Glutathione Reductase: Solvent Equilibrium and Kinetic Isotope Effects†

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ABSTRACT: Glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG). The kinetic mechanism is ping-pong, and we have investigated the rate-limiting nature of proton-transfer steps in the reactions catalyzed by the spinach, yeast, and human erythrocyte glutathione reductases using a combination of alternate substrate and solvent kinetic isotope effects. With NADPH or GSSG as the variable substrate, at a fixed, saturating concentration of the other substrate, solvent kinetic isotope effects were observed on V but not V/K. Plots of V_m vs mole fraction of D_2O (proton inventories) were linear in both cases for the yeast, spinach, and human erythrocyte enzymes. When solvent kinetic isotope effect studies were performed with DTNB instead of GSSG as an alternate substrate, a solvent kinetic isotope effect of 1.0 was observed. Solvent kinetic isotope effect measurements were also performed on the asymmetric disulfides GSSNB and GSSNP by using human erythrocyte glutathione reductase. The K_m values for GSSNB and GSSNP were 70 μM and 13 μM, respectively, and V values were 62 and 57% of the one calculated for GSSG, respectively. Both of these substrates yield solvent kinetic isotope effects greater than 1.0 on *both* V and V/K and linear proton inventories, indicating that a single proton-transfer step is still rate limiting. These data are discussed in relationship to the chemical mechanism of GSSG reduction and the identity of the proton-transfer step whose rate is sensitive to solvent isotopic composition. Finally, the solvent equilibrium isotope effect measured with yeast glutathione reductase is 4.98, which allows us to calculate a fractionation factor for the thiol moiety of GSH of 0.456.

Glutathione reductase catalyzes the NADPH[1]-dependent reduction of oxidized glutathione. The kinetic mechanism of the enzyme from yeast has been studied by using steady-state and pre-steady-state kinetic measurements, and the rates of the isolated half-reactions have been shown to be consistent with a ping-pong kinetic mechanism (Williams, 1976). Chemical mechanisms have been proposed on the basis of spectroscopic pH titrations of the yeast enzyme (Arscott et al., 1980) and of the crystallographic analysis of the structure of several free and substrate-bound forms of the human erythrocyte enzyme (Pai & Schulz, 1983). One proton-transfer step may be part of the reductive half-reaction, while two one-proton transfer steps are involved in the production of two GSH molecules from GSSG during the oxidative half-reaction. On the basis of the three-dimensional structure of the enzyme, His467 and Tyr114 have been implicated as potential proton donors in the oxidative half-reaction, while protonation of the flavin N(1) position may not be required during the reductive half-reaction since an α-helix is properly oriented and might alone be able to stabilize the negative charge on the flavin. Unfortunately, crystallographic analyses fail to provide direct information on proton-transfer steps that occur during catalysis.

To further elucidate the chemical mechanism of glutathione reductase, we have carried out solvent kinetic isotope effect measurements in the presence of NADPH and GSSG or several symmetric and asymmetric disulfide analogues of GSSG. The use of symmetric and asymmetric disulfides, in which at least one of the two product mercaptans does not require protonation upon reduction and release from the enzyme active site, demonstrated that a single, one-proton transfer step, which occurs during oxidized glutathione reduction, is partially rate limiting in the overall reaction. Finally, the measurement of the solvent equilibrium isotope effect for the reaction catalyzed by yeast glutathione reductase has allowed the calculation of a fractionation factor for the thiol moiety of reduced glutathione of 0.456.

Experimental Procedures

Spinach (type VI) and yeast (type IV) glutathione reductase were purchased from Sigma and were greater than 95% pure by SDS–PAGE using Coomassie Blue G-350 staining. Human erythrocyte glutathione reductase was the generous gift of Dr. Emil F. Pai (Max-Planck Institute for Medical Research, Heidelberg, FRG). Glucose-6-phosphate dehydrogenase from yeast (type VII), GSSG, GSH, DTNB, DTNP, NADPH, and all buffer components were purchased from Sigma. D_2O (>99.8 atom % excess) was from Bio-Rad or ICN and was distilled (bp 101 °C, uncor) before use.

Preparation of Disulfides. GSSNB was prepared by a modification of the method of Mannervik (1970). DTNB (75 mg, 203.6 μmol) was dissolved in 5 mL of water by titrating the suspension to pH 6.5 with 0.2 N NaOH. GSH (87.6 mg,

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[1] Abbreviations: FPLC, fast protein liquid chromatography; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTNP, 2,2′-dithiobis(5-nitropyridine); GSSG, oxidized glutathione; GSH, reduced glutathione; GSSNB, glutathione–5–thio–2–nitrobenzoic acid mixed disulfide; GSSNP, glutathione–2-thio-4-nitropyridine mixed disulfide; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NADP, oxidized β-nicotinamide adenine dinucleotide phosphate; TEA, triethanolamine; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; 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.

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285 μmol) was dissolved in 1 mL of water and added in 50-μL aliquots to the DTNB solution. The pH of the solution was maintained at 6.5 by addition of 0.2 N NaOH after each addition of GSH. The pale yellow solution turned orange, and after the addition of GSH, the reaction mixture was stirred at room temperature for an additional 2 h.

The mixture (10 mL) was then injected onto a Mono Q anion-exchange column (Pharmacia) equilibrated with 10 mM TEA, pH 7.7, in 1-mL aliquots. Elution of the desired product, measured by absorbance at 300 nm, was accomplished by using a linear 0–1 M KCl gradient in 10 mM TEA, pH 7.7, and the product eluted between 130 and 170 mM KCl. The peak was ninhydrin positive, absorbed at 412 nm upon addition of DTT, and was pure as judged by analytical rechromatography on Mono Q. Peak fractions were pooled, and the concentration of GSSNB was calibrated by using yeast glutathione reductase in the presence of excess NADPH and by the reduction of the disulfide with DTT, using ϵ_{412} = 13 600 M^{-1} cm^{-1} (Ellman, 1959).

GSSNP was synthesized in a similar manner. DTNP (37.2 mg, 1.2 mmol) was dissolved in 5 mL of acetone. GSH (30.7 mg, 1 mmol) was dissolved in 1 mL of water and titrated to pH 8. GSH was added in aliquots into the DTNP solution, and the pH of the solution was maintained at pH 6.5 with 0.2 N NaOH. After the addition of GSH was complete, the reaction mixture was stirred at room temperature for an additional 2 h.

The solvent was removed by rotary evaporation, and the residue was resuspended in 10 mM TEA-HCl, pH 7.7 buffer. The suspension was filtered, and the orange filtrate was loaded onto a 1 × 20 cm DEAE column, equilibrated with 10 mM TEA-HCl buffer, pH 7.7, and eluted with a linear gradient of KCl (0–1 M) in 10 mM TEA, pH 7.7. The desired product was eluted at 200 mM salt. The fractions containing GSSNP were pooled and further purified by using a Mono Q column exactly as described above for GSSNB. The product was ninhydrin positive, absorbed at 386 nm after reduction with DTT, and was greater than 90% pure as judged by analytical rechromatography on a Mono Q column. GSSNP concentration was calibrated by using yeast glutathione reductase in the presence of excess NADPH and by reduction with DTT, using ϵ_{386} = 14 000 M^{-1} cm^{-1} (Grassetti & Murray, 1969).

Solvent Equilibrium Isotope Effect. Stock solutions of NADPH (10 mM), GSSG (300 mM), GSH (300 mM, pH 8.1), and NADP (100 mM) were prepared. NADPH concentrations were calibrated by using yeast glutathione reductase in the presence of excess GSSG. GSSG concentrations were calibrated by using yeast glutathione reductase in the presence of excess NADPH. NADP concentrations were calibrated by using yeast glucose-6-phosphate dehydrogenase in the presence of excess glucose 6-phosphate. GSH concentrations were calibrated by reduction of DTNB using ϵ_{412} = 13 600 M^{-1} cm^{-1} (Ellman, 1959). These solutions were added to their approximate equilibrium concentration in 300 mM TAPS buffer, pH 8.1, in a final volume of 5 mL. This solution was equilibrated at 25 °C in a water bath. A 300-μL aliquot of this mixture (10× mixture) was diluted to 3 mL with water in a 3-mL cuvette, and 10 μL of concentrated yeast glutathione reductase (40 mg/mL) was added. The changes in absorbance at 340 nm were used to adjust the concentrations of substrates or products in the stock mixture, until upon addition of enzyme, no changes in absorbance at 340 nm were observed. A set of reaction mixtures were then made up in 3-mL cuvettes at different percentages of D_2O, ranging from 10 to 90%, by addition of various volumes of H_2O and D_2O to 0.3 mL of the 10× mixture. Upon addition of enzyme, the increase in absorbance at 340 nm was monitored until a new equilibrium was established. Changes in the concentration of NADPH, and thus the other reactants, were calculated from the change in absorbance at 340 nm by using ϵ_{340} = 6220 M^{-1} cm^{-1} for NADPH. A new equilibrium constant was calculated by using the equation:

$$([NADP] - x)([GSH] - 2x)^2/([NADPH] + x) \times ([GSSG) + x) = K_{eq} \quad (1)$$

where x is equal to the calculated change in NADPH concentration at each D_2O concentration. The calculated K_{eq} values were then plotted against the corresponding mole fraction of D_2O. The solvent equilibrium isotope effect and the fractionation factor for GSH were calculated as discussed under Results.

Kinetic Procedure and Preparation of Isotopic Solvents. Buffers were prepared by titrating H_2O or D_2O solutions containing 300 mM TAPS to pH or pD (pH meter reading + 0.4) 8.1 with KOH in H_2O or D_2O, respectively. NADPH (10 mM) in H_2O was prepared fresh daily. GSSG (300 mM) was prepared and stored at 4 °C.

Glutathione reductase reaction rates were measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm with a Gilford 260 spectrophotometer equipped with thermospacers attached to a constant-temperature circulating water bath maintained at 25 °C. Rates of reduction of DTNB, GSSNB, and GSSNP were measured spectrophotometrically by monitoring their reduction at 412, 412, and 386 nm, respectively.

Reaction mixtures containing NADPH, disulfide substrate, and the desired mole fraction of D_2O, prepared volumetrically from stocks of H_2O and D_2O buffer solutions, were prepared in 3-mL cuvettes. The cuvettes were thermally equilibrated at 25 °C in a water bath, and reactions was initiated by the addition of a small amount (<20 μL) of cold enzyme. Initial velocities were measured at various mole fractions of D_2O with either varying NADPH or varying disulfide substrate concentrations, in the presence of a saturating concentration of the other substrate. The kinetic parameters V and V/K were determined, and calculated values of V were plotted against mole fraction of D_2O. The resulting proton inventories were used to determine the number of protons transferred and the solvent kinetic isotope effects on V (^{D_2O}V).

pH Profiles. The following buffers (300 mM) were prepared by titrating their acid forms, dissolved in water to the desired pH with 2 N KOH, and were used at 100 mM concentrations at the stated pH to allow for overlap: PIPES (6.6–7.4), HEPES (7.2–8.2), TAPS (8.1–9.0), CHES (8.9–9.9), and CAPS (9.8–10.9). Initial velocity studies were performed at the above pH values with the human erythrocyte glutathione reductase with varying concentrations of GSSG and fixed, saturating concentrations of NADPH. The kinetic parameters, V and V/K_{GSSG}, were determined at each pH value from 6.6 to 10.9 by using eq 2, and their log values were then plotted against the pH values, determined by insertion of a combined microelectrode into the cuvette after the initial velocity assays were carried out.

Data Analysis. Reciprocal initial velocities were plotted against the reciprocal of the variable substrate concentration, and the data were fitted to appropriate rate equations by the least-squares method, assuming equal variance of the velocities v. The Fortran programs of Cleland (1979) were modified to run on the Vax 11/780 operating system. Individual saturation curves were fitted to eq 2, and the initial velocities obtained by varying the concentration of D_2O were fitted to

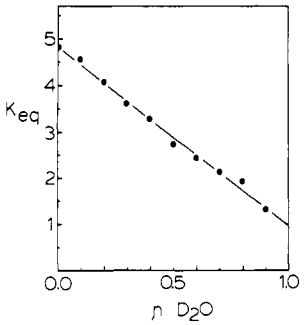


FIGURE 1: Solvent equilibrium isotope effect using yeast glutathione reductase. The equilibrium constants calculated at several percentages of D_2O at pH 8.1 are plotted against the mole fraction of D_2O. The experimental points are best fit by a straight line assuming a value of $^{D_2O}K_{eq}$ of 4.98.

eq 3 and 4, which assume solvent kinetic isotope effects on V only and on both V and V/K, respectively. In eq 2–4, A is

$$v = VA/(K + A) \quad (2)$$

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

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_V)] \quad (4)$$

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. pH profiles in which the log of the parameter plotted decreased above pK_1 with a slope of −1 were fitted to eq 5, while pH profiles in which the log of the parameter plotted decreased above pK_1 with a slope of −1, and above pK_2 with a slope of −2, were fitted to eq 6, assuming independent pK values. In eq 5 and 6, Y is the parameter being fitted and c is the pH-independent plateau value.

$$\log Y = \log [c/(1 + K_1/H)] \quad (5)$$

$$\log Y = \log [c/(1 + K_1/H + K_1K_2/H^2)] \quad (6)$$

RESULTS

Solvent Equilibrium Isotope Effect and GSH Fractionation Factor. The solvent equilibrium isotope effect was determined by using yeast glutathione reductase. A plot of the equilibrium constants determined at various mole fractions of D_2O yielded a straight line with a $^{D_2O}K_{eq} = 4.98$ (Figure 1).[2] This value represents the product of the fractionation factors for the substrates divided by the product of the fractionation factors for the products. For glutathione reductases, the substrates whose fractionation factors must be considered are the protons of water ($\phi = 1.058$; Friedman & Shiner, 1966) and NADPH ($\phi = 0.98$; Cleland, 1980), while the product whose fractionation factor must be considered is GSH (ϕ_{GSH}). Since two molecules of GSH are produced from the substrate GSSG, the fractionation factor of GSH appears in the equation as a squared term. Solution of the equation $^{D_2O}K_{eq} = \phi_{NADPH}\phi_{H^+}/(\phi_{GSH})^2$ yields a value of 0.456 for the fractionation factor of the glutathione thiol moiety.

pH Profiles of the Kinetic Parameters. When GSSG was varied at saturating levels of NADPH (over $20K_{NADPH}$ at all pH's) at pH values between 6.6 and 10.8 and the data were fitted to eq 2, the V and V/K_{GSSG} pH profiles shown in Figure 2 were obtained with human erythrocyte glutathione reductase. The maximum velocity decreases at high pH above a pK of 8.98 ± 0.06, while V/K_{GSSG} decreases at alkaline pH above two pK values of 7.80 ± 0.06 and 8.62 ± 0.07.

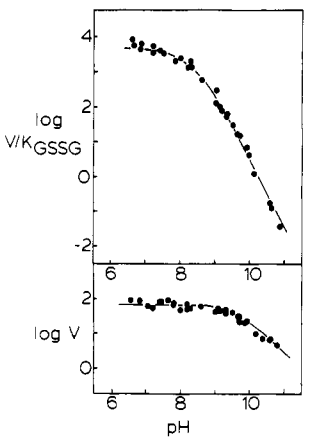


FIGURE 2: pH profiles of V and V/K_{GSSG} determined with the human erythrocyte glutathione reductase. The pH dependence of the maximum velocity was determined with GSSG as the variable substrate at a fixed, saturating concentration of NADPH (0.1–0.2 mM). Upper panel: The V/K_{GSSG} profile decreases with the deprotonation of two groups, and the smooth curve represents the best fit of the experimental data to eq 6 assuming $pK_1 = 7.80 \pm 0.06$ and $pK_2 = 8.62 \pm 0.07$. Lower panel: The V profile decreases with the deprotonation of a single group, and the smooth curve represents the best fit of the experimental data to eq 5 assuming $pK = 8.98 \pm 0.06$.

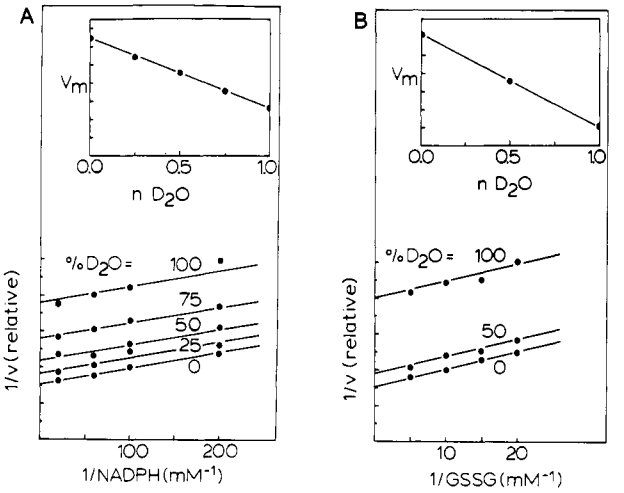


FIGURE 3: Solvent kinetic isotope effects on the human erythrocyte glutathione reductase reaction. (A) Steady-state initial velocity measurements of the reaction by using varying concentrations of NADPH and a fixed, saturating concentration of GSSG (3 mM). (B) Steady-state initial velocity measurements of the reaction by using varying concentrations of GSSG and a fixed, saturating concentrations of NADPH (0.1 mM). In both cases only V is sensitive to the isotopic composition of the solvent, and the insets show the calculated maximum velocities plotted against the mole fraction of D_2O. The linear proton inventories yielded values of $^{D_2O}V_{NADPH} = 2.5 \pm 0.1$ and $^{D_2O}V_{GSSG} = 2.6 \pm 0.1$.

Solvent Kinetic Isotope Effects and Proton Inventories Using NADPH and GSSG. Solvent kinetic isotope effect measurements were performed with homogeneous preparations of the yeast, spinach, and human erythrocytes glutathione reductases at pH 8.1. When NADPH was used as the variable substrate at a fixed, saturating concentration of GSSG, parallel reciprocal plots were obtained for all three enzymes as the mole fraction of D_2O was varied (shown only for the human erythrocyte enzyme, Figure 3A). When V was plotted against the mole fraction of D_2O, linear proton inventories were obtained for all three enzymes which yielded $^{D_2O}V_{NADPH}$ solvent

[2] The nomenclature used throughout is similar to that originally proposed by Northrop (1975). Thus, $^{D_2O}V_{NADPH}$ represents the solvent kinetic isotope effect on the maximum velocity V_m determined by using NADPH as the variable substrate at a saturating concentration of GSSG. Similarly, $^{D_2O}V/K_{GSSNB}$ represents the solvent kinetic isotope effect on V/K determined by using GSSNB as the variable substrate at a saturating concentration of NADPH.

kinetic isotope effect values of 1.9 ± 0.1, 2.8 ± 0.1, and 2.5 ± 0.1 for the yeast, spinach, and human erythrocyte glutathione reductases, respectively. Identical solvent kinetic isotope effect studies were performed on the three enzymes by using GSSG as the variable substrate at a fixed, saturating concentration of NADPH and again yielded parallel reciprocal plots for all three enzymes as the mole fraction of D_2O was varied (shown only for the human erythrocyte enzyme, Figure 3B). Plots of calculated V values against mole fraction of D_2O yielded straight lines for all three enzymes with $^{D_2O}V_{GSSG}$ values of 1.9 ± 0.1, 2.5 ± 0.1, and 2.6 ± 0.1 for the yeast, spinach, and human erythrocyte enzymes, respectively.

Solvent Kinetic Isotope Effect and Proton Inventory Using DTNB. Solvent kinetic isotope effect measurements on the NADPH-dependent reduction of DTNB were performed with the human erythrocyte glutathione reductase. DTNB is a poor substrate and exhibits a maximum velocity only 4% of that measured in the presence of GSSG. A steady-state K_{DTNB} of 2 mM was determined at pH 8.1, but because of the observed substrate inhibition exhibited by DTNB, solvent kinetic isotope effects were determined at a fixed, nonsaturating concentrations of DTNB, 0.1 mM ($0.05K_m$), and at a fixed, saturating concentration of NADPH, 0.1 mM. The velocity of DTNB reduction was independent of solvent isotope composition (data not shown), and identical results were obtained when DTNB reduction was determined at a fixed concentration of 2 mM (K_m level, data not shown).

SKIE and Proton Inventory Using GSSNB and GSSNP. Solvent kinetic isotope effect measurements were performed with the human erythrocyte glutathione reductase in the presence of the asymmetric mixed disulfides of glutathione and TNB (GSSNB) or TNP (GSSNP) at a saturating concentration of NADPH (0.1 mM). Steady-state K_m values of 70 μM and 13 μM were determined for GSSNB and GSSNP, respectively, while the calculated maximal velocities were 62 and 57% of the one determined in the presence of GSSG. Plots of the reciprocal initial velocities vs reciprocal disulfide concentration determined at various mole fractions of D_2O revealed solvent kinetic isotope effects greater than 1 on both V and V/K (shown for GSSNB, Figure 4). Plots of V_m against mole fraction of D_2O were linear (shown for GSSNB) with a ^{D_2O}V = 3.4 ± 0.8 for GSSNB and 3.2 ± 1.2 for GSSNP. Plots of V/K against the mole fraction of D_2O were also linear and allowed the calculation of $^{D_2O}V/K_{GSSNB}$ = 3.7 ± 0.4 and $^{D_2O}V/K_{GSSNP}$ = 2.4 ± 0.8.

Discussion

The three-dimensional structure of human erythrocyte glutathione reductase has been determined to 1.5-Å resolution (Karplus & Schulz, 1987), and chemical mechanisms for the oxidative half-reaction have been proposed on the basis of spectroscopic pH titrations of the yeast enzyme (Arscott et al., 1981) and crystallographic analysis of several reaction intermediates and enzyme–substrate complexes (Pai & Schulz, 1983) with the human erythrocyte enzyme. In the proposed mechanisms, the oxidative half-reaction involves two one-proton transfer steps to the reduced glutathione products. The first proton transfer between protonated His467 and the first glutathione thiolate anion occurs after or concomitant with the reductive cleavage of oxidized glutathione to form the enzyme–glutathione mixed disulfide. The second proton-transfer step to the second glutathione thiolate anion occurs after attack by Cys63 on the Cys58–glutathione mixed disulfide to re-form the enzyme disulfide.

In this study, we have determined solvent kinetic isotope effects (Venkatasubban & Schowen, 1981) on the reaction catalyzed by glutathione reductase to gain information about the involvement of proton transfers as rate-determining steps in the reaction.

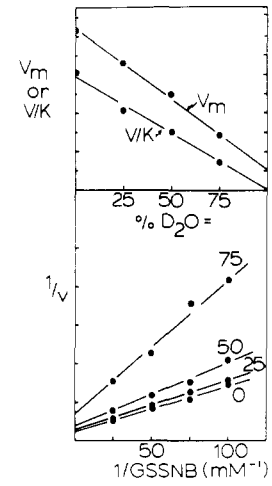


FIGURE 4: Solvent kinetic isotope effects using the human erythrocyte glutathione reductase with GSSNB as alternate substrate. Double-reciprocal plot (lower panel) of the initial velocity by using varying concentrations of GSSNB and a fixed, saturating concentration of NADPH (0.1 mM) is shown. Both V and V/K solvent kinetic isotope effects are observed, and the upper panel shows the calculated V and V/K values plotted against the mole fraction of D_2O. The linear proton inventories were used to calculate $^{D_2O}V_{GSSNB}$ = 3.4 ± 0.8 and $^{D_2O}V/K_{GSSNB}$ = 3.7 ± 0.4.

Solvent Equilibrium Isotope Effects. Ground-state fractionation factors (ϕ) define the preference of a molecule with one exchangeable hydrogenic position for deuterium over protium relative to the solvent's preference for deuterium over protium (Schowen, 1972; Schowen, 1978). Ground-state fractionation factors for carbon, nitrogen, oxygen, and sulfur compounds with one exchangeable hydrogenic position are known, and of particular interest is the unique behavior of sulfur with a ground-state fractionation factor between 0.40 and 0.46. This low value describes sulfur's strong preference for protons over deuterons. Fractionation factors have been directly determined for hydrogen sulfide (Pohl, 1961) and ethanethiol (Hobden et al., 1939) by exchange in isotopically labeled solvent followed by mass spectrometry. The determination of the fractionation factor for the catalytic cysteine in papain (Szawelski & Wharton, 1981) and the presumptive cysteines in proline racemase (Fisher et al., 1986) have been calculated from solvent kinetic isotope effect data.

In the reaction catalyzed by glutathione reductase, 1 mol of GSSG is converted to 2 mol of GSH. GSH is predicted to exhibit a ground-state fractionation factor of between 0.40 and 0.46. The determination of the solvent equilibrium isotope effect exhibited by yeast glutathione reductase has allowed us to precisely and directly determine a value for the fractionation factor of the thiol group on GSH. By measuring the equilibrium constant at various mole fractions of D_2O, we obtained a $^{D_2O}K_{eq}$ of 4.98. As presented under Results, we calculated a ground-state fractionation factor for GSH of 0.456, in excellent agreement with values for thiol fractionation factors obtained by others.

pH Profiles of the Kinetic Parameters. All solvent kinetic isotope effect studies have been performed at pH(D) 8.1, and it was important to rule out the possibility that the observed V solvent kinetic isotope effects were due to changes in V with pH, since changes in solvent isotopic composition have demonstrated effects on the ionization behavior of enzymatic side chains. We thus determined the V and V/K_{GSSG} pH profiles using the human erythrocyte glutathione reductase between

6.6 and 10.8, as shown in Figure 2.

V is constant over much of the pH range and decreases as a single acidic group, with a pK of 8.98, is deprotonated. In the structure of crystalline human erythrocyte glutathione reductase, several groups are present in the glutathione binding site that could act as proton donors, including Tyr114 and His467. The latter group has been proposed (Pai & Schulz, 1983) as a candidate for the group which must be protonated for catalysis of GSSG reduction and acts as a proton donor to the first product glutathione thiolate anion.

The V/K_{GSSG} pH profile decreases above pK values of 7.80 (pK_1) and 8.62 (pK_2). The V/K_{GSSG} profile represents the ionization behavior of groups on GSSG and free enzyme, in this case the two-electron-reduced enzyme, EH_2. The only ionizable groups on GSSG with pK values in this range are the α-amino group of the γ-glutamyl moiety. The pK of this amino group has been reported to be between 8.9 and 9.6 (Jung et al., 1972), and thus we tentatively identify pK_2 as being the GSSG α-amino group. The protonated group with pK of 7.80 observed in the V/K_{GSSG} profile must be an enzymatic side chain. Regardless of the chemical identity of the groups observed in the V and V/K pH profiles, it seems unlikely that the solvent kinetic isotope effects that we observe at pH 8.1 are due to solvent isotopic composition dependent pK changes of catalytically important groups, since in D_2O th pK values should be higher than in H_2O.

Solvent Kinetic Isotope Effects and Proton Inventories. By use of low-temperature, rapid-reaction studies it has been shown that the rate of reduction of the yeast enzyme by NADPH is 1.8 times faster than the rate of reduction of GSSG by EH_2 at 5 °C (Williams, 1976). The reductive half-reaction involves hydride transfer from the reduced pyridine nucleotide to FAD, followed by electron transfer to the catalytic disulfide, forming the thiol–thiolate pair. If a rate-limiting proton-transfer step to FAD during transient reduction or protonation of the cysteine thiolate anions formed during reduction of the enzymatic disulfide were occurring, we might observe solvent kinetic isotope effects greater than 1 on V/K when NADPH was used as the variable substrate. Solvent kinetic isotope effect measurements and proton inventories were performed with the yeast, spinach, and human erythrocyte enzymes by varying the NADPH concentrations at a fixed saturating GSSG concentration. Solvent kinetic isotope effects of greater than 1.0 on V, but not on V/K, were observed with all three enzymes, and all proton inventories were linear. $^{D_2O}V_{NADPH}$ for the yeast, spinach, and human erythrocyte enzymes are 1.9 ± 0.1, 2.8 ± 0.1, and 2.5 ± 0.1, respectively (Figure 3A).

The magnitude of the solvent kinetic isotope effects observed on V was similar with the three enzymes to those determined by using NADPH, when GSSG was used as the variable substrate, at saturating concentrations of NADPH. Proton inventories were linear and yielded $^{D_2O}V_{GSSG}$ = 1.9 ± 0.1 for the yeast enzyme, 2.5 ± 0.1 for the spinach enzyme, and 2.6 ± 0.1 for the human enzyme. The lack of a V/K solvent kinetic isotope effect observed when either NADPH or GSSG is the variable substrates does not allow us to localize this proton-transfer step within either of the two half-reactions. We therefore determined solvent kinetic isotope effects for symmetric and asymmetric alternate disulfide substrates.

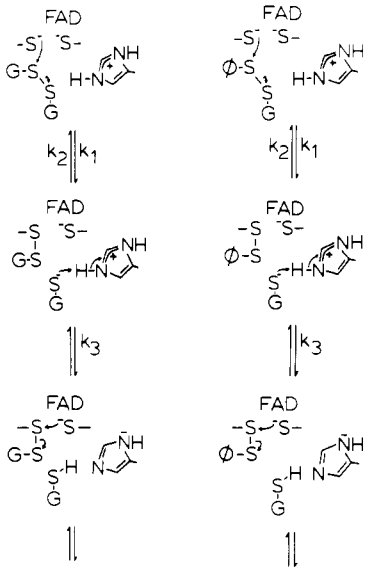
Since the crystal structure of the human erythrocyte enzyme has been determined, subsequent solvent kinetic isotope effect studies with alternate reducible substrates were performed by using the human enzyme. DTNB, a symmetric disulfide that is capable of stabilizing a developing negative charge on the sulfurs after reductive cleavage of the disulfide, was used as an alternate substrate in solvent kinetic isotope effect studies on the human enzyme. The predicted result is that this symmetric disulfide would not exhibit a solvent kinetic isotope effect. The data demonstrated that there was no solvent kinetic isotope effect when NADPH-dependent DTNB reduction was measured by using the human enzyme.

Solvent Kinetic Isotope Effects Using Asymmetric Disulfides. Proton inventories for both GSSNB and GSSNP are linear, and extrapolated V solvent kinetic isotope effects, ^{D_2O}V, are 3.4 ± 0.8 and 3.2 ± 1.2, respectively. These are within experimental error of the value for ^{D_2O}V seen when GSSG is used as substrate, 2.6 ± 0.1. These data suggest that the same rate-limiting proton transfer is being observed when these three substrates are used, and for these asymmetric disulfides, it is clear that the proton transfer is to the glutathione thiolate anion rather than to the thiophenolate. Since, a priori, we do not know the orientation of the binding of these asymmetric disulfides to the active site, the V solvent kinetic isotope effect data alone do not allow us to experimentally distinguish which of the two proton steps is observed in the proton inventory experiments.

The asymmetric disulfides uniquely exhibit V/K solvent kinetic isotope effects, and $^{D_2O}V/K$ is, within experimental error, equal to ^{D_2O}V for both GSSNB and GSSNP. We define two disulfide half-molecule binding sites, II and I [corresponding to the nomenclature of Pai and Schulz (1983)], where II is the site whose product will leave first and I is the site whose product will form an intermediate mixed enzyme–product disulfide. If GSSNB were bound in an orientation in which the glutathione portion was in site I, then thionitrobenzoate would be the first product released and the observed proton transfer must occur to the glutathione thiolate anion after or concomitant with Cys63 attack on the mixed Cys58–glutathione disulfide. Alternatively, if GSSNB were bound in an orientation in which the thionitrobenzoate portion was in site I, the glutathione thiolate anion would be the first product released, and the observed proton transfer would occur between the enzyme and the glutathione thiolate anion after or concomitant with Cys63 attack on the substrate. The presence of a $^{D_2O}V/K$ when GSSNB and GSSNP are used as substrates, and the approximate equality of this value with $^{D_2O}V_{GSSNB,GSSNP}$ and $^{D_2O}V_{GSSG}$, suggests that these substrates bind in the second orientation and that, for all substrates, the rate-limiting proton transfer is occurring to the glutathione anion in site II. Further, the disulfide interchange reaction which ruptures the substrate disulfide, releasing the glutathione thiolate anion, must be reversible for the solvent kinetic isotope effect to be seen on V/K. Finally, if GSSNB were bound in an orientation such that the thionitrobenzoate were the first product released, the proton-independent release of thionitrobenzoate would likely be an irreversible step, and one would predict that no $^{D_2O}V/K$ would be observed for asymmetric disulfide binding in that orientation.

Chemical Mechanism and Rate-Limiting Proton-Transfer Step. A chemical mechanism for GSSG and asymmetric disulfide reduction catalyzed by human erythrocyte glutathione reductase is shown in Scheme I. While we limit this discussion to the human enzyme, it is likely that this scheme describes protonic movements in the yeast and spinach enzymes as well, on the basis of the similarities of the solvent kinetic isotope effect behavior exhibited by all three enzymes. The overall rate-limiting step(s) has (have) been shown to occur in the second half-reaction for the yeast glutathione reductase (Williams, 1976), and our solvent kinetic isotope effect data, obtained by using NADPH as the variable substrate with all

Scheme I: Proposed Mechanism of Rate-Determining Proton-Transfer Step in the Oxidative Half-Reaction of Human Glutathione Reductase and Binding Orientation of Asymmetric Disulfides[a]



[a] The proton donor to GSH_{II} is shown as His467, on the basis of the proposal of Pai and Schulz (1983).

three enzymes, corroborate this interpretation and further localize the rate-limiting step as a single proton-transfer step. For GSSG and the human enzyme (left side of Scheme I), we propose that this rate-limiting step is the protonation of the first glutathione thiolate anion (by His467), k_3, after disulfide exchange and substrate disulfide cleavage, k_1. To explain the lack of a $^{D_2O}V/K$ when GSSG is the variable substrate, the substrate disulfide cleavage must precede proton transfer and the glutathione thiolate anion must partition predominantly toward protonation rather than toward nucleophilic attack on the mixed enzyme–glutathione disulfide, $k_3 \gg k_2$.[3]

When GSSNB or GSSNP are used as substrate for the human enzyme, the appearance of a $^{D_2O}V/K$ suggests that these substrates bind in the orientation shown in Scheme I (right side) and that substrate disulfide cleavage is reversible ($k_3 \sim k_2$). The released glutathione thiolate anion can partition toward protonation (perhaps by His467) or toward reformation of the substrate disulfide. The enzyme–substrate binding interactions that govern the binding orientations of the asymmetric disulfides at the active site of the human enzyme are unknown, but the aromatic ring of Tyr114 is positioned adjacent to the glutathione I binding site.

[3] The partitioning of the glutathione thiolate anion toward protonation versus attack on the enzyme–glutathione mixed disulfide need not be larger than 10 to reduce $^{D_2O}V/K$ to a value experimentally indistinguishable from 1.0.

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