Glutathione Reductase: Comparison of Steady-State and Rapid Reaction Primary Kinetic Isotope Effects Exhibited by the Yeast, Spinach, and *Escherichia coli* Enzymes†

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Received January 10, 1990; Revised Manuscript Received March 20, 1990

ABSTRACT: Kinetic parameters for NADPH and NADH have been determined at pH 8.1 for spinach, yeast, and *E. coli* glutathione reductases. NADPH exhibited low K_m values for all enzymes (3–6 μM), while the K_m values for NADH were 100 times higher (~400 μM). Under our experimental conditions, the percentage of maximal velocities with NADH versus those measured with NADPH were 18.4, 3.7, and 0.13% for the spinach, yeast, and *E. coli* enzymes, respectively. Primary deuterium kinetic isotope effects were independent of GSSG concentration between K_m and $15K_m$ levels, supporting a ping-pong kinetic mechanism. For each of the three enzymes, NADPH yielded primary deuterium kinetic isotope effects on V_{max} only, while NADH exhibited primary deuterium kinetic isotope effects on both V and V/K. The magnitude of $^{D}V/K_{NADH}$ at pH 8.1 is 4.3 for the spinach enzyme, 2.7 for the yeast enzyme, and 1.6 for the *E. coli* glutathione reductase. The experimentally determined values of $^{T}V/K_{NADH}$ of 7.4, 4.2, and 2.2 for the spinach, yeast, and *E. coli* glutathione reductases agree well with those calculated from the corresponding $^{D}V/K_{NADH}$ using the Swain–Schaad expression. This suggests that the intrinsic primary kinetic isotope effect on NADH oxidation is fully expressed. In order to confirm this conclusion, single-turnover experiments have been performed. The measured primary deuterium kinetic isotope effects on the enzyme reduction half-reaction using NADH match those measured in the steady state for each of the three glutathione reductases. These data support the conclusions that NADH dissociates from the binary E–NADH complex more rapidly than it transfers a hydride ion to flavin and that with NADH, intrinsic primary deuterium kinetic isotope effects are observed for all three enzymes. The substantial differences in the magnitudes of the intrinsic deuterium kinetic isotope effects on hydride transfer may reflect differences in the transition-state structures for the hydride-transfer step catalyzed by the three enzymes.

Glutathione reductase (EC 1.6.4.2) catalyzes the reversible, reduced pyridine nucleotide dependent reduction of oxidized glutathione, to form 2 mol of reduced glutathione:

$$NAD(P)H + H^+ + GSSG \rightleftharpoons NAD(P)^+ + 2GSH \quad (1)$$

The enzyme is widely distributed, and its in vivo function is to maintain a high GSH/GSSG ratio (K_{eq} = 1100, pH 7; Mapson & Isherwood, 1963). Homogeneous preparations from spinach (Hailiwell & Foyer, 1978), yeast (Racker, 1955), and *E. coli* (Williams et al., 1967) have been obtained. All glutathione reductases share extensive structural and mechanistic similarities, which extend to the other flavoprotein reductases: lipoamide dehydrogenase (EC 1.6.4.3; Williams, 1976), mercuric reductase (EC 1.16.1.1; Fox & Walsh, 1982), and trypanathione reductase (EC 1.6.4.*X*; Shames et al., 1986). The structure of human erythrocyte glutathione reductase has been determined and refined to 1.54-Å resolution (Thieme et al., 1981; Karplus & Schulz, 1987) and a detailed chemical mechanism for the reduction of oxidized glutathione has been proposed on the basis of the structures of enzyme–substrate complexes (Pai & Schulz, 1983; Karplus et al., 1989). The amino acid sequence of the *E. coli* glutathione reductase has been determined from the sequence of its structural gene (Greer & Perham, 1986) and shown to be highly homologous to the amino acid sequence of the human erythrocyte glutathione reductase. The kinetic mechanism of the yeast enzyme appears to be ping-pong on the basis of steady-state (Massey & Williams, 1965) and rapid reaction (Williams, 1976) kinetic studies, although alternative ternary complex mechanisms have been proposed to occur at high GSSG concentrations (Mannervik et al., 1981). The overall catalytic sequence can be represented as the sum of a reductive half-reaction

$$E + NAD(P)H + H^+ \rightleftharpoons EH_2 + NAD(P)^+$$

and an oxidative half-reaction

$$EH_2 + GSSG \rightleftharpoons E + 2GSH$$

Solvent kinetic isotope effect studies have revealed that the rate-limiting step in the oxidative half-reaction is the transfer of a single proton from a histidine residue (His-467′ in the human erythrocyte enzyme) to the first reduced glutathione thiolate product (Wong et al., 1988). The protonation state of this imidazole side chain is critical in catalysis, since deprotonation of this residue above its pK_a of 9.2 abolishes catalytic activity in the human erythrocyte and yeast glutathione reductase (Wong & Blanchard, 1989).

We report here the determination of primary deuterium kinetic isotope effects exhibited by the spinach, yeast, and *E. coli* glutathione reductases on the oxidation of NADPH and

† This work was supported by Grant GM33449 awarded by the N.I.H. and the Sinsheimer Scholar Foundation (to J.S.B.), by Grant GM20877 awarded by the N.I.H. (to D.P.B.), and by the Albert Cass Traveling Fellowship (to K.K.W.).

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0006-2960/90/0429-5790$02.50/0

NADH in steady-state experiments and on NADH oxidation in stopped-flow experiments of the reductive half-reaction. The comparison of steady-state primary deuterium and tritium kinetic isotope effects exhibited by NADH suggests that the intrinsic kinetic isotope effect is observed in $^{D}V/K_{NADH}$ with all three enzymes. This observation is strongly supported by an independent determination of the primary deuterium kinetic isotope effects on enzyme reduction by NADH for all three glutathione reductases using stopped-flow spectrophotometry.

Experimental Procedures

Materials. Spinach (type VI) and yeast (type IV) glutathione reductases were purchased from Sigma and were judged greater than 95% pure on the basis of high-performance gel-permeation chromatography on Superose 12B (Pharmacia) and from SDS–PAGE[1] using Coomassie Blue staining. Glutathione reductase from *E. coli* K-12 was purified from 500 g of cells (Grain Processing Corporation) by osmotic lysis, ammonium sulfate precipitation, Fast Q (Pharmacia) anion-exchange chromatography, Blue dye affinity chromatography (Amicon), 2′,5′-ADP agarose affinity chromatography, and finally high-performance anion-exchange chromatography on Mono Q (Pharmacia). A typical yield from 500 g of cells was 10 mg of homogeneous enzyme as judged by SDS–PAGE stained with Coomassie Blue. Glutathione reductase preparations were dialyzed against 10 mM TEA·HCl buffer, pH 7.8, containing 0.3 mM EDTA. Protein concentrations were determined after dialysis by using the Bio-Rad protein assay and bovine plasma γ-globulin as standard.

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), yeast hexokinase, and glutamate dehydrogenase were from Sigma. [1-^{2}H]-D-Glucose (>98 atm % excess ^{2}H) was purchased from Omicrom Biochemicals (Ithaca, NY). [1-^{3}H]-D-Glucose was purchased from Amersham.

Preparation of Reduced Pyridine Nucleotides. The procedures described below were routinely used for the preparation of the 4*S* 4-^{2}H reduced pyridine nucleotides. One-milliliter solutions of the oxidized pyridine nucleotides (5–25 mM) in 10 mM TEA·HCl, pH 7.8, were purified on a FPLC Mono Q anion-exchange column equilibrated in 10 mM TEA·HCl buffer, pH 7.8, and eluted isocratically with the same buffer as described previously (Orr & Blanchard, 1984). This prepurification was essential for obtaining sufficiently pure reduced pyridine nucleotides.

The oxidized nucleotides were reduced in a total volume of 5 mL in the presence of either 1-protio-, 1-deuterio-, or 1-tritioglucose 6-phosphate (20–40% excess over pyridine nucleotide) by the action of *L. mesenteroides* glucose-6-phosphate dehydrogenase in 10 mM TEA·HCl buffer, pH 7.8. The reaction was monitored spectrophotometrically and the pH was maintained at 7.7–8.0 by the addition of 1 N KOH. When the reaction was completed, the solutions were filtered through an Amicon YM10 ultrafiltration membrane and diluted to 10 mL with water, and the reduced pyridine nucleotides were purified as described previously (Orr & Blanchard, 1984). Peak fractions were diluted 1:50 into 10 mM TEA buffer, pH 7.8, and their spectra were recorded on a Cary 219 recording spectrophotometer. Fractions that had $A_{260}/A_{340} \leq 2.3$ were pooled. The concentrations of these solutions were determined enzymatically by incubation with glutamate dehydrogenase, in the presence of excess α-ketoglutarate and ammonia, and the total change in absorbance was monitored at 340 nm. Solutions of reduced pyridine nucleotides were used within 24 h. [1-^{3}H]-D-Glucose 6-phosphate was prepared from [1-^{3}H]-D-glucose by phosphorylation with MgATP and yeast hexokinase. [1-^{3}H]-D-Glucose 6-phosphate was purified by adsorption to Dowex 1-X8 (Cl^{-} form, 200–400 mesh) and elution with 10 mM HCl (Bartlett, 1959). Peak radioactive fractions were pooled, concentrated by rotary evaporation, and neutralized to pH 6.5 with KOH. Tritiated reduced pyridine nucleotides were used immediately for tritium isotope effect studies.

Initial Velocity and Deuterium Isotope Effect Studies. Initial velocities were measured by monitoring the decrease in absorbance at 340 nm of the reduced pyridine nucleotide (ϵ_{340} = 6220 M^{-1} cm^{-1}) in a Gilford 260 spectrophotometer. Temperature was maintained at 25 ± 0.1 °C with a circulating water bath and thermospacers.

Determination of Primary Tritium Isotope Effects. For the routine determination of primary tritium isotope effects, 3-mL reaction mixtures containing 80–150 μM (4*S*)-[4-^{3}H]NADH and 3 mM glutathione in 10 mM TEA·HCl buffer, pH 7.8, were equilibrated at 25 °C. One hundred microliter aliquots were removed before the addition of enzyme and at various times after the addition of enzyme. These aliquots were directly injected onto a Mono Q column, and $^{3}H_2O$ was separated from NAD and residual (4*S*)-[4-^{3}H]-NADH. The reaction was continuously monitored spectrophotometrically. After 20–30% reaction, additional enzyme was added to ensure complete oxidation of the reduced pyridine nucleotide, the cuvette was tightly stoppered, and the reaction was allowed to proceed to completion. Three to five replicate analyses of the 100% reaction mixture were performed. The Mono Q column eluates were monitored at 260 nm and 1-mL fractions were collected directly into scintillation vials. Hydrofluor scintillation fluid (4.5 mL) was added, and the fractions were analyzed on an LKB Rack Beta liquid scintillation counter. Fractions containing $^{3}H_2O$ were counted two or three times and were corrected for nonenzymatic release of ^{3}H into the solvent. The primary tritium kinetic isotope effect was calculated from

$$^{T}(V/K) = \log (1.0 - F_1)/\log (1.0 - F_2) \quad (2)$$

where

$$F_1 = (A_{340(0)} - A_{340(t)})/(A_{340(0)} - A_{340(\infty)}) \quad (3)$$

and

$$F_2 = (DPM_t - DPM_0)/(DPM_\infty - DPM_0) \quad (4)$$

DPM_t is the disintegrations per minute (dpm) of $^{3}H_2O$ at time t, DPM_∞ is the average of the dpm of $^{3}H_2O$ at 100% conversion, and DPM_0 is the dpm of $^{3}H_2O$ released nonenzymatically. This equation does not require that the dpm of $^{3}H_2O$ be corrected for the percentage of the complete reaction and dilution of the released ^{3}H into unlabeled water (Blanchard & Englard, 1983).

Solvent Isotope Exchange. To ensure that the release of tritium from (4*S*)-[4-^{3}H]NADH was tightly coupled to enzyme reduction, we determined whether solvent tritium could exchange into NADH during net oxidation of NADH. Re-

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; TEA·HCl, triethanolamine hydrochloride; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; GSSG, oxidized glutathione; GSH, reduced glutathione; EDTA, ethylenediaminetetraacetic acid; NADH, reduced β-nicotinamide adenine dinucleotide; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; EH_2, two electron reduced enzyme; EH_4, four electron reduced enzyme; k_{red}, rates of enzyme reduction measured at 525 nm; ^{D}V, primary deuterium kinetic isotope effect on the maximum velocity; $^{D}V/K$, primary deuterium kinetic isotope effect on V/K.

Table I: Kinetic Parameters Measured for NADPH and NADH with Yeast, Spinach, and *E. coli* Glutathione Reductases[a]

	NADPH			NADH			
enzyme	V_m[b]	V/K	K_m (μM)	V_m[b]	rel V[c] (%)	V/K	K_m (μM)
spinach	100.8	27.2	3.7	18.6	18.40	0.0500	386
yeast	74.8	12.7	5.9	2.8	3.74	0.0070	408
E. coli	30.9	6.4	4.8	0.04	0.13	0.0001	384

[a] All data were obtained in 100 mM TAPS buffer, pH 8.1, and at 3.75 mM GSSG at 25 °C. [b] Maximum velocities are expressed as μmol min^{-1} mg^{-1}. The standard errors of the calculated parameters were less than 10% in all cases. [c] Relative $V = (V_{NADH}/V_{NADPH}) \times 100$.

action mixtures (0.5 mL) were prepared that contained NADH (150–200 μM) and GSSG (3 mM) in 10 mM TEA, pH 7.8, containing 7×10^{10} dpm of ^3H_2O/mL. Fifty-microliter aliquots were removed before the addition of yeast enzyme and at regularly spaced intervals after the addition of enzyme, corresponding to 5–60% reaction. These aliquots were placed in Rittenberg flasks, immediately frozen, and lyophilized to remove the bulk of the ^3H_2O. The residue was taken up in 550 μL of water and injected onto a Mono Q column, and the residual tritiated water was separated from NADH, as described above. The column eluate was monitored at 260 nm and collected directly into scintillation vials. The NADH peak was counted, and the percent reaction was determined from the ratio of absorbance at 260 nm of NAD and NADH in the column eluate.

Rapid Reaction Deuterium Kinetic Isotope Effect Studies. Rapid reaction experiments were performed in a stopped-flow apparatus designed for anaerobic work and coupled to a Nova minicomputer system (Beaty & Ballou, 1981). Glutathione reductase at a concentration of 1.25–3.00 mg/mL (corresponding to 25–60 μM active sites based on a subunit molecular weight of 50000 Da) in 100 mM TAPS, pH 8.1, was made anaerobic in a tonometer by repeated evacuation and flushing with oxygen-free nitrogen. NADH and (4*S*)-[4-^{2}H]NADH solutions (0.5–4 mM) in 100 mM TAPS, pH 8.1, were made anaerobic by bubbling with oxygen-free nitrogen for 5–10 min. Equal volumes of the enzyme and reduced pyridine nucleotides were rapidly mixed at 25 °C, and the reduction reaction was recorded by monitoring the formation of the charge-transfer complex of two electron reduced enzyme (EH_2) at 525 nm. Reactions were performed in duplicate at each substrate concentration.

Data Analysis. Reciprocal initial velocities from steady-state experiments were plotted against the reciprocal of the reduced pyridine nucleotide concentration; the data were fitted to the appropriate rate equation by the least-squares method, assuming equal variances for the velocity values. The Fortran programs of Cleland (1979) were modified to run on a Vax 11/780 operating system. Individual saturation curves were fitted to eq 5 to determine the kinetic parameters (V and V/K)

$$v = VA/(K + A) \quad (5)$$

$$v = VA/[K + A(1.0 + F_iE_V)] \quad (6)$$

$$v = VA/[K(1.0 + F_iE_{V/K}) + A] \quad (7)$$

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_V)] \quad (8)$$

for substrates for the three enzymes. Isotope effects were calculated by comparing the initial velocities measured with 4-^{1}H and 4*S* 4-^{2}H reduced pyridine nucleotides using eqs 6–8, which applies to situations where isotope effects on V only, on V/K only, or on both V and V/K, respectively, are exhibited. In eqs 6–8, A is the reduced pyridine nucleotide concentration and F_i is the fraction of deuterium label in the reduced pyridine nucleotide substrate (F_i = 0.0 and 1.0 for hydrogen- and deuterium-containing substrate, respectively), while $E_V = {}^DV - 1.0$ and $E_{V/K} = {}^DV/K - 1.0$. The most appropriate equation for each case was chosen on the basis of the lowest value of the standard errors of the fitted parameters.

Table II: Effect of Oxidized Glutathione Concentration on the Primary Isotope Effects Exhibited by the Spinach, Yeast, and *E. coli* Glutathione Reductases

nucleotide substrate	[GSSG] (mM)	fit to eq 6 ^DV	fit to eq 7 $^DV/K$
	Spinach Glutathione Reductase		
NADPH	3.75	1.44 ± 0.03	
	0.10	1.44 ± 0.03	
NADH	3.75		4.34 ± 0.14
	0.10		4.86 ± 0.32
	Yeast Glutathione Reductase		
NADPH	1.00	1.50 ± 0.01	
	0.10	1.28 ± 0.03	
	0.05	1.32 ± 0.05	
NADH	3.75		2.52 ± 0.09
	0.10		2.44 ± 0.06
	E. coli Glutathione Reductase		
NADPH	3.00	1.28 ± 0.3	
	0.10	1.11 ± 0.5	
NADH	3.00		1.62 ± .25
	0.10		1.63 ± .02

Stopped-flow spectrophotometric traces were analyzed as a sum of exponential processes (Mastrigt, 1977). Data for the formation of EH_2 or EH_4 were first fitted to a single exponential and then to multiple exponentials. The appropriateness of the fits was determined by visual inspection of the experimental and calculated traces and plots of the residuals. The reciprocal of the reaction rate constants was plotted versus the reciprocal of the NADH concentration. Primary deuterium kinetic isotope effects on enzyme reduction were determined by fitting the data to eq 8, where $E_V = E_{V/K}$.

Results

Reduced Pyridine Nucleotide Specificity of Glutathione Reductases. The steady-state kinetic parameters for NADPH and NADH observed with the yeast, spinach, and *E. coli* glutathione reductases are shown in Table I. All of these studies were performed in 100 mM TAPS, pH 8.1; the kinetic parameters measured in 10 mM TEA HCl, pH 7.8, were unchanged.

Dependence of Primary Deuterium Kinetic Isotope Effects on Glutathione Concentration. When NADPH and (4*S*)-[4-^{2}H]NADPH were used as variable substrates with the spinach enzyme, only small primary deuterium kinetic isotope effects on V were observed. In contrast, when NADH and (4*S*)-[4-^{2}H]NADH were used as the variable substrates, pronounced changes in both the slopes and intercepts of the reciprocal plots were observed. Qualitatively similar results were obtained with the spinach and *E. coli* enzymes. The dependence of the primary deuterium kinetic isotope effect on NAD(P)H oxidation on the concentration of oxidized glutathione was evaluated by using the yeast, spinach, and *E. coli* enzymes. As shown in Table II, the experimental kinetic isotope effects are essentially invariant when the concentration

Table III: Primary Deuterium Kinetic Isotope Effects[a]

enzyme	NADPH fit to eq 6 ^DV	NADPH fit to eq 8 ^DV	NADPH fit to eq 8 $^DV/K$
spinach	1.44 ± 0.03	1.44 ± 0.04	0.97 ± 0.18
yeast	1.50 ± 0.02	1.50 ± 0.02	0.98 ± 0.11
E. coli	1.27 ± 0.03	1.33 ± 0.07	0.90 ± 0.11

enzyme	NADH fit to eq 7 $^DV/K$	NADH fit to eq 8 ^DV	NADH fit to eq 8 $^DV/K$
spinach	4.34 ± 0.14[b]	1.67 ± 0.63[b]	3.99 ± 0.34[b]
	4.04 ± 0.25[c]	1.44 ± 0.78[c]	3.66 ± 0.69[c]
yeast	2.52 ± 0.09[b]	1.21 ± 0.65[b]	2.45 ± 0.23[b]
	2.73 ± 0.06[c]	1.00 ± 0.33[c]	2.73 ± 0.15[c]
E. coli	1.62 ± 0.10[b]	1.51 ± 0.22[b]	1.32 ± 0.68[b]
	1.73 ± 0.04[c]	1.75 ± 0.14[c]	0.80 ± 1.50[c]

[a] Determined at a saturating concentration of GSSG (3 mM). [b] 100 mM TAPS, pH 8.1. [c] 10 mM TEA·HCl, pH 7.8.

of GSSG is varied between 0.05 and 3.75 mM ($K_{m,GSSG}$ = 45–65 μM for all three enzymes, this work).

Solvent Isotope Exchange. At all percentages of reaction, negligible (<100 dpm above background; <0.1% of the specific radioactivity of the ^3H_2O used in the experiment) solvent-derived tritium could be measured in the residual, reisolated NADH (data not shown). These data suggest that, under our experimental conditions, NADH oxidation is essentially irreversible and that the tritium release method used below to determine $^TV/K$ is valid.

Primary Deuterium Kinetic Isotope Effects for Spinach, Yeast, and E. coli Glutathione Reductase. At saturating levels of GSSG, primary deuterium kinetic isotope effects were obtained for NADPH and NADH by using the spinach, yeast, and *E. coli* enzymes. Initial velocities of NAD(P)H and (4*S*)-[4-^{2}H]NAD(P)H oxidation were fitted to eqs 6 and 8 for NADPH and to eqs 7 and 8 for NADH. For all enzymes using NADPH as substrate, only ^DV was significantly greater than 1.0 by comparison of the fits of the data to eqs 6 and 8 (Table III). For all three enzymes, $^DV/K_{NADH}$ was significantly greater than 1.0 by comparison of the fit of the data to eqs 7 and 8. Primary deuterium kinetic isotope effects with NADH were obtained under two different conditions: 100 mM TAPS, pH 8.1, and 10 mM TEA·HCl, pH 7.8. Since no large differences in the results under these conditions are observed, we could compare deuterium and tritium V/K kinetic isotope effects for NADH oxidation under identical reaction conditions (i.e., 10 mM TEA·HCl, pH 7.8).

Primary Tritium Kinetic Isotope Effects for Spinach, Yeast, and E. coli Glutathione Reductases. The primary tritium kinetic isotope effects exhibited by spinach, yeast, and *E. coli* glutathione reductases using (4*S*)-[4-^{3}H]NADH as reductant were determined by measuring the tritium transferred to the flavin, and subsequently exchanged into solvent, at various percentages of reaction. The chromatographic method developed for the separation of ^3H_2O and (4*S*)-[4-^{3}H]NADH required that the reaction be run under low ionic strength conditions (10 mM TEA·HCl, pH 7.8). (4*S*)-[4-^{3}H]NADH (120 μM) was reacted with each of the three glutathione reductases in the presence of 3 mM GSSG, and the ^3H_2O was separated from unreacted substrate by ion-exchange chromatography on a Mono Q column. Multiple determinations of $^TV/K_{NADH}$ for each enzyme were made by comparing the amount of ^3H_2O released at low (<30%) and at high (100%) percentages of reaction.

Figure 1 shows the results of experiments where primary tritium kinetic isotope effects were measured by using

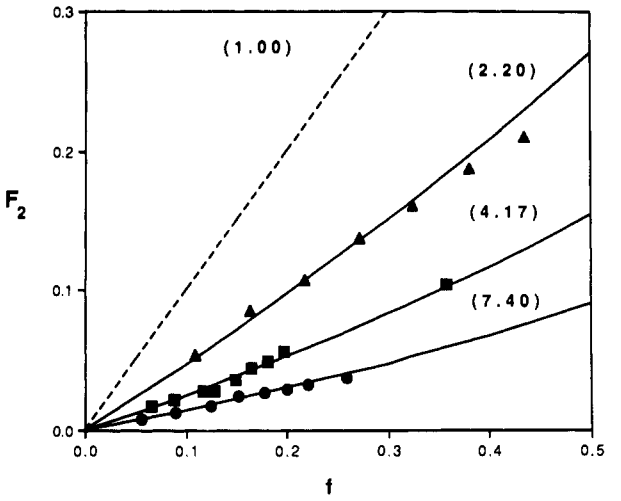

FIGURE 1: Primary tritium kinetic isotope effects exhibited by spinach, yeast, and *E. coli* glutathione reductases using (4*S*)-[4-^{3}H]NADH at 3 mM GSSG in 10 mM TEA·HCl, pH 7.8. The lines were drawn by using eq 2 and values of the tritium isotope effect are shown in parentheses.

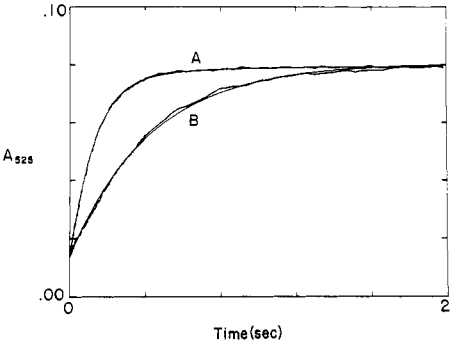

FIGURE 2: Rapid reaction primary deuterium kinetic isotope effect on the reduction of spinach glutathione reductase using 0.25 mM NADH (A) or (4*S*)-[4-^{2}H]NADH (B). Enzyme reduction was monitored by the increase in absorbance at 525 nm as a function of time. The rough curves are the experimental traces, while the smooth curves represent the fits of experimental data with k_H = 6.91 s^{-1} and k_D = 2.34 s^{-1}.

(4*S*)-[4-^{3}H]NADH. $^TV/K_{NADH}$ values were 7.26 ± 0.34, 4.17 ± 0.28, and 2.20 ± 0.14 for the spinach, yeast, and *E. coli* enzymes, respectively.

Deuterium Kinetic Isotope Effects on Enzyme Reduction. Representative stopped-flow, 525-nm absorbance traces for the reduction of spinach glutathione reductase by either 0.25 mM NADH (curve A) or 0.25 mM (4*S*)-[4-^{2}H]NADH (curve B) are shown in Figure 2. The two electron reduced form of spinach glutathione reductase undergoes additional reduction to form EH_4, where absorbance at both 525 and 450 nm is lost. This requires that the traces be fitted to at least two exponentials, with the first, fast phase (26.0 s^{-1} extrapolated to an infinite NADH concentration) representing enzyme reduction to form EH_2 and with a second, slower phase (~0.05 s^{-1} extrapolated to an infinite NADH concentration) representing the decrease in A_{525} accompanying EH_4. The formation of EH_4 is also subject to a primary deuterium kinetic isotope effect (~2–4), but this very slow reaction is not kinetically significant in normal catalysis and was not rigorously analyzed. When the fast, rising phase (formation of EH_2) is fitted to two exponential events, greater than 90% of the amplitude derives from the faster phase, which corresponds closely to the rates obtained from fits to a single exponential. Only marginal improvements in the fits were obtained by using two exponentials and therefore we have used the data from single

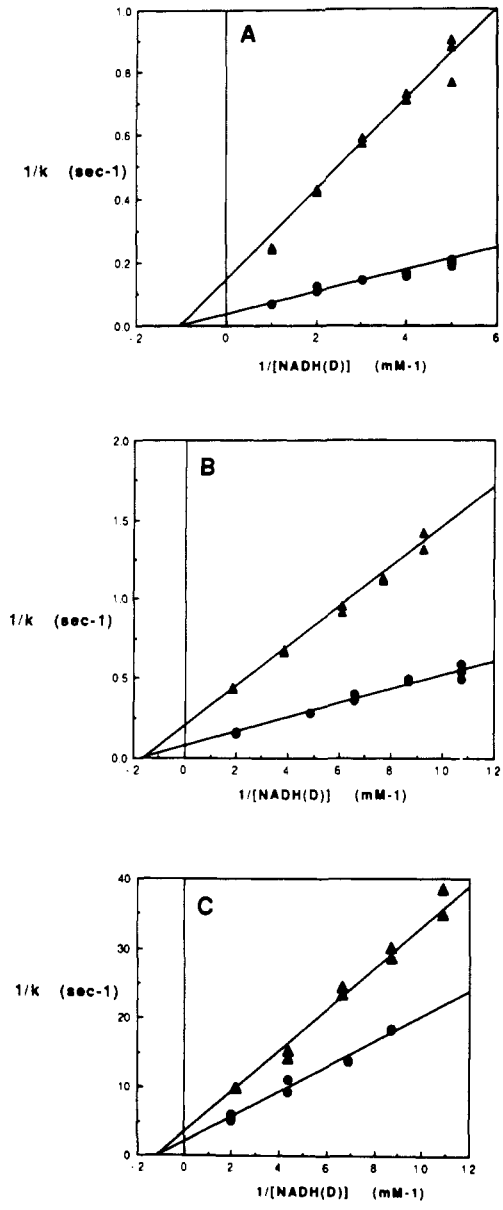

FIGURE 3: Primary deuterium kinetic isotope effects on enzyme reduction exhibited by the spinach (A), yeast (B), and *E. coli* (C) glutathione reductases in 100 mM TAPS, pH 8.1, measured under rapid reaction conditions.

exponential fits. The *E. coli* enzyme, like the spinach glutathione reductase, is reduced by NADH to form EH_2 and then slowly reduced by a second molecule of NADH to form EH_4. These data are well fitted to the sum of two exponential processes, representing the rapid formation of EH_2 and the slower formation of EH_4. The two electron reduced form of yeast glutathione reductase is stable to further reduction, and these data were adequately fitted to a single exponential process. In all cases, an additional criterion for the appropriateness of the rate constants obtained from the stopped-flow data was the analysis of the plots of reciprocal rate constants versus the reciprocal of the NADH or (4*S*)-[4-^{2}H]NADH concentrations. The reciprocal plots shown in Figure 3 were obtained by using the spinach (A), yeast (B), and *E. coli* (C) glutathione reductases, and these data were fitted to eq 8, assuming identical isotope effects on the slopes and intercepts. Primary deuterium kinetic isotope effects on enzyme reduction by NADH were determined to be 3.99 ± 0.13, 2.78 ± 0.07, and 1.64 ± 0.05 for the spinach, yeast, and *E. coli* glutathione reductases, respectively, compared to the values for the primary deuterium kinetic isotope effects of 4.34 ± 0.14, 2.52 ± 0.09, and 1.62 ± 0.10 measured in steady-state experiments.

Discussion

A large body of literature concerning structural and mechanistic properties of the yeast glutathione reductase exists [reviewed in Williams (1976)], and a three-dimensional structure of the human erythrocyte glutathione reductase is available (Thieme et al., 1981; Karplus & Schulz, 1987). The structures of the enzyme–NADPH and enzyme–NADH complexes have recently been determined (Pai et al., 1988), and the structures of some reaction intermediates are known in detail (Pai & Schulz, 1983; Karplus et al., 1989). In addition, groups involved in reversible electron storage (Cys-58 and Cys-63) and at least one group involved in the GSSG reduction half-reaction (His-467′) have been determined. The *E. coli* enzyme has been cloned and sequenced recently (Greer & Perham, 1986), and its amino acid sequence shows a high degree of homology to the sequence of the human enzyme. Site-directed mutagenesis of the *E. coli* enzyme revealed changes in the kinetic mechanism as a result of a single amino acid substitution (Berry et al., 1989). Rapid reaction studies on the yeast enzyme indicate that enzyme reduction by NADPH occurs in three steps (Huber & Brandt, 1980), identified as (a) the very rapid formation of a charge-transfer Michaelis complex between NADPH and oxidized enzyme, followed by (b) hydride transfer to form the reduced dithiol form of the enzyme, presumable via a reduced flavin intermediate, and (c) dissociation of NADP and formation of the stable two electron reduced enzyme, EH_2. On the other hand, less is known about the kinetic properties of the *E. coli* (Berry et al., 1989) or spinach glutathione reductases. The availability of several sources of highly purified enzyme has allowed us to measure steady-state and rapid reaction kinetic isotope effects on the oxidation of reduced pyridine nucleotide substrates catalyzed by spinach, yeast, and *E. coli* glutathione reductases and to probe species-dependent differences among them.

Kinetic Parameters of NADPH and NADH. All steady-state and rapid reaction kinetic studies have been performed at pH values between 7.8 and 8.1, a region where there is no change in V or V/K for NAD(P)H (Wong & Blanchard, 1989). The presence of a 2′-phosphate at C2′ of the adenosylribose moiety of the pyridine nucleotide is important in the binding of the substrate to the enzyme. This is demonstrated by the 100-fold increase in the K_m values for NADH as a reductant when compared to NADPH, for each of the three enzymes used in this study. This is in agreement with the structural studies on pyridine nucleotide binding to human erythrocyte glutathione reductase (Pai et al., 1988).

There are substantial species-dependent differences in the ability of the three enzymes to use NADH as a reductant. Thus, the spinach, yeast, and *E. coli* enzymes exhibit maximal velocities with NADH that are 18, 3.7, and 0.13% of the maximal velocity with NADPH, respectively. The different steady-state kinetic behavior of NADH and NADPH is highlighted below when primary deuterium kinetic isotope effects on NADPH and NADH oxidation are compared.

Isotope Effect Dependence on GSSG Concentration. In most kinetic isotope effect experiments, the concentration of the unvaried, unlabeled substrate is held at a saturating level. Stopped-flow spectrophotometric analysis of the isolated half-reactions has previously demonstrated that the kinetic mechanism of the yeast enzyme can be fully described by a ping-pong kinetic mechanism (Williams, 1976). However, the report (Mannervik et al., 1980) of a change in kinetic mechanism from ping-pong at low GSSG concentrations to a ter-

nary complex mechanism at high glutathione concentrations and the observation of a switch from ping-pong to sequential kinetic mechanism upon replacement of Tyr-177 with a serine or glycine residue in *E. coli* glutathione reductase (Berry et al., 1989) led us to perform the experiments whose results are shown in Table II. For the well-characterized yeast enzyme, there is no significant difference in ^{D}V or $^{D}V/K$, when isotope effects are measured by using NAD(P)H at glutathione concentrations between 0.05 and 1 mM (K_{GSSG} = 45–65 μM for the three enzymes, this work). The spinach and *E. coli* enzymes show a similar independence of observed kinetic isotope effect on glutathione concentration when either NADPH or NADH are used as substrates. These results are consistent with a ping-pong kinetic mechanism, since the variation of a V/K kinetic isotope effect with the concentration of a second substrate is expected only for ordered or random, but not ping-pong, kinetic mechanisms (Klinman et al., 1980). Considering that the pyridine nucleotide and glutathione binding sites observed in the crystal structure of the human enzyme are topographically distinct and distant, these results suggest that even if ternary complexes do form, the rates of the individual half-reactions are essentially unaffected by substrate occupancy at the other site.

Modeling of the Reductive Half-Reaction. In this study, to model the overall reaction mechanism, we have assumed a ping-pong kinetic mechanism without kinetically significant ternary complex formation. This assumption is supported by our findings of GSSG-independent primary kinetic isotope effects, as well as other studies (Massey & Williams, 1965; Williams, 1976; Berry et al., 1989). For the mechanism below, the following equations can be derived by using the net rate constants method (Cleland, 1975):

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

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

$$^{D}V/K_{NAD(P)H} = \frac{^{D}k_3 + c_f + c_r{}^{D}K_{eq}}{1.0 + c_f + c_r} \quad (9)$$

$$^{D}V_{NAD(P)H} = \frac{^{D}k_3 + c_{vf} + c_r{}^{D}K_{eq}}{1.0 + c_{vf} + c_r} \quad (10)$$

where

$$c_f = (k_3/k_2) \quad (11)$$

$$c_r = (k_4/k_5) \quad (12)$$

$$c_{vf} = (k_3/k_5 + k_3/k_{11} + k_3/k_9\,(1.0 + k_{10}/k_{11})) \quad (13)$$

and where $^{D}K_{eq} = k_{3H}k_{4D}/k_{3D}k_{4H}$ and has a value of 0.98 (Cleland, 1980). Starred rate constants represent the isotope-sensitive steps. The commitment factors, c_f, c_r, and c_{vf}, represent ratios of rate constants whose values will affect the magnitude of the observed primary deuterium kinetic isotope effects on V, ^{D}V or V/K, $^{D}V/K$.

Isotope Effect Analysis: NADPH. Because of the wealth of independent kinetic evidence, we shall only discuss our isotope effect results measured with the yeast enzyme in this analysis. When primary deuterium kinetic isotope effects were determined by using NADPH and the spinach, yeast, and *E. coli* enzymes, only very small isotope effects on V_{max}, ^{D}V were observed (~1.3–1.5; see Table III), while no statistically significant effects were observed on $^{D}V/K$. Since for the yeast enzyme there is independent evidence that the intrinsic isotope effect on the C–H(^{2}H) bond-breaking step for NADPH is ≥2.7 (Huber & Brandt, 1980), we interpret our ^{D}V results as supporting suppression of ^{D}V due to substantial values for the forward, c_{vf}, and reverse, c_r, commitment factors when NADPH is used as substrate. The rate constant ratio, k_4/k_5 will become significant as the rate of NADP dissociation (k_5) decreases, and Massey and Williams (1965) have suggested that NADP, produced during enzyme reduction, remains tightly bound to two electron reduced yeast glutathione reductase. Bulger and Brandt (1971) calculated a $K_{i,NADP}$ of 70 μM, on the basis of steady-state and stopped-flow kinetic data. Additionally, the c_{vf} term may be responsible for some suppression of the intrinsic kinetic isotope effect. Williams (1976) has shown that the rate of enzyme reduction by NADPH, k_3, is 1.8 times faster than the rate of EH_2 oxidation by GSSG, k_9 (5250 and 2900 min^{-1}, respectively, at 5 °C). For an intrinsic primary deuterium kinetic isotope effect of 2.7 on k_3 (Huber & Brandt, 1980), this minimum value of c_{vf} (the ratio of the forward catalytic rate constants of the two half-reactions, k_3/k_9) would predict an observed $^{D}V_{NADPH}$ of 1.6, close to the value that we measured experimentally.

The observation that $^{D}V/K_{NADPH}$ is equal to 1.0 suggests that NADPH partitions predominantly through catalysis, rather than dissociating from the enzyme. This is supported by the very low steady-state K_m for NADPH (see Table I) and the higher values of V_{max} relative to those measured with NADH as substrate. The same analysis of isotope effects exhibited by NADPH should be valid for the spinach and *E. coli* glutathione reductases.

Isotope Effect Analysis: NADH. NADH exhibits distinctly different isotope effect behavior than NADPH for all three enzymes. We measured large $^{D}V/K$ and ^{D}V (footnote 2) for NADH, and NADH dissociates from the E–NADH complex more rapidly than it undergoes catalysis with all of the glutathione reductases tested. Bulger and Brandt (1971) have proposed that the formation of a productive Michaelis complex is rate-limiting with NADH for the yeast enzyme. Our steady-state K_m and maximum-velocity data using NADH as a substrate for all three glutathione reductases support this.

We thus sought to determine the intrinsic primary deuterium kinetic isotope effect on NADH oxidation, $^{D}k_3$, catalyzed by the spinach, yeast, and *E. coli* glutathione reductases. In the first method, we compared steady-state deuterium and tritium V/K kinetic isotope effects (Northrop, 1975). In the second method, we directly measured the primary deuterium kinetic isotope effects on the reduction of the enzyme in the stopped-flow spectrophotometer. The results of the steady-state determinations with the spinach enzyme are shown in Table III and Figure 1 and yield values of $^{D}V/K_{NADH}$ of 4.04 ± 0.30 in 10 mM TEA, pH 7.8, and a value of $^{T}V/K_{NADH}$ of 7.24 ± 0.34 under identical conditions, which is consistent with the value predicted by the Swain–Schaad relationship ($^{T}k = {}^{D}k^{1.44}$; Swain et al., 1958) from the experimentally determined $^{D}V/K$. The results of similar steady-state determinations of the deuterium and tritium kinetic isotope effect for NADH oxidation are also shown in Table III and Figure 1 for the yeast

[2] Fits of the steady-state deuterium kinetic isotope effect data to eq 8 where ^{D}V and $^{D}V/K$ were not required to be equal gave good results by visual inspection but had larger standard errors in the fitted parameters than fits to either eq 7 or eq 8, constrained such that $^{D}V/K = {}^{D}V$. The determination of steady-state deuterium kinetic isotope effects exhibited by human erythrocyte glutathione reductase using NADH clearly shows that for this enzyme these data are best fit to eq 8, assuming $^{D}V/K = {}^{D}V$ (Sweet & Blanchard, unpublished results). This is the expected behavior for rate-limiting hydride transfer.

and *E. coli* enzymes, respectively. In both of these cases, values for $^{T}V/K_{NADH}$ calculated by using the Swain–Schaad relationship and experimentally determined values of $^{D}V/K_{NADH}$ match closely the experimentally determined $^{T}V/K_{NADH}$. Because of the inherent limitations on the precision of the steady-state primary deuterium and tritium kinetic isotope effect determinations and the favorable spectroscopic properties of the three glutathione reductases, we sought to confirm our findings by using the rapid reaction method to directly measure the primary deuterium kinetic isotope effect on the formation of EH_2.

The data shown in Figure 3 clearly demonstrate no effect of deuterium substitution on K_m (K_d). This is the expected result, and the data yield primary deuterium kinetic isotope effects on enzyme reduction, $^{D}k_3$, of 3.99, 2.78, and 1.64, for the spinach, yeast, and *E. coli* glutathione reductase, respectively. These values correspond closely to $^{D}V/K_{NADH}$ values for the three enzymes determined by steady-state methods.

These data are consistent with our conclusion that the observed steady-state $^{D}V/K_{NADH}$ is equal to the *intrinsic* primary deuterium kinetic isotope effect on reduced pyridine nucleotide oxidation, $^{D}k_3$. Our value of 2.7 for the primary deuterium kinetic isotope effect on hydride transfer catalyzed by yeast glutathione reductase can be compared to the same value obtained for enzyme reduction by NADPH in rapid reaction studies of the yeast enzyme (Huber & Brandt, 1980).

Conclusions

Steady-state and pre-steady-state measurements of primary kinetic isotope effects exhibited by glutathione reductases from spinach, yeast, and *E. coli* have allowed us to determine species-dependent similarities and differences among these homologous enzymes. Analysis of the dependence of $^{D}V/K$ on GSSG concentration has allowed us to confirm the similarity in the kinetic mechanisms of the spinach, yeast, and *E. coli* glutathione reductases, which appears to be ping-pong in all cases, and is in agreement with previous steady-state, rapid reaction and crystallographic studies on the yeast and human erythrocyte enzymes.

All three enzymes have low steady-state K_m values for NADPH (3–6 μM) and 100-fold higher steady-state K_m values for NADH, reflecting the contribution of the 2′-phosphate moiety to binding. Furthermore, only ^{D}V is observed when NADPH is used as reductant, while both ^{D}V and $^{D}V/K$ are observed when NADH is used as reductant. For all three enzymes this is consistent with a small partitioning through catalysis, k_3, relative to substrate dissociation, k_2, when NADH is used as a reductant. There is a striking difference in the magnitude of the intrinsic primary deuterium kinetic isotope effect measured by using NADH exhibited by the spinach, yeast, and *E. coli* glutathione reductases.

The complete primary sequences of the human erythrocyte (Krauth-Siegel et al., 1982) and *E. coli* (Greer & Perham, 1986) glutathione reductases have been reported, and are very homologous, especially in catalytically essential regions. Although complete primary sequence data are not yet available for the yeast or spinach glutathione reductases, it would appear that conservative and limited amino acid substitutions could be responsible for significant changes in both the energetics of the reaction and the stabilization of the transition state for hydride transfer. Our findings of differences in the intrinsic primary kinetic isotope effect exhibited by spinach, yeast, and *E. coli* glutathione reductase catalyzing the *same* reaction between the *same* redox partners are in accord with theories on the strong modulating power of homologous enzymes on the energy levels of common reaction intermediates and transition states due to specific interactions between active site residues and bound substrates (Jones et al., 1986). Experiments are in progress to more precisely define the reason for the striking differences in intrinsic primary deuterium kinetic isotope effect on reduced pyridine nucleotide oxidation catalyzed by the three glutathione reductases, although one appealing and testable hypothesis would be that each of the three enzymes stabilizes a different transition state for the hydride transfer between NADH and FAD.

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