Pig Heart Lipoamide Dehydrogenase: Solvent Equilibrium and Kinetic Isotope Effects†

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ABSTRACT: Lipoamide dehydrogenase is a flavoprotein which catalyzes the reversible oxidation of dihydrolipoamide, $Lip(SH)_2$, by NAD^+. The ping-pong kinetic mechanism involves stable oxidized and two-electron-reduced forms. We have investigated the rate-limiting nature of proton transfer steps in both the forward and reverse reactions catalyzed by the pig heart enzyme by using a combination of alternate substrates and solvent kinetic isotope effect studies. With NAD^+ as the variable substrate, and at a fixed, saturating concentration of either $Lip(SH)_2$ or DTT, inverse solvent kinetic isotope effects of 0.68 ± 0.05 and 0.71 ± 0.05, respectively, were observed on V/K. Solvent kinetic isotope effects on V of 0.91 ± 0.07 and 0.69 ± 0.02 were determined when $Lip(SH)_2$ or DTT, respectively, was used as reductant. When $Lip(SH)_2$ or DTT was used as the variable substrate, at a fixed concentration of NAD^+, solvent kinetic isotope effects of 0.74 ± 0.06 and 0.51 ± 0.04, respectively, were observed on V/K for these substrates. Plots of the kinetic parameters versus mole fraction D_2O (proton inventories) were linear in all cases. Solvent kinetic isotope effect measurements performed in the reverse direction using NADH as the variable substrate showed equivalent, normal solvent kinetic isotope effects on V/K_{NADH} when oxidized lipoamide, lipoic acid, or DTT were present at fixed, saturating concentrations. Solvent kinetic isotope effects on V were equal to 1.5–2.1. When solvent kinetic isotope effect measurements were performed using the disulfide substrates lipoamide, lipoic acid, or DTT as the variable substrates, normal kinetic isotope effects on V/K of 1.3–1.7 were observed. Using DTNB as the variable substrate, an inverse kinetic isotope effect of 0.41 ± 0.02 was observed on V/K with no solvent kinetic isotope effect on V. The solvent equilibrium isotope effect was determined to be 0.438, which allows us to calculate a fractionation factor for the thiol moieties of $Lip(SH)_2$ of 0.526. These data suggest that, during enzyme reduction by NADH, a single proton transfer between protonated His450′ and Cys45 is slowed in D_2O. Oxidation of the reduced enzyme by disulfide substrates is also sensitive to solvent isotopic composition, and these data suggest that a single proton transfer between protonated His450′ and the thiolate anion of the mixed enzyme–substrate disulfide is rate-limiting.

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.8.1.4)[1] is a ubiquitously distributed enzyme component of the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine reductase multienzyme complexes. It functions physiologically to catalyze the NAD-dependent oxidation of the dihydrolipoyl cofactors that are covalently linked to the acyltransferase components of these multienzyme complexes. The enzyme has been isolated from numerous sources and shown to be capable of catalyzing the reversible oxidation of free dihydrolipoamide by NAD^+.

$$NAD^+ + \text{Lipoamide}(SH)_2 \rightleftharpoons NADH + \text{Lipoamide}(S)_2 + H^+$$

Steady-state (Massey et al., 1960; Reed, 1973; Koike & Koike, 1976) and pre-steady-state (Massey et al., 1960; Massey & Veeger, 1961) kinetic analyses have demonstrated that lipoamide dehydrogenase catalysis proceeds via a ping-pong kinetic mechanism in which the enzyme shuttles between an oxidized (E) and 2-electron-reduced (EH_2) form. The latter exhibits the classical charge transfer absorbance band centered at 530 nm (Massey et al., 1960) typical of members of the flavoprotein reductase family: glutathione reductase (EC 1.6.4.2; Schulz et al., 1978), mercuric ion reductase (EC 1.16.1.1; Fox & Walsh, 1982), thioredoxin reductase (EC 1.6.4.5; Holmgren, 1980), trypanothione reductase (EC 1.6.4.8; Shames et al., 1986), and NADH peroxidase (EC 1.11.1.1; Dolin, 1975). On the basis of spectroscopic pH titrations of the pig heart enzyme, it has been proposed that the two-electron reduction of E is accompanied by the transfer of two protons (Matthews & Williams, 1976), with one proton residing on the active site thiols and with the other present on an adjacent basic residue (Matthews et al., 1977).

The lipoamide dehydrogenase genes from *Escherichia coli* (Stephens et al., 1983) and pig heart (Otulakowski & Robinson, 1987) have been cloned and sequenced. There is substantial primary sequence homology between lipoamide dehydrogenase and glutathione reductase at all positions in the gene (Allison et al., 1988), and all residues which have been implicated in the chemical mechanism of glutathione reductase are strictly conserved in lipoamide dehydrogenase. The active site disulfide, which is responsible for reversible electron storage and oxidation of substrate dithiols, is composed of Cys45 and Cys50. The amino-terminal cysteine residue has been proposed

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[1] Abbreviations: $Lip(S)_2$, lipoamide; $Lip(SH)_2$, dihydrolipoamide; NAD^+, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; DTT, reduced dithiothreitol; DTT_{ox}, oxidized dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TEA, triethanolamine; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(cyclohexylamino)propanesulfonic acid.

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to participate in substrate disulfide interchange (Thorpe & Williams, 1976a), while Cys50 participates in the charge transfer complex with the oxidized flavin in the two-electron-reduced enzyme (Thorpe & Williams, 1976b, 1981). The base noted above, which is protonated in EH_2, has been identified as His450′ in analogy with the same residue in glutathione reductase. The recent report of the 2.8-Å resolution structure of lipoamide dehydrogenase from *Azotobacter vinelandii* (Schierbeek et al., 1989) has confirmed all of these previous identifications based on primary sequence alignments.

We report here the investigation of solvent kinetic isotope effects on the reaction catalyzed by pig heart lipoamide dehydrogenase. Similar studies on the related flavoprotein, glutathione reductase, have demonstrated that a single proton transfer from His467′ to the glutathione thiolate anion is rate-limiting in the overall reaction (Wong et al., 1988). The structural and mechanistic information on the pig heart lipoamide dehydrogenase, and the ability to measure the reaction in both the forward, and reverse, reactions, permits the unique kinetic characterization of proton transfers in the reactions catalyzed by lipoamide dehydrogenase. The similarities in the reactions catalyzed by lipoamide dehydrogenase and other members of the flavoprotein reductase family enable us to make novel comparisons of the chemical mechanisms catalyzed by related members of the family.

Experimental Procedures

Materials. Porcine heart (type III) lipoamide dehydrogenase was purchased from Sigma and dialyzed against 10 mM TEA (pH 7.8) and 1.5 μM FAD before use. NADH was purchased from Pharmacia. NAD^+ was purchased from Boehringer Mannheim. DTT was from Research Organics. Lipoamide, lipoic acid, DTNB, *Leuconostoc mesenteroides* (type XXIV) glucose-6-phosphate dehydrogenase, *Streptococcus faecalis* NADH peroxidase, EDTA, and all other buffer components were from Sigma. D_2O (>99.8 atom % excess) was purchased from ICN and Cambridge Isotope Laboratories and was distilled (bp 101 °C, uncorrected) before use.

Synthesis of Substrates. Dihydrolipoamide was prepared by the reduction of lipoamide with sodium borohydride as described by Reed et al. (1958) and was crystallized from benzene and petroleum ether (2.5:1). The cyclic disulfide of DTT was prepared by oxidation of DTT in the presence of DTNB. DTNB (220 mg, 555 μmol) was dissolved in 2 mL of 10 mM TEA (pH 8) by titrating the suspension with 0.2 N KOH. DTT (46.8 mg, 303 μmol) was dissolved in 3 mL of the same buffer and added in 100-μL aliquots to the DTNB solution. The solution was maintained at pH 8 by the addition of 0.2 N KOH after each addition of DTT. After all the DTT was added, the reaction mixture was stirred for an hour and then applied onto either a DEAE or Fast Q (Pharmacia) column. Fractions containing oxidized DTT, identified by the characteristic 230–350-nm ultraviolet absorbance (Cleland, 1964), were pooled, lyophilized, and stored desiccated.

Solvent Equilibrium Isotope Effects. Stock solutions of NADH (50 mM), lipoamide (25 mM in absolute ethanol), NAD^+ (100 mM), and dihydrolipoamide (10 mM, 33% ethanol) were prepared. NADH concentrations were calibrated using *S. faecalis* NADH peroxidase in the presence of excess H_2O_2. NAD^+ concentrations were calibrated by using *L. mesenteroides* glucose-6-phosphate dehydrogenase in the presence of excess glucose 6-phosphate. Dihydrolipoamide concentrations were calibrated using porcine heart lipoamide dehydrogenase in the presence of excess NAD^+. Lipoamide concentrations were not calibrated. These stock solutions were added in their approximate equilibrium concentrations to 300 mM TAPS buffer (pH 8.0) to a final volume of 5 mL. This solution was then equilibrated in a 37 °C water bath. A 300-μL aliquot of this 10× mixture was diluted to 3 mL with H_2O in a 3-mL quartz cuvette, and 20 μL of concentrated lipoamide dehydrogenase (10 mg/mL) was added. The change in absorbance was used to adjust the concentration of substrates and products in the 10× mixture, so that, upon addition of enzyme, no changes in the absorbance at 340 nm were demonstrated. Reaction mixtures containing different mole fractions of D_2O were then prepared in 3-mL cuvettes by the addition of the appropriate volumes of H_2O and D_2O to 300 μL of the 10× mixture. Enzyme was added to each reaction mixture, and the increase in absorbance was monitored for each reaction until a new equilibrium was established. Changes in the concentration of NADH, and thus in the other reactants, were calculated from the changes in absorbance at 340 nm using $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$ for NADH. New equilibrium constants were calculated for each mole fraction of D_2O from

$$K_{eq}' = \frac{[NADH + x][LipS_2 + x]}{[NAD^+ - x][Lip(SH)_2 - x]}$$

where x is the calculated change in NADH concentration at each mole fraction of D_2O. A plot of the calculated K_{eq}' values against mole fraction of D_2O was then used to determine the solvent equilibrium isotope effect, $^{D_2O}K_{eq}$, and the fractionation factor(s) of the thiol moieties of $Lip(SH)_2$, ϕ_{SH}, as discussed under Results.

Solvent Kinetic Isotope Effects. 300 mM TAPS buffers containing 3 mM EDTA were prepared in either H_2O or D_2O at pH or pD (pH meter reading + 0.4) 8.0 by titrating with 4 N KOH dissolved in H_2O or D_2O, respectively. Buffers were used at 100 mM concentrations for enzyme assays, and all reactants were prepared daily in He-saturated solvents noted below to maintain anaerobiosis. NADH was prepared in D_2O and calibrated by endpoint assay using *S. faecalis* NADH peroxidase. NAD^+ was prepared in H_2O and was calibrated using *L. mesenteroides* glucose-6-phosphate dehydrogenase. Oxidized lipoamide and lipoic acid solutions were prepared volumetrically in absolute ethanol and 33% ethanol, respectively. Solutions of dihydrolipoamide, prepared in 36% ethanol, and DTT, prepared in 10 mM TEA (pH 7.8), were calibrated by reduction of DTNB using $\epsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$ (Ellman, 1959). DTNB, prepared in 10 mM TEA (pH 7.8) was calibrated in the presence of excess DTT. Oxidized DTT, prepared in 10 mM TEA (pH 8), was calibrated using $\epsilon_{283} = 273\ M^{-1}\ cm^{-1}$ (Cleland, 1964).

Lipoamide dehydrogenase assays were performed spectrophotometrically by monitoring the oxidation or reduction of NADH or NAD^+ at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) with a Gilford 260 spectrophotometer equipped with thermospacers attached to a constant temperature water bath maintained at 37 °C. The rate of reduction of DTNB was measured spectrophotometrically at 412 nm. Quartz cuvettes of 1-cm light path were used throughout, and the total volume of each assay was 3 mL.

Reaction mixtures containing NADH (or NAD^+), disulfide (or dithiol) substrate, and the desired mole fraction of D_2O were prepared volumetrically from H_2O and D_2O buffer stock solutions. Each reaction mixture was thermally equilibrated in a 37 °C water bath, and each reaction was initiated by the addition of enzyme. Initial velocities were measured at various mole fractions of D_2O with either varying NADH (or NAD^+) or disulfide (or dithiol) substrate concentrations, at a fixed saturating concentration of the other substrate. The kinetic

parameters V and V/K were determined at each mole fraction of D_2O. The calculated V and V/K values were plotted against mole fraction of D_2O, and the resulting proton inventories were used to determine the number of protons undergoing transfer and to determine the kinetic isotope effects on V (^{D_2O}V) and V/K ($^{D_2O}V/K$).

pH Profiles. pH profiles were performed as described by Wong and Blanchard (1989). The buffers were used at 100 mM concentrations at the stated pH values: PIPES (6.0–7.2), HEPES (7.0–8.2), TAPS (7.8–9.0), and CHES (8.9–9.6). Initial velocity measurements were performed using lipoamide dehydrogenase by varying the concentration of NADH at a fixed saturating concentration of lipoamide. The kinetic parameters V and V/K_{NADH} were determined at each pH, and their log values were plotted against the pH value determined by the insertion of a microelectrode into the cuvette immediately after each assay was performed.

Data Analysis. Reciprocal initial velocities were plotted against reciprocal variable substrate concentrations, and the data were fitted to the appropriate rate equations using the Fortran programs of Cleland (1979). The individual saturation curves were fitted to eq 1, and the initial velocities obtained by varying the mole fraction of D_2O were fitted to eq 2, where A is the variable substrate concentration, F_i is the mole fraction of D_2O, and E_V and $E_{V/K}$ are the isotope effect minus 1 for V and V/K, respectively. Data for pH profiles that showed a decrease in log V or log V/K with a slope of −1 as pH was increased were fitted to eq 3, where y is the parameter to be fitted, and C is the pH-independent plateau value.

$$v = VA/(K + A) \quad (1)$$

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_V)] \quad (2)$$

$$\log y = \log [C/(1 + K/[H^+])] \quad (3)$$

Results

Solvent Equilibrium Isotope Effect and Lip(SH)$_2$ Fractionation Factor. The solvent equilibrium isotope effect was determined using pig heart lipoamide dehydrogenase. A plot of the calculated equilibrium constants at various mole fractions of D_2O yielded a calculated value for $^{D_2O}K_{eq}$ of 0.438. This value represents the product of fractionation factors for the products divided by the fractionation factors of the substrates (Quinn & Sutton, 1991), i.e.

$$H_2O + Lip(SH)_2 + NAD^+ \rightleftharpoons LipS_2 + NADH + H_3O^+$$

$$D_2O + Lip(SD)_2 + NAD^+ \rightleftharpoons LipS_2 + NADD + D_3O^+$$

$$^{D_2O}K_{eq} = \frac{^{H}K_{eq}}{^{D}K_{eq}} = \frac{[D_2O][Lip(SD)_2]^2[NADH][H_3O^+]}{[H_2O][Lip(SH)_2]^2[NADD][D_3O^+]} \quad (4)$$

$$^{D_2O}K_{eq} = \frac{\phi_{H_2O}\phi_{Lip(SH)_2}^{\ 2}}{\phi_{NADH}\phi_{H_3O^+}} \quad (5)$$

The reactants whose fractionation factors must be considered are the protons of water [ϕ_{H_2O} = 1.058 (Friedman & Shiner, 1966)], the protons of hydronium ion [$\phi_{H_3O^+}$ = 0.69 (Schowen, 1978)], NADH [ϕ_{NADH} = 0.97 (Cleland, 1980)], and Lip(SH)$_2$. Solution of eq 5 yields a value of 0.526 for the fractionation factor for the thiol moieties of dihydrolipoamide.

pH Profiles of the Kinetic Parameters. Initial velocity measurements were performed using NADH as the variable substrate, at saturating levels of oxidized lipoamide between pH 6.33 and 9.62. The data were fitted to eq 3, and the V and V/K_{NADH} pH profiles are shown in Figure 1. The maximum velocity decreases at alkaline pH as a group exhibiting a pK_a value of 8.29 ± 0.06 is deprotonated, and V/K_{NADH} decreases as a group exhibiting a pK_a value of 8.53 ± 0.09 is deprotonated.

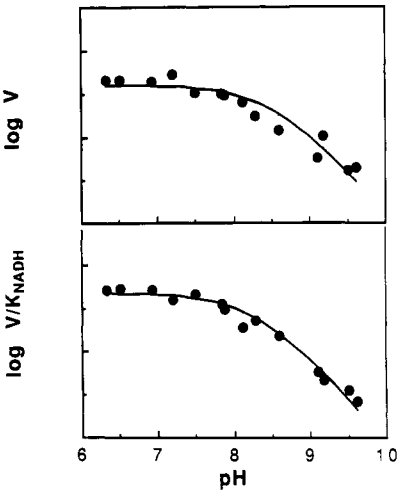


FIGURE 1: pH profiles of V and V/K_{NADH} determined with pig heart lipoamide dehydrogenase. The pH dependence of the maximum velocity was determined with NADH as the variable substrate at a fixed, saturating concentration of Lip(S)$_2$. (Lower panel) V/K_{NADH} profile decreases with the deprotonation of one group with a pK of 8.53 ± 0.09. (Upper panel) V profile decreases with the deprotonation of a single group with a pK of 8.29 ± 0.06.

Table I: Kinetic Parameters and Solvent KIE's for the Backward Reaction

reducible substrate	K_m (mM)[a]	relative V_{max} (%)[b]	^{D_2O}V	$^{D_2O}V/K_{NADH}$	$^{D_2O}V/K_{RSSR}$
lipoamide	3.08	100.0	1.9 ± 0.2[c]	2.3 ± 0.7	
			2.1 ± 0.3[d]		1.3 ● 0.1
lipoic acid	1.49	0.7	1.7 ± 0.2[c]	21. ± 0.5	
			1.5 ± 0.3[d]		1.7 ± 0.1
oxidized DTT	0.13	0.1	1.7 ± 0.2[c]	2.0 ± 0.3	
			2.0 ± 0.3[d]		1.4 ± 0.1
DTNB	2.37	0.4	1.02 ± 0.08[d]	nd[e]	0.41 ± 0.02

[a] All data were obtained in 100 mM TAPS buffer, pH 8.0, at 25 °C. The K_m for NADH was determined to be 24 μM under these conditions. [b] Relative velocities were determined by comparing the reciprocal plots of each analogue to that of lipoamide, determined under identical experimental conditions. [c] Determined using NADH as the variable substrate. [d] Determined using the disulfide as the variable substrate. [e] The solvent kinetic isotope effect on V/K_{NADH} was not determined under saturating DTNB conditions.

Solvent Kinetic Isotope Effects and Proton Inventories Using NADH and Lipoamide. Solvent kinetic isotope effect measurements were performed using NADH as the variable substrate at a fixed saturating concentration of lipoamide. Plots of the reciprocal initial velocities vs reciprocal NADH concentrations determined at various mole fractions of D_2O showed normal kinetic isotope effects on both V and V/K. Plots of V_m and V/K (determined by linear regression) against mole fraction of D_2O were linear with $^{D_2O}V_{NADH}$ = 1.9 ± 0.2 and $^{D_2O}V/K_{NADH}$ = 2.3 ± 0.7. Solvent kinetic isotope effect measurements performed with lipoamide as the variable substrate also yielded normal effects and linear proton inventories with $^{D_2O}V_{lipoamide}$ = 2.1 ± 0.3 and $^{D_2O}V/K_{lipoamide}$ = 1.3 ± 0.1 (Table I).

Solvent Kinetic Isotope Effects and Proton Inventories Using NADH and Alternate Disulfide Substrates. Solvent kinetic isotope effect measurements were performed using NADH as the variable substrate and at fixed saturating concentrations of lipoic acid or oxidized DTT (Table I). Normal solvent kinetic isotope effects and linear proton inventories were observed on both V and V/K with $^{D_2O}V_{NADH}$

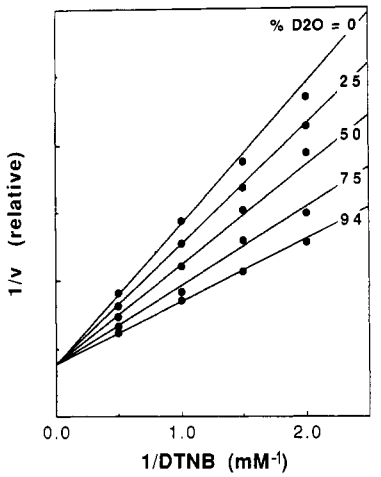


FIGURE 2: Solvent kinetic isotope effect on the LDH-catalyzed backward reaction. Steady-state initial velocity measurements of the reaction using varying concentrations of DTNB at a fixed, saturating concentration of NADH (150 μM). An inverse SKIE on V/K is observed, and a plot of this kinetic parameter against mole fraction of D_2O yielded a linear proton inventory with $^{D_2O}V/K_{DTNB}$ = 0.41 ± 0.02.

= 1.7 ± 0.2 and $^{D_2O}V/K_{NADH}$ = 2.1 ± 0.5 for lipoic acid and $^{D_2O}V_{NADH}$ = 1.7 ± 0.2 and $^{D_2O}V/K_{NADH}$ = 2.0 ± 0.3 for oxidized DTT. Solvent kinetic isotope effect measurements were also performed with lipoic acid, oxidized DTT, or DTNB as the variable substrate. Steady-state K_m values of 1.49, 0.13, and 2.37 mM were determined for lipoic acid, oxidized DTT, and DTNB, respectively, while the calculated maximal velocities were 0.7, 0.1, and 0.4% of that measured in the presence of lipoamide (Table I). Double-reciprocal plots determined at various mole fractions of D_2O demonstrated normal solvent kinetic isotope effects on both V and V/K with lipoic acid or oxidized DTT as the saturating substrates. Plots of the V and V/K values against mole fraction of D_2O were linear with $^{D_2O}V_{lipoic}$ = 1.5 ± 0.3 and $^{D_2O}V/K_{lipoic}$ = 1.7 ± 0.1 for lipoic acid and $^{D_2O}V_{DTTox}$ = 2.0 ± 0.3 and $^{D_2O}V/K_{DTTox}$ = 1.4 ± 0.1 for oxidized DTT. An inverse solvent kinetic isotope effect of 0.41 ± 0.02 was exhibited on $^{D_2O}V/K_{DTNB}$; no ^{D_2O}V effect was observed (Figure 2).

Solvent Kinetic Isotope Effects and Proton Inventories Using NAD⁺ and Dihydrolipoamide. Solvent isotope effect measurements were determined using pig heart lipoamide dehydrogenase at pH 8.0. When NAD^+ was used as the variable substrate at a fixed saturating concentration of dihydrolipoamide, V and V/K effects were observed in the double-reciprocal plots as the mole fraction of D_2O was varied. When the calculated V and V/K values were plotted against mole fraction of D_2O, linear proton inventories were obtained which yielded an inverse $^{D_2O}V_{NAD^+}$ solvent kinetic isotope effect of 0.91 ± 0.07 and an inverse $^{D_2O}V/K_{NAD^+}$ of 0.68 ± 0.05. Identical solvent kinetic isotope effect measurements were performed using dihydrolipoamide as the variable substrate at a fixed saturating concentration of NAD^+. V and V/K effects were again observed in the double-reciprocal plots as the mole fraction of D_2O was varied. Plots of the calculated V and V/K values against mole fraction of D_2O again yielded linear proton inventories with $^{D_2O}V_{Lip(SH)2}$ = 0.94 ± 0.02 and $^{D_2O}V/K_{Lip(SH)2}$ = 0.74 ± 0.06 (Table II).

Solvent Kinetic Isotope Effects and Proton Inventories Using NAD⁺ and DTT. Solvent kinetic isotope effect measurements were performed using NAD^+ as the variable substrate at a fixed saturating concentration of DTT. Double-reciprocal plots determined at various mole fractions of D_2O demonstrated inverse solvent kinetic isotope effects on both V and V/K. Plots of the calculated V and V/K values against mole fraction of D_2O were linear with $^{D_2O}V_{NAD^+}$ = 0.69 ± 0.02 and $^{D_2O}V/K_{NAD^+}$ = 0.71 ± 0.05. Identical solvent kinetic isotope effect measurements were also performed with DTT as the variable substrate. A steady-state K_m of 348 mM was determined for DTT, and the calculated maximal velocity was 0.02% of that determined in the presence of dihydrolipoamide. Plots of the reciprocal initial velocities versus reciprocal DTT concentrations determined at various mole fractions of D_2O also revealed solvent kinetic isotope effects on V and V/K. Plots of the calculated V and V/K values against mole fraction of D_2O were linear with $^{D_2O}V_{DTT}$ = 0.69 ± 0.02 and $^{D_2O}V/K_{DTT}$ = 0.51 ± 0.04 (Table II).

Table II: Kinetic Parameters and Solvent KIE's for the Forward Reaction

oxidizable substrate	K_m (mM)[a]	relative V_{max} (%)[b]	^{D_2O}V	$^{D_2O}V/K_{NAD^+}$	$^{D_2O}V/K_{RSH}$
dihydrolipoamide	0.19	100.0	0.91 ± 0.07[c] 0.94 ± 0.02[d]	0.68 ± 0.05	0.74 ± 0.06
reduced DTT	348	0.02	0.69 ± 0.02[c] 0.69 ± 0.02[d]	0.71 ± 0.05	0.51 ± 0.04

[a] All data were obtained in 100 mM TAPS buffer, pH 8.0, at 25 °C. The K_m for NAD^+ was determined to be 140 μM under these conditions. [b] Relative velocities were determined by comparing the reciprocal plots of the analogue to that of dihydrolipoamide, determined under identical experimental conditions. [c] Determined using NAD^+ as the variable substrate. [d] Determined using the dithiol as the variable substrate.

Discussion

This laboratory has previously investigated the effect of solvent isotopic substitution on the reactions catalyzed by both glutathione reductase (Wong et al., 1988) and NADH peroxidase (Stoll & Blanchard, 1991). In both of these flavoprotein reductases, solvent isotopic substitution has a unique effect on the reaction occurring in the oxidative half-reaction. In the case of glutathione reductase, the proton transfer step whose rate was slowed in D_2O was the proton transfer from His467′ to the first glutathione thiolate anion, while in the case of NADH peroxidase, a proton transfer from some acidic enzyme group to hydrogen peroxide was proposed to be slowed in D_2O. On this basis, the two-electron-reduced forms of both of these enzymes was suggested to be monoprotonated, i.e., EH^- versus EH_2, at the pH values where the experiments were performed. In the present case, there is compelling evidence that the two-electron-reduced enzyme exists as EH_2 (Matthews et al., 1979). This fact, plus the ease with which both the forward and reverse reactions could be determined at the appropriate pH values (in contrast to glutathione reductase or NADH peroxidase), led us to perform this solvent kinetic isotope effect analysis.

Lipoamide dehydrogenase, like other flavoprotein reductases, is capable of being overreduced to EH_4 forms by pyridine nucleotide substrates, and *E. coli* lipoamide dehydrogenase is particularly susceptible to this because of the values and closeness of the redox potentials for the E/EH_2 couple (−264 mV) and the EH_2/EH_4 couple (−317 mV; Wilkinson & Williams, 1979). In contrast, the same redox potentials exhibited by the pig heart enzyme are −280 and −346 mV, respectively (Matthews & Williams, 1976), and significant overreduction to EH_4 is not encountered. The thorough stopped-flow kinetic analysis of the reaction between oxidized lipoamide dehydrogenase and dihydrolipoamide and reduced lipoamide dehydrogenase and lipoamide has been reported (Matthews et al., 1977, 1979). These studies included the analysis of the pH dependence of this half-reaction in both

directions, which confirmed the earlier report of the pH dependence of the overall reaction in both directions. These data together suggested that, in the forward direction, a group exhibiting a pK of 6.3 was required to be unprotonated and that the protonation of this group abolished activity in the pyridine nucleotide half-reaction and had no effect on the rate of substrate dithiol oxidation. In the reverse reaction, a single group exhibiting a pK value of 7.9 was shown to be required in a protonated state, and deprotonation of this group decreased the reduction of the substrate disulfide. In the steady-state pH studies presented here, a group exhibiting a similar pK value of 8.29 is also required to be protonated, and deprotonation of this group results in a loss of catalytic activity. In human erythrocyte glutathione reductase (Wong & Blanchard, 1989) and *Trypanosoma congolense* trypanothione reductase (Leichus et al., unpublished results), groups exhibiting similar pK values, whose deprotonation decreases V, have been attributed to a His′–Glu′ ion pair which functions as a proton donor to the substrate disulfide during its cleavage, and the group observed in the V profile presented here may similarly be attributed to the conserved His450′–Glu455′ ion pair in lipoamide dehydrogenase (Otulakowski & Robinson, 1987). These studies and earlier stopped-flow studies of the reaction of lipoamide dehydrogenase with NADH and NAD^+ (Massey et al., 1960) suggested that the rate-limiting steps were in the half-reaction of the enzyme with disulfide–dithiol substrates.

In both the forward and reverse reactions catalyzed by pig heart lipoamide dehydrogenase, we have measured solvent kinetic isotope effects on the maximum velocity and on the V/K values for pyridine nucleotide and disulfide–dithiol substrates. $^{D_2O}V/K$ values represent the solvent isotopic sensitivity of reactions occurring in the half-reaction in which chemistry is being performed on the variable substrate, while ^{D_2O}V may be viewed as reflecting the solvent isotopic sensitivity of all proton transfer steps, particularly those occurring in the slower of the two half-reactions. As is true of any isotope effect analysis of an enzyme-catalyzed reaction, ratios of rate constants, which describe the binding and chemical steps that make up catalysis, will influence the magnitude of experimentally determined values of the solvent kinetic isotope effects. These ratios, termed commitment factors, can be attenuated by the use of alternate substrates which exhibit kinetic parameters that are different than those exhibited by the normal substrate, resulting in altered magnitudes of the isotope effect. The chemical mechanism for the reaction catalyzed by lipoamide dehydrogenase involves many proton transfer steps between substrates, enzymic thiols, and the histidine base, only some of which are of kinetic importance. The analysis of the solvent kinetic isotope effects reported here relies on the unique fractionation behavior of protonated mercaptans (Schowen, 1978), with their strong preference for protons over deuterons. Thus, during disulfide reduction and protonation, rates decrease as the percentage of D_2O increases, resulting in normal isotope effects. Conversely, during dithiol oxidations, rates will increase in increasing percentages of D_2O, resulting in inverse isotope effects.

Fractionation Factor for the Thiol Moieties of Lipoamide. The equilibrium constant for the reaction catalyzed by lipoamide dehydrogenase is shifted in D_2O from its value in H_2O due to the nonunitary values for the fractionation factors of substrates and products. The species whose fractionation factor dominates the solvent equilibrium isotope effect is dihydrolipoamide, since its two thiols at C6 and C8 are converted to the disulfide in the product, lipoamide. The measured value of K_{eq} is dependent on the mole fraction of D_2O and yields a value for $^{D_2O}K_{eq}$ (0.438) which allows us to calculate the fractionation factor for dihydrolipoamide of 0.526. This value is in excellent agreement with the value determined similarly for glutathione [0.456 (Wong et al., 1988)], and for other mercaptans (Pohl, 1961). It is unknown whether there are measurable differences in the fractionation factors of primary and secondary thiols, although lipoamide contains both a primary thiol at C8 and a secondary thiol at C6.

Solvent Kinetic Isotope Effects Exhibited by NADH and Reducible Disulfide Substrates. The reaction being investigated in these studies is the sum of the following two half-reactions:

$$E + NADH + H^+ \rightleftharpoons E{\cdot}NADH{\cdot}H^+ \rightleftharpoons EH_2{\cdot}NAD^+ \rightleftharpoons EH_2 + NAD^+ \quad (6)$$

$$EH_2 + Lip(S)_2 \rightleftharpoons EH_2{\cdot}Lip(S)_2 \rightleftharpoons E{\cdot}Lip(SH)_2 \rightleftharpoons E + Lip(SH)_2 \quad (7)$$

The solvent kinetic isotope effect on V/K_{NADH}, $^{D_2O}V/K_{NADH}$, reflects only those proton transfer steps in the reductive half-reaction (reaction 6). Under the initial velocity conditions used to measure the solvent kinetic isotope effect, there is little back-reaction of EH_2 and NAD^+ to form NADD, and thus any isotope effect will be due to the proton transfers accompanying two-electron enzymic disulfide reduction via the flavin. These protons have been suggested to be localized on the disulfide interchange thiol, Cys45, and on His450′. Thus a major prototautomer for the pig heart lipoamide dehydrogenase EH_2 species (Sahlman & Williams, 1989a) is

FAD

Cys50–S^-···H–His^+–450′

Cys45–S–H

As seen in Table I, $^{D_2O}V/K_{NADH}$ is independent of the reducible disulfide used and is approximately equal to 2.2. In all cases, replotting the V/K value versus the mole fraction of D_2O yielded linear proton inventories (Venkatasubban & Schowen, 1981) indicative of a single proton being transferred in the step which is sensitive to solvent isotopic composition. This suggests that a kinetically significant proton transfer is occurring in the reductive half-reaction, in contrast to the situation observed with glutathione reductase (Wong et al., 1988) or NADH peroxidase (Stoll & Blanchard, 1991). This argues that NADH does not exhibit a large commitment to catalysis under these conditions, since a large commitment factor would reduce the size of the solvent kinetic isotope effect toward one. We interpret our V/K_{NADH} solvent kinetic isotope effect as reflecting a slow proton transfer step which is sensitive to solvent isotopic composition. A likely proton transfer step would be one in which, after reduction of the enzymic disulfide, Cys45–Cys50, one of the thiols would acquire a proton (or deuteron) from the base.

The solvent kinetic isotope effect on V/K_{RSSR}, $^{D_2O}V/K_{RSSR}$, reflects only those proton transfer steps occurring in the oxidative half-reaction (reaction 7). In this reaction, two protons are transferred from EH_2 to the disulfide substrate, forming E and the dithiol form of the substrate. As seen in Table I, the values of $^{D_2O}V/K_{RSSR}$ depend only slightly on the nature of the reducible substrate and are relatively small and normal for all disulfides, except DTNB. In all cases, replotting the V/K value versus the mole fraction of D_2O yielded linear proton inventories (Venkatasubban & Schowen, 1981) indicative of a single proton being transferred in the step which is sensitive to solvent isotopic composition. Independent

evaluation of the rate constants for this half-reaction using oxidized lipoamide (Matthews et al., 1979) argues that chemistry is 7 times slower than the release of either the substrate or product from the binary complexes. Since lipoamide, which exhibits a maximum velocity at least 100 times greater than any of the other reducible substrates tested (Table I), does not appear to exhibit a large commitment to catalysis, it seems unlikely that the other substrates tested exhibit substantial commitments to catalysis which would reduce the magnitude of the observed solvent kinetic isotope effect from the intrinsic value for the proton transfer step.

DTNB, when used as the reducible substrate exhibits unique solvent kinetic isotope effects on both V/K and V. The reduction of DTNB does not require the transfer of protons to the thiolate product, since the anion is resonance stabilized by the *para*-nitro group. The large inverse value for $^{D_2O}V/K_{DTNB}$, and the linear dependence of V/K_{DTNB} on the mole fraction of D_2O, requires that a single proton be removed from a group with a ground state fractionation factor that is large and inverse, i.e., a thiol. The thiol whose deprotonation is being measured using DTNB must be an enzymic thiol, and the value of $^{D_2O}V/K_{DTNB}$ represents the fractionation factor of an enzymic thiol, presumably either Cys45 or Cys50. The value of 0.41 that we assign to the fractionation factor of the enzymic thiol is in excellent agreement with the previously reported value for the fractionation factor of the catalytic cysteine of papain (Szawelski & Wharton, 1981).

The solvent kinetic isotope effect on the maximum velocity, ^{D_2O}V, represents the solvent isotopic compositional dependence of the overall reaction and includes solvent kinetic isotope effects on all steps of kinetic significance. Considering the very large number of potentially slow proton transfer steps in the overall reaction catalyzed by lipoamide dehydrogenase, the interpretation of any value for ^{D_2O}V is daunting. However, the ability of lipoamide dehydrogenase to reduce alternative substrates with lowered thiol pK values simplifies this problem. One such substrate, DTNB, exhibits a unitary value for ^{D_2O}V, a result which suggests that the rate-limiting proton transfer step in the overall reaction is, in fact, localized in the half-reaction in which substrate disulfide is reduced. This result is in accord with previous determinations of the rates of the individual half-reactions using rapid reaction methods (Massey et al., 1960; Matthews & Williams, 1977).

The normal values for ^{D_2O}V exhibited by lipoamide, lipoic acid, and oxidized DTT and the linear proton inventories exhibited by all of these substrates thus reflect a solvent kinetic isotope effect on a step in the oxidative half-reaction which involves the protonation of one of the thiolate anions of the product species, produced concomitant with disulfide reduction. This proton is presumably transferred from the protonated His450′ observed in the V profile discussed above. The equivalence of the values of ^{D_2O}V exhibited by the various disulfide substrates suggests that the same or similar proton transfer steps are rate-limiting in the reactions of each of these substrates.

Solvent Kinetic Isotope Effects Exhibited by NAD and Dithiol Substrates. The reaction being investigated in these studies is the sum of the two half-reactions:

$$E + Lip(SH)_2 \rightleftharpoons E{\cdot}Lip(SH)_2 \rightleftharpoons EH_2{\cdot}Lip(S)_2 \rightleftharpoons EH_2 + Lip(S)_2 \quad (8)$$

$$EH_2 + NAD^+ \rightleftharpoons EH_2{\cdot}NAD^+ \rightleftharpoons E{\cdot}NADH{\cdot}H^+ \rightleftharpoons E + NADH + H^+ \quad (9)$$

All solvent kinetic isotope effects measured in this direction are inverse, immediately suggesting that the proton transfers whose rates are being increased in D_2O involve the deprotonation of thiols.

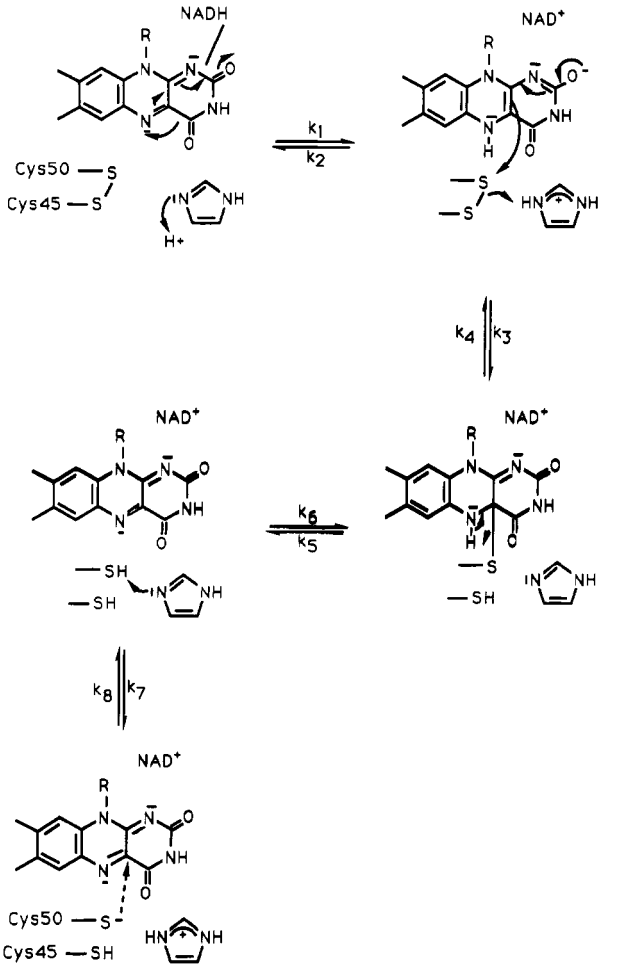
The solvent kinetic isotope effects on V/K_{NAD^+}, $^{D_2O}V/K_{NAD^+}$, are independent of the chemical identity of the oxidizable substrate and are the result of a single proton being transferred. $^{D_2O}V/K_{NAD^+}$ reflects only those proton transfer steps in the oxidative half-reaction (reaction 9), and the most likely step affected by the solvent isotopic composition is the deprotonation of the enzymic thiol, Cys45, during enzyme disulfide formation. This will be discussed further below. One might expect that $^{D_2O}V/K_{NAD^+}$ should exhibit opposite, but equal, solvent isotopic behavior to $^{D_2O}V/K_{NADH}$, yet this behavior is not observed (Table II) since this would predict values of $^{D_2O}V/K_{NAD^+}$ closer to 0.45. However, enzyme reduction by NADH involves transfer of a hydride ion from NADH to flavin, while enzyme oxidation by NAD^+ involves transfer of a deuteride ion from flavin to NAD. These steps are thus not the microscopic reverse of each other, and one cannot assume that the magnitudes of the solvent kinetic isotope effects in opposite direction will be equal and opposite.

$^{D_2O}V/K_{R(SH)2}$ reflects proton transfer steps only in the reductive half-reaction (reaction 8). The inverse solvent kinetic effects observed for dihydrolipoamide and DTT are due to the deprotonation of a single thiol and cannot be due to the protonation of one of the enzymic thiols formed during reduction, since this would result in a normal solvent kinetic effect. The magnitude of the V/K effect is dependent on the nature of the oxidizable substrate, with dihydrolipoamide exhibiting a smaller inverse effect than the extremely poor substrate, DTT. This is presumably the result of the larger commitment to catalysis of dihydrolipoamide, relative to DTT, and it would appear that the commitment is an external one, rather than an internal one, since DTT is a stronger reductant [−366 mV at pH 8.1 (Cleland, 1964)] than dihydrolipoamide [−287 mV at pH 7 (Massey, 1960; Sanadi et al., 1959)].

While ^{D_2O}V is essentially independent of the nature of the reducible disulfide substrate in the backward reaction, ^{D_2O}V in the forward direction is dependent on the nature of the dithiol substrate. Dihydrolipoamide exhibits a ^{D_2O}V of 0.94, while reduced DTT exhibits a ^{D_2O}V of 0.69 (Table II). One possible explanation for these results is that, using dihydrolipoamide as substrate, the overall rate of reaction is partially limited by the rate of release of NADH from the E·NADH complex. NADH has been shown to be a potent product inhibitor of the *E. coli* enzyme (Wilkinson & Williams, 1981), and the analysis of the *E. coli* catalyzed reaction in the forward direction can only be accomplished using rapid-reaction methods (Sahlman & Williams, 1989b). When reduced DTT is used as the substrate, however, the maximum velocity is 5000 times slower than when dihydrolipoamide is used as substrate, and the reduction of enzyme by DTT becomes partially rate-limiting and faster than NADH release as evidenced by the large, inverse solvent isotope effect on V. The lack of equivalence of ^{D_2O}V and $^{D_2O}V/K_{DTT}$ suggests that some rate limitation is still present in the pyridine nucleotide half-reaction even with this very slow substrate.

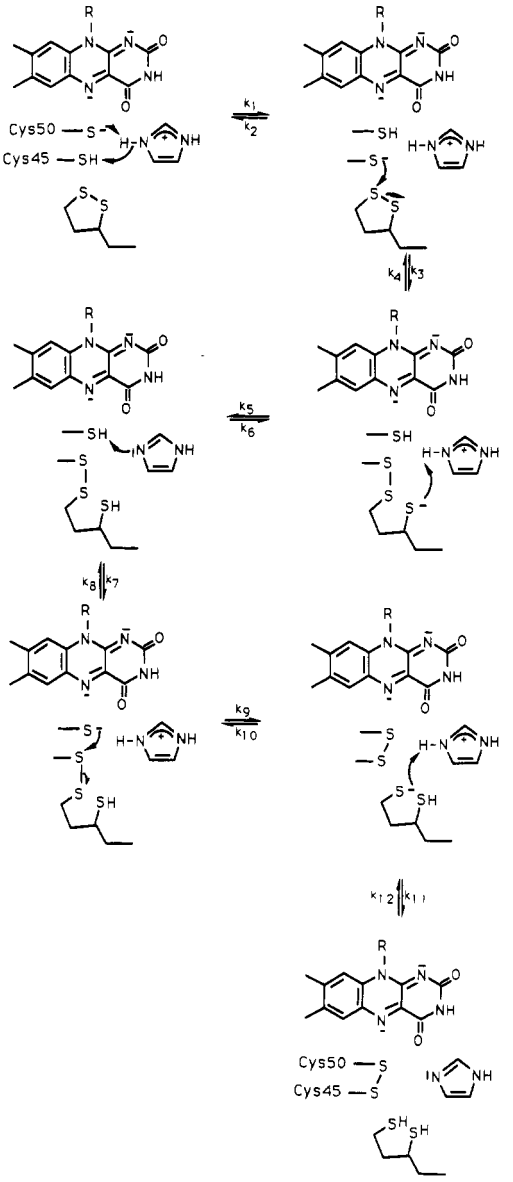
Chemical Mechanism and Identification of Solvent-Sensitive Steps. Scheme I presents a chemical mechanism for the half-reaction involving pyridine nucleotide substrates which is consistent with these solvent kinetic isotope effect data. By analogy to glutathione reductase (Pai et al., 1988), NADH presumably binds in a stacked orientation to the *re* side of the isoalloxazine ring, and with the 4*S* hydrogen pointed toward the N5 position of the flavin. Hydride transfer, k_1, generates the C2 enolate, which is 6.4 Å from His450′ (Mattevi et al.,

Scheme I: Proposed Chemical Mechanism of the Pyridine Nucleotide Half-Reaction Catalyzed by Lipoamide Dehydrogenase



1991), and may induce the protonation of the imidazole ring. The reduction of the Cys45–Cys50 disulfide is accomplished via the transient formation of a Cys50–C4a flavin adduct (Thorpe & Williams, 1981), k_3, and with the protonation of Cys45 by the now protonated imidazole. Breakdown of this Cys50–C4a flavin adduct, k_5, generates one of the prototautomers of EH_2, which is converted to another prototautomer in a step which is presumably rapid. The collapse of the Cys50–C4a flavin adduct was considered as a possible step which might be sensitive to solvent isotopic composition; however, the hydrogen atom at N5, which was transferred from the 4*S* position of NADH, is probably unable to rapidly exchange with solvent when NAD^+ is bound. Thus, in the direction of NADH oxidation, N5 would be protonated, while in the direction of NAD^+ reduction, N5 would be deuterated. We propose that k_3 and k_4 are the steps which are sensitive to solvent isotopic composition. In the direction of NADH oxidation, the rate of protonation of Cys45 should be slowed in D_2O and exhibit a normal solvent kinetic isotope effect, while in the direction of NAD^+ reduction, the rate of deprotonation of Cys45 should be increased in D_2O and exhibit an inverse solvent kinetic isotope effect. The rates of steps involved in the prototautomerism, k_7 and k_8, would be expected to exhibit opposite effects from those observed. The opposite, but nonequivalent, V/K solvent kinetic isotope effects exhibited during NADH oxidation and NAD^+ reduction are presumably the result of the solvent inaccessibility of the N5 flavin locus when pyridine nucleotide substrates are bound. In the reduction of NAD^+, the inverse solvent kinetic isotope effect resulting from the proton transfer represented by k_4 might be

Scheme II: Proposed Chemical Mechanism of the Reduced Enzyme–Substrate Disulfide Exchange Half-Reaction Catalyzed by Lipoamide Dehydrogenase



decreased by the presence of a normal deuterium kinetic isotope effect on the transfer of the deuteride ion from [N5-^{2}H] $FADH^-$ to NAD^+, k_2.

Scheme II presents a chemical mechanism for the half-reaction involving disulfide exchange between E/EH_2 and the dithiol–disulfide substrate and represents an expanded version of an earlier proposed mechanism (Matthews et al., 1977). Substrate disulfide binding results in a shift of the prototautomeric equilibrium and allows Cys45 to become nucleophilic, k_1. Nucleophilic attack on the substrate disulfide results in the formation of the mixed enzyme–substrate disulfide, k_3, and release of the thiolate anion. The protonation of this thiolate by protonated His450′, k_5, is presumably the step which is slow and sensitive to isotopic composition. This same step has been suggested to be rate-limiting in the reaction catalyzed by glutathione reductase (Wong et al., 1988), and the data reported here for lipoamide dehydrogenase suggest that the protonation of a substrate thiol is rate-limiting in the direction of disulfide substrate reduction and that the reverse of this step, thiol deprotonation, is rate-limiting in the direction of dithiol oxidation. The data obtained using DTNB confirm that ^{D_2O}V is reflective of substrate thiol protonation/deprotonation, since

with this substrate no ^{D_2O}V is observed. Deprotonation of Cys50, k_7, enables this thiol to attack the mixed Cys45–substrate disulfide, k_9, reforming the enzyme disulfide and releasing the product after a final proton transfer from His450′, k_{11}. The requirement for a proton transfer from His450′ to the first thiolate anion product and the subsequent deprotonation of Cys50 by His450′ would appear to be crucial in preventing the formation of a stable Cys45–substrate disulfide. This scheme accounts for the results obtained in this study, and previous studies of the protonation states of the oxidized and two-electron-reduced enzyme (Matthews et al., 1977, 1979; Sahlman & Williams, 1989a). It also is in accord with the most recent structural data for the *A. vinelandii* lipoamide dehydrogenase (Schierbeek, 1989; Mattevi et al., 1991), in particular, in depicting the relative orientation of the groups proposed in this study to participate in catalysis, and the results of site-directed mutagenesis studies (Benen et al., 1991; Williams et al., 1991).

The flavoprotein reductases have been shown to share an impressive number of isotopic similarities; however, an important difference has been documented in the present study. While glutathione reductase (Wong et al., 1988), trypanothione reductase (Leichus et al., unpublished results), and NADH peroxidase (Stoll & Blanchard, 1991) exhibit no solvent isotope effects on the pyridine nucleotide half-reaction, lipoamide dehydrogenase clearly does. The explanation for this difference may be the protonation state of the two-electron-reduced enzyme, which is EH_2 in the case of lipoamide dehydrogenase but likely to be EH^- in the other three enzymes. This difference may be the underlying reason for the opposite physiological roles of lipoamide dehydrogenase, which functions to reduce NAD^+ at the expense of the protein-bound dihydrolipoyl moiety, and the other reductases, which function to reduce their disulfide and peroxide substrates at the expense of NADH.

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