Human Erythrocyte Glutathione Reductase: Chemical Mechanism and Structure of the Transition State for Hydride Transfer†

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ABSTRACT: Kinetic parameters and primary deuterium kinetic isotope effects for NADH and five pyridine nucleotide substrates have been determined at pH 8.1 for human erythrocyte glutathione reductase. $^{D}V/K_{NADH}$ and ^{D}V are equal to 1.4 and are pH independent below pH 8.1, but ^{D}V decreases to 1.0 at high pH as a group exhibiting a pK of 8.6 is deprotonated. This result suggests that as His-467′ is deprotonated, the rate of the isotopically insensitive oxidative half-reaction is specifically decreased and becomes rate-limiting. For all substrates, equivalent V and V/K primary deuterium kinetic isotope effects are observed at pH values below 8.1. The primary deuterium kinetic isotope effect on V, but not V/K, is sensitive to solvent isotopic composition. The primary tritium kinetic isotope effects agree well with the corresponding value calculated from the primary deuterium kinetic isotope effects by using the Swain–Schaad relationship. This suggests that the primary deuterium kinetic isotope effects observed in these steady-state experiments are the intrinsic primary deuterium kinetic isotope effects for hydride transfer. The magnitude of the primary deuterium kinetic isotope effect is dependent on the redox potential of the pyridine nucleotide substrate used, varying from ~1.4 for NADH and −320 mV reductants to 2.7 for thioNADH to 4.2–4.8 for 3-acetylpyridine adenine dinucleotide (3APADH). The α-secondary tritium kinetic isotope effects also increase as the redox potential of the pyridine nucleotide substrate becomes more positive. Together, these data indicate that the transition state for hydride transfer is very early for NADH and becomes later for thioNADH and 3APADH, as predicted by Hammond's postulate. The temperature dependence of the kinetic parameters and the primary deuterium kinetic isotope effects have been determined for NADH, thioNADH, and 3APADH. The energies of activation increase as the redox potential of the substrate becomes more positive. In all cases, the isotope effect on the energies of activation and the Arrhenius preexponential factors are close to unity. These data suggest that quantum mechanical tunneling is not significant in the hydride transfer reaction. A chemical mechanism for the reductive half-reaction of human erythrocyte glutathione reductase is proposed on the basis of these data.

Glutathione reductase (EC 1.6.4.2) is a member of the family of flavoprotein reductases, which contain a redox-active disulfide moiety at the active site. This disulfide is critically involved in both reversible two-electron storage and in transferring reducing equivalents to GSSG[1] [reviewed in Williams (1976)]. The human erythrocyte enzyme used in this study carries out two kinetically distinct reactions: a reductive half-reaction in which the enzyme is reduced by NAD(P)H and an oxidative half-reaction in which the enzyme is oxidized concomitant with GSSG reduction. These reactions serve to maintain a high GSH/GSSG ratio in the red blood cell.

Human erythrocyte glutathione reductase has been crystallized and its three-dimensional structure has been determined (Thieme et al., 1981) and refined to 1.54-Å resolution (Karplus & Schulz, 1987). The structures of complexes between NADPH, and various analogues and fragments of NADPH, and the human erythrocyte enzyme have been determined at a resolution of 3 Å; NADPH binds in an extended conformation and induces a conformational change that is required for the stacking of the nicotinamide ring between the isoalloxazine ring and Tyr-197 (Pai et al., 1988). A chemical mechanism has been proposed on the basis of spectroscopic analysis (Massey & Williams, 1965; Arscott et al., 1981) and the X-ray crystallographic analysis of enzyme–substrate complexes (Pai & Schulz, 1983; Karplus et al., 1989; Janes & Schulz, 1990). Steady-state (Massey & Williams, 1965) and rapid-reaction kinetic studies of the yeast enzyme (Williams, 1976) have provided overwhelming evidence for a ping-pong kinetic mechanism.

We have previously reported the pH dependence of the kinetic parameters exhibited by the human erythrocyte enzyme and have shown that the V/K profiles for reduced pyridine nucleotides decrease above pH 9 as a group (pK ~ 8.9–9.3) is deprotonated. V similarly decreases at high pH as a group (pK = 9.2), which was identified as the His-467′–Glu-472′ ion pair, is deprotonated (Wong & Blanchard, 1989). This ionization prevents the rate-limiting protonation of the first

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[1] Abbreviations: GSSG, oxidized glutathione; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NADH, reduced β-nicotinamide adenine dinucleotide; tNADH, reduced thionicotinamide adenine dinucleotide; NHDH, reduced nicotinamide hypoxanthine dinucleotide; NGDH, reduced nicotinamide guanine dinucleotide; 3PAADH, reduced 3-pyridinealdehyde nicotinamide adenine dinucleotide; 3APADH, reduced 3-acetylpyridine nicotinamide adenine dinucleotide; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TEA-HCl, triethanolamine hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; FPLC, fast protein liquid chromatography.

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glutathione thiolate anion product (Wong et al., 1988). We have also demonstrated, using steady-state and rapid reaction methods, that the intrinsic primary deuterium kinetic isotope effect on hydride transfer is observed when NADH is used as reductant for the spinach, yeast, and *E. coli* glutathione reductases (Vanoni et al., 1990). In this paper, we focus on the mechanism and transition-state structure for hydride transfer that occurs in the reductive half-reaction catalyzed by human erythrocyte glutathione reductase.

Materials and Methods

Human erythrocyte glutathione reductase was a generous gift from Dr. Emil F. Pai (Max-Planck-Institut, Heidelberg, FRG), and malic enzyme from *Ascaris summ* was a gift from Dr. Paul F. Cook (Texas College of Osteopathic Medicine, Fort Worth, TX). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), yeast hexokinase, creatine kinase, tNAD, 3PAAD, 3APAD, NHD, NGD, GSSG, G6P, and buffers were purchased from Sigma. NAD and NADP were from Boehringer Mannheim. D_2O (>99.8 atom % excess) was from ICN and was distilled before use. [1-^{3}H]Glucose, [4-^{3}H]NAD, and [*adenosine*-^{14}C]NAD were purchased from Amersham. [1-^{2}H]Glucose (>98 atom % excess ^{2}H) was purchased from Omicrom Biochemicals. [1-^{2}H]G6P and [1-^{3}H]G6P were synthesized and purified as previously described (Vanoni et al., 1990).

[2-^{3}H]Malate was synthesized in a 10-mL solution containing 200 mM oxaloacetic acid, titrated to pH 7.6 with 2 N NaOH, by the addition of NaB^3H_4 at 0 °C. The reaction mixture was allowed to react for 1.5 h, and solid $NaBH_4$ (76 mg) was added. After an additional hour, the reaction mixture was acidified with 2 N HCl to pH 4. The pH of the solution was raised to 7.6 with 2 N NaOH, and the solution was applied to a Dowex 1 × 8 column (formate form) and was eluted with a linear 0–4 N ammonium formate gradient. The product eluted as a sharp, symmetric peak at approximately 2 N ammonium formate. The peak fractions were pooled, lyophilized, taken up in 20 mL of water, and enzymatically calibrated by using malic enzyme and excess NAD.

Preparation of 4S-Labeled Reduced Nucleotides. The oxidized forms of all nucleotides were purified on a FPLC Mono-Q anion-exchange column as described previously (Orr & Blanchard, 1984). All 4*S*-deuterated and -tritiated nucleotides were enzymatically prepared by reduction of the oxidized nucleotides with *L. mesenteroides* glucose-6-phosphate dehydrogenase as described previously (Vanoni et al., 1990). For each reduced nucleotide, fractions that had appropriate absorbance ratios (i.e., tNADH, $A_{260nm}/A_{395nm} \leq 1.3$; 3PAADH, $A_{260nm}/A_{358nm} \leq 1.5$; 3APADH, $A_{260nm}/A_{363nm} \leq 1.5$; NHDH, $A_{249nm}/A_{338nm} \leq 2.0$; NGDH, $A_{260nm}/A_{340nm} \leq 2.3$) were pooled.

Preparation of Reduced [4R-^{3}H]Pyridine Nucleotides. [4*R*-^{3}H, *adenosine*-^{14}C]NADH was synthesized from [4-^{3}H]NAD and [*adenosine*-^{14}C]NAD in the presence of [^{1}H]-glucose 6-phosphate and *L. mesenteroides* glucose-6-phosphate dehydrogenase. Both [4*R*-^{3}H]tNADH and [4*R*-^{3}H]3APADH were prepared from [2-^{3}H]malate and tNAD or 3APAD by the action of malic enzyme from *A. suum*. 4*R*-Tritiated reduced nucleotides were purified and calibrated as described above.

Initial Rate Studies. For the determination of primary deuterium kinetic isotope effects, the initial rate of the reaction was determined at a saturating concentration of GSSG (3 mM) and at varied concentrations of reduced pyridine nucleotide (25–400 μM) by measuring the decrease in absorbance of each reduced (protio or deuterio) nucleotide with a Gilford model 260 spectrophotometer. All reactions were performed in 1-mL cuvettes, and the desired temperature (10–40 °C) was maintained by using thermospacers and a constant temperature circulating water bath. Rates were determined at the following wavelengths for the following nucleotides: NADH, 340–370 nm; tNADH, 390 nm; 3PAADH, 390 nm; 3APADH, 390 nm; NHDH, 338 nm; NGDH, 365 nm. Reciprocal initial velocities were plotted against the reciprocal of reduced pyridine nucleotide concentration, and the data were fitted by the least-squares method and by using the kinetics programs of Cleland (1979). Primary deuterium kinetic isotope effect data were fit to eq 1, 2, or 3, which assume equal isotope effects on V and V/K, different isotope effects on V and V/K, and isotope effects on V only.

$$v = VA/[(K + A)(1.0 + F_iE)] \quad (1)$$

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

$$v = VA/[K + A(1.0 + F_iE_V)] \quad (3)$$

where F_i is the fraction of deuterium label (= 1.0), E is the isotope effect minus one on V and V/K, $E_{V/K}$ is the isotope effect minus one on V/K, and E_V is the isotope effect minus one on V. Primary deuterium kinetic isotope effects were determined at pH values between 6 and 10.5 in the following buffers at the pH values stated to allow for overlap: MES (pH 6.2–6.8), PIPES (pH 6.5–7.2), TAPS (pH 7.5–8.5), CHES (pH 9.0–10.0), and CAPS (pH 10.0–11.0). These buffers were also prepared in D_2O by first dissolving each buffer in a minimum amount of D_2O, lyophilizing each, and then redissolving in D_2O, and titrating with KOD to the desired pD (pH meter reading + 0.4). The pH values of all buffers were measured with a Radiometer PHM84 pH meter equipped with a combined microelectrode. The buffer solutions were filtered through a 0.22-μm membrane. GSSG solutions (30 mM) in H_2O and D_2O were freshly prepared and stored at 0 °C.

The pH dependence of the primary deuterium kinetic isotope effects were fit to

$$\log y = \log [Y_L + Y_H(K/H^+)/(1.0 + K/H^+)] \quad (4)$$

where y is the parameter whose pH dependence is being determined and Y_L and Y_H are the values of y at low and high pH.

The effect of solvent isotopic composition on the primary deuterium isotope effects was determined by using NADH and [4*S*-^{2}H]NADH at 25 °C in a range of pH values from 6.5 to 10.5. The primary deuterium kinetic isotope was determined in 0% and 100% D_2O at the stated pH values.

Determination of Tritium Isotope Effects. A 4-mL reaction mixture containing 100-140 μM reduced [4*S*-^{3}H]pyridine nucleotide and 3 mM GSSG in 10 mM TEA-HCl was thermally equilibrated at 25 °C. Before the enzyme was added, several 100-μL aliquots of the reaction mixture were withdrawn, and the nucleotides were separated by chromatography on a FPLC Mono-Q anion-exchange column equilibrated with 10 mM TEA-HCl (pH 7.8) and eluted with a 0–1 M NaCl gradient. Fractions of 1 mL were collected directly into 20-mL glass scintillation vials, to which 15 mL of Hydroflour scintillation fluid was added. The radioactivity of each fraction was analyzed to obtain the nonenzymatic background. At various time points after the addition of enzyme, approximately equal to 5, 10, 15, 20, 30, and 60 % conversion, 100-μL aliquots were removed, and labeled products were purified as described above. The absorbance was monitored during the reaction at the indicated wavelengths: 340 nm (NADH, NHDH, and NGDH), 395 nm (tNADH), 360 nm (3PAADH), and 363 nm (3APADH). ^3H_2O released from the C-4*S* position was

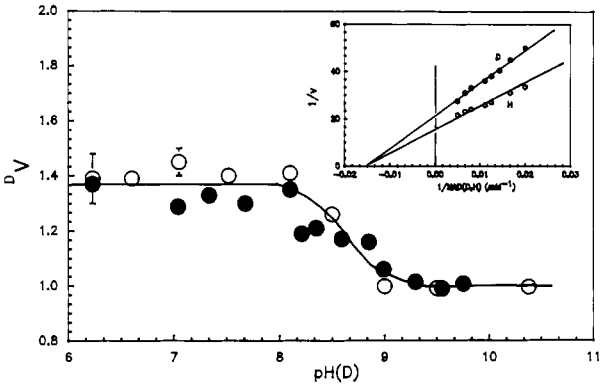


FIGURE 1: Primary deuterium kinetic isotope effects determined as a function of pH(D). Primary deuterium kinetic isotope effects were determined at each pH(D) value with NADH as reductant, and the experimental values of ^{D}V are plotted versus pH (○) or pD (●). The solid line through the data is a fit of the pH and pD combined data to eq 4 and yielded a pK value of 8.6 ± 0.3 for a group whose deprotonation causes ^{D}V to equal 1.0. (Inset) Representative primary data obtained at pH 8.1.

cleanly separated from [^{3}H]NADH and the radioactivity was quantitated. $^{T}V/K$ was determined by using

$$^{T}V/K = \log (1.0 - f)/\log (1.0 - \mathrm{dpm_f}/\mathrm{dpm_{100}}) \quad (5)$$

where f is the fractional percent conversion of the reaction, $\mathrm{dpm_f}$ is the dpm of ^3H_2O at f, and $\mathrm{dpm_{100}}$ is the dpm of ^3H_2O at 100% conversion (Blanchard & Englard, 1983). The above protocol was also used to determine α-secondary tritium kinetic isotope effects, where the specific activity of isolated [4-^{3}H]tNAD and [4-^{3}H]3APAD were measured. The α-secondary tritium kinetic isotope effect using [4R-^{3}H, *adenine*-^{14}C]NADH was performed according to the same protocol, but the ^{3}H/^{14}C ratio of the isolated [4-^{3}H, *adenine*-^{14}C]NAD was used to calculate $^{\alpha\text{-}T}V/K$ by using

$$^{T}V/K = \log (1.0 - f)/\log (1.0 - f\mathrm{dpm_f}/\mathrm{dpm_{100}}) \quad (6)$$

where the symbols are defined as before.

Calculations of the C-4S–H bond order from values of the α-secondary kinetic isotope effect were performed by using the BEBOVIB program (Sims et al., 1977; Sims & Lewis, 1984) running on a Silicon Graphics 4D80 and by using the crystallographically determined coordinates for NAD$^+$ (Reddy et al., 1981).

RESULTS

pH Dependence of the Primary Deuterium Kinetic Isotope Effect. Primary deuterium kinetic isotope effects, with NADH and [4S-^{2}H]NADH as the variable substrates, were performed at different pH values between 6.2 and 10.5. At all pH values below pH 8.1, equivalent primary deuterium kinetic isotope effects were observed on V and V/K. The primary deuterium kinetic isotope effects on V and V/K (only ^{D}V is plotted) are small and equal to 1.4 between pH 6.2 and 8.1, but ^{D}V decreases to a value of 1.0 at higher pH values (Figure 1, open circles). $^{D}V/K$ is greater than 1.0 at pH values greater than 8.1, but precise values are difficult to obtain. When the data were fitted to eq 4, a value of 8.6 ± 0.3 was obtained for the pK value of a group whose deprotonation causes ^{D}V to decrease to a value of 1.0. The primary deuterium kinetic isotope effects were also measured in D_2O at different pD values between 6.2 and 9.8 (Figure 1, filled circles). The magnitude and pH dependence of the primary deuterium kinetic isotope effect were not significantly changed in D_2O with NADH as substrate. All subsequent analyses were performed at pH(D) 8.1.

When tNADH or 3APADH and their 4S-deuterated isotopomers were used as substrates, a significant difference in the values of the V and V/K primary deuterium kinetic isotope effects was observed in D_2O, but not in H_2O. For tNADH, ^{D}V was equal to $^{D}V/K$ and had a value of 2.7 in H_2O. In D_2O, ^{D}V was less than $^{D}V/K$, yielding fitted values of 1.8 and 2.7, respectively (data not shown). When 3APADH was used as a substrate in H_2O, ^{D}V was equal to $^{D}V/K$, and both had values of 4.2. In D_2O, ^{D}V was equal to 2.3, while $^{D}V/K$ maintained its value of 4.2, as in H_2O (Figure 2).

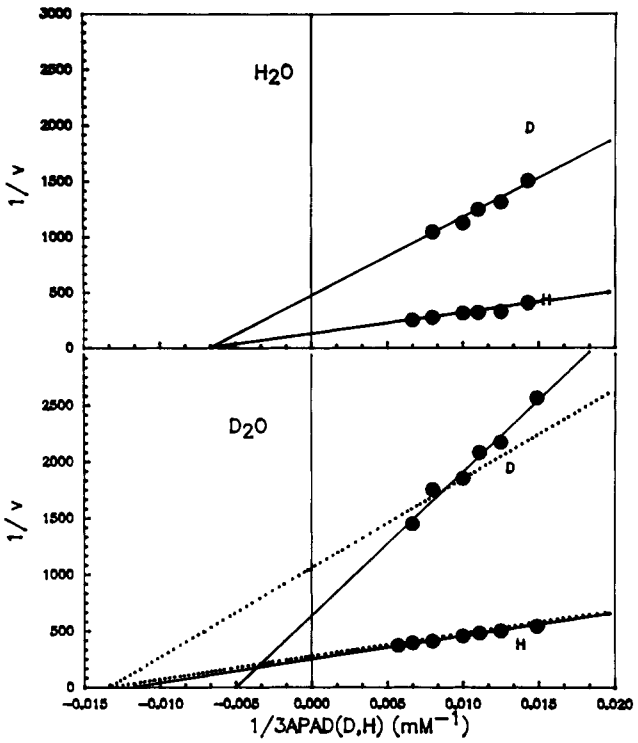


FIGURE 2: Primary deuterium kinetic isotope effect exhibited by 3APADH in H_2O (top panel) and 100% D_2O (bottom panel). The experimental data obtained in H_2O are best fit to eq 1 (solid line), in which equivalent isotope effects on V and V/K are observed. The experimental data obtained in 100% D_2O are best fit to eq 2 (solid line), in which isotope effects on V and V/K are not constrained to be equal. For comparison, the fit of these data to eq 1 is shown by the dotted lines.

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects, with NADH as the variable substrate, were performed at pH 8.1, and the results are shown in Figure 3. Only the intercepts of the reciprocal plot were affected by solvent isotopic composition, and the replot of the maximum velocity versus mole fraction of D_2O was linear and yielded a value of 2.4 ± 0.1 for ^{D_2O}V (inset, Figure 3). The expected deviation from linearity for a diprotonic transition state was calculated by using the Gross–Butler equation and is shown for comparison in the inset to Figure 3 as a dashed line.

Kinetic Parameters and Primary Kinetic Isotope Effect for Reduced Pyridine Nucleotide Substrates. The kinetic parameters for the reduced pyridine nucleotide substrates are presented in Table I, along with experimental values of $^{D}V/K$ and $^{T}V/K$. Also presented in the table are values of $^{T}V/K$ calculated from the Swain–Schaad relationship ($^{D}k^{1.442} = {}^{T}k$; Swain et al., 1985) by using the experimentally determined $^{D}V/K$ values. Equivalent primary deuterium kinetic isotope effects were observed on V and V/K in H_2O for all substrates tested at pH 8.1. The redox potentials of the pyridine nucleotide substrates and the reported redox potential for yeast glutathione reductase of −235 mV (Arscott et al., 1990) were used to calculate the equilibrium constants for enzyme reduction by using the Nernst equation. When the natural log of this calculated equilibrium constant was plotted versus the

Table I: Kinetic Parameters and Primary Kinetic Isotope Effects Exhibited by Pyridine Nucleotide Substrates[a]

nucleotide substrate	redox potential (mV)	rel V[b]	K_m (μM)	$^{D}V/K$	$^{T}V/K$ (exp)	$^{T}V/K$ (calc)
NADH	−320	1.0	44	1.35 ± 0.02	1.40 ± 0.13	1.50–1.57
NHDH	−320	0.20	19	1.18 ± 0.05	1.41 ± 0.06	1.19–1.35
NGDH	−320	0.12	33	1.19 ± 0.05	1.35 ± 0.16	1.17–1.39
tNADH	−285	0.09	107	2.67 ± 0.03	4.76 ± 0.15	4.05–4.17
3PAADH	−262	0.011	1009	3.93 ± 0.03	7.11 ± 0.13	7.10–7.26
3APADH	−258	0.0005	1000	4.81 ± 0.12	9.54 ± 0.22	9.26–9.95

[a] Kinetic data were obtained in 100 mM TAPS, pH 8.1, and at 3.0 mM GSSG at 5 °C. The values of $^{D}V/K$ and $^{T}V/K$ presented here were obtained in 10 mM TEA-HCl, pH 7.8, and at 3.0 mM GSSG at 25 ° C. Values of $^{D}V/K$ in 100 mM TAPS were determined to be within 10% of the values shown in the table. [b] NADH exhibits a specific activity of 26 μmol min^{-1} mg^{-1}, as compared to the reported specific activity with NADPH as a reductant of 235 μmol min^{-1} mg^{-1} (Krohne-Ehrich et al., 1977). The standard errors of the calculated values of V and K were less than 10% in all cases.

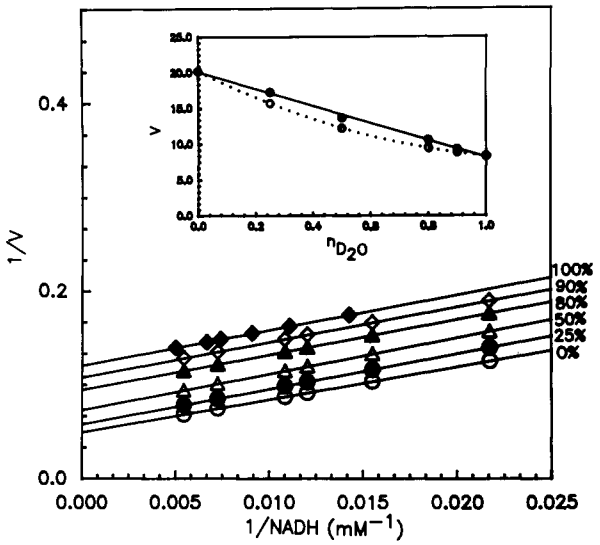


FIGURE 3: Solvent kinetic isotope effect on NADH oxidation. Initial velocities were obtained at various mole fractions of D_2O at pH(D) 8.1. The experimental points are shown, and the solid lines represent the fit of all the data to eq 3. (Inset) Replot of V_{max} versus mole fraction D_2O. The data yield a value 2.4 ± 0.1 for ^{D_2O}V. The solid line is drawn for a monoprotonic transition state, while the dashed line represents the expected behavior for a diprotonic transition state calculated by using the Gross–Butler equation.

natural log of the maximum velocity, a good correlation (r^2 = 0.906) was obtained.

α-Secondary Tritium Kinetic Isotope Effect. α-Secondary tritium kinetic isotope effects were determined by using the [4*R*-^{3}H]-labeled reduced pyridine nucleotide substrates. As seen in Table II, the magnitude of the secondary kinetic isotope effect increases as the reducing power of the substrate is decreased. From these values and the calculated value of $^{\alpha\text{-}T}K_{eq}$ for the oxidation of [4*R*-^{3}H]NADH to NAD^{3}H of 1.18 ($1.12^{1.442}$; Cook et al., 1980), we have calculated the bond order of the primary C-4*S*–H bond, which yields the experimental value for the α-secondary tritium kinetic isotope effect (Sims & Lewis, 1984).

Temperature Dependence of the Kinetic Parameters and the Primary Deuterium Kinetic Isotope Effect. The analysis of the temperature dependence of the kinetic parameters and the primary deuterium kinetic isotope effect exhibited by these reduced pyridine nucleotide substrates with different redox potentials were determined between 10 and 40 °C. For all substrates tested, linear Arrhenius plots were obtained from the plots of log V/K values versus the reciprocal absolute temperature (data not shown), and activation energies were determined from the slopes of these lines. As seen in Table III, there is a systematic increase in the energy of activation as the redox potential of the reductant becomes more positive

Table II: α-Secondary Tritium Kinetic Isotope Effect Exhibited by Human Erythrocyte Glutathione Reductase

nucleotide substrate	redox potential (mV)	$^{\alpha\text{-}T}V/K$	bond order (C-4*S*–H)[b]
NADH	−320	1.012 ± 0.005	0.96
tNADH	−285	1.054 ± 0.055	0.81
3APADH	−258	1.088 ± 0.043	0.69

[a] All data were obtained in 100 mM TAPS, pH 8.1, and at 3.0 mM GSSG at 25 °C. [b] Bond orders for the C-4*S*–H bond were calculated with a value of 1.18 for $^{\alpha\text{-}T}K_{eq}$ (Cook et al., 1980) and the BEBOVIB program, assuming that the motions of the primary, H_s, and secondary, H_r, hydrogens are coupled.

and in all cases the deuterated substrate exhibits a higher energy of activation. The primary deuterium kinetic isotope effects are thus temperature dependent and increase slightly with increasing temperature. The ratio of energy of activation and the values of log V/K extrapolated to infinite temperature for the protio- and [4*S*-^{2}H]-labeled reduced pyridine nucleotides yield values for $^{D}E_a$ and ^{D}A; the primary deuterium kinetic isotope effects on the activation energy and Arrhenius preexponential factor. Both of these values are close to unity for these three substrates (Table III).

Discussion

Glutathione reductase represents one of the most thoroughly studied flavoproteins due to its favorable spectroscopic properties and its stability and catalytic reactivity in the crystalline state. The kinetic mechanism is ping-pong (Massey & Williams, 1965), and the overall reaction has been demonstrated to be kinetically described as the sum of two half-reactions (Williams, 1975); the reductive half-reaction (eq 6) and the oxidative half-reaction (eq 7).

$$NAD(P)H + E \rightleftharpoons EH^- + NAD(P)^+ \quad (6)$$

$$EH^- + H^+ + GSSG \rightleftharpoons E + 2GSH \quad (7)$$

When NADPH is used as a reductant with the yeast enzyme, the oxidative half-reaction is 2–3 times slower than the reductive half-reaction (Williams, 1976), and a proton transfer from His 467′ to the first glutathione thiolate anion has been proposed to be rate-limiting in this half-reaction (Wong et al., 1988). However, the reductive half-reaction becomes rate-limiting for the spinach, yeast, and *E. coli* glutathione reductases when NADH is used *in lieu* of NADPH as reductant (Bulger & Brandt, 1971; Vanoni et al., 1990).

The three-dimensional structure of glutathione reductase from human erythrocytes has been determined (Thieme et al., 1981), and a detailed chemical mechanism for the overall reaction catalyzed by glutathione reductase has been proposed on the basis of the structures of various stable catalytic intermediates formed by the addition of substrates and products

Table III: Primary Deuterium Kinetic Isotope Effect Exhibited by Pyridine Nucleotide Substrates[a]

nucleotide substrate	redox potential (mV)	E_a (kcal/mol)	^DE_a	A_H/A_D
NADH		13.08 ± 0.65		
	−320		0.95 ± 0.07	0.96 ± 0.10
[4*S*-2H]NADH		13.85 ± 0.32		
tNADH		14.49 ± 0.48		
	−285		0.91 ± 0.05	1.12 ± 0.11
[4*S*-^{2}H]tNADH		15.94 ± 0.38		
3APADH		15.73 ± 0.40		
	−258		0.94 ± 0.04	1.00 ± 0.03
[4*S*-^{2}H]APADH		16.67 ± 0.40		

[a] V/K values for each nucleotide substrate were determined at temperatures between 10 and 40 °C. The van't Hoff plots were linear in all cases and the slope of each line was used to calculate E_a, the energy of activation, while the intercept of the line was used to calculate A, the Arrhenius preexponential factor.

to the crystals (Pai & Schulz, 1983). A refined structure at 1.54-Å resolution has recently been reported (Karplus & Schulz, 1987), as have the high-resolution structures of binary complexes of the enzyme and numerous nucleotides (Pai et al., 1988). These data permit a detailed analysis of the transition-state structure for hydride transfer and for the chemical mechanism of the reductive half-reaction. Studies of the transition state for hydride transfer catalyzed by formate dehydrogenase (Blanchard & Cleland, 1980; Hermes et al., 1984) and alcohol dehydrogenase (Scharschmidt et al., 1984; Cha & Klinman, 1989) have been reported but have lacked the high-resolution structural information available for human erythrocyte glutathione reductase.

pH Dependence of the Primary Deuterium Kinetic Isotope Effect. The pH dependence of the kinetic parameters exhibited by human erythrocyte glutathione reductase have demonstrated that the maximum velocity of the reaction decreases as a general acid is protonated above its pK of 9.2 (Wong & Blanchard, 1989). This general acid was proposed to be His-467′, whose function is to protonate the glutathione thiolate anion formed during the formation of the mixed disulfide between Cys-58 and oxidized glutathione (Pai & Schulz, 1983). We thus determined the pH dependence of the primary deuterium kinetic isotope effect by using NADH and its 4*S*-deuterated isotopomer as substrates.[2] Equivalent isotope effects were observed on both V/K and V at pH values less than 8.1, and the magnitude of these effects was small and equal to 1.4. At higher pH values, ^DV decreased to 1.0 (Figure 1, open circles), but $^DV/K$ remained greater than 1.0. Similar data were obtained when D_2O was substituted for H_2O as the solvent (Figure 1, filled circles). These data yield a pK value of 8.6 for the group whose deprotonation causes the isotope effect on V to decrease to 1.0, which is similar to the previously reported pK value of 9.2 observed in the V pH profiles (Wong & Blanchard, 1989). We thus suggest that the decrease in the magnitude of the primary deuterium kinetic isotope effect on V, above the pK value of 8.6, is due to the deprotonation of His-467′, with the oxidative half-reaction becoming increasingly rate-limiting at higher pH values. This would result in the primary deuterium kinetic isotope effect on the maximum velocity to decrease to the experimentally observed, limiting value of 1.0.

Solvent and Multiple Kinetic Isotope Effects. The reductive half-reaction catalyzed by spinach, yeast, and *E. coli* glutathione reductases has been proposed to be rate-limiting when NADH is substituted for NADPH as a reductant (Bulger & Brandt, 1971; Vanoni et al., 1990). The human erythrocyte glutathione reductase exhibits the lowest degree of nucleotide specificity for NADPH, with NADH exhibiting a $V_m \approx 11\%$ that of NADPH and a steady-state K_m value only 10 times higher than that of NADPH (44 vs 4 μM). The original proposal for the transfer of the 4*S* hydrogen atom, as a proton, to Lys-66, followed by rapid electron transfer to flavin (Pai & Schulz, 1983), has been recently questioned since direct hydride transfer from reduced 5-deazaflavin-reconstituted erythrocyte glutathione reductase to NAD^+ has been demonstrated (Manstein et al., 1986).

The crystallographically determined structure of the E·NAD(P)H complex of erythrocyte glutathione reductase shows the reduced nicotinamide ring to be positioned between the isoalloxazine ring of FAD and the phenol ring of Tyr-197 (Pai et al., 1988). The C4′ position of NADH is 3.5 Å from the presumptive hydride acceptor position of FAD, N5 of the isoalloxazine ring, and only 2.6 Å from the ε-amino group of Lys-66 and thus is appropriately positioned to act as a proton acceptor. To distinguish between a direct hydride transfer mechanism and a proton/two-electron mechanism, we performed the solvent kinetic isotope effect and multiple isotope effect experiments described below.

As seen in Figure 3, no solvent kinetic isotope effect was observed on V/K_{NADH}, suggesting that no proton transfers are involved in enzyme reduction and that hydride transfer occurs directly between NADH and the isoalloxazine ring. Further, when primary deuterium kinetic isotope effects were determined with NADH and [4*S*-^{2}H]NADH in H_2O and D_2O, no significant change in $^DV/K_{NADH}$ was observed. These data also suggest that, during flavin reduction, hydride ion transfer is not accompanied by a proton transfer to N1 of the isoalloxazine ring to form $FADH_2$ (see below).

However, a solvent kinetic isotope effect on V of 2.4 was observed with NADH as the variable substrate. Replotting the intercepts versus the mole fraction of D_2O yielded a linear proton inventory (Venkattasubban & Schowen, 1981), indicative of a single proton transfer step in the reaction. Calculating the expected proton inventory for a diprotonic transition state yielded a curved proton inventory, which did not fit the data satisfactorily. Similar values for ^{D2O}V have been reported for the reactions catalyzed by glutathione reductases from spinach, yeast, and *E. coli* when NADPH is used as reductant (Wong et al., 1988), which was interpreted as suggesting a slowing of the oxidative half-reaction in D_2O. This interpretation was reinforced by independent rapid reaction studies which have shown that the reductive half-reaction catalyzed by yeast glutathione reductase using NADPH as a reductant is 2.8 times faster than the oxidative half-reaction (Williams, 1976).

[2] When NADPH and its 4*S* isotopomer are used as substrates, small primary deuterium kinetic isotope effects are observed on V (≈1.2) and no statistically significant isotope effects are observed on V/K (= 1.0). These data suggest that the binary E·NADPH complex partitions predominantly through catalysis as has been observed for the spinach, yeast, and *E. coli* glutathione reductases (Vanoni et al., 1990).

When NADH is used as a reductant for the human erythrocyte glutathione reductase, the rate of the overall reaction is reduced by an order of magnitude from its value when NADPH is used as reductant, reflecting the decreased rate of the reductive half-reaction relative to the rate of the oxidative half-reaction. In the case of yeast glutathione reductase, it has been shown that the rate of enzyme reduction is identical with the steady-state turnover rate when NADH is used as reductant (Bulger & Brandt, 1971). We would not expect to observe a solvent kinetic isotope effect on V/K_{NADH} in a case in which no proton transfers were occurring in the reductive half-reaction, or on V, if k_{red} were much slower (10 times) than k_{ox}. For a ping-pong kinetic mechanism, the reciprocal of the maximum velocity is equal to the sum of the reciprocal of the rate constants for the reductive and oxidative half-reactions. Solution of this equality to obtain a solvent kinetic isotope effect of 2.4 on V requires that the reductive half-reaction be at least two times slower than the oxidative half-reaction. These arguments predict that the primary deuterium kinetic isotope effect on V will decrease in 100% D_2O, as the rate of the oxidative half-reaction is specifically slowed and approaches the rate of the reductive half-reaction. The magnitude of $^DV/K_{NADH}$, however, must be unaffected by solvent isotopic composition if no proton transfer steps occur in the reductive half-reaction, since only rate constants involving steps in the reductive half-reaction appear in the expression for V/K. Exactly such behavior has been reported for the multiple isotope effects observed with the related flavoprotein NADH peroxidase (Stoll & Blanchard, 1991).

The primary deuterium kinetic isotope effects measured with NADH are too small to convincingly demonstrate the multiple isotope effect. On the other hand, the primary deuterium kinetic isotope effects exhibited by reduced pyridine nucleotide substrates with less negative potentials are significantly larger. We selected 3APADH and its 4S isotopomer for multiple isotope effect analysis. In H_2O, ^DV and $^DV/K_{3APADH}$ are equivalent and equal to 4.2. However, in D_2O, ^DV decreases to a value of 2.3, while $^DV/K_{3APADH,D_2O}$ maintains its value at 4.2 (Figure 2). Using the model and equations previously described for glutathione reductase[3] (Vanoni et al., 1990), we can explain this result. The commitment factor that controls the expression of ^DV relative to the value of the intrinsic primary deuterium kinetic isotope effect for hydride transfer, $^Dk_{red}$, is the ratio of the two catalytic rate constants, k_{red}/k_{ox}. If we assume that no solvent kinetic isotope effect is manifested in the reductive half-reaction (as evidenced by no change in $^DV/K_{3APADH}$) and that only the rate of the oxidative half-reaction is slowed in D_2O, then one can see that ^DV will decrease in D_2O relative to its value in H_2O, since the commitment factor in H_2O, $k_{red}/k_{ox,H_2O}$, is smaller than the corresponding value in D_2O, $k_{red}/k_{ox,D_2O}$. Since the commitment factor that controls the expression of $^DV/K_{3APADH}$ does not contain rate constants from chemical steps in the oxidative half-reaction, the magnitude of $^DV/K_{3APADH}$ should be insensitive to solvent isotopic composition, as observed. This provides a sensitive control for these multiple isotope effect studies.

Pyridine Nucleotide Analogue Studies. The sensitivity of enzymatic hydride transfer reactions to the redox potential of the hydride ion donor and acceptor is well-established. Examples of this type of analysis include formate dehydrogenase (Hermes et al., 1983), alcohol and aldehyde dehydrogenase (Scharschmidt et al., 1984), and adrenodoxin reductase (Light & Walsh, 1980). In the latter case, the turnover number and isotope effect on V using [4S-^{2}H]NADPH are well correlated with the redox potential of flavin analogues reconstituted into the apoflavoprotein. In the present studies, the kinetic parameters and both deuterium and tritium primary deuterium kinetic isotope effects were determined for six reduced pyridine nucleotide substrates (Table I). NADH, NHDH, and NGDH have identical redox potentials, differing only in the nature of the substituents on the adenosyl ring, and exhibit steady-state K_m values which are similar to that of NADH and only 5- and 8-fold reduced maximum velocities, respectively. When analogues whose nicotinamide 3′-substituents differ are tested as substrates, the steady-state K_m values increase significantly, and the maximum velocities decrease precipitously as the redox potential of the substrate becomes less negative.

These data also suggested that the rate of enzyme reduction was rate-limiting and that it might be possible to correlate these kinetic parameters with the equilibrium constant for enzyme reduction. Reported values of the midpoint potential for flavoprotein reductases range from −269 mV for mercuric reductase (Fox & Walsh, 1982) to −280 mV for lipoamide dehydrogenase (Matthews & Williams, 1976). However, the midpoint potential of yeast glutathione reductase has recently been reported as being much lower than the other reductases (−235 mV; Arscott et al., 1990). Using this value as the midpoint potential for human erythrocyte glutathione reductase, we can calculate the equilibrium constant for enzyme reduction for the six substrates tested using the Nerst equation and find that there is a reasonable correlation ($r^2 = 0.906$) between the value of ln V_m and the value of ln K_{eq}.[4]

Transition-State Analysis. The magnitude of the primary deuterium kinetic isotope effects exhibited by these substrates appears to be exquisitely sensitive to the redox potential of the individual substrate. Thus, NADH, NHDH, and NGDH exhibit small primary deuterium kinetic isotope effects of 1.18–1.35, while substrates with increasingly positive redox potentials exhibit increasingly large kinetic isotope effects. The experimentally determined values of the corresponding primary tritium kinetic isotope effects are statistically indistinguishable from values calculated from the deuterium kinetic isotope

[3] The model of the reaction catalyzed by glutathione reductase is shown below (Vanoni et al., 1990)

$$E_{ox} + NAD(P)H \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot NAD(P)H \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red}\cdot NAD(P)^+ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_{red} + NAD(P)^+$$

$$E_{red} + GSSG \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_{red}\cdot GSSG \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_{ox}\cdot 2GSH \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_{ox} + 2GSH$$

where k_3 is the catalytic rate for the reductive half-reaction, k_{red}, and is sensitive to isotopic substitution at the C-4S position of the reduced pyridine nucleotide substrate, and k_9 is the catalytic rate for the oxidative half-reaction, k_{ox}, and is sensitive to solvent isotopic composition. The equations describing the isotope effects on $^DV/K_{NADH}$ and ^DV have been derived and shown to be

$$^DV/K_{NAD(P)H} = \frac{^Dk_3 + c_f + c_r{}^DK_{eq}}{1.0 + c_f + c_r}$$

$$^DV_{NAD(P)H} = \frac{^Dk_3 + c_{vf} + c_r{}^DK_{eq}}{1.0 + c_{vf} + c_r}$$

where

$$c_f = k_3/k_2$$

$$c_r = k_4/k_5$$

$$c_{vf} = k_3/k_5 + k_3/k_{11} + k_3/k_9\,(1.0 + k_{10}/k_{11})$$

The rate constant ratio which dominates c_{vf} is k_3/k_9 [see Stoll and Blanchard (1990)].

[4] This correlation is insensitive to the exact value of the midpoint potential for E/EH^- used in the calculation.

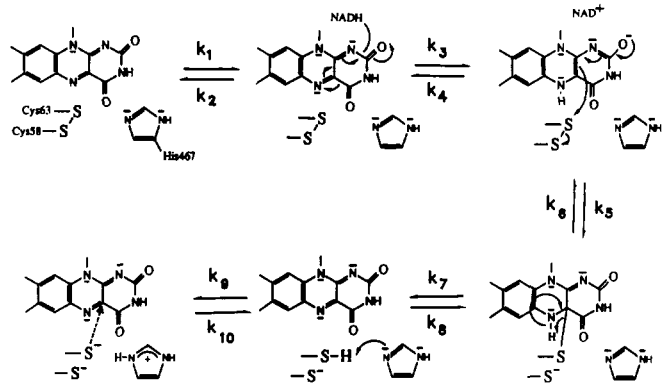
effects by using the Swain–Schaad relationship ($^{T}k = {}^{D}k^{1.442}$; Swain et al., 1954). The agreement between the calculated and experimental values of the primary tritium kinetic isotope effects argues that the intrinsic kinetic isotope effect on hydride transfer is largely being observed in these steady-state experiments. While small values of the commitment factor for NADH may not be rigorously excluded by our data, previous studies comparing such steady-state values of the primary kinetic isotope effects on NADH oxidation with values determined for enzyme reduction in rapid reaction studies (Vanoni et al., 1990) lend support to this conclusion.

The correlation between the thermodynamic properties and the transition-state structure of a reaction can be derived from the analysis of the predictions from Hammond postulate (Hammond, 1955): for an exergonic reaction, the transition state will resemble the substrates, and as the reaction becomes more isoenergetic, the transition state will become increasingly symmetric. In these studies, the strong reductants (−320 mV) exhibit small primary deuterium kinetic isotope effects, indicative of an early asymmetric transition state (Westheimer, 1961). The values of the primary deuterium kinetic isotope effect increase as the redox potential of the substrates becomes more positive, indicating the increasingly symmetric disposition of the hydride ion between its pyridine nucleotide donor and flavin acceptor in the transition state. Even in the case of the least potent reductant tested, 3APADH, the transition state has not become later than symmetric, suggesting that the redox potential of human erythrocyte glutathione reductase may be similar to that of the yeast enzyme, i.e., more positive than −258 mV.

A second sensitive indicator of the transition-state structure for enzyme-catalyzed hydride transfer involving pyridine nucleotides is the analysis of α-secondary kinetic isotope effects. In the oxidation of NADH by glutathione reductase, the 4*S* hydrogen is transferred as a hydride ion to the isoalloxazine ring, while the carbon to which the 4*R* hydrogen is bound experiences a change in hybridization, going from sp^3 hybridized in NADH to sp^2 hybridized in NAD. The magnitude of the α-secondary deuterium kinetic isotope effect can be estimated from the value of the α-secondary deuterium equilibrium isotope effect on NADH oxidation of 1.12 (Cook et al., 1980). The α-secondary kinetic isotope effect has as its limiting values: 1.0 for a very early transition state in which no change in hybridization has occurred and 1.12 for a very late transition state in which C4 is completely sp^2 hybridized. As seen in Table II, the α-secondary tritium kinetic isotope effect determined with NADH is 1.01, indicative of an early transition state for NADH oxidation. With tNADH and 3APADH, the α-secondary tritium kinetic isotope effects become larger, indicative of successively later transition states with more rehybridization of C4′ of the substituted nicotinamide ring.

The analysis of the primary and secondary kinetic isotope effects exhibited by reduced pyridine nucleotide substrates discussed above relies on the assumption that there are no redox-dependent changes in the relative distance or geometry of the redox partners. Human erythrocyte glutathione reductase represents an unique experimental system in this regard, since the positions of the enzyme-bound reduced pyridine nucleotide substrates used in these studies are known to high resolution (Pai et al., 1988). A conformational change is elicited by the binding of pyridine nucleotides, and Tyr-197, which forms a hydrogen bond between the phenol hydrogen and N10 of the isoalloxazine ring of enzyme-bound FAD in the oxidized enzyme, swings away from the ring and assumes

Scheme I: Proposed Chemical Mechanism for the Reductive Half-Reaction of Human Erythrocyte Glutathione Reductase



a position that is coplanar with the isoalloxazine ring and 8 Å away. The nicotinamide ring inserts between these two aromatic ring systems in the precatalytic binary complex. In the structures of 10 binary enzyme–pyridine nucleotide complexes, the nicotinamide rings of the different nucleotides are superimposable with root mean square deviations of less than ~0.3 Å. Although the X-ray crystallographically determined positions represent average ground-state structures, it appears unlikely that the differences in primary and secondary kinetic isotope effects that we observe for the various pyridine nucleotide substrates are the result of large changes in the geometry of, or distance between, the redox partners during catalysis.

Temperature Dependence of the Kinetic Isotope Effect. The recent report of hydride ion tunneling in the reaction catalyzed by liver alcohol dehydrogenase (Cha et al., 1989), and previous suggestions of the contribution of tunneling to the behavior of kinetic isotope effects (Hermes et al., 1984), led us to examine the temperature dependence of the primary deuterium kinetic isotope effects using NADH, tNADH, and 3APADH as representative substrates. In all cases, there was a linear dependence of V/K on the reciprocal absolute temperature over the range 10–40 °C (data not shown). These plots were used to calculate the energies of activation for the three substrates and their 4*S* isotopomers. In all cases, the deuterated isotopomers yielded slightly higher values of E_a. These nonphosphorylated substrates exhibit significantly higher energies of activation than NADPH (E_a = 9.0 ± 0.4 kcal/mol) or GSSG (E_a = 10.9 ± 2.0 kcal/mol), and the values of the E_a of these nonphosphorylated substrates are well correlated with the redox potential of substrates examined. The primary deuterium kinetic isotope effects for all three substrates increase with increasing temperature, resulting in a modest inverse deuterium isotope effect on the energies of activation. However, these values are all close to unity and cannot support a conclusion that tunneling contributes significantly to the kinetic isotope effect. Extrapolation of the linear van't Hoff plots to infinite temperature allow a rough approximation to be made of the Arrhenius preexponential factor, A, and the ratio of this factor for the hydrogen- and deuterium-containing substrates. The values that we obtain for A_H/A_D using these three substrates are clearly within the classical limits ($0.7 < A_H/A_D < 1.3$; Kwart, 1982) and again argue against hydride ion tunneling. Similar data for the temperature dependence of V support this conclusion.

Chemical Mechanism of the Reductive Half-Reaction. The chemical mechanism for the reduction of human erythrocyte glutathione reductase is proposed in Scheme I. In the first step, NADH binds to the enzyme and the nicotinamide ring

stacks between the isoalloxazine and Tyr-197 rings. In the second step, k_3, a hydride ion is directly transferred from the 4*S* position of the nicotinamide to the N5 position of the isoalloxazine ring. We suggest that the negatively charged hydride ion being transferred is electrostatically stabilized by the close proximity of the positively charged ϵ-amino group of Lys-66, which is maintained in its protonated form by ion pairing with the carboxylate anion of Glu-201. We draw the reduced flavin as the C2 enolate anion for several reasons. The absence of a solvent kinetic isotope effect on $^{D}V/K_{NADH}$ suggests that no protons are transferred to FAD concomitant with hydride transfer. Secondly, a long α-helix is appropriately located to stabilize the developing negative charge at N1/C2 by a charge–dipole interaction (Pai & Schulz, 1983). Thirdly, model studies of hydride transfers between pyridine nucleotide and flavin analogues suggest the formation of a postcatalytic $NAD^+/FADH^-$ ionic complex (Blankenhorn, 1976). Lastly, compelling mutagenesis studies of the highly homologous mercuric reductase supports the intermediate formation of $FADH^-$ (Miller et al., 1990). We suggest that this hydride transfer step is rate-limiting in the overall reaction when 2′-dephospho reduced pyridine nucleotides are used as substrates for erythrocyte glutathione reductase, as has been described for the yeast enzyme (Bulger & Brandt, 1971). No compelling evidence has been presented in support of the observation of reduced flavin species ($FADH^-$ or $FADH_2$) during turnover with any native flavoprotein reductase (Huber & Brandt, 1980), and we therefore assume all subsequent steps to be fast.

The transfer of reducing equivalents from $FADH^-$ to the redox-active disulfide of Cys-58–Cys-63 has been proposed to occur as shown in k_5, by the formation of a flavin C4a–Cys-63 covalent bond (Arscott et al., 1981), with rupture of the Cys-58–Cys-63 disulfide bond and the release of the Cys-58 thiolate anion. Direct spectroscopic evidence from the trapping of this intermediate at low pH values again comes from the mutagenesis studies of mercuric reductase (Miller et al., 1990). This intermediate breaks down as shown in k_7, and a rapid proton transfer from Cys-63 ($pK \approx 4$ in yeast glutathione reductase; Sahlman & Williams, 1989) to His-467′ ($pK \approx 8.5$–9.2 in yeast and human erythrocyte glutathione reductase; Sahlman & Williams, 1989; Wong & Blanchard, 1989) results in the stable reduced enzyme. The sulfur atom of Cys-63 is 3.1 Å from the C4a position of the isoalloxazine ring in this complex (Pai & Schulz, 1983), and a charge transfer interaction, denoted by the dashed line, is responsible for the absorbance shoulder at 525–530 nm, characteristic of the two-electron-reduced forms of flavoprotein reductases. An increasing body of information suggests that this chemical mechanism for enzyme reduction may apply to all flavoprotein reductases.

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