# Kinetic Isotope Effect Analysis of the Reaction Catalyzed by *Trypanosoma congolense* Trypanothione Reductase†

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ABSTRACT: African trypanosomes are devoid of glutathione reductase activity, and instead contain a unique flavoprotein variant, trypanothione reductase, which acts on a cyclic derivative of glutathione, trypanothione. The high degree of sequence similarity between trypanothione reductase and glutathione reductase, as well as the obvious similarity in the reactions catalyzed, led us to investigate the pH dependence of the kinetic parameters, and the isotopic behavior of trypanothione reductase. The pH dependence of the kinetic parameters $V$, $V/K$ for NADH, and $V/K$ for oxidized trypanothione has been determined for trypanothione reductase from *Trypanosoma congolense*. Both $V/K$ for NADH and the maximum velocity decrease as single groups exhibiting p$K$ values of 8.87 ± 0.09 and 9.45 ± 0.07, respectively, are deprotonated. $V/K$ for oxidized trypanothione, $T(S)_2$, decreases as two groups exhibiting experimentally indistinguishable p$K$ values of 8.74 ± 0.03 are deprotonated. Variable magnitudes of the primary deuterium kinetic isotope effects on pyridine nucleotide oxidation are observed on $V$ and $V/K$ when different pyridine nucleotide substrates are used, and the magnitude of $^{D}V$ and $^{D}(V/K)$ is independent of the oxidized trypanothione concentration at pH 7.25. Solvent kinetic isotope effects, obtained with 2′,3′-cNADPH as the variable substrate, were observed on $V$ only, and plots of $V$ versus mole fraction of $D_2O$ (i.e., proton inventory) were linear, and yielded values of 1.3–1.6 for $^{D_2O}V$. Solvent kinetic isotope effects obtained with alternate pyridine nucleotides as substrates were also observed on $V$, and the magnitude of $^{D_2O}V$ decreases for each pyridine nucleotide as its maximal velocity relative to that of NADPH oxidation decreases. These data are compared to similar data obtained for glutathione reductase and other flavoprotein reductases.

Trypanothione reductase catalyzes the reduced β-nicotinamide adenine dinucleotide phosphate (NADPH)[1]-linked reduction of trypanothione, $N^1$,$N^8$-bis(glutathionyl)spermidine, $T(S)_2$, a glutathione analog unique to trypanosomatid parasites:

$$NADPH + H^+ + T(S)_2 \rightleftharpoons NADP^+ + T(SH)_2$$

The reduced trypanothione formed is capable of undergoing rapid nonenzymatic disulfide exchange with intracellular disulfides, including glutathione. This enzyme, therefore, serves the function of glutathione reductase in trypanosomatid parasites, the causative agents of African sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodiense*) and Chagas disease (*Trypanosoma cruzi*) in humans, and nagana (*Trypanosoma congolense* and *Trypanosoma brucei*) in cattle.

The enzymes from *Crithidia fasciculata*, *T. cruzi*, and *T. congolense* have been purified (Shames et al., 1986; Krauth-Siegel et al., 1987; Sullivan et al., 1989), and their genes have been isolated and sequenced (Sullivan & Walsh, 1991; Shames et al., 1988). The overexpressed *T. congolense* enzyme (Sullivan et al., 1989) is a dimer made up of identical 55-kDa subunits, each containing an FAD cofactor and a reducible active-site disulfide. Titration of the enzyme with 1 equiv of NADPH produces the two-electron-reduced enzyme, which exhibits the characteristic "charge transfer" spectrum (Sullivan et al., 1989) exhibited by other flavoprotein reductases such as glutathione reductase, lipoamide dehydrogenase, mercuric ion reductase, thioredoxin reductase, and NADH peroxidase [see Williams (1976) for a review]. Alignment and overlay of the primary sequence of *T. congolense* trypanothione reductase with that of human erythrocyte glutathione reductase reveal 41% amino acid sequence identity, with essentially all of the catalytically important residues conserved between the two enzymes (Shames et al., 1988; Karplus et al., 1989; Karplus & Schulz, 1989). The active-site disulfide, which functions in the reduced form in the temporary storage of electrons and in the reduction of trypanothione, is composed of Cys52 and Cys57. The carboxyl-terminal cysteine residue, Cys57, by analogy with glutathione reductase, is thought to participate in the "charge transfer" interaction with the flavin

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[1] Abbreviations: $T(S)_2$, oxidized trypanothione; $T(SH)_2$, reduced trypanothione; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NHDPH, reduced β-nicotinamide hypoxanthine dinucleotide phosphate; tNADPH, reduced β-thionicotinamide adenine dinucleotide phosphate; 3APADPH, reduced β-3-acetylpyridine adenine dinucleotide phosphate; 3′NADPH, reduced β-3′-nicotinamide adenine dinucleotide phosphate; 2′,3′-cNADPH, reduced β-2′,3′-cyclic nicotinamide adenine dinucleotide phosphate; NADH, reduced β-nicotinamide adenine dinucleotide; tNADH, reduced β-thionicotinamide adenine dinucleotide; NHDH, reduced β-nicotinamide hypoxanthine dinucleotide; NGDH, reduced β-nicotinamide guanine dinucleotide; 3APADH, reduced β-3-acetylpyridine adenine dinucleotide; ATP, adenosine triphosphate; G6P, glucose 6-phosphate; GSSG, oxidized glutathione; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TEA-HCl, triethanolamine hydrochloride; PIPES, 1,4-piperazinediethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; FPLC, fast protein liquid chromatography.

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cofactor in the reduced form of the enzyme, while Cys52 is thought to participate in disulfide interchange. His461′, by analogy with His467′ in glutathione reductase, is the active-site base, and Glu466′ hydrogen-bonds to His461′ and orients the imidazole ring for effective catalysis (Karplus & Schulz, 1987; Wong & Blanchard, 1989). Crystals of the *T. congolense* (Sullivan et al., 1990) and *T. cruzi* (Krauth-Siegel et al., 1987) trypanothione reductases have been produced, and a three-dimensional structure of the *C. fasciculata* enzyme reveals that these catalytically important residues are located in positions identical to that of human glutathione reductase (Kuriyan et al., 1991).

The obvious structural and mechanistic similarities between trypanothione reductase and glutathione reductase have encouraged us to perform the studies reported here to evaluate the groups involved in catalysis, and rate-limiting steps in the reaction catalyzed by *Trypanosoma congolense* trypanothione reductase.

## Experimental Procedures

*Materials.* The cloned, overexpressed trypanothione reductase from *T. congolense* was isolated as previously described (Sullivan et al., 1989). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), hexokinase from yeast, glutathione reductase from bakers' yeast, NADH peroxidase from *Streptococcus faecalis*, D-[1-$^2$H]glucose (97 atom % $^2$H), all oxidized pyridine nucleotides, ATP, G6P, GSSG, $H_2O_2$, and all buffer components were purchased from Sigma. Oxidized trypanothione was purchased from Bachem (Philadelphia, PA). D-[1-$^3$H]Glucose was purchased from Amersham. $D_2O$ (>99.8 atom % excess) was from Cambridge Isotope Laboratory and was distilled (bp 101 °C, uncor) before use.

*Preparation of (4S)-Labeled Reduced Nucleotides.* The oxidized forms of all nucleotides were purified using a Mono Q (Pharmacia) anion-exchange column as previously described (Orr & Blanchard, 1984). All (4*S*)-protonated, -deuterated, and -titrated nucleotides were prepared by enzymatic reduction of the oxidized nucleotides with *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase using [1-$^1$H]-, [1-$^2$H]-, or [1-$^3$H]G6P, respectively, as previously described (Vanoni et al., 1990). For each nucleotide, fractions with the appropriate absorbance ratios (i.e., tNADH, tNADPH, $A_{260}/A_{395} \leq 1.3$; 3APADH, $A_{260}/A_{363} \leq 1.5$; NHDH, NHDPH, $A_{249}/A_{340} \leq 2.0$; NADH, NGDH, NADPH, 2′,3′-cNADPH, 3′NADPH, $A_{260}/A_{340} \leq 2.3$) were pooled. The concentrations of the phosphorylated and nonphosphorylated nucleotides were determined by enzymatic end-point assays with bakers' yeast glutathione reductase or *S. faecalis* NADH peroxidase, respectively.

*pH Profiles.* The determination of pH profiles was performed as described by Wong and Blanchard (1989); 300 mM aliquots of the following stock buffers were prepared by titrating their acid forms to the desired pH with KOH, and filtered through a 0.22-μm Millipore filter. The buffers were used in 100 mM concentrations at the stated pH in the assays: PIPES (6.2–7.0), TAPS (7.8–8.9), CHES (9.3–9.5), CAPSO (9.9–10.2), CAPS (10.5). Trypanothione reductase reaction rates were determined at the pH values stated by varying the concentration of $T(S)_2$ or NADH at a fixed, saturating concentration of the other substrate. Initial velocities were measured by monitoring the oxidation of NAD(P)H at 340 nm ($\epsilon_{340}$ = 6220 $M^{-1}$ $cm^{-1}$) on a Gilford Model 260 spectrophotometer equipped with thermospacers attached to a constant-temperature circulating water bath maintained at 25 °C. The kinetic parameters $V$, $V/K$ for $T(S)_2$, and $V/K$ for NADH were determined at each pH, and their log values plotted against pH, as determined by the insertion of a combined microelectrode into the curvette after each assay was performed.

*Primary Deuterium Kinetic Isotope Effects.* Initial velocity experiments were performed in 1-mL cuvettes containing 10 μM DTNB, fixed, saturating concentrations of $T(S)_2$ (75 μM), and varied concentrations of (4*S*)-4-$^1$H- or (4*S*)-4-$^2$H-labeled reduced pyridine nucleotide. Trypanothione reductase was assayed by monitoring the reduction of DTNB at 412 nm ($\epsilon_{412}$ = 13 600 $M^{-1}$ $cm^{-1}$; Ellman, 1959).

*Determination of Primary Tritium Kinetic Isotope Effects.* A 4-mL reaction mixture containing 80 μM [(4*S*)-4-$^3$H]-2′,3′-cNADPH and 375 μM $T(S)_2$ in 10 mM TEA-HCl buffer, pH 7.25, was thermally equilibrated at 25 °C. Several 100-μL aliquots were removed from the reaction mixture, and $^3H_2O$ was separated from 2′,3′-cNADP$^+$ and [(4*S*)-4-$^3$H]-2′,3′-cNADPH on a Mono Q column, equilibrated with 10 mM TEA-HCl buffer, pH 7.25, and eluted with a 0–1 M NaCl gradient. Two-milliliter fractions were collected directly into 20-mL scintillation vials, to which 15 mL of Hydrofluor (National Diagnostics) scintillation cocktail was added. The radioactivity of each fraction was quantitated using an LKB Rack Beta liquid scintillation counter to obtain the nonenzymatic background. At various time points after the addition of enzyme (5–35% conversion), 100-μL aliquots were removed, and labeled products were separated as described above. After 35% conversion, additional enzyme and 120 μM DTNB were added to ensure complete oxidation of 2′,3′-cNADPH. Several 100% conversion aliquots were analyzed for complete oxidation of [(4*S*)-4-$^3$H]-2′,3′-cNADPH. Fractions containing $^3H_2O$ were counted at least 5 times and were corrected for nonenzymatic release of $^3$H into solvent. The primary tritium kinetic isotope effect, $^T(V/K)$, was calculated using eq 1 where $f$ is

$$^T(V/K) = \log (1.0 - f)/[\log (1.0 - DPM_f/DPM_{100})] \quad (1)$$

the fractional percent conversion of 2′,3′-cNADPH to 2′,3′-cNADP$^+$, $DPM_f$ is the dpm of $^3H_2O$ at $f$, and $DPM_{100}$ is the dpm of $^3H_2O$ at 100% conversion (Blanchard & Englard, 1983).

*Solvent Kinetic Isotope Effects.* The 300 mM TAPS buffers were prepared in $H_2O$ and 99.8% $D_2O$ at pH or pD (pH reading + 0.4) 7.25 by titrating with KOH dissolved in $H_2O$ or $D_2O$; 2 M TAPS buffer was prepared in $H_2O$ at pH 7.0. Buffers were used in 100 mM concentrations for enzyme assay. All substrate solutions were prepared daily; $T(S)_2$ concentrations were calibrated by end-point assay using *T. congolense* trypanothione reductase, and reduced pyridine nucleotide concentrations were calibrated by end-point assay using bakers' yeast glutathione reductase.

Reaction mixtures containing $T(S)_2$, pyridine nucleotide, and the desired mole fraction of $D_2O$ were prepared volumetrically from $H_2O$ and $D_2O$ buffer stock solutions. Initial velocities were measured at various mole fractions of $D_2O$ either by varying the 2′,3′-cNADPH concentration at a fixed, saturating $T(S)_2$ concentration or by using saturating concentrations of both $T(S)_2$ and reduced pyridine nucleotide. The kinetic parameters $V$ and/or $V/K$ were determined at each mole fraction of $D_2O$. The calculated $V$ values were plotted against mole fraction of $D_2O$, and the resulting proton inventories were used to determine the solvent kinetic isotope effect on $V$, $^{D_2O}V$.

*Data Analysis.* Reciprocal initial velocities from steady-state experiments were plotted against the reciprocal of the variable substrate concentrations, and the data were fitted to the appropriate rate equations using the FORTRAN programs

of Cleland (1979). The individual saturation curves were fitted to eq 2. Data for pH profiles that showed a decrease in log $V$ or log $V/K$ with a slope of −1.0 as the pH was increased were fitted to eq 3. Data for pH profiles that showed a decrease in log $V/K$ with a slope of −2.0 as the pH was increased were fitted to eq 4. In eq 3 and 4, $y$ is the parameter to be fitted, and $C$ is the pH-independent plateau value.

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

$$\log y = \log [C/(1.0 + K/[H^+])] \quad (3)$$

$$\log y = \log [C/(1.0 + K_1[H^+] + K_1K_2/[H^+]^2)] \quad (4)$$

Isotope effects on the oxidation of reduced pyridine nucleotides were fitted to eq 5–8, which assume equal isotope effects on $V$ and $V/K$, different isotope effects on $V$ and $V/K$,

$$v = VA/[(K + A)(1.0 + F_iE)] \quad (5)$$

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

$$v = VA/[K + A(1.0 + F_iE_V)] \quad (7)$$

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

isotope effects on $V$ only, and isotope effects on $V/K$ only. In eq 2 and 5–8, $A$ is the variable substrate concentration, $F_i$ is the fraction of deuterium label ($F_i$ = 0.0 and 1.0 for hydrogen- and deuterium-containing pyridine nucleotide substrates, respectively), $E$ is the isotope effect minus 1 on $V$ and $V/K$, $E_{V/K}$ is the isotope effect minus 1 on $V/K$, and $E_V$ is the isotope effect minus 1 on V.

Solvent kinetic isotope effects, using reduced pyridine nucleotide as the variable substrate, were fitted to eq 7 where $F_i$ is the mole fraction of $D_2O$ solvent. Solvent kinetic isotope effects, obtained at saturating concentrations of reduced pyridine nucleotide and $T(S)_2$, were determined by linear regression analysis of the experimental $V$ values against $F_i$.

## Results

*Effect of pH on the Kinetic Parameters.* The pH profile shown in Figure 1 (top) was obtained when NADH concentration was varied at a fixed, saturating concentration of $T(S)_2$ (150 μM, $K_m$ = 14.9 μM) at pH values between 5.8 and 10.7. NADH ($K_m$ = 48 μM) was used because the low $K_m$ value for the natural substrate NADPH ($K_m$ = 0.88 μM) made it difficult to accurately determine $V/K$ for this substrate. A decrease in activity is observed at high pH values as a single group with a p$K$ value of 8.87 ± 0.09 is deprotonated.

The middle and bottom pH profiles shown in Figure 1 were obtained when the concentration of $T(S)_2$ was varied at saturating concentrations of NADPH at pH values between 6.2 and 10.2. The maximum velocity (bottom) decreases at high pH values as a single group with a p$K$ vlaue of 9.45 ± 0.07 is deprotonated. The experimental data for the pH dependence of $V/K$ for $T(S)_2$ were fitted to both eq 3 and eq 4. The profile was best fit to eq 4, and shows a decrease in activity as two groups exhibiting experimentally indistinguishable p$K$ values of 8.74 ± 0.03 are deprotonated. All subsequent studies were performed at pH 7.25, a pH value where no pH-dependent changes in any of the kinetic parameters were observed.

*Kinetic Parameters and Primary Kinetic Isotope Effect for Reduced Pyridine Nucleotide Substrates.* The effect of trypanothione concentration on the primary deuterium kinetic isotope effect exhibited by the alternate pyridine nucleotide 2′,3′-cNADP(H,D) was determined at pH 7.25. Parallel reciprocal velocity patterns were observed when either reciprocal concentrations of [(4$S$)-4-$^1$H]- or [(4$S$)-4-$^2$H]-2′,3′-cNADPH

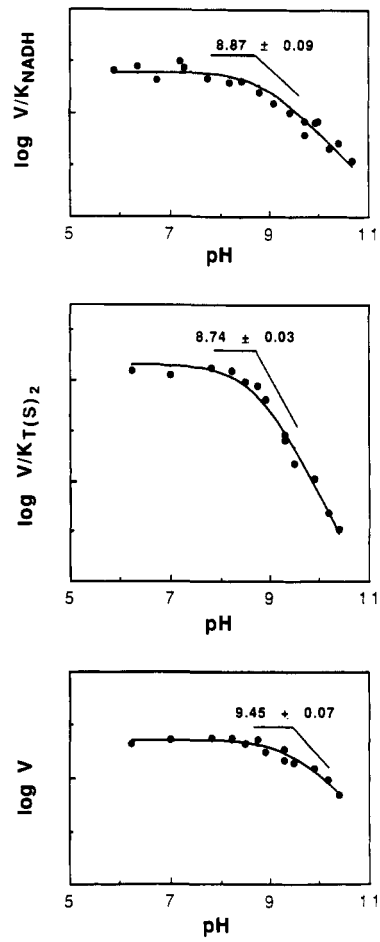



FIGURE 1: Effect of pH on the kinetic parameters $V$, $V/K$ for NADH, and $V/K$ for $T(S)_2$. The individual experimental points are fits of the data to eq 2, and the smooth curves for the pH profiles $V$ and $V/K$ for NADH are fits of the data to eq 3. The $V/K$ pH profile for $T(S)_2$ was fit to eq 4. The $V/K$ profile for NADH (top) decreases as a single enzymic group with a p$K$ value of 8.87 ± 0.09 is deprotonated. The $V/K$ profile for $T(S)_2$ (middle) decreases as two groups with experimentally indistinguishable p$K$ values of 8.74 ± 0.03 are deprotonated. The $V$ profile (bottom) shows a decrease in activity as a group exhibiting a p$K$ value of 9.45 ± 0.07 is deprotonated.

were varied (data not shown), suggesting a ping-pong kinetic mechanism for trypanothione reductase. The experimentally determined kinetic isotope effects are essentially invariant when the concentration of $T(S)_2$ is varied between 15 and 180 μM (data not shown). All subsequent kinetic isotope effect studies were performed in the presence of 75 μM $T(S)_2$.

Kinetic parameters and primary deuterium kinetic isotope effects were determined for various reduced pyridine nucleotide substrates and are presented in Table I. Initial velocities of (4$S$)-4-$^1$H- and (4$S$)-4-$^2$H-labeled reduced pyridine nucleotide oxidation were fitted to eq 5–8, and the best fit for each pyridine nucleotide was chosen primarily on the basis of visual inspection of the experimental data and the calculated fits, and also on the lowest value of the standard errors of the kinetic parameters. Nonunitary primary deuterium kinetic isotope effects were observed on $V$ and $V/K$ for all substrates tested at pH 7.25, except NHDPH and NADPH. Primary deuterium kinetic isotope effects with 2′,3′-cNADPH were obtained under two different conditions: in 100 mM HEPES, pH 7.25, and in 10 mM TEA-HCl, pH 7.25. Since no large differences in the magnitude of the isotope effect were observed under these conditions (2.85 ± 0.07 versus 2.68 ± 0.12, respectively), comparisons between deuterium and tritium $V/K$ kinetic isotope effects for 2′,3′-cNADPH oxidation under

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

| nucleotide substrate | redox potential (mV) | rel $V$ (%) | $K_m$ ($\mu$M) | $^{D}V$ | $^{D}(V/K)$ |
|---|---|---|---|---|---|
| NHDPH | −320 | 152 | 0.81 ± 0.04 | 1.16 ± 0.02 | 0.95 ± 0.05[b] |
| NADPH | −320 | 100 (def.) | 0.88 ± 0.05 | 1.01 ± 0.01 | 1.01 ± 0.01[c] |
| tNADPH | −285 | 35.6 | 1.0 ± 0.2 | nd[d] | nd |
| 3′NADPH | −320 | 1.26 | 6.3 ± 0.9 | 1.97 ± 0.12 | 4.90 ± 0.14[b] |
| 3APADPH | −258 | 12.5 | 10.7 ± 0.6 | nd | nd |
| NADH | −320 | 2.76 | 47.9 ± 6.4 | 1.45 ± 0.01 | 1.45 ± 0.01[c] |
| 2′,3′-cNADPH | −320 | 8.44 | 103.9 ± 10.2 | 2.85 ± 0.07 | 2.85 ± 0.07[c] |
| tNADH | −285 | 0.34 | 100.6 ± 0.7 | 4.67 ± 1.04 | 6.90 ± 0.94[b] |
| NHDH | −320 | 1.79 | 142.5 ± 15.1 | 1.27 ± 0.06 | 2.45 ± 0.11[b] |
| NGDH | −320 | 1.64 | 187.0 ± 7.2 | 2.30 ± 0.03 | 2.30 ± 0.03[c] |
| 3APADH | −258 | 0.11 | 535.0 ± 33.4 | 4.06 ± 0.72 | 6.89 ± 0.70[b] |

[a] All data were obtained at 25 °C in 100 mM $K^+$ HEPES, 75 $\mu$M $T(S)_2$, and 10 $\mu$M DTNB, pH 7.25. $K_{m,T(S)_2}$ = 14.9 ± 0.2 $\mu$M under these conditions. Maximal velocities were determined to ±5%. [b] Fitted to eq 6 where $^{D}V$ and $^{D}(V/K)$ are not equal. [c] Fitted to eq 5 where $^{D}V$ and $^{D}(V/K)$ are equal. [d] Not determined.

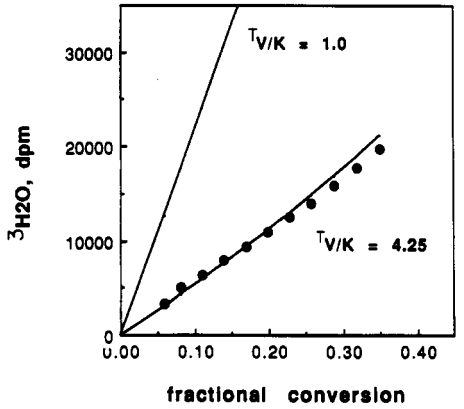



FIGURE 2: Primary $^{T}(V/K)$ measured by analyzing the $^3H_2O$ formed in the trypanothione reductase reaction. The *T. congolense* enzyme was incubated in a system containing [(4*S*)-4-$^3$H]-2′,3′-cNADPH, and at the intervals indicated, aliquots were removed, and $^3H_2O$ was isolated and counted. The experimental points are shown, and the line drawn is a fit of the data to eq 1 with $^{T}(V/K)$ = 4.25. A fit with $^{T}(V/K)$ = 1.0 is shown for comparison.

identical reaction conditions (i.e., 10 mM TEA-HCl, pH 7.25) were made.

*Primary Tritium Kinetic Isotope Effects.* The primary tritium kinetic isotope effect using [(4*S*)-4-$^3$H]-2′,3′-cNADPH as reductant was determined by measuring the transfer of tritium to the flavin, and subsequent exchange into solvent, at various percentages of reaction. $^3H_2O$ was separated from unreacted [(4*S*)-4-$^3$H]-2′,3′-cNADPH by anion-exchange chromatography on a Mono Q column. Multiple determinations of $^{T}(V/K)$ for 2′,3′-cNADPH were made by comparing the amount of $^3H_2O$ released at low (<35%) and high (100%) percentages of reaction. A value of 4.25 ± 0.32 was calculated for $^{T}(V/K)$ for 2′,3′-cNADPH as seen in Figure 2.

*Solvent Kinetic Isotope Effects.* Solvent kinetic isotope effect measurements were performed with trypanothione reductase between pH(D) 7.0 and 7.25. When 2′,3′-cNADPH was used as the variable substrate at a fixed, saturating concentration of $T(S)_2$ at pH 7.25, a solvent kinetic isotope effect on $V$, but not on $V/K$, was observed as the mole fraction of $D_2O$ was varied (data not shown). When the experimental data were fitted to eq 7, a linear proton inventory was obtained which yielded a $^{D_2O}V$ solvent kinetic isotope effect value of 1.30 ± 0.02 for 2′,3′-cNADPH.

Since only $^{D_2O}V$ isotope effects are observed using 2′,3′-cNADPH as the variable substrate for trypanothione reductase, subsequent solvent kinetic isotope effect measurements were performed at pH 7.0 using saturating concentrations of both $T(S)_2$ and pyridine nucleotide. Plots of $V$ values against the mole fraction of $D_2O$ were essentially linear for all pyridine nucleotides studied, and the magnitude of $^{D_2O}V$ in each case was determined from the ratio of the $V$ values at $F_i$ = 0.0 and 1.0. Values obtained for $^{D_2O}V$ correlated with the maximum velocities exhibited by each pyridine nucleotide substrate (Table II).

Table II: Dependence of the Solvent Kinetic Isotope Effect on the Rate of the Reductive Half-Reaction[a]

| nucleotide | $K_m$ ($\mu$M) | rel $V$ (%) | $^{D_2O}V$ |
|---|---|---|---|
| NHDPH | 0.81 | 152 | 3.81 ± 0.27 |
| NADPH | 0.88 | 100 (def.) | 3.60 ± 0.18 |
| tNADPH | 1.0 | 35.6 | 2.60 ± 0.18 |
| 3APADPH | 4.2 | 12.5 | 2.36 ± 0.10 |
| 2′,3′-cNADPH | 104 | 8.44 | 1.64 ± 0.07 |
| 3′NADPH | 6.3 | 1.26 | 1.60 ± 0.04 |

[a] All data were obtained at 25 °C in 100 mM $K^+$HEPES, 150 $\mu$M $T(S)_2$, and 100 or 200 $\mu$M pyridine nucleotide, pH 7.0. Plots of $V$ versus mole fraction of $D_2O$ were drawn for each pyridine nucleotide, and $^{D_2O}V$ values were determined by linear regression of the experimental data. Errors were determined by propagating the errors of the slope and the $V$ values at mole fraction $D_2O$ = 0.0 and 1.0.

## Discussion

Trypanothione reductase plays a key role in oxidative stress management in the parasitic trypanosomatids *T. cruzi*, *T. congolense*, *C. fasciculata*, and *Leishmania*, and is an obvious target for rational drug design due to its unique substrate specificity. Sequence comparisons with the related host enzyme glutathione reductase suggest that essentially all of the catalytically important residues are retained in trypanothione reductase. The wealth of structural (Karplus et al., 1989; Karplus & Schulz, 1989), mechanistic (Williams, 1976; Wong & Blanchard, 1989), and isotopic (Wong et al., 1988; Vanoni et al., 1990) studies on glutathione reductase and the recent report of the 2.4-Å resolution structure of the *C. fasciculata* trypanothione reductase (Kuriyan et al., 1991) make the present comparison with the *T. congolense* trypanothione reductase appropriate and pertinent.

*pH Profiles of the Kinetic Parameters.* Due to the submicromolar $K_m$ value exhibited for NADPH by trypanothione reductase, we elected to perform the analysis of the pH dependence of $V/K$ using NADH. In the related glutathione reductase, it has been shown that NADPH and NADH exhibit similar $V/K$ pH profiles (Wong & Blanchard, 1989). The $V/K$ pH profile for NADH decreases at high pH as a group with a p$K$ value of 8.87 ± 0.09 is deprotonated. This pH dependence reflects the ionization behavior of a group on either NADH or free oxidized enzyme which affects binding of NADH to enzyme. Since NADH does not have any titratable groups in this pH range, the $V/K$ pH profile for NADH must reflect the deprotonation of a group on the free enzyme. In human erythrocyte glutathione reductase, a group exhibiting a similar p$K$ value of 8.8, whose deprotonation decreases $V/K$

for NADH, has been attributed (Wong & Blanchard, 1989) to an arginine residue (Arg218) which makes extensive contacts with the adenine and adenosyl ribose rings of bound pyridine nucleotide (Pai et al., 1988). The p$K$ value observed for trypanothione reductase may similarly be attributed to a conserved arginine residue (Arg182) in the pyridine nucleotide binding domain of the enzyme.

The $V/K$ pH profile for $T(S)_2$ decreases at high pH as two groups exhibiting indistinguishable p$K$ values of 8.74 ± 0.03 are deprotonated. This p$K$ may reflect the ionization of groups on $T(S)_2$ or on the two-electron-reduced enzyme which are important in $T(S)_2$ binding to $EH_2$. This $V/K$ pH profile for $T(S)_2$ is similar to that previously reported for glutathione reductase, and in that case, two groups exhibiting p$K$ values of 7.8 and 8.6 were reported to be protonated for optimal binding (Wong et al., 1989). The interactions between bound GSSG and human glutathione reductase have been reported, as have kinetic parameters for glutathione analogs (Janes & Schulz, 1990). These data suggest that the most important binding interaction occurs between the $\alpha$-amino group of GluII and Glu473′. The solution p$K$ values of the $\alpha$-amino groups of GSSG have been reported to be 8.7–9.2 (Jung et al., 1972), and thus one of the groups whose deprotonation is likely to be observed in the $V/K$ pH profiles for GSSG and $T(S)_2$ is the $\alpha$-amino group of GSSG and $T(S)_2$, respectively. A second important interaction in glutathione reductase occurs between the $\alpha$-carboxyl group of GluII and Lys67, which is conserved in trypanothione reductase. Deprotonation of this enzymic group would presumably decrease the affinity of both glutathione reductase and trypanothione reductase for their respective substrates. It is important to note that the p$K$ values observed in these $V/K$ pH profiles are not necessarily intrinsic p$K$ values but may be displaced by the commitment to catalysis of these substrates (Cleland, 1990).

The $V$ pH profile for trypanothione reductase decreases as a single group exhibiting a p$K$ value of 9.45 ± 0.07 is deprotonated, resulting in a loss of catalytic activity. In human erythrocyte glutathione reductase, a group with a p$K$ value of 9.2, whose deprotonation decreases $V$, was suggested to be the His467′–Glu472′ ion pair (Wong & Blanchard, 1989) which is close to the sulfur atom of the bound glutathione disulfide (Janes & Schulz, 1990), and is responsible for the rate-limiting protonation of the first glutathione thiolate anion product (Wong et al., 1988). The group that is observed in the $V$ profile exhibited by trypanothione reductase may similarly be attributed to the conserved His461′–Glu466′ ion pair in trypanothione reductase, and it presumably functions as a proton donor to trypanothione during disulfide cleavage in the oxidative half-reaction.

*Kinetic Parameters of Reduced Pyridine Nucleotide Substrates.* All steady-state kinetic studies have been performed between pH 7.0 and 7.25, where there are no pH-dependent changes in the maximum velocity or the $V/K$ values for substrates. The presence of a phosphate group at the adenosyl-ribose moiety of the pyridine nucleotide is important in the binding of the substrate to the enzyme. This is demonstrated by the dramatic increase in the $K_m$ values for non-phosphorylated nucleotides as reductants when compared to NADPH or NHDPH. Interestingly, the isomeric 3′-monophosphate 3′NADPH binds surprisingly tightly, and can clearly be accommodated in the pyridine nucleotide binding pocket, yet is an ineffective reductant as evidenced by its relative maximal velocity (~1%). The cyclic derivative 2′,3′-cNADPH exhibits a $K_m$ value greater than 100 times higher than the 2′-monophosphate substrates, and 16 times higher than the 3′-monophosphate substrate. The 2′-dephospho substrates NADH, NHDH, and NGDH, which differ only in the nature of the substituents on the adenosyl ring, exhibit high $K_m$ values which are 50–200 times higher than NADPH. The 2′-dephosphopyridine nucleotide substrates tNADH and 3APADH, which differ in the nature of the substituents on the nicotinamide ring, and therefore, redox potential, exhibit even larger $K_m$ values. The $V_{max}$ value exhibited by NHDPH is 150% that of NADPH, perhaps reflecting the faster rate of dissociation of $NHDP^+$ compared to $NADP^+$. All other pyridine nucleotide substrates exhibit maximum velocities which are from 3 to 900 times slower than NADPH.

*Modeling of the Reaction Mechanism.* The trypanothione reductases which have been purified from *C. fasciculata* (Shames et al., 1986), *T. cruzi* (Krauth-Siegel et al., 1987), and *T. congolense* (Sullivan et al., 1989) have been shown to undergo two-electron reduction to form a stable intermediate, termed $EH_2$, with the spectroscopically identifiable signature of a charge-transfer complex between an enzymic thiolate and oxidized flavin. In common with other flavoprotein reductases, such as glutathione reductase, lipoamide dehydrogenase, and mercuric reductase, enzyme reduction by reduced pyridine nucleotides results in the reductive cleavage of an enzymic disulfide to form a pair of cysteine thiols (Cys52 and Cys57 in *T. congolense* trypanothione reductase). These data, in conjunction with studies which demonstrate that the initial velocity pattern obtained using 2′,3′-cNADPH and $T(S)_2$ is parallel, support the ping-pong kinetic mechanism for trypanothione reductase:

$$E_{ox} + NADPH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot NADPH \underset{k_4^*}{\overset{k_3^*}{\rightleftharpoons}} E_{red}\cdot NADP^+ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_{red} + NADP^+$$

$$E_{red} + T(S)_2 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_{red}\cdot T(S)_2 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_{ox}\cdot T(SH)_2 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_{ox} + T(SH)_2$$

Using the net rate constant method (Cleland, 1975), the expressions for $V$ and $V/K$ for NADH can be determined, and from these, the appropriate expressions for the primary deuterium kinetic isotope effects exhibited by [(4$S$)-4-$^2$H]-NADPH on $V$ ($^DV$) and $V/K$ for NADPH [$^D(V/K)_{NADPH}$] can be shown to be

$$^D(V/K)_{NADPH} = \frac{^Dk_3 + c_f + c_r{}^DK_{eq}}{1.0 + c_f + c_r}$$

$$^DV = \frac{^Dk_3 + c_{Vf} + c_r{}^DK_{eq}}{1.0 + c_{Vf} + c_r}$$

where $c_f = k_3/k_2$, $c_r = k_4/k_5$, $c_{Vf} = k_3/k_5 + k_3/k_{11} + k_3/k_9(1.0 + k_{10}/k_{11})$, and $^DK_{eq} = k_{3H}k_{4D}/k_{3D}k_{4H}$ and has a value of 0.98 (Cleland, 1980). Asterisked rate constants represent the isotope-sensitive steps when (4$S$)-4-$^2$H-labeled reduced pyridine nucleotide substrates are used. 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 effect on $V$ or $V/K$.

*Primary Deuterium and Tritium Kinetic Isotope Effect Analysis.* When primary deuterium kinetic isotope effects were determined using (4$S$)-4-$^1$H-labeled and (4$S$)-4-$^2$H-labeled NADPH or NHDPH as variable substrates, small $^DV$'s were observed, with no statistically significant $^D(V/K)$ (Table I). These findings are similar to previous results obtained by Sullivan et al. (1991) for trypanothione reductase and those observed for human erythrocyte glutathione using NADPH

as substrate (Vanoni et al., 1990). The lack of an isotope effect on $V/K$ is caused by large values for the forward ($c_f$) and/or reverse ($c_r$) commitment factors for these substrates. A large value for the reverse commitment ($c_r = k_4/k_5$) can result from the slow dissociation of product ($k_5$), and the faster maximum velocity exhibited by NHDPH compared to NADPH suggests that $NADP^+$ release might be partially rate-limiting. A large $c_f$ term ($k_3/k_2$) would suggest that both NADPH and NHDPH partition through catalysis effectively, and only slowly dissociate from the E·nucleotide complex. This is supported by the submicromolar $K_m$ values exhibited by NADPH and NHDPH (Table I). Small isotope effects were also observed on $V$ for these two substrates, and it would appear that suppression of the intrinsic deuterium kinetic isotope effect is likely due to a large forward commitment, $c_{Vf}$. A large $c_{Vf}$ term would suggest that the rate of enzyme reduction by NADPH or NHDPH ($k_3$) is faster than the rate of enzyme oxidation by $T(S)_2$ ($k_9$), as is the case for glutathione reductase (Vanoni et al., 1990) and NADH peroxidase (Stoll & Blanchard, 1991). We have obtained additional evidence in support of this conclusion, as described below.

Isotope effects on both $V$ and $V/K$ have been measured for other pyridine nucleotide substrates with redox potentials of −320 mV (Table I). The larger values of $^D(V/K)$ suggest that these alternate pyridine nucleotides dissociate from the E· nucleotide complex more rapidly than they transfer hydride ion to the flavin. There is a reasonable correlation between the steady-state $K_m$ values and the magnitude of $^D(V/K)$ exhibited by each nucleotide, suggesting that $c_f$, rather than $c_r$, is the major contributor to the expression of $^D(V/K)$. Of particular interest, the magnitude of $^D(V/K)_{3'NADPH}$ is unusually large when compared to the magnitude of $^D(V/K)$ exhibited by the other pyridine nucleotides (4.9 versus ≤2.8). This large value may be a reflection of the differential orientation of the nicotinamide ring of this substrate in the precatalytic complex as the result of the phosphate group being in the 3′-position rather than the 2′-position; this will be further discussed below. For all these pyridine nucleotides tested, the experimentally determined values of $^DV$ are statistically greater than unity. The maximum velocities exhibited by these pyridine nucleotide substrates are 12 to 900 times slower than the physiologically relevant substrate, NADPH. Since the rate of the oxidative half-reaction ($k_9$) is insensitive to the chemical nature of the hydride ion-donating nucleotide, these data suggest that the rate of the reductive half-reaction ($k_3$) is specifically, and incrementally, attenuated when increasingly slower pyridine nucleotide substrates are used. For both the related flavoproteins human erythrocyte glutathione reductase (Vanoni et al., 1990) and NADH peroxidase (Stoll & Blanchard, 1991), evidence has been presented which suggests that the dominant term in $c_{Vf}$ is $k_3/k_9$, the ratio of the catalytic rate constants for enzyme reduction by pyridine nucleotide substrate and enzyme oxidation by disulfide substrate. These isotope effect data for trypanothione reductase are entirely consistent with this interpretation, and additional evidence will be presented below which confirms this interpretation.

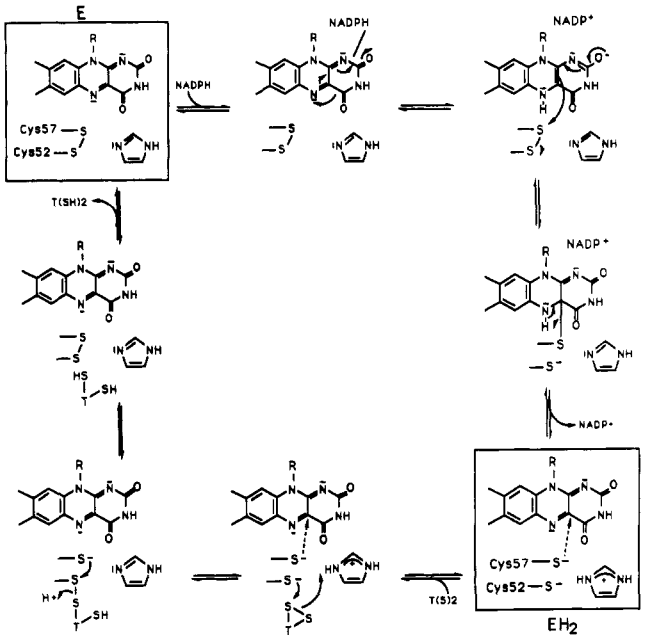
One prediction of the analysis presented above is that for substrates in which the reductive half-reaction has been slowed sufficiently to become predominantly or completely rate-limiting, the primary deuterium and tritium kinetic isotope effects on $V/K$ should be related by the Swain–Schaad approximation ($^Tk = {}^Dk^{1.442}$; Swain et al., 1958). We chose to perform tritium kinetic isotope effects using 2′,3′-cNADPH, since its $K_m$ value is greater than 100 times the corresponding value for NADPH, the maximum velocity is reduced 12-fold from NADPH, and its redox potential is identical to the redox potential of NADPH. The experimentally determined value of the primary tritium kinetic isotope effect [$^T(V/K)_{2',3'\text{-cNADPH}} = 4.25 \pm 0.32$] is statistically indistinguishable from the value calculated from the primary deuterium kinetic isotope effect [$^D(V/K)_{2',3'\text{-cNADPH}} = 2.85 \pm 0.07$] using the Swain–Schaad relationship ($2.85^{1.442} = 4.52$) obtained under the same experimental conditions. This agreement between the calculated and experimental values of the primary tritium kinetic isotope effect suggests that the intrinsic kinetic isotope effect on hydride transfer is observed in this experiment and that the reductive half-reaction is largely rate-limiting. The magnitude of this intrinsic kinetic isotope effect is similar to that determined for the yeast glutathione reductase (2.7) from steady-state and pre-steady-state studies (Vanoni et al., 1990). The observation that $^D(V/K)_{3'NADPH}$ is greater than $^D(V/K)_{2',3'\text{-cNADPH}}$, however, suggests that the intrinsic isotope effect for hydride transfer is greater for 3′NADPH due to its presumably different orientation in the active site. Experiments are underway to directly determine the intrinsic kinetic isotope effect for hydride transfer.

The largest primary deuterium kinetic isotope effects were observed using nucleotide substrates whose redox potentials are more positive than NADPH. The large values for the deuterium kinetic isotope effects exhibited by tNADH and 3APADH suggest that the transition state for hydride ion transfer between the pyridine nucleotide donor and flavin acceptor is sensitive to the redox potential of the donor, as has been observed with human erythrocyte glutathione reductase (Sweet & Blanchard, 1991). A more complete description of the transition state for hydride ion transfer catalyzed by trypanothione reductase will be the subject of a subsequent study.

*Solvent Kinetic Isotope Effect Analysis.* The reductive half-reaction catalyzed by trypanothione reductase involves hydride transfer from reduced pyridine nucleotide to FAD, followed by electron transfer to the catalytic disulfide, forming the dithiol/dithiolate pair. If a slow proton transfer step to FAD or to the cysteine thiolates occurs, a solvent kinetic isotope effect greater than unity on $V/K_{nucleotide}$ might be observed. No solvent kinetic isotope effect on $V/K$ and only a small $^{D_2O}V$ of 1.30 ± 0.02 (fitted to eq 7) were observed when 2′,3′-cNADPH was used as the variable substrate. Replotting the maximum velocities versus the mole fraction of $D_2O$ yielded a linear proton inventory (Venkatassuban & Schowen, 1981), indicative of a single proton transfer step occurring somewhere in the overall reaction. These observations are similar to those previously reported for glutathione reductase using either NADPH (Wong et al., 1988) or NADH (Sweet & Blanchard, 1991).

The oxidative half-reaction catalyzed by trypanothione reductase involves re-formation of the redox-active disulfide, concomitant with irreversible reductive cleavage of the substrate disulfide and protonation of the thiolate moieties of reduced trypanothione. Proton transfer steps occurring in this half-reaction would be expected to result in large solvent kinetic isotope effects on $V$ in the case when the rate of the oxidative half-reaction was appreciably slower than the rate of the reductive half-reaction. In support of this, the solvent kinetic isotope effect observed for trypanothione reductase when NADPH was used as substrate was reported as 3.0 ± 0.1 (Sullivan et al., 1991), a value substantially higher than that determined here for 2′,3′-cNADPH. Since no solvent $V/K$ isotope effects were observed when 2′,3′-cNADPH was used as the variable substrate, we performed all subsequent solvent kinetic isotope effects in the presence of saturating amounts

Scheme I: Proposed Chemical Mechanism for the Reaction Catalyzed by Trypanothione Reductase




($\geq 10K_m$ levels) of both substrates, and determined the rates of reduced pyridine nucleotide oxidation as a function of the mole fraction of $D_2O$.[2] As seen in Table II, there is a smooth attenuation of the magnitude of $^{D_2O}V$, which correlates with the maximum velocity exhibited by the reduced pyridine nucleotide, as one proceeds from NHDPH to 3′NADPH. These data confirm our earlier interpretation of the substrate-dependent magnitude of the primary deuterium kinetic isotope effects, obtained using (4*S*)-4-$^2$H-labeled substrates. For substrates which exhibit high maximum velocities, such as NADPH and NHDPH, the rate of the reductive half-reaction is faster than the rate of the oxidative half-reaction, causing the primary deuterium kinetic isotope effect on hydride transfer to be attenuated. However, the solvent kinetic isotope effect on $V$ is maximal under these conditions, due to the kinetically significant single proton transfer occurring uniquely in the oxidative half-reaction. We propose, by analogy to glutathione reductase (Wong et al., 1988), that this proton transfer occurs between His461′ and the thiolate anion of the mixed enzyme–trypanothione disulfide intermediate. This is shown in Scheme I which details a proposed chemical mechanism for the enzyme-catalyzed reaction. Our solvent kinetic isotope effect data then argue that protonation of the first thiolate anion formed is a slow step in the oxidative half-reaction.

As reduced pyridine nucleotides which exhibit increasingly lower maximum velocities are analyzed for their isotopic behavior, the primary deuterium kinetic isotope effects on hydride transfer increase, while the solvent deuterium kinetic isotope effects decrease. This opposite behavior results from the increasingly rate-limiting nature of the reductive half-reaction, which decreases the kinetic significance of the proton transfer step occurring uniquely in the oxidative half-reaction. This "titration" of the solvent kinetic isotope effect should be generally applicable to enzymatic systems which display ping-pong kinetics, and which exhibit similar isotopic sensitivity.

[2] For a reaction exhibiting only a solvent kinetic isotope effect on $V$ when the initial velocity is determined at variable concentrations of a substrate at several fixed percentages of $D_2O$, the determination of solvent kinetic isotope effects yields valid values of $^{D_2O}V$ and linear proton inventories at substrate concentrations $\geq 10K_m$ levels. At lower substrate concentrations ($\leq 3$–$5K_m$ levels), the plots of $V$ versus mole fraction of $D_2O$ are concave upward and yield values for $^{D_2O}V$ that are less than the true value determined by extrapolation to infinite substrate concentrations.

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