol. The data are consistent with a minimal chemical mechanism in which the lactate hydroxyl proton is not in flight in the transition state for C−H bond cleavage and there is an internal equilibrium involving the lactate-bound enzyme prior to C−H bond cleavage which is sensitive to solution conditions. Removal of the hydroxyl proton may occur in this pre-equilibrium.

Flavocytochrome b_2 from *Saccharomyces cerevisiae* catalyzes the oxidation of L-lactate to pyruvate. The enzyme is a member of a family of homologous flavoenzymes catalyzing similar oxidations of α-hydroxy acids to keto acids; this family includes the enzymes glycolate oxidase, lactate monooxygenase, lactate oxidase, mandelate dehydrogenase, and 2-hydroxyacid oxidase (*1*−*4*). Each of the identical 57 500 M_r subunits in the tetrameric flavocytochrome b_2 contains a heme b_2 and an FMN (*5*). In the course of the reaction, a hydride equivalent is first transferred from L-lactate to the FMN; this is followed by two single-electron transfers to the heme and then to a final acceptor (Scheme 1) (*6*−*8*). The final electron acceptor in vivo is cytochrome *c*, but this can be replaced by a variety of acceptors in vitro.

The crystal structure of the enzyme with pyruvate bound has been determined (*9*, *10*), allowing identification of potential catalytic residues (*10*−*12*). The carboxylate of pyruvate is bound by Arg376 and Tyr143, suggesting similar roles for these residues in the binding of the α-hydroxy acid substrate. In support of this, mutation of Arg376 to lysine decreases the activity by at least 3 orders of magnitude (*13*), while mutation of Tyr143 decreases the *V*/*K* value for lactate several-fold (*14*). Lactate can be placed into the active site

Scheme 1: Kinetic Mechanism of Flavocytochrome b_2



in place of pyruvate in two alternative orientations (*15*). In one (Scheme 2, path a), the α-proton of lactate is closest to His373 and the hydroxyl proton is close to Tyr254. In the second (Scheme 2, path b), lactate is oriented such that the hydroxyl proton is closest to His373. Mutation of His373 to glutamine decreases the rate of lactate oxidation by at least 10^5 (*16*), consistent with a role for this residue as an active site base. The two possible orientations of lactate make different mechanistic predictions (Scheme 2). Abstraction of the substrate α-hydrogen by His373 would form a carbanion. Alternatively, abstraction of the hydroxyl hydrogen by this residue would be consistent with direct transfer of the α-hydrogen to the FMN as a hydride. Experimental studies of flavocytochrome b_2 and the other members of this flavoenzyme family have been interpreted as favoring either a carbanion mechanism (*17*) or a hydride transfer mechanism (*18*, *19*).

The oxidation of lactate to pyruvate requires removal of both the hydrogen bound to the α-carbon and the hydroxyl proton. The relative timing of these bond cleavages differs in the two mechanisms depicted in Scheme 2. If the reaction proceeds via a carbanion intermediate, the α-proton would

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* To whom correspondence should be addressed: Department of Biochemistry and Biophysics, 2128 TAMU, College Station, TX 77843-2128. Phone: (979) 845-5487. Fax: (979) 845-4946. E-mail: fitzpat@tamu.edu.

‡ Department of Biochemistry and Biophysics.

§ Department of Chemistry.

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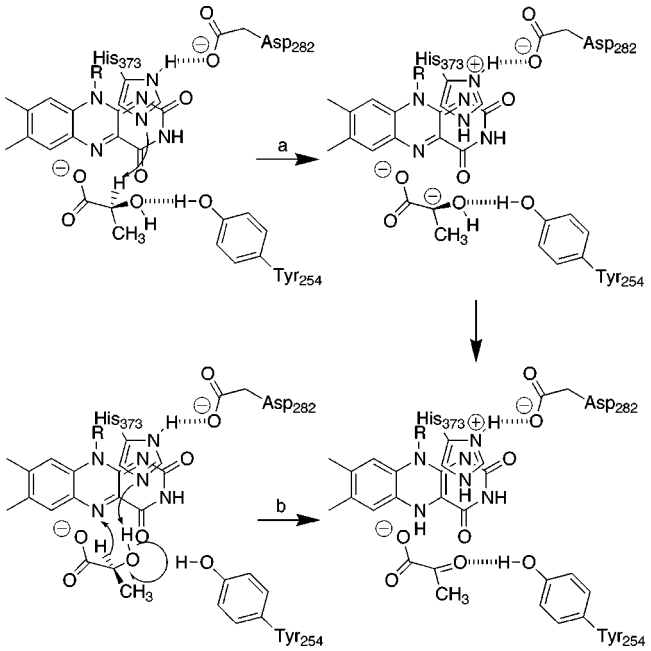
Scheme 2: Proposed Mechanisms for Lactate Dehydrogenation by Flavocytochrome b_2[a]



[a] (a) Carbanion mechanism, in which His373 abstracts the α-proton from lactate to form a carbanion. This intermediate reacts with the FMN, producing reduced flavin and pyruvate. (b) Hydride transfer mechanism in which His373 abstracts the hydroxyl proton as the α-proton is transferred as a hydride directly to the FMN in a concerted manner. The figure was adapted from refs *14* and *15*.

be abstracted first, with cleavage of the oxygen−hydrogen bond occurring in a subsequent step. In contrast, the bond cleavages could be concerted in a hydride transfer mechanism. In the work described herein, primary deuterium and solvent kinetic isotope effects have been used to probe the order of bond cleavage in the flavocytochrome b_2 reaction to provide further insight into the mechanism. In addition to experiments with the wild-type enzyme, the analyses were carried out with a mutant protein lacking the heme domain, to eliminate effects due to electron transfer between the cofactors, and with the active site mutant D282N, a protein for which chemical steps appear to be rate-limiting (*20*).

EXPERIMENTAL PROCEDURES

Materials. Sodium L-[2-^{2}H]lactate (98%) and deuterium oxide (99.9%) were purchased from Cambridge Isotope Co. (Andover, MA). L-Lactate and D,L-lactate were from Sigma Chemical Corp. (St. Louis, MO). Hydroxyapatite was from Bio-Rad Laboratories (Hercules, CA). All other reagents were of the highest purity commercially available.

DNA Manipulation. Custom oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer by the Gene Technology Laboratory of Texas A&M University. Restriction endonucleases were from New England Biolabs Inc. *Pfu* DNA polymerase was obtained from Stratagene USA. Plasmids were purified using kits from Qiagen Inc. The plasmid pDSb$_2$ (*13*), which contains the region encoding wild-type flavocytochrome b_2, and a plasmid encoding flavocytochrome b_2 D282N were generous gifts from F. Lederer.

The DNA fragment encoding mature flavocytochrome b_2 was removed from pDSb$_2$ by digestion with *Eco*RI and *Hin*dIII, and placed by ligation into pET21d which had been digested with *Eco*RI and *Hin*dIII. This construct was called pEFCB3. Site-directed mutagenesis (*21*) with the oligonucleotide 5′-AGCGGGCGAAATCTTTTGTTTAT**CCATGG**CC-AGTTTCGGCTCGTTGTC-3′ was used to introduce a new *Nco*I restriction site into pEFCB3 so that the ATG of the restriction site coincided with the codon for methionine 6 of the mature enzyme. The resulting plasmid, pEFCB4, was digested with *Nco*I and incubated with T4 DNA ligase. The plasmid found to have lost the sequence between the *Nco*I site of pET21b and the newly introduced *Nco*I site (pEFCB5) was utilized for expression of the wild-type enzyme.

Flavocytochrome b_2 lacking the 100 amino-terminal amino acid residues comprising the heme domain was constructed by incorporating an additional *Nco*I recognition site at position 300 of the gene in pEFCB5 using the QuikChange protocol (Stratagene) with oligonucleotides 5′-GCTCCTG-GTGAAA**CCATGG**AAGATATCGC-3′ and 5′-GCGATAT-CTT**CCATGG**TTTCACCAGGAGC-3′. The resulting mutagenesis mixture was used to transform XL1-Blue cells. Mutated plasmids were detected by electrophoretic analysis of restriction digests of plasmids, using *Nco*I and *Hin*dIII. A plasmid containing the additional *Nco*I site (pEFCB6) was digested with *Nco*I and then ligated with T4 DNA ligase. This ligation mixture was used to transform XL1-Blue cells. Resulting colonies were screened for the correct construct using the polymerase chain reaction. Isolated colonies were picked and duplicated on a second agar plate, and the remaining cells were dispersed in 100 μL of 10 mM Tris-HCl (pH 8.0). The samples were boiled for 5 min, chilled on ice, and centrifuged for 5 min. The supernatants were used as templates for a PCR, using Taq polymerase from Stratagene USA and oligonucleotide primers that flanked the entire flavocytochrome b_2 gene. Colonies yielding products with a flavocytochrome b_2 gene 300 bp shorter than the wild-type gene were selected for plasmid isolation and DNA sequencing. A plasmid containing the truncated coding region, pEFCBΔheme, was introduced into competent *Escherichia coli* BL21(DE3) cells. Small cultures were stored as 10% glycerol stocks at −80 °C.

Cells Strains and Growth. Cells from permanent stocks containing the appropriate plasmid were streaked on LB agar plates containing 100 μg/mL ampicillin; a single colony was used to inoculate a 50 mL liquid culture. After incubation of the latter overnight at 37 °C, 10 mL was used to inoculate a 1 L culture of LB broth containing 100 μg/mL ampicillin. When the A_{600} value of the culture reached 0.8, isopropyl β-D-thioglucanopyranoside was added to a final concentration of 500 μM. Incubation was continued for 6 h, at which time cells were harvested by centrifugation for 1 h at 5000*g*.

Enzyme Purification. For purification of the wild-type enzyme, cell pellets were resuspended in 10 mM Tris-HCl, 10 mM EDTA, 0.2 mg/mL lysozyme, 20 mM D,L-lactate, and 1 mM phenylmethanesulfonyl fluoride (pH 7.5) at 4 °C, using 10 mL/g. The cell suspension was stirred for 1 h at 4 °C and then centrifuged at 12000*g* for 1 h at 4 °C. The resulting supernatant was brought to 30% saturation with ammonium sulfate, followed by centrifugation at 10000*g* for 30 min. This supernatant was brought to 70% saturation with ammonium sulfate. The pellet produced by centrifugation was resuspended in 100 mL of 10 mM potassium phosphate, 1 mM EDTA, and 20 mM D,L-lactate (pH 7.5) and loaded

Table 1: Steady-State Kinetic Parameters and Primary Isotope Effects in the Oxidation of Lactate by Wild-Type and Mutant Flavocytochromes b_2[a]

enzyme	V (s^{-1})	$V/K_{lactate}$ (mM^{-1} s^{-1})	$K_{lactate}$ (mM)	^{D}V	$^{D}(V/K)$
wild-type	372 ± 12	2300 ± 215	0.16 ± 0.02	2.9 ± 0.1	3.0 ± 0.3
flavin domain	200 ± 7	543 ± 32	0.36 ± 0.03	3.7 ± 0.3	3.5 ± 0.2
D282N	2.2 ± 0.1	15 ± 1	0.15 ± 0.02	4.9 ± 0.3	4.3 ± 1.1

[a] Conditions: 100 mM potassium phosphate, 1 mM EDTA, and 1 mM ferricyanide for the wild-type and D282N enzymes and 3 mM ferricyanide for the flavin domain at pH 7.5 and 25 °C.

onto a hydroxyapatite column previously equilibrated with the same buffer. The enzyme was eluted with a linear gradient from 10 to 300 mM potassium phosphate. Purification of the flavin domain was carried out as described by Balme et al. (*22*). The D282N enzyme was purified as described by Gondry et al. (*23*). Enzyme concentrations were determined using an ϵ_{423} value of 183 mM^{-1} cm^{-1} and an ϵ_{413} value of 129.5 mM^{-1} cm^{-1} for the reduced and oxidized full length enzymes, respectively (*24*), and an ϵ_{453} value of 11.1 mM^{-1} cm^{-1} for the flavin domain (*25*). Purified enzymes were stored in 100 mM potassium phosphate, 1 mM EDTA, and 20 mM D,L-lactate (pH 7.5) at −70 °C.

To remove the lactate used for storage, on the day of use an aliquot was precipitated with 70% ammonium sulfate in 100 mM potassium phosphate and 1 mM EDTA (pH 7.5) at 4 °C. The pellet obtained by centrifugation at 15000*g* for 5 min was resuspended in 100 mM potassium phosphate and 1 mM EDTA (pH 7.5). This procedure was repeated twice. The final enzyme concentration was about 3 μM. This treatment also resulted in oxidation of the cofactors. For stopped-flow experiments, enzyme was passed through a Sephadex G-25 column in 100 mM potassium phosphate and 1 mM EDTA (pH 7.5) to remove the lactate.

Enzyme Assays. Initial rate assays were performed in 100 mM potassium phosphate, 1 mM EDTA, and 1 mM potassium ferricyanide (pH 7.5) at 25 °C, with varied concentrations of L-lactate or L-[2-^{2}H]lactate, following the decrease in absorbance at 420 nm, and using an ϵ_{420} value for ferricyanide of 1.04 mM^{-1} cm^{-1}. For the flavin domain, 3 mM potassium ferricyanide was used. The steady-state kinetic parameters given in Table 1 for the flavin domain were obtained by varying the concentrations of both potassium ferricyanide and L-lactate. Concentrations of lactate were determined by the end point assay with wild-type flavocytochrome b_2. For pH studies, the buffer was 50 mM Bis-Tris at pH 5.5−7.0, 150 mM HEPES at pH 7.0−8.5, and 50 mM ethanolamine at pH 8.5−10.0.[1] All assays were carried out in the presence of 1 mM potassium ferricyanide at 25 °C. pD profiles were determined in the same buffers made up using 99.9% deuterium oxide and adjusting the pD with DCl and NaOD as needed, adding 0.4 to the value indicated by the pH meter (*27*). Steady-state kinetic solvent isotope effects and proton inventories were determined in 100 mM phosphate and 1 mM EDTA (pH 7.5 or pD 8.0) with 1 mM potassium ferricyanide for the wild-type and D282N enzymes and 3 mM for the flavin domain at 25 °C. For the proton inventory experiments, the desired atom fraction of D_2O was achieved by combining appropriate volumes of assay components made up in H_2O or D_2O. Assays were initiated by the addition of 5 μL of enzyme in H_2O to a final volume of 1 mL.

The effects of viscosity on steady-state kinetic parameters were measured in 100 mM potassium phosphate and 1 mM EDTA (pH 7.5) at 25 °C using 1 mM potassium ferricyanide for the wild-type enzyme and 3 mM for the flavin domain. The values for the relative viscosities of glycerol-containing solutions were taken from Weast (*28*) and adjusted for 25 °C.

For rapid reaction analyses, flavin reduction was monitored on an Applied Photophysics SX.18MV stopped-flow spectrophotometer. Enzyme in 100 mM potassium phosphate and 1 mM EDTA (pH 7.5) was mixed with an equal volume of the same buffer containing varied concentrations of lactate at 25 °C. The reaction of the wild-type enzyme was monitored at 438 nm, a heme isosbestic point, while that of the flavin domain was monitored at 452 nm (*22*). To determine solvent isotope effects, the enzyme in aqueous buffer was mixed with an equal volume of lactate in D_2O-containing buffer.

Data Analysis. Kinetic data were fit to eqs 1−8 using Kaleidagraph (Adelbeck Software, Reading, PA), KinetAsyst (IntelliKinetics, State College, PA), or Igor (Wavemetrics, Lake Oswego, OR). Initial rate data when only one substrate was varied were fit to the Michaelis−Menten equation. When both lactate and ferricyanide were varied, the data were fit to eq 1. Primary deuterium and solvent isotope effects were calculated using eqs 2−4. Equation 2 is for separate isotope effects on the V_{max} and $V/K_{lactate}$ values. Equation 3 is for an isotope effect on the V_{max} value only. Equation 4 is for an isotope effect on the $V/K_{lactate}$ value only. In each, F_i is the fraction of heavy atom substitution in the substrate, E_V is the isotope effect on the V_{max} value minus 1, and E_{VK} is the isotope effect on the $V/K_{lactate}$ value minus 1. The pH profiles were obtained by fitting the V_{max} and $V/K_{lactate}$ values obtained at each pH to eq 5; here, K_1 and K_2 are the dissociation constants of the ionizable groups, and C is the pH-independent value of the specific kinetic parameter. Proton inventory data were fit to eq 6, where n is the atom fraction of D_2O, ^nk is the solvent isotope effect at that D_2O content, and ^{D_2O}k is the solvent isotope effect in 100% D_2O. Stopped-flow traces were fit to eq 7 which describes a biphasic exponential decay, where λ_1 and λ_2 are first-order rate constants, t is time, and A_a, A_b, and A_c are the absorbances of species A−C, respectively. The lactate concentration

[1] Since it has been reported that the activity of flavocytochrome b_2 is sensitive to ionic strength and buffer composition (*26*), the effects of a number of buffers on the activity were determined in preliminary experiments. The most commonly used buffers for this enzyme have been Tris and phosphate. In our hands, the enzyme is inhibited by concentrations of Tris of >100 mM at pH 7.5 (results not shown). The effect of phosphate is more complex. As the concentration of phosphate increases from 10 to 100 mM, the V_{max} value decreases slightly and the $V/K_{lactate}$ value increases by almost 2-fold. In contrast, if the ionic strength is increased up to 0.75 using KCl with 10 mM potassium phosphate as the buffer, the V_{max} value decreases about 4-fold and the $V/K_{lactate}$ value decreases almost 8-fold as the ionic strength increases. Both the V_{max} and $V/K_{lactate}$ values are independent of the concentration of HEPES at concentrations of >100 mM at pH 7.5. At pH 5.8, the V_{max} and $V/K_{lactate}$ values are independent of the concentration of Bis-Tris up to 250 mM. At pH 9.2, the V_{max} and $V/K_{lactate}$ values change by less than 10% at concentrations of ethanolamine up to 100 mM. Consequently, these last three buffers were used to determine the effects of pH on the kinetic parameters. We also determined the pH profiles in a buffer composed of 100 mM ACES, 50 mM ethanolamine, and 50 mM Tris-HCl. The pK_a values obtained with this buffer were the same.

dependence of the rapid phase, which corresponds to flavin reduction, was analyzed using eq 8 (*7*), where k_{obs} is the observed rate of reduction of the flavin, k_{red} is the limiting rate of reduction at saturating substrate concentrations, and K_d is the concentration of substrate at which the rate of reduction is one-half the maximum value. To calculate isotope effects on rapid reaction kinetic parameters, the observed rates of reduction were fit to eq 2, using the k_{obs} values in place of the initial rates.

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VA}{K_m[1 + F_i(E_{vk})] + A[1 + F_i(E_v)]} \quad (2)$$

$$v = \frac{VA}{K_m + A[1 + F_i(E_v)]} \quad (3)$$

$$v = \frac{VA}{K_m[1 + F_i(E_{vk})] + A} \quad (4)$$

$$\log Y = \log \frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \quad (5)$$

$$1/^{n}k = 1 - n + (n/^{D_2O}k_1) \quad (6)$$

$$A_{total} = (A_a - A_c)e^{\lambda_1 t} + \frac{\lambda_1(A_b - A_c)}{\lambda_1 - \lambda_2}e^{-\lambda_2 t} - e^{-\lambda_1 t} + A_c \quad (7)$$

$$k_{obs} = \frac{k_{red}A}{K_d + A} \quad (8)$$

RESULTS

Wild-Type Flavocytochrome b_2. As a measure of the extent to which the V_{max} and $V/K_{lactate}$ values reflect the rate of the cleavage of the carbon−hydrogen bond of lactate, primary kinetic isotope effects were determined with [2-^{2}H]lactate. Ferricyanide was used as the electron acceptor in these analyses because the oxidative half-reaction becomes partially rate-limiting when cytochrome *c* is used (*14*). The concentration of ferricyanide was kept at least 50-fold higher than its K_M value to ensure that reduction was irreversible in the steady state. For the mechanism of Scheme 1, the $V/K_{lactate}$ value includes the rates of steps for the reaction of free enzyme and lactate to form the reduced enzyme−pyruvate complex. The $^DV_{max}$ and $^D(V/K_{lactate})$ values for wild-type flavocytochrome b_2 are close to 3 at pH 7.5 (Table 1). The primary isotope effects are independent of pH; at pH 5.2, the $^DV_{max}$ and $^D(V/K_{lactate})$ values are 3.0 ± 0.2 and 3.0 ± 0.4, respectively, while at pH 9.2, the values are 2.9 ± 0.2 and 3.0 ± 0.5, respectively. The values are within the range of values previously determined under slightly different conditions (*7*, *14*, *15*).

Solvent isotope effects were determined to probe the timing of steps involving solvent exchangeable protons, such as the cleavage of the lactate oxygen−hydrogen bond. As a necessary step in the measurement of solvent isotope effects on the flavocytochrome b_2 reaction, the pH dependencies of the V_{max} and $V/K_{lactate}$ values for the wild-type enzyme were determined over the accessible pH range. The $V/K_{lactate}$−pH profile of wild-type flavocytochrome b_2 is bell-shaped, consistent with the involvement of two ionizable groups in the free enzyme (Figure 1A). One group with a pK_a value of 5.4 ± 0.1 must be unprotonated for activity, and one with a pK_a value of 8.7 ± 0.1 must be protonated for activity. The V_{max}−pH profile is also bell-shaped, reflecting apparent pK_a values of 5.0 ± 0.2 and 9.4 ± 0.1 for the groups which must be unprotonated and protonated for catalysis, respectively (Figure 1B). These values are in good agreement with previously published values obtained under different conditions (*29*, *30*).

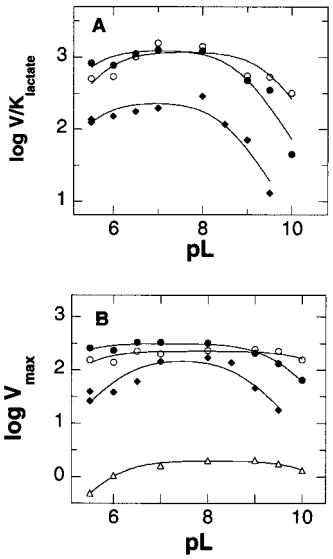


FIGURE 1: pL dependence of wild-type and mutant flavocytochromes b_2. Initial rates were determined with ferricyanide as the electron acceptor as described in Experimental Procedures. Panel A shows the $V/K_{lactate}$ profile in H_2O for the wild-type (●) and flavin domain (◆) enzymes and in D_2O for the wild-type enzyme (○). Panel B shows the V_{max} profile in H_2O for the wild-type (●), D282N (△), and flavin domain (◆) enzymes, and in D_2O for the wild-type enzyme (○). The lines are fits of the data to eq 5.

Table 2: pK_a Values for Wild-Type and Mutant Flavocytochromes b_2[a]

enzyme	parameter	solvent	pK_a	pK_a
wild-type	V	H_2O	5.0 ± 0.2	9.4 ± 0.1
	V	D_2O	5.3 ± 0.1	10.4 ± 0.1
	$V/K_{lactate}$	H_2O	5.4 ± 0.1	8.7 ± 0.1
	$V/K_{lactate}$	D_2O	5.8 ± 0.2	9.4 ± 0.2
flavin domain	V	H_2O	6.2 ± 0.2	8.5 ± 0.2
	$V/K_{lactate}$	H_2O	5.5 ± 0.3	8.4 ± 0.2
D282N	V	H_2O	5.9 ± 0.1	10.2 ± 0.1

[a] Conditions: 150 mM HEPES, 50 mM Bis-Tris, 50 mM ethanolamine, and 1 or 3 mM ferricyanide at pH 5.5−10.0 and 25 °C.

When the pH dependence is carried out in D_2O, all of the pK_a values in the V_{max} and $V/K_{lactate}$ profiles are shifted upward, as expected (Figure 1 and Table 2). These analyses show that the V_{max} and $V/K_{lactate}$ values are independent of pL at pH 7.5 and pD 8.0. Therefore, we chose pH 7.5 and pD 8.0 to measure the solvent isotope effects. With the wild-type enzyme, there is a normal effect on the V_{max} value of 1.38 ± 0.07 and a slightly inverse effect on the $V/K_{lactate}$ value of 0.90 ± 0.04 (Table 3). Similar values with slightly lower precision could be calculated from the complete pL dependencies.

Two approaches were taken to determine if the solvent isotope effects with the wild-type enzyme were on the step

Table 3: Solvent Isotope Effects in the Oxidation of Lactate by Wild-Type and Mutant Flavocytochromes b_2[a]

enzyme	$^{D_2O}(V/K_{lactate})$	^{D_2O}V	eq	χ^2
wild-type	0.90 ± 0.04	1.38 ± 0.07	2	236
		1.35 ± 0.03	3	265
	1.52 ± 0.34		4	3185
flavin domain	0.44 ± 0.05	1.18 ± 0.05	2	1784
		0.93 ± 0.08	3	10333
	0.55 ± 0.06		4	3416
D282N	1.06 ± 0.14	0.97 ± 0.04	2	0.099
		0.98 ± 0.04	3	0.101
	1.00 ± 0.16		4	0.104

[a] Conditions: 100 mM potassium phosphate, 1 mM EDTA, and 1 or 3 mM ferricyanide at pH 7.5 or pD 8.0 and 25 °C.

in which the lactate carbon−hydrogen bond is cleaved. In the first, the solvent isotope effects were measured with [2-^{2}H]lactate under the conditions used with the nondeuterated substrate. This gave values for $^{D_2O}(V/K_{lactate})_D$ of 0.87 ± 0.1 and for $(^{D_2O}V)_D$ of 1.1 ± 0.03. The decrease in the magnitude of the ^{D_2O}V value with deuterated lactate is consistent with the solvent isotope effect on this parameter arising from a different step than that generating the primary isotope effect (*31*). The magnitudes of the $^{D_2O}(V/K_{lactate})$ values are too small to draw conclusions.

As an alternative approach, isotope effects were determined for the rate of reduction of the flavin by lactate using a stopped-flow spectrophotometer. When lactate is mixed with flavocytochrome b_2 in the absence of an electron acceptor, complete reduction of the FMN occurs in an apparently irreversible first-order process. Direct measurements of FMN reduction eliminate contributions from subsequent electron-transfer steps between FMN and heme b_2 and between heme b_2 and ferricyanide. In this experiment, the wild-type enzyme exhibited a $^Dk_{red}$ of 5.4 (Table 4). The solvent isotope effect on the rate of reduction was measured by mixing enzyme in aqueous buffer with an equal volume of lactate in D_2O in the stopped-flow spectrophotometer. No significant solvent isotope effect on the limiting rate of reduction of the flavin could be detected (Table 4).

Isolated Flavin Domain. The lack of a solvent isotope effect on the rate of flavin reduction suggests that the solvent isotope effect on the V_{max} value for the wild-type enzyme occurs on a step after flavin reduction. As a test of this hypothesis, isotope effects were determined with a mutant protein lacking the heme domain, an enzyme previously described by Balme et al. (*22*). While the ability of this enzyme to utilize cytochrome *c* as an electron acceptor is nearly eliminated by the truncation, the effects on kinetic parameters with ferricyanide as electron acceptor are much less. The $V/K_{lactate}$ and V_{max} values decrease several-fold, and there is an increase in the value of the primary isotope effects on these parameters (Table 1), in agreement with the previous characterization (*22*). The pH dependence of steady-state kinetic parameters was also determined with this mutant protein. The $V/K_{lactate}$−pH profile for the flavin domain is indistinguishable from that of the wild-type enzyme (Figure 1A and Table 2). This was expected since catalytic steps involved in $V/K_{lactate}$ should not require the heme domain. The V_{max} profile is still bell-shaped, but the pK_a values are shifted inward compared to those of the wild-type enzyme (Figure 1B and Table 2).

Solvent isotope effects were determined with the flavin domain at pH 7.5 and pD 8. The data were fit best by assuming isotope effects on both the $V/K_{lactate}$ and V_{max} values (Table 3). The solvent isotope effect on the V_{max} value is normal and smaller than that seen with the wild-type enzyme, consistent with most of the effect with the latter being due to steps in the oxidative half-reaction. Surprisingly, there was a relatively large inverse effect on the $V/K_{lactate}$ value. The solvent isotope effect on the limiting rate of reduction of the flavin by lactate was also determined directly by stopped-flow methods. As was the case with the wild-type enzyme, there was no significant solvent isotope effect on this parameter (Table 4). A large inverse effect was seen on the apparent second-order rate constant for reduction; this parameter should be equivalent to the steady-state $V/K_{lactate}$.

Proton Inventories. Proton inventories, in which kinetic parameters are determined in mixtures of H_2O and D_2O, can often provide insight into the origin of solvent isotope effects (*27*). Consequently, such analyses were carried out with wild-type flavocytochrome b_2 and the isolated flavin domain. With both enzymes, the V_{max} proton inventory shows a linear dependence of the solvent isotope effect on the fraction of D_2O (Figure 2A), although the small magnitude of the effect with the flavin domain precludes ruling out more complex behavior. The $V/K_{lactate}$ proton inventories for both enzymes are also linear, although inverse (Figure 2B).

Viscosity Effects. Small solvent isotope effects can arise because the viscosity of D_2O is greater than that of H_2O by 23% at 25 °C (*32*). This will decrease the rate of diffusion-limited steps such as substrate association and product dissociation. The effects of viscosity on the V_{max} and $V/K_{lactate}$ values of the wild-type enzyme and the flavin domain were determined with glycerol as the viscogen. The V_{max} values for both enzymes decreased slightly as the viscosity of the medium increased. A plot of the normalized increase in the V_{max} value as a function of the normalized increase in viscosity allows calculation of the extent to which diffusion limits the V_{max} value. As shown in Figure 3A, the slope of the line for wild-type flavocytochrome b_2 is 0.32 ± 0.03, while that for the flavin domain is 0.15 ± 0.02. Thus, a viscosity-sensitive step such as product release partially limits the V_{max} value for both wild-type flavocytochrome b_2 and the isolated flavin domain.

The effects of viscosity on the $V/K_{lactate}$ values are more complex with both enzymes. The $V/K_{lactate}$ for the wild-type enzyme increases slightly with increasing concentrations of glycerol (Figure 3B). The flavin domain is activated up to 2-fold by moderate concentrations of glycerol (10−20%) (Figure 3B).

Characterization of D282N Flavocytochrome b_2. The primary isotope effects on the $V/K_{lactate}$ and V_{max} values for wild-type flavocytochrome b_2 and the flavin domain could be decreased from the intrinsic values on the chemical step by contributions to these kinetic parameters from steps other than that in which the lactate C−H bond is cleaved. One approach to minimizing this problem is to utilize substrates for which the chemistry is slowed so that catalysis becomes more rate-limiting. An alternative approach is to use modified enzymes in which the rate of the chemical step of interest has been decreased. In the active site of flavocytochrome b_2, His373 is appropriately placed to act as an active site base (*11*). However, mutagenesis of His373 to glutamine

Table 4: Rapid Reaction Kinetic Parameters for the Oxidation of Lactate by Wild-Type and Mutant Flavocytochromes b_2[a]

enzyme	k_{red} (s^{-1})	$k_{red}/K_{lactate}$ (mM^{-1} s^{-1})	$K_{lactate}$ (mM)	$^{D}k_{red}$	$^{D_2O}k_{red}$[b]	$^{D_2O}k_{red}/K_{lactate}$[b]
wild-type	520 ± 19	1100 ± 78	0.47 ± 0.05	5.4 ± 0.4	1.0 ± 0.10	1.1 ± 0.2
flavin domain	227 ± 6	785 ± 64	0.30 ± 0.02	3.5 ± 0.2	1.02 ± 0.02	0.6 ± 0.1

[a] Conditions: 100 mM potassium phosphate and 1 mM EDTA at pH 7.5 or pD 8.0 and 25 °C. [b] Calculated assuming a linear dependence of the isotope effect on the mole fraction of D_2O.

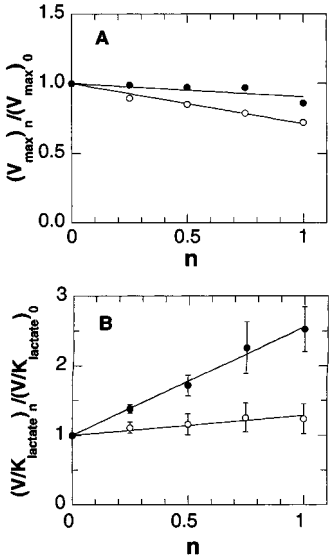


FIGURE 2: Proton inventories for wild-type flavocytochrome b_2 (○) and the flavin domain (●). The observed isotope effects on the V_{max} (A) and $V/K_{lactate}$ (B) values are plotted as a function of the mole fraction of D_2O. The lines are fits of the data to eq 6.

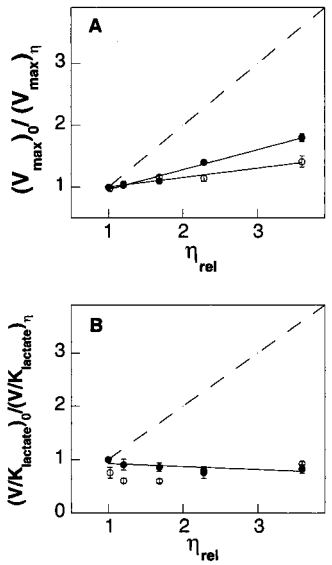


FIGURE 3: Effects of viscosity on the steady-state kinetic parameters for wild-type flavocytochrome b_2 (●) and the flavin domain (○). Panel A shows the effect on the V_{max} value and panel B the effect on the $V/K_{lactate}$ value when the viscosity is increased with glycerol. The dashed line has a slope of 1 and indicates the expected results for a fully diffusion-limited reaction.

results in an enzyme with no detectable activity (*16*), ruling out this mutant for further analyses. The side chain of Asp282 is appropriately placed for an electrostatic interaction with the imidazole ring of His373 (*10*), and disruption of this interaction by mutagenesis of Asp282 has been reported to decrease both the V_{max} and $V/K_{lactate}$ values by 70−100-fold (*20*). Consequently, the D282N enzyme was selected as a protein in which the rate of the C−H bond cleavage step was likely to be significantly decreased, simplifying interpretation of isotope effects.

In agreement with the previous work (*20*), we found that the D282N mutation decreases both the V_{max} and $V/K_{lactate}$ values by about 2 orders of magnitude (Table 1). The primary isotope effects on both kinetic parameters also increase, consistent with cleavage of the lactate C−H bond becoming rate-limiting. The V_{max}−pH profile for the D282N enzyme shows a bell-shaped curve with two ionizable groups (Figure 1B). The pK_a values are shifted upward compared to that of the wild-type enzyme (Table 2). The $V/K_{lactate}$−pH profile was not determined due to the low activity of this enzyme. It was not possible to follow the flavin reduction by rapid reaction methods, as noted previously, due to electron transfer between the redox centers in the different subunits reoxidizing the flavin more rapidly than it is reduced by lactate (*20*).

Solvent isotope effects on the V_{max} and $V/K_{lactate}$ values were determined with the D282N enzyme under the conditions used for the wild-type enzyme (pH 7.5 and pD 8.0). Neither isotope effect was significantly different from 1, although the precision of the $^{D_2O}V/K_{lactate}$ value was not as good due to the low activity of this enzyme (Table 3). Thus, with a mutant in which C−H bond cleavage is rate-limiting, there is no evidence for an exchangeable proton in flight in the transition state for cleavage of the lactate C−H bond.

DISCUSSION

Despite a great deal of mechanistic and structural analysis, the mechanism by which flavoproteins oxidize α-hydroxy acids to the corresponding keto acids remains controversial. The majority of the data are readily accommodated by removal of the α-hydrogen as a proton to form a carbanion (*17*). The various members of this family have been shown to varying extents to catalyze halide elimination from *β*-substituted substrates and to be inactivated by acetylenic substrate analogues. In addition, flavocytochrome b_2 will catalyze transfer of tritium from [2-^{3}H]lactate to 3-bromo- and 3-chloropyruvate, forming both labeled pyruvate and the respective labeled lactate; this reaction has been interpreted in favor of partitioning of a carbanion intermediate (*33*). The homologous active sites of these enzymes contain a conserved histidine residue properly placed to abstract a proton from the substrate (*34*, *35*). In all cases, mutagenesis of this histidine decreases the rate of substrate oxidation by 3−5 orders of magnitude, consistent with a role as an active site base. However, neither the mechanistic nor the structural data provide unambiguous support for a carbanion mechanism. Halide elimination can be accommodated by nucleophilic displacement of the halide by a hydride from the N(5) position of the reduced flavin. If the exchange of this proton with solvent is hindered, the transhydrogenation reaction can be similarly rationalized. Inactivation by acetylenic com-

pounds could involve attack of an active site nucleophile on the enzyme-bound product. A structure is not available for a member of this family with substrate bound due to the chemical reactivity of such a complex. Instead, structures with bound keto acid product have been used to model the enzyme−substrate complex. The results of such modeling studies are ambiguous, in that the hydroxy acid can be modeled with the conserved histidine near either the α-proton or the hydroxyl proton (*15*). The former orientation is consistent with formation of a carbanion, while the latter is more suggestive of transfer of the α-hydrogen as a hydride. The dramatic effects of mutagenesis of this residue are consistent with either role.

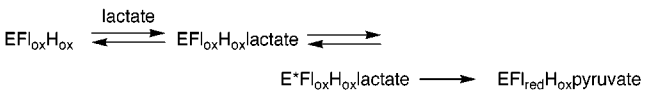
The experiments described here were designed to address the mechanism of lactate oxidation by flavocytochrome b_2 by focusing on the relative timing of the cleavage of the lactate C−H and O−H bonds. Primary deuterium kinetic isotope effects provide a probe of the status of the C−H bond, while solvent isotope effects can be used to probe the status of the O−H bond. With wild-type flavocytochrome b_2, both the ^{D}V and $^{D}V/K_{lactate}$ values are about 3, establishing that cleavage of the lactate C−H bond is at least partially rate-limiting for both the reductive half-reaction and for overall turnover. The solvent isotope effect on the V_{max} value could arise from any of the first-order steps in Scheme 1. The decrease in the ^{D_2O}V value when [2-^{2}H]lactate is the substrate is consistent with the solvent isotope effect arising from a step different from that for the primary deuterium isotope effect. The rapid reaction kinetic parameters confirm this conclusion in a more direct fashion. The limiting rate of reduction of the flavin, which should be a direct measure of k_3 in Scheme 1, shows a larger primary isotope effect of the magnitude expected for the intrinsic effect, but no solvent isotope effect. These data are consistent with no change in the O−H bond order in the transition state for C−H bond cleavage in the wild-type enzyme.

If the solvent isotope effect on the V_{max} value for the wild-type enzyme does not arise from lactate oxidation, it must be due to subsequent steps in the oxidative half-reaction. The data are consistent with several steps contributing to the ^{D_2O}V value. Part of the effect is due to the increased viscosity of D_2O compared to H_2O. The V_{max} value for the wild-type enzyme is about 30% limited by viscosity. This effect could be due to product release steps or conformational changes. The viscosity contribution to the solvent isotope effect can be calculated to be about 1.09, with the remainder of the effect due to other steps in the oxidation of the reduced enzyme by ferricyanide.

The behavior of the isolated flavin domain confirms the conclusions drawn from the wild-type enzyme. In this case, the reduced enzyme reacts directly with the oxidant, without first transferring the electrons to the heme b_2. Again, there is no significant solvent isotope effect on the limiting rate of flavin reduction by lactate. Both the solvent isotope effect and the effect of viscosity on the V_{max} value for the flavin domain are attenuated from the wild-type value. This is readily explained by the loss of contributions from steps in the oxidative half-reaction.

The analyses of both the wild-type enzyme and the flavin domain required the use of rapid reaction kinetics to measure the isotope effects on lactate oxidation without interference from other steps. An alternative approach is to utilize mutant proteins in which the rate of lactate oxidation is decreased sufficiently that C−H bond cleavage is fully rate-limiting for turnover. His373 is appropriately placed in the active site of flavocytochrome b_2 to act as a base during catalysis (*15*). Mutagenesis of His373 decreases turnover by at least 5 orders of magnitude (*16*), eliminating mutation of this residue from consideration. However, mutagenesis of Asp282, which interacts with His373, has the desired effect, decreasing both the V_{max} and $V/K_{lactate}$ values by 2 orders of magnitude and increasing the isotope effects on these parameters to the values seen in the reductive half-reaction of the wild-type enzyme (*14*).

Scheme 3: Alternative Kinetic Mechanism for the Reductive Half-Reaction of Flavocytochrome b_2



The properties of the D282N enzyme are consistent with a sufficient decrease in the rate of lactate C−H bond cleavage that this step becomes fully rate-limiting. There is no solvent isotope effect on the V_{max} value for this enzyme, confirming the conclusion drawn from the wild-type enzyme and the flavin domain that there is no change in the bond order of the lactate O−H bond in the transition state for C−H bond cleavage. These results confirm the critical role of His373 as an active site base and illustrate the utility of using appropriately mutated enzymes to make chemical steps in enzyme-catalyzed reactions manifest.

While the isotope effects on the V_{max} values of the wild-type and mutant enzymes are consistent with the expectations of the carbanion mechanism shown in Scheme 2, the effects on the $V/K_{lactate}$ values establish that the situation is more complex. With wild-type flavocytochrome b_2, there is a small but significant inverse solvent isotope effect on the $V/K_{lactate}$ value. This increases markedly in the flavin domain. In addition, the $V/K_{lactate}$ value for the flavin domain shows an unusual increase in activity in the presence of low concentrations of glycerol. A reasonable explanation for these results is that there is an internal equilibrium after formation of the enzyme−lactate complex but before C−H bond cleavage, as shown in Scheme 3. This equilibrium is shifted by the presence of either glycerol or D_2O. Both of these solvents affect the stability of proteins favorably, and in both cases, the effect has been attributed to changes in solvation (*36*, *37*). Thus, it is reasonable that they could have similar effects on conformational equilibria. Other possible explanations for inverse solvent isotope effects are the presence of a thiol or metal-bound water; both of these possibilities can be ruled out for flavocytochrome b_2. The NAD malic enzyme from *Ascaris suum* provides a previous example in which an inverse solvent isotope effect could be reproduced by the addition of glycerol; this result was similarly attributed to an effect on enzyme conformation (*38*). Indeed, the results described here for flavocytochrome b_2 reinforce the recommendation of Karsten et al. (*38*) that an evaluation of viscosity effects is prudent for all solvent isotope effect studies.

The solvent inventories for the $V/K_{lactate}$ values are consistent with a single exchangeable proton being responsible for the inverse solvent isotope effect. If the inverse effect is due to a change in a conformational equilibrium,

an effect due to multiple protons is often expected (*32*). The precision of the $V/K_{lactate}$ values is such that a small degree of curvature cannot be ruled out. Moreover, the possibility that the apparent linearity arises from compensating isotope effects must be considered, with an inverse effect on enzyme conformation being opposed by a normal kinetic effect on O−H bond cleavage. A previous study of the *Bacillus* lactate dehydrogenase provides a useful comparison with the present results with flavocytochrome b_2, since both enzymes catalyze the interconversion of lactate and pyruvate. The pyridine nucleotide-dependent lactate dehydrogenase would be expected to utilize a hydride transfer mechanism. With the *Bacillus* enzyme, in the direction of pyruvate reduction the primary deuterium isotope effect on the V/K_{NADH} value is 5.5, while the solvent isotope effect on the same kinetic parameter is unity or slightly inverse (*39*). Xie et al. concluded that the simplest explanation for these results is that O−H bond cleavage and hydride transfer do not occur in the same step. However, they pointed out that the data could not rule out a normal isotope effect from O−H bond cleavage which is opposed by a multiproton effect arising from an enzyme conformational change. The same possibility must be considered in the case of flavocytochrome b_2.

In summary, the data presented here with flavocytochrome b_2 support the conclusion that there is no solvent isotope effect on the step in catalysis in which the lactate C−H bond is cleaved. This is based on both the results of rapid kinetic analyses and the results with a mutant in which carbon−hydrogen bond cleavage is fully rate-limiting. A reasonable conclusion from these data is that the hydroxyl proton of lactate is not in flight in the transition state for cleavage of the lactate carbon−hydrogen bond. This is fully consistent with the predictions of a carbanion mechanism for this enzyme. However, the data cannot rule out an alternative mechanism in which the hydroxyl proton is removed in an equilibrium coupled to an enzyme conformational change which occurs prior to cleavage of the carbon−hydrogen bond by hydride transfer.

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REFERENCES

1. Urban, P., Chirat, I., and Lederer, F. (1988) *Biochemistry 27*, 7365−7371.
2. Giegel, D. A., Williams, C. H., Jr., and Massey, V. (1990) *J. Biol. Chem. 265*, 6626−6632.
3. Tsou, A. Y., Ransom, S. C., Gerlt, J. A., Buechter, D. D., Babbitt, P. C., and Kenyon, G. L. (1990) *Biochemistry 29*, 9856−9862.
4. Yorita, K., Aki, K., Ohkuma-Soyejima, T., Kokubo, T., Misaki, H., and Massey, V. (1996) *J. Biol. Chem. 271*, 28300−28305.
5. Bright, H. J., and Appleby, M. (1969) *J. Biol. Chem. 244*, 3625−3634.
6. Capeillere-Blandin, C., Bray, R. C., Iwatsubo, M., and Labeyrie, F. (1975) *Eur. J. Biochem. 54*, 549−566.
7. Pompon, D., Iwatsubo, M., and Lederer, F. (1980) *Eur. J. Biochem. 104*, 479−488.
8. Daff, S., Ingledew, W. J., Reid, G. A., and Chapman, S. K. (1996) *Biochemistry 35*, 6345−6350.
9. Xia, Z.-X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F., and Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2629−2633.
10. Xia, Z., and Mathews, F. S. (1990) *J. Mol. Biol. 212*, 837−863.
11. Lederer, F., and Mathews, F. S. (1987) in *Flavins and Flavoproteins, 1987* (Edmondson, D. E., and McCormick, D. B., Eds.) pp 133−142, Walter de Gruyter and Co., Berlin.
12. Lindqvist, Y., and Brändèn, C.-I. (1989) *J. Biol. Chem. 264*, 3624−3628.
13. Black, M. T., White, S. A., Reid, G. A., and Chapman, S. K. (1989) *Biochem. J. 258*, 255−259.
14. Rouviere-Fourmy, N., Capeillere-Blandin, C., and Lederer, F. (1994) *Biochemistry 33*, 798−806.
15. Dubois, J., Chapman, S. K., Mathews, F. S., Reid, G. A., and Lederer, F. (1990) *Biochemistry 29*, 6393−6400.
16. Gaume, B., Sharp, R. E., Manson, F. D. C., Chapman, S. K., Reid, G. A., and Lederer, F. (1995) *Biochimie 77*, 621−630.
17. Lederer, F. (1996) in *Flavins and Flavoproteins 1996* (Stevenson, K. J., Massey, V., and Williams, C. H., Eds.) pp 545−553, University of Calgary Press, Calgary, AB.
18. Stenberg, K., Clausen, T., Lindqvist, Y., and Macheroux, P. (1995) *Eur. J. Biochem. 228*, 408−416.
19. Yorita, K., Janko, K., Aki, K., Ghisla, S., Palfey, B. A., and Massey, V. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9590−9595.
20. Gondry, M., and Lederer, F. (1996) *Biochemistry 35*, 8587−8594.
21. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−380.
22. Balme, A., Brunt, C. E., Pallister, R. L., Chapman, S. K., and Reid, G. A. (1995) *Biochem. J. 309*, 601−605.
23. Gondry, M., Diêp Lê, K. H., Manson, F. D. C., Chapman, S. K., Mathews, F. S., Reid, G. A., and Lederer, F. (1995) *Protein Sci. 4*, 925−935.
24. Labeyrie, F., Baudras, A., and Lederer, F. (1978) *Methods Enzymol. 53*, 238−256.
25. Iwatsubo, I., Mevel-Ninio, M., and Labeyrie, F. (1977) *Biochemistry 16*, 3558−3566.
26. Rao, K. S., and Lederer, F. (1998) *Protein Sci. 7*, 1531−1537.
27. Schowen, K. B., and Schowen, R. L. (1982) *Methods Enzymol. 89*, 551−606.
28. Weast, R. C. (1981) in *CRC Handbook of Chemistry and Physics*, Chemical Rubber Publishing Company (CRC Press), Boca Raton, FL.
29. Suzuki, H., and Ogura, Y. (1970) *J. Biochem. 67*, 291−295.
30. Hinkson, J. W., and Mahler, H. R. (1963) *Biochemistry 2*, 216−220.
31. Hermes, J. D., Roeske, C. A., O'Leary, M. H., and Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
32. Quinn, D. M., and Sutton, L. D. (1991) in *Enzyme mechanism from isotope effects* (Cook, P. F., Ed.) Chapter 3, pp 73−126, CRC Press, Boca Raton, FL.
33. Urban, P., and Lederer, F. (1985) *J. Biol. Chem. 260*, 11115−11122.
34. Diêp Lê, K. H., and Lederer, F. (1991) *J. Biol. Chem. 266*, 20877−20881.
35. Illias, R. M., Sinclair, R., Robertson, D., Neu, A., Chapman, S. K., and Reid, G. A. (1998) *Biochem. J. 333*, 107−115.
36. Makhatadze, G. I., Clore, G. M., and Gronenborn, A. M. (1995) *Nat. Struct. Biol. 2*, 852−855.
37. Gekko, K., and Timasheff, S. N. (1981) *Biochemistry 20*, 4667−4676.
38. Karsten, W. E., Lai, C.-J., and Cook, P. F. (1995) *J. Am. Chem. Soc. 117*, 5914−5918.
39. Xie, M., Seravalli, J., Huskey, W. P., Schowen, K. B., and Schowen, R. L. (1994) *Bioorg. Med. Chem. Lett. 2*, 691−695.

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