Redox Potentials for Yeast, *Escherichia coli* and Human Glutathione Reductase Relative to the NAD^+/NADH Redox Couple: Enzyme Forms Active in Catalysis†

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ABSTRACT: The flavoenzyme glutathione reductase catalyzes the NADPH-dependent reduction of glutathione disulfide, yielding two molecules of glutathione. The oxidation−reduction potentials, E_{ox}/EH_2 (two-electron reduced enzyme), for yeast, *Escherichia coli*, and human glutathione reductase have been determined between pH 6.0 and 9.8 relative to the nonphysiological substrate couple NAD^+/NADH and were found to be −237, −243, and −227 mV (±5 mV) at pH 7.0 and 20 °C, respectively. The potential as a function of pH demonstrated slopes of −51, −45, and −42 mV/pH unit, respectively, at low pH and −37, −31, and −34 mV/pH unit, respectively, at high pH. The change in slope indicated pK_a values of 7.4, 8.5, and 7.6, respectively. The slopes indicate that two protons are associated with the two-electron reduction of E_{ox} at low pH and that only one proton is involved with the two-electron reduction of E_{ox} at high pH, provided that the effects of nearby titratable residues are considered in the data analysis. The influence of four such groups, Cys^{50}, Cys^{45}, $His^{456'}$, and either Tyr^{107} or the flavin-(N3), has been included (residue numbering refers to the yeast sequence). The enzyme loses activity upon deprotonation of the acid−base catalyst at high pH. Since the pK_a ascribed to the EH_2-to-EH^- ionization is lower than the pK_a of the acid−base catalyst, both the EH_2 and EH^- forms of glutathione reductase must be catalytically active, in contrast to the closely related enzyme lipoamide dehydrogenase, for which only EH_2 is active.

Glutathione reductase catalyzes the reversible transfer of electrons between NADPH and glutathione disulfide with the production of two molecules of glutathione. In many cells, it is the most important of a group of enzymes that maintain thiol−disulfide homeostasis, and its product, glutathione, is the substrate of glutathione peroxidase *(1)*. Glutathione reductase belongs to the pyridine nucleotide−disulfide oxidoreductase family that includes lipoamide dehydrogenase and thioredoxin reductase. The active enzyme is a dimer (M_r = 102 000). The X-ray structures of human erythrocyte and *Escherichia coli* glutathione reductase have been refined at high resolution *(2−4)*. Electron transfer involves a redox active disulfide and FAD on each monomeric unit in both glutathione reductase and the closely related enzyme lipoamide dehydrogenase. In the two-electron reduced enzyme at equilibrium, a charge-transfer complex predominates; one of the nascent thiols (as a thiolate), referred to as the charge-transfer or proximal thiol, is the donor, and FAD is the acceptor in this complex (Scheme 1) *(1)*. The charge-transfer species can be detected by its absorbance beyond 540 nm, where the oxidized and fully reduced enzyme lack absorbance. The other thiol in EH_2 interacts with glutathione disulfide and is referred to as the interchange (or distal) thiol. Physiologically, glutathione reductase catalyzes the oxidation of pyridine nucleotide, whereas lipoamide dehydrogenase catalyzes the reduction of pyridine nucleotide. The two enzymes share many structural and mechanistic properties as outlined above, but an important part of understanding their distinct physiological reactions lies in determining their differences.

One of the differences has to do with the dependence of the oxidation−reduction potential (E_{ox}/EH_2)[1] on pH, and a continuing study of that dependence in glutathione reductase is the subject of this paper. In lipoamide dehydrogenase, this dependence showed that two protons as well as two electrons are involved in reduction of the enzyme to its active species, EH_2, below pH 7.6 *(5)*. Because one of the thiols produced in the reduction must be present largely as the thiolate, the uptake of two protons suggested the presence of an acid−base catalyst *(5)*. The acid−base catalyst, a histidine residue closely linked to a glutamate, is seen in the crystal structures of glutathione reductase and lipoamide dehydrogenase *(2, 6)*. (In the discussion that follows, the acid−base catalyst is considered to be the His−Glu pair as a unit.) The close juxtaposition of the active center acid−base catalyst and the two nascent thiols in EH_2 indicate that these three groups will be closely linked; indeed, in EH_2, the three dissociable groups share two protons (Scheme 1).

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[1] Abbreviations: E_{ox}, oxidized enzyme; EH_2, two-electron reduced enzyme; EH_4, four-electron reduced enzyme; EH_3^+ and EH^-, the further protonated and deprotonated forms of EH_2; E_t, enzyme total; NAD_i, initial NAD; NAD_t, total NAD; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; DTT, 1,4-dithiothreitol.

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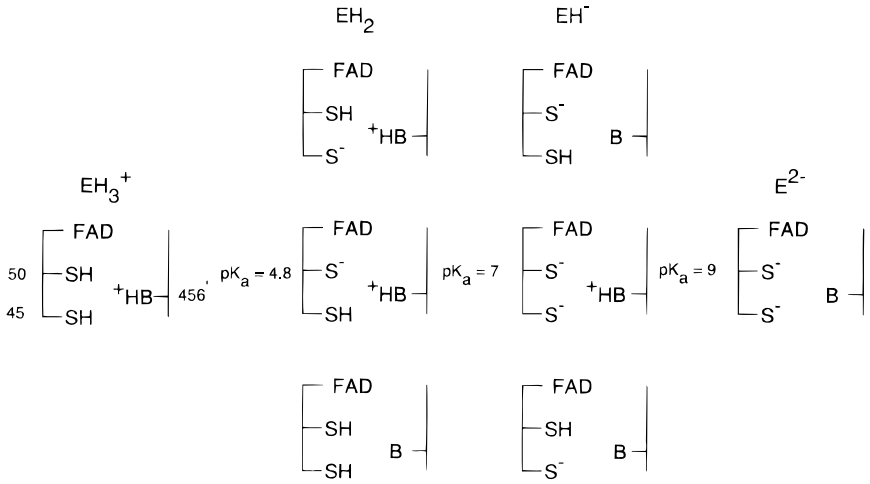
Scheme 1. Forms of Two-Electron Reduced Glutathione Reductase as a Function of pH[a]



[a] The residue numbers for the yeast sequence are beside EH_3^+; the prime indicates that the active site contains residues from both polypeptide chains. All possible species given three ionizing residues are shown. The middle row contains the species thought to predominate at any given pH.

In addition, two other ion pairs are near the flavin, bringing to seven the total number of titratable side chains surrounding the isoalloxazine ring. The eight amino acid residues are conserved in glutathione reductase and lipoamide dehydrogenase, both in the sequence and in three dimensions *(6−7)*. The glutathione binding site contains a tyrosine residue that has been assigned a role assisting the acid catalyst *(8)*. Given the large number of titratable groups in the active site, association of macroscopic pK_a values with specific residues must remain tentative.

The pH dependence of the spectral properties in the visible region, the steady-state kinetic properties, and the E_{ox}/EH_2 redox potential have yielded pK_a values (Scheme 1). A pK_a value of 4.8 (EH_3^+-to-EH_2 transition) in yeast glutathione reductase has been assigned to the charge-transfer thiol on the basis of its absorbance behavior in enzyme having the interchange thiol alkylated *(9)*, and a pK_a value of 9.2 (EH^--to-E^{2-} transition) has been assigned to the acid−base catalyst from both spectral and kinetic properties *(9−10)*. The EH_2-to-EH^- transition of the yeast enzyme could be associated with pK_a values of 6.2−8.4 *(9−10)*. Although lipoamide dehydrogenase cycles in catalysis only between E_{ox} and EH_2 *(5)*, glutathione reductase from yeast appears to cycle either between E_{ox} and EH_2 or, less efficiently, between E_{ox} and EH^- *(11)*, which is yet another difference between these enzymes.

A preliminary estimate of the redox potential (E_{ox}/EH_2) for glutathione reductase from yeast was made as a function of pH *(12)*. A pK_a of 7.6 was determined from the change of slope in the plot of turnover number/K_m as a function of pH. The ratio turnover number/K_m in the forward and reverse directions can be related to the equilibrium constant via the Haldane relationship. The value of the potential, relative to the potential of the physiological $NADP^+$/NADPH couple, at pH 7.0 was −255 mV at 25 °C (−248 mV corrected to 20 °C) *(12)*. In work from this laboratory, a pK_a of 7.4 was determined from the change in slope of the plot of the redox potential, relative to the potential of the nonphysiological NAD^+/NADH couple, as a function of pH; a midpoint potential of −237 mV at pH 7.0 and 20 °C was calculated *(11)*. The slopes observed below and above this pK_a value indicated that enzyme reduction involved changes of E_{ox} to EH_2 and E_{ox} to EH^-, respectively. The quantitative differences between these two studies should be viewed in the light of the recently reported redox potential, E_{ox}/EH_2, for lipoamide dehydrogenase. The potential depends markedly on the reference couple used. The physiological pyridine nucleotide NADH, the nonphysiological pyridine nucleotide NADPH, or dye couples were shown to have differential binding to oxidized and reduced forms of the enzyme, resulting in differences in enzyme potentials for the three references *(13)*.

Glutathione reductase from yeast has been more thoroughly studied than any other species. In an effort to better understand the acid−base dynamics in catalysis, we have determined the pH dependence of the oxidation−reduction potential for *E. coli* and human glutathione reductase for which high-resolution X-ray structures are available *(2−4)* and compared it with that of the yeast enzyme, for which a high resolution structure does not yet exist. The data analysis has specifically considered the influence of nearby titratable groups on the pH dependence.

EXPERIMENTAL PROCEDURES

Materials. Yeast glutathione reductase grade IV, NADH grade III, and NAD^+ grade VC were purchased from Sigma Chemical Co. All other reagents were of the highest quality available. The yeast glutathione reductase was further purified by chromatography on calcium phosphate gel with cellulose *(14)*. *E. coli* glutathione reductase was obtained from the high-expression plasmid pKGR3 transformed into *E. coli* strain TG1 *(15)*. The plasmid, cell line, and an initial sample of the enzyme, as well as the cell line SG5, were generously supplied by Prof. Richard N. Perham, University of Cambridge. The plasmid pUB302 containing the gene for human glutathione reductase *(16)* was the generous gift of Prof. Heiner Schirmer, Heidelberg University.

Cell Growth and Purification of E. coli Enzyme. The contents of two lawn plates were transferred to two 200 mL cultures of Terrific Broth *(17)* with 100 μg/mL ampicillin and were grown with shaking for 2 h at 37 °C. The cultures were then transferred to a Labline high-density fermentor

with an additional 2 L of media plus 100 mL of 20% glucose. The culture was monitored (A_{600nm}) to determine saturation, which was at approximately 4.5 h. Cells were harvested by centrifugation (40−60*g*), resuspended in 20 mM Tris pH 7.6, 25 °C, 0.3 mM EDTA, 0.1% PMSF (starting buffer), and disrupted by sonication. Streptomycin sulfate was added at 2% w/v, and the solution was centrifuged for 1 h at 45 000 rpm. The supernatant containing *E. coli* glutathione reductase was equilibrated by dialysis at 4 °C with starting buffer, centrifuged at 15 000 rpm for 30 min, and applied to a 5 cm × 10 cm Pharmacia Q-Sepharose anion exchange column equilibrated with the same buffer without PMSF. The column was developed stepwise with 0, 0.1, 0.3, and 0.5 M NaCl added to the buffer. Fractions containing glutathione reductase were combined and precipitated with ammonium sulfate (80% saturation). The precipitate was resuspended in 20 mM Na/K phosphate pH 7.6, 0.3 mM EDTA and dialyzed at 4 °C against the same buffer. Aliquots containing 1−2 μmol of enzyme were applied to a 2 cm × 20 cm Pharmacia 2′,5′-ADP-Sepharose 4B affinity column equilibrated in the same buffer. The column was developed stepwise with 0, 0.1, 0.3, and 0.5 M NaCl. Fractions containing pure glutathione reductase were combined and concentrated with 80% ammonium sulfate. The yield of *E. coli* enzyme was 4−5 μmol.

Cell Growth and Purification of Human Enzyme. Cells containing human enzyme were grown overnight at 37 °C in 6 × 1 L of 2 × YT media containing 100 μg/mL ampicillin. The cells were harvested by centrifugation (∼40*g*), resuspended in 10 mM Na/K phosphate pH 7.6, 0.3 mM EDTA, 0.1% PMSF (starting buffer) and disrupted by sonication. The solution was then centrifuged for 45 min at 15 000 rpm, followed by precipitation with 30% ammonium sulfate. The solution was centrifuged for 1 h at 15 000 rpm. The supernatant containing human glutathione reductase was equilibrated by dialysis against starting buffer, centrifuged for 30 min at 15 000 rpm, and applied to a 2 cm × 20 cm Pharmacia 2′,5′-ADP-Sepharose 4B affinity column equilibrated in the same buffer without PMSF. The column was developed with a linear gradient to 1 M NaCl in four column volumes. Fractions containing pure glutathione reductase were combined and concentrated with 80% ammonium sulfate. Yield of human enzyme was ca. 1 μmol.

The purity of each enzyme was verified by SDS−PAGE and comparison of activity and spectral ratios to published values. Concentrated stocks of yeast and *E. coli* enzyme were stored in 25 mM Na/K phosphate pH 7.6, 0.3 mM EDTA. Large supplies were kept frozen. Human enzyme was stored in 25 mM Na/K phosphate pH 6.9, 100 mM KCl, 0.5 mM EDTA, and large supplies were stored as ammonium sulfate (80%) suspensions. Changes to desired pH and buffer concentrations were made by diluting the highly concentrated stock of enzyme with the desired buffer.

Titrations. Each enzyme was titrated anaerobically with NADH in the presence of NAD^+. Enzyme was contained in a cuvette having a syringe port and a stopcock *(18)*. NAD^+ was added, and the solution was made anaerobic using alternating cycles of vacuum and nitrogen. A separate solution of NADH containing ∼1 equiv/20 μL was also made anaerobic. A gastight Hamilton syringe was used to titrate the anaerobic solution of NADH into the enzyme−NAD^+ solution. The mixture was allowed to equilibrate at 20 °C between additions. Equilibration was determined by following the absorbance change at 540 nm until no further change could be detected. Preliminary determinations of the redox potential for the EH_2/EH_4 couple were performed in the absence of NAD^+.

Buffers. Na/K phosphate buffer was used for titrations from pH 5.9 to 7.9. Tris/HCl buffers were used for titrations from pH 8.0 to 8.8, and glycine/NaOH buffers were used for titrations from pH 9.0 to 10.0. Buffer dependence was examined in human enzyme by substitution with HEPES, pH 7.2, and in *E. coli* enzyme by substitution of PIPES, pH 6.8, and HEPES, pH 7.2. All buffer concentrations were ca. 70 mM. No effort was made to maintain constant ionic strength. All buffers contained 0.3 mM EDTA. Solutions of NADH were made in 5 mM unneutralized Tris. Enzyme concentrations were typically 12−25 μM. Concentration dependence of enzyme was examined by tripling the enzyme concentration; yeast enzyme was examined at pH 7.16 and 8.43, and *E. coli* enzyme was examined at pH 9.25. Concentrations of NAD^+ varied from a 250-fold excess over flavin at pH 5.9 to a 25-fold excess at pH 10.

RESULTS

The glutathione−glutathione disulfide couple is an important redox modulator in most cells, existing at a higher concentration than any other thiol/disulfide couple *(19)*. The redox potential is −234 mV at pH 7 and corrected to 20 °C *(1)*. Under normal cellular conditions where the NADPH concentration is high and peroxide production is low, the couple is maintained largely in the reduced state by glutathione reductase *(19)*. The redox potential of the enzyme (E_{ox}/EH_2) is of interest for a fuller understanding of the system.

NADH and NAD^+ do not form detectable complexes with glutathione reductase in either the E_{ox} or EH_2 states. The K_d value for the binding of NADH to E_{ox} has been shown to be greater than 400 μM. Moreover, complexes of NAD^+ or NADH with EH_2 are not spectrally detectable, whereas complexes between $NADP^+$ or NADPH are readily detectable *(20)*. The redox potential of NADH is well established at −309 mV (pH 7.0, 20 °C, ionic strength 0.1) *(21)*. The NAD^+/NADH couple is therefore appropriate for the determination of the redox potential of glutathione reductase, provided concentrations of NAD^+ appropriate for each pH are included. Reduction of the oxidized enzyme by NADH to the two-electron level is relatively rapid and forms the spectrally well characterized charge-transfer intermediate *(22, 23)*.

Figure 1 shows a typical redox titration of glutathione reductase by NADH in the presence of NAD^+. The increase in absorbance at 540 nm indicates the formation of the thiolate−FAD charge-transfer complex. The increasing absorbance at 340 nm reflects the addition of excess NADH. The isosbestics at 445 and 505 nm indicate that E_{ox} and EH_2 are essentially the only enzyme species present. A plot of the extinction value at 540 nm versus [NADH] for each experiment conformed to a rectangular hyperbola with less than 3% standard error (Figure 2). Extrapolation to the extinction value of fully formed EH_2 can be determined from

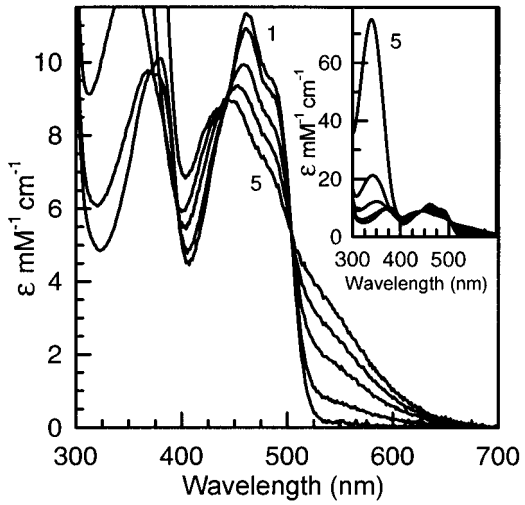


FIGURE 1: Reduction of *E. coli* glutathione reductase with NADH in the presence of NAD^+. Reduction was performed under anaerobic conditions at 20 °C. The enzyme (22.4 μM) and NAD^+ (0.54 mM) were in 70 mM Tris/HCl, pH 8.36, 0.3 mM EDTA. Curve 1, no NADH; curve 2, 5.49 μM NADH; curve 3, 24.4 μM NADH; curve 4, 58.5 μM NADH; curve 5, 216 μM NADH.

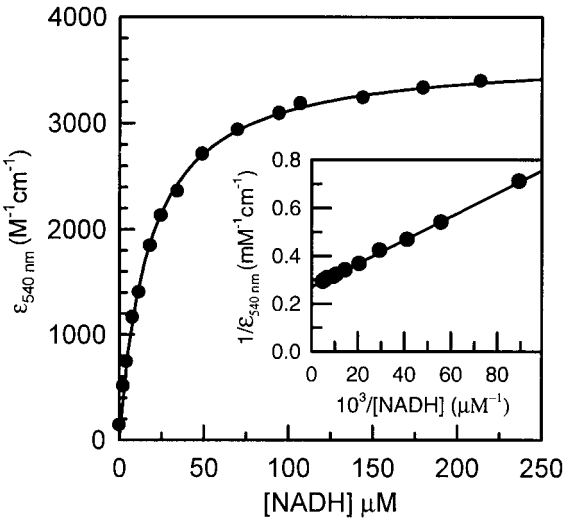


FIGURE 2: The charge-transfer complex characteristic of EH_2 formation as a function of the NADH concentration in the presence of NAD^+. The concentration of NAD^+ was 0.54 mM, and that of free NADH was determined by the increase in absorbance at 358 nm, the isosbestic for E_{ox}/EH_2. Conditions were as described in Figure 1. Inset, double reciprocal plot of the titration data.

either the rectangular hyperbola or the resulting reciprocal plot (Figure 2, inset). This method was in good agreement with extinction coefficients for EH_2 of the yeast enzyme determined from reduction with dithionite at all pH values *(22)*. It should be noted that, in these titrations in the presence of NAD^+, some excess NADH is present at each point in the titration (even with only 0.25 equiv of NADH, Figure 1, spectrum 2); the isosbestic at 358 nm observed in dithionite titrations is missing (see below).

The equilibrium equation for the reaction E_{ox} + NADH ↔ EH_2 + NAD is defined in eq 1. Values for each

$$K_{eq} = \frac{[EH_2][NAD_t]}{[E_{ox}][NADH]} \quad (1)$$

component throughout the titration were determined using

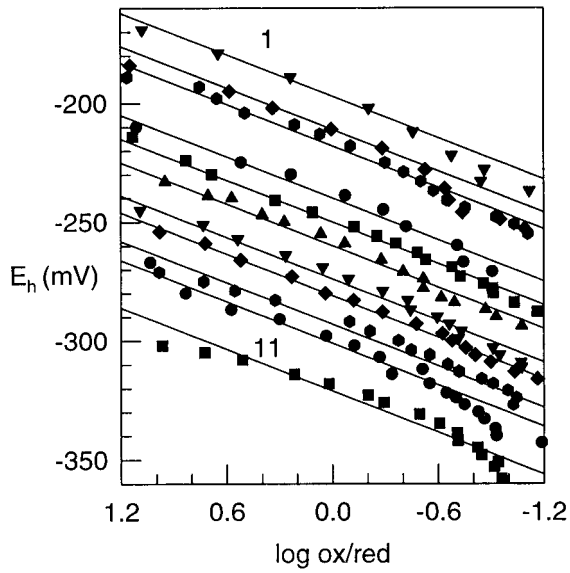


FIGURE 3: Nernst plots for the reduction of human glutathione reductase in the presence of NAD^+. The solid line drawn through each data set represents a theoretical two-electron slope. The data sets correspond to the following pH values at 20 °C: 1, 6.32; 2, 6.60; 3, 6.83; 4, 7.33; 5, 7.57; 6, 7.87; 7, 8.25; 8, 8.52; 9, 8.81; 10, 9.11; 11, 9.62.

eqs 2−5. Because NADH will hydrolyze below pH 7.0, it

$$[EH_2] = \frac{(A_{540obs} - A_{540E_{ox}})}{\Delta\epsilon_{540EH_2}} \quad (2)$$

$$[E_{ox}] = [E_t] - [EH_2] \quad (3)$$

$$[NADH] = \frac{(A_{358obs} - A_{358E_{ox}})}{\epsilon_{358NADH}} \quad (4)$$

$$[NAD_t] = [NAD_i] + [EH_2] \quad (5)$$

is desirable to measure its concentration directly rather than by difference. Therefore, spectral titrations monitor 358 nm (where the spectra of E_{ox} and EH_2 are isosbestic as determined from dithionite titrations) for equilibrium amounts of NADH using an extinction coefficient of 4.4 mM^{-1} cm^{-1} (eq 4). The amount of NAD^+ that must be added initially (NAD_i) depends on the pH because the redox potentials of the pyridine nucleotide and enzyme couples are somewhat separated and this separation increases as the pH is lowered (eq 5 and Figure 4).

Equation 6 gives the Nernst relationship for the two-

$$E_h = E_m(E_{ox}/EH_2) + \left(\frac{RT}{nF}\right)\ln\frac{[E_{ox}]}{[EH_2]} \quad (6)$$

electron reduction of the enzyme (E_{ox}/EH_2). The system potential, E_h, is set by the NAD^+/NADH ratio, leading to the relationship of eq 7 where K_{eq} is the equilibrium constant

$$E_m(E_{ox}/EH_2) = E_m(NAD^+/NADH) + \left(\frac{RT}{nF}\right)\ln K_{eq} \quad (7)$$

for the reaction of the NAD^+/NADH couple with the E_{ox}/EH_2 couple. The system potential for each experiment was plotted against the log([E_{ox}]/[EH_2]). The data for human

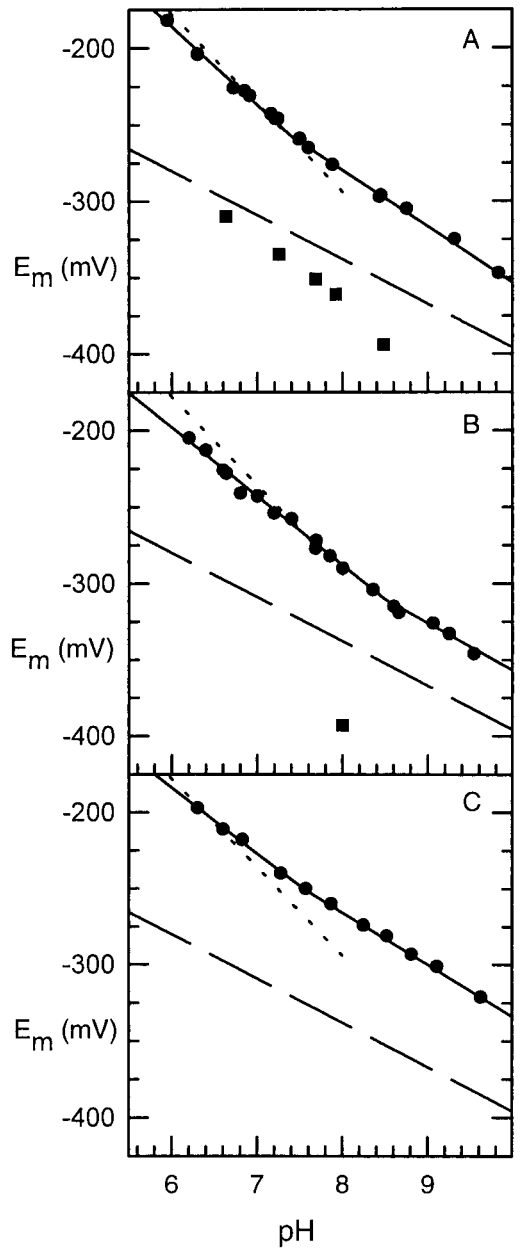


FIGURE 4: The pH dependence of the redox potential, E_m (E_{ox}/EH_2), of glutathione reductase. Data for the yeast enzyme is in panel A, for *E. coli* enzyme is in panel B, and for human enzyme is in panel C. The solid circles represent midpoint potentials for each data set. The dashed lines represent the NAD/NADH redox potential, $E_{m7} = -309$ mV at 20 °C *(21)*, and illustrate a −29 mV/pH unit slope (one proton). The dotted lines represent the GSSG/2GSH redox potential, $E_{m7} = -234$ mV at 20 °C, and illustrate a −58 mV/pH unit slope (two protons). (GSSG and GSH have pK_a values above pH 8 that would alter the slope.) *(1)* The filled squares represent preliminary data for the EH_2/EH_4 redox couple.

enzyme are shown in Figure 3 and give a slope indicating a two-electron reduction ($n = 2$). Similar plots of the yeast and *E. coli* enzymes have been shown as preliminary data and are not presented here *(11, 24)*.

A plot of the midpoint potential (E_m) determined from each experiment versus pH is shown in Figure 4. The E_{ox}/EH_2 redox potential at pH 7.0 for each enzyme is summarized in Table 1. It can be seen in Figure 4 that the data define two slopes and a pK_a (Table 1). Slopes of −29 or −58 mV/pH unit (at 20 °C) are predicted depending on whether the number of protons associated with a two-electron change is one or two, respectively. A change in slope then indicates a pK_a on the enzyme because NAD^+ and NADH are known to have no ionizations in the pH range of these experiments. Moved by insightful reviews, we have subjected the data in Figure 4A to further analysis (Figure 5). The data are very well modeled (simulated) by an equation assuming two pK_a values on E_{ox} (the base and either the Tyr residue or the flavin) and three pK_a values on the reduced enzyme (the base and the two redox active thiols) (Table 2). The equation used in the model (eq 8) is of the form of Clark's general

$$E_m = E_0 + \left(\frac{RT}{nF}\right) \times \ln\left[\frac{[H^+]^4 + K_{r1}[H^+]^3 + K_{r1}K_{r2}[H^+]^2 + K_{r1}K_{r2}K_{r3}[H^+]^2}{[H^+]^2 + K_{o1}H^+ + K_{o1}K_{o2}}\right] \quad (8)$$

equation *(21)*. The slope responds to a pK_a on E_{ox} with a change that is opposite that for a pK_a on reduced enzyme. K_{r1}, K_{r2}, and K_{r3} are the dissociation constants of the reduced enzyme, and K_{o1} and K_{o2} are on E_{ox}. The redox potential at pH 0, E_o, is taken as +135 mV and was determined empirically by allowing the simulated data to fall exactly on the experimental data. This value furthermore falls between two reasonable assumptions: to use either the theoretical slope of −58 mV/pH unit or the observed slope of −51 mV/pH unit and the experimentally determined E_{m7} of −237 mV. This further analysis follows closely the work of O'Donnell and Williams *(30)*. Very few studies have offered a demonstration of the statement that divergence from the slopes of −58 and −29 mV/pH unit predicted by theory reflects other groups near the flavin having redox-dependent pK_a values. Although the analysis may still be incomplete, it indicates that all titratable groups near the redox active group of interest must be considered. The model data correlates well with the experimental data within the pH range studied and also suggests that an additional dissociation constant at a very basic pH value is involved on the EH_2 species. Thus, the first term of eq 8, the hydrogen ion concentration, is raised to the fourth power. An appreciation for the subtleties of redox measurements and of equations which involve multiple pK_a values on both oxidized and reduced species is demonstrated graphically in an early primer by Clark *(31)* and again in a later reference: "...equations cannot as yet provide comprehensive and definitive descriptions of these systems;" *(21)*.

Approximate redox potentials for the EH_2/EH_4 couples of glutathione reductase from yeast and *E. coli* were determined in the absence of added NAD^+ (Table 1). The yeast enzyme, in the presence of 10−30 equiv of NADH and ca. 16 h of equilibration, demonstrated nearly 60% formation of EH_4. After appropriate modifications to eqs 1−7, several measurements for the yeast EH_2/EH_4 couple were determined from pH 6.6 to 8.5 (Figure 4a). Measurement of the EH_2/EH_4 redox potential of *E. coli* enzyme was performed with a single experiment at pH 8.0 with 20 equiv of NADH. The reaction was equilibrated for 12 h and showed ca. 8% formation of EH_4, indicating a much lower EH_2/EH_4 redox potential than that for yeast enzyme (Table 1). The redox potential for EH_2/EH_4 for human enzyme was attempted at

Table 1: Slopes, pK_a Values, and Redox Potentials Derived from the Plots of E_m vs pH Redox Data for Glutathione Reductase

type of data	yeast	*E. coli*	human
slope at low pH, mV/pH unit	−51	−45	−42
slope at high pH, mV/pH unit	−37	−31	−34
E_{m7}(E_{ox}/EH_2 20 °C), mV	−237 ± 5	−243 ± 5	−227 ± 5
E_m(EH_2/EH_4 20 °C), mV	−360 (pH 7.9)	−393 (pH 8.0)	<−393 (pH 8.2)
pK_a (change in slope)	7.4	8.5	7.6

Table 2: Comparison of pK_a Values Derived from Redox, Spectral and Kinetic Data for Glutathione Reductase

	yeast				*E. coli*			human		
type of data	pK_1	pK_2	pK_3	ref	pK_1	pK_2	ref	pK_2	pK_3	ref
redox measurements		7.4				8.5		7.6		
redox inactivation	4.7	7.5		25	5.8	7.3	26			
charge transfer (540 nm)	4.8	7.1	9.2	9	[5.7]	[8.0]	27			
	4.8	7.4		22						
	[3.7]		[9.2]	9						
V_{max}, vary GSSG		6.2	9.2	*b*		[6.3, 7.6]	28		9.0	29
V_{max}/K_{GSSG}			8.4, 8.8	*b*		[7.4]	28	7.8	8.6	29

[a] All data are from studies with wild type enzyme with the exception of those in square brackets, which are from enzyme modified either chemically or by site-directed mutagenesis. Ref 9 reports data where the interchange thiol of yeast enzyme was modified by alkylation; Ref *27* reports data from *E. coli* C42A, interchange thiol modified; Ref *28* reports data from *E. coli* H439A, acid−base catalyst modified. [b] Wong and Blanchard, personal communication.

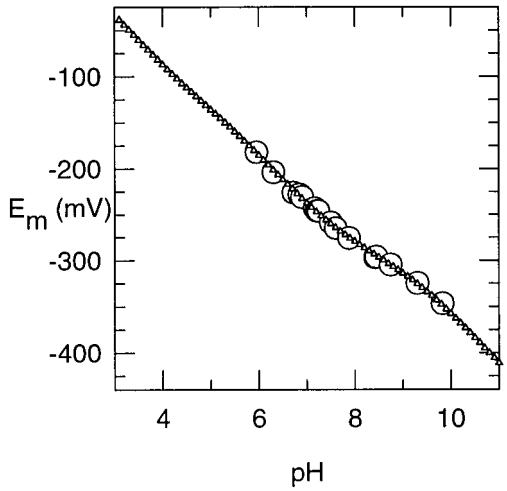


FIGURE 5: The pH dependence of the redox potential, E_m (E_{ox}/EH_2), for yeast glutathione reductase overlaid with simulated data. Equation 8 from the text was used to produce the simulated data (small open triangles) with the following values: E_o, +135 mV; pK_{o1}, 5.2; pK_{o2}, 9.4; pK_{r1}, 4.6; pK_{r2}, 7.5; pK_{r3}, 8.7. pK_{ox} represents a pK on the oxidized enzyme, and pK_r is a pK on the reduced species.

pH 8.2. After equilibration with ca. 45 equiv of NADH for nearly 50 h, loss of 540 nm absorbance indicating the conversion of EH_2 to EH_4 was not detected. Estimates of the EH_2/EH_4 redox potential for human enzyme would appear to be even lower than that estimated for *E. coli* enzyme (Table 1).

DISCUSSION

Glutathione reductase and lipoamide dehydrogenase catalyze identical reactions, but the direction of substrate flux is opposite: pyridine nucleotide oxidation and disulfide reduction in the case of glutathione reductase and dithiol oxidation and pyridine nucleotide reduction in lipoamide dehydrogenase. The base catalyst in lipoamide dehydrogenase, with a pK_a of approximately 8, sequentially deprotonates both thiols of the bound dihydrolipoamide in an obligatory E_{ox}-to-EH_2 mechanism *(5)*. In contrast, the acid catalyst in glutathione reductase, with a pK_a of approximately 9, protonates the first departing molecule of glutathione in an EH^--to-E_{ox} mechanism *(29)* and could protonate both glutathione molecules in an EH_2-to-E_{ox} mechanism *(1)*. The pK_a value near 8 revealed in the present work and ascribed to the EH_2-to-EH^- transition, being below the pK_a near 9 of the acid catalyst that controls activity, shows that both EH_2 and EH^- are active in catalysis by glutathione reductase, a striking difference compared with lipoamide dehydrogenase.

The E_{m7} of −237 mV at 20 °C for the enzyme from yeast compares reasonably well with the value of −248 mV (corrected to 20 °C) arrived at through steady-state kinetics using the Haldane relationship *(12)*. The small difference in the reported potentials may have been due to the inclusion of 10 mM DTT when measuring the kinetics of the back reaction. The DTT was thought to maintain the glutathione in the reduced state, but we have found that DTT is effective in reducing the mixed disulfide between the enzyme and glutathione.[2]

The E_{m7} for *E. coli* enzyme was determined to be −243 mV at 20 °C (Table 1). The intersection of the two slopes in the pH-dependence study indicates an apparent pK_a of 8.5. This is clearly higher than pK_a values determined by other methods that are associated with the EH_2-to-EH^- transition (Table 2). The attribution of the apparent pK_a of 8.5 to a specific transition cannot be made with the available data with any certainty. However, in the *E. coli* enzyme, as in glutathione reductase from yeast, both thiols appear to be deprotonated in EH^-, that is, the single proton resides primarily on the base (see below). A preliminary study with excess NADH in the absence of NAD^+ indicated that the redox potential for the EH_2/EH_4 couple is approximately −393 mV at pH 8.0 for the *E. coli* enzyme at 20 °C.

The E_{m7} for human enzyme was determined to be −227 mV at 20 °C (Table 1). The intersection of the two slopes

[2] Arscott, L. D., Veine, D. M., and Williams, C. H., Jr., unpublished results.

in the pH-dependence study indicates an apparent pK_a of 7.6. Kinetic studies have shown evidence of pK_a values of 7.8, 8.6, and approximately 9.0 (Table 2). Thus, in human glutathione reductase, as had been found for the enzyme from yeast or *E. coli*, a pK_a on the enzyme at a value less than the pK_a of the base indicates that the enzyme can cycle between E_{ox} and EH^-, as well as between E_{ox} and EH_2. Preliminary studies of the redox potential for the EH_2/EH_4 couple of human enzyme with excess NADH in the absence of NAD^+ at pH 8.2 suggest that the value is lower than that determined for *E. coli* (−393 mV at pH 8.0 at 20 °C).

Table 1 lists the slopes from the plots of E_m vs pH for glutathione reductase from yeast, *E. coli*, and human erythrocytes. These slopes deviate from those expected for a two-electron reduction involving two protons (−58 mV/pH unit, 20 °C) or one proton (−29 mV/pH unit, 20 °C) (*1*, *21*). The deviation from theory indicates the presence of other dissociable groups whose pK_a values are linked to the redox state of the enzyme *(21)*. The constellation of dissociable aminoacyl groups surrounding the flavin was mentioned at the beginning of this paper. Deviations from the expected slopes have been observed in thioredoxin reductase and lipoamide dehydrogenase *(30, 32)* However, considering only the nascent thiols and the acid−base catalyst (see below), the two-electron reduction in the range below the pK_a appears to involve two protons (E_{ox}-to-EH_2) and reduction at higher pH involves one proton (E_{ox}-to-EH^-).

The association of macroscopic pK_a values with the ionization of specific amino acid residues in the protein can be made more exact by comparing pK_a values determined from several types of data and by considering the effect of removing one titrating group on the apparent pK_a values of the remaining groups *(1, 9)*. A notable exception to this generalization should be cited *(33)*. The three types of data considered in Table 2 are enzyme inactivation by substrate, the spectral characteristics of EH_2, and steady-state kinetics. All of these are associated with ionizations on reduced enzyme (EH_2). Thus, macroscopic pK_a values associated with the pH dependence of these properties can be grouped into three broad ranges (Table 2). The data for the yeast enzyme are most complete and will be considered here. The lowest pK_a of 4.8 has been attributed to ionization of the flavin-interacting thiol, Cys^{50} *(1, 9)*. The pK_a of the interchange thiol, Cys^{45}, has been associated with the range of pK_a values of 6.2−7.5 *(1, 9)*.[3] The highest pK_a, in a range of 8.4−9.2, has been attributed to the acid−base catalyst, $His^{456'}$ *(1, 9)*.[3] In this simple model with three pK_a values, it has been argued that these represent the following transitions: EH_3^+ to EH_2 to EH^- to E^{2-} as shown in Scheme 1 *(1)*.

Failure of this analysis of the redox potential experiments to detect a pK_a in the range of 8.4−9.2 indicates that the species in Scheme 1 for two-electron reduced enzyme are not complete and do not fully explain slopes that differ from those predicted by theory (Figure 4A). The analysis in Figure 5, based on eq 8, takes into account ionizations on E_{ox} as well as on reduced enzyme *(21)*. It should be noted that the pK_a of the acid−base catalyst has been assumed to be near 5 on E_{ox} by analogy with lipoamide dehydrogenase *(34)*. The model also assumed a pK_a of 9.4 on E_{ox} attributed either to the Tyr^{107} in the glutathione binding site *(8)* or to the flavin-(N3). Such a treatment simulates the data more exactly within the pH range studied and yields slopes of −51 mV/pH unit and −37 mV/pH unit as given in Table 1.

This study of the effect of pH on the redox potential, E_{ox}/EH_2, of glutathione reductase from yeast, *E. coli*, and human erythrocytes has revealed a pK_a on each that can be associated with the EH_2-to-EH^- transition. More detailed analysis has suggested that deviations from theory are indeed due to at least four nearby titratable groups: Cys^{50}, Cys^{45}, $His^{456'}$, and either Tyr^{107} or the flavin-(N3). This will facilitate a study in progress on the effect of pH on the equilibria between E_{ox} and GSH and between EH_2 and GSSG and the involvement of a mixed disulfide in these reactions.[2]

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