Human Erythrocyte Glutathione Reductase: pH Dependence of Kinetic Parameters†

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ABSTRACT: Human erythrocyte glutathione reductase catalyzes the pyridine nucleotide dependent reduction of oxidized glutathione (GSSG). The pH dependence of the kinetic parameters V and V/K for three reduced pyridine nucleotide substrates, the K_i's for three competitive inhibitors (versus NADPH), and the temperature dependence of the V pH profile have been determined. Below pH 8, V and V/K for NADPH, 2',3'-cyclic-NADPH, and NADH are pH independent. In the basic pH region, both V and V/K for the three substrates are pH dependent. All three of the V profiles decrease with increasing pH as a group with a pK_a of approximately 9.2 is titrated. The V/K profiles for NADPH, 2',3'-cyclic-NADPH, and NADH decrease at high pH as a group with a pK_a of greater than 9.8, 8.9, and 8.8, respectively, is deprotonated. The K_i's for ATP-ribose and 2',5'-ADP are pH independent below pH 8 but increase in the basic region as a group with a pK_a of about 8.8 and 8.5, respectively, is deprotonated. The K_i of AADP is pH independent between pH 6 and 9. These studies suggest that binding interactions between the 2'-phosphate of NADPH and the enzyme are predominately nonionic. The temperature dependence of the pK observed in all V pH profiles allows the calculation of an enthalpy of ionization of 3.2 kcal/mol for this group. The high pK and low enthalpy of ionization suggest that the protonation state of the His-467'–Glu-472' ion pair observed in the structure of human erythrocyte glutathione reductase influences proton-transfer steps occurring in the oxidative half-reaction.

Glutathione reductase (EC 1.6.4.2) is an FAD-containing enzyme that catalyzes the pyridine nucleotide dependent reduction of oxidized glutathione (GSSG). The human erythrocyte enzyme is a homodimer of 104 800 daltons (Worthington & Rosemeyer, 1975) and functions to maintain a high GSH/GSSG ratio in the red blood cell. The kinetic mechanism of glutathione reductase is ping-pong (Williams & Massey, 1965; Icen, 1967), with NADPH[1] binding first and reducing the enzyme. The two electron reduced enzyme (EH_2) then binds GSSG, which is converted to 2 mol of GSH concommitant with enzyme oxidation. The enzyme from human erythrocytes has been crystallized, and its structure has been determined (Theime et al., 1981) and refined to 1.54-Å resolution (Karplus & Schulz, 1987). A chemical mechanism has been proposed on the basis of pH titrations of the yeast enzyme (Arscott et al., 1980) and X-ray crystallographic analysis of free and substrate-bound complexes of human erythrocyte glutathione reductase (Pai & Schulz, 1983).

NADPH binds in an extended conformation, with the nicotinamide ring parallel and adjacent to the isoalloxazine ring of FAD. The 2'-phosphate of NADPH provides important interactions between the nucleotide and the enzyme, since NADH has a greater than 50-fold higher K_m than NADPH. In the reduction of GSSG, 2 mol of protons must be transferred to the 2 mol of product, reduced glutathione. An acid, thought to be histidine-467'[2] has been suggested (Pai & Schulz, 1985) from the crystal structure as the proton donor for the first reduced glutathione product.

Two questions pertinent to the reaction mechanism have been investigated in this study. The first was to define the types of interactions involved in the binding of the 2'-phosphate of NADPH. The second was to identify the enzymic side chain that provides protons to the leaving glutathione anions. Studies of the pH dependence of the kinetic parameters indicate that the ionic contribution to binding between the 2'-phosphate of NADPH and Arg-218, His-219, and Arg-224 present in the 2'-phosphate binding site of human erythrocyte glutathione reductase is small. From the temperature variation studies, we suggest that the protonation state of the His-467'–Glu-472' ion pair determines the proton-donating ability of the enzyme in the oxidative half-reaction.

Materials and Methods

Chemicals. NADPH, NADH, 2',3'-cyclic-NADP, GSSG, AADP, ATP-ribose, 2',5'-ADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides* type XXIV), glutathione reductase (bakers' yeast, type IV), and all buffers were purchased from Sigma. Human erythrocyte glutathione reductase was the generous gift of Dr. Emil F. Pai (Max Planck Institute for Medical Research, Heidelberg, FRG).

Synthesis of 2',3'-Cyclic-NADPH. 2',3'-Cyclic-NADP (6 mg) was purified on a Mono Q (Pharmacia) anion-exchange

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[1] Abbreviations: NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NADH, reduced β-nicotinamide adenine dinucleotide; 2',3'-cyclic-NADP, β-nicotinamide adenine dinucleotide 2',3'-cyclic monophosphate; AADP, 3-aminopyridine adenine dinucleotide phosphate; ATP-ribose, 2'-monophosphoadenosine 5'-diphosphoribose; 2',5'-ADP, adenosine 2',5'-bisphosphate; G6P, glucose 6-phosphate; FPLC, fast protein liquid chromatography; TEA, triethanolamine; TAPS, 3-[[tris(hydroxymethy)methyl]amino]propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 2,2'-piperazine-1,4-diylbis(ethanesulfonic acid); CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

[2] Primed amino acid residues indicate that the primed residue is derived from the other subunit of the dimer.

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column equilibrated with 10 mM TEA-HCl, pH 7.7 (Orr & Blanchard, 1984). The column was developed with a linear gradient of 0–1 M KCl. 2′,3′-Cyclic-NADP eluted at 70 mM KCl. The nucleotide was reduced by using glucose-6-phosphate dehydrogenase from *L. mesenteroides* and excess glucose 6-phosphate in 20 mM TEA-HCl, pH 7.7. The reaction was monitored spectrophotometrically at 370 nm, and the pH was maintained at pH 7.7 by addition of 0.2 N KOH. Upon completion of the reaction, the mixture was ultrafiltered through an Amicon PM-10 membrane. The filtrate was injected onto a Mono Q anion-exchange column equilibrated with 10 mM TEA-HCl, pH 7.7, and 2′,3′-cyclic-NADPH was eluted with a linear gradient of KCl (0–1 M) in 10 mM TEA-HCl, pH 7.7. The 2′,3′-cyclic-NADPH eluted at 200 mM KCl, and the concentration was calibrated by using yeast glutathione reductase in the presence of excess GSSG. The compound was used the same day following purification.

Kinetic Procedures. Glutathione reductase reaction rates were measured spectrophotometrically by monitoring the oxidation of the reduced pyridine nucleotide substrate at 340 nm with a Gilford 260 spectrophotometer equipped with thermospacers and connected to a constant-temperature circulating water bath maintained at the appropriate temperatures. Reaction mixtures containing the reduced pyridine nucleotide, GSSG, and 100 mM buffer were prepared in 3-mL cuvettes and thermally equilibrated at 25 °C in the water bath (5 min). The reactions were initiated by the addition of a small amount of enzyme (<20 μL).

pH Studies. Buffers were prepared by titrating their acid forms, dissolved in double-distilled water, to the desired pH with 2 N KOH and were filtered through a 0.22-μm filter. Buffers were used at a final concentration of 100 mM at the stated pH to allow for overlap: Bis-Tris (5.8–6.9), MES (6.0–6.6), BES (6.2–7.2), PIPES (6.6–7.4), HEPES (7.0–8.2), TAPS (8.1–9.0), CHES (8.9–9.9), and CAPS (9.8–10.9). The kinetic parameters V and V/K for the variable substrate were determined at each pH, and their log values were plotted against pH, determined by insertion of a combined microelectrode into the cuvette after the initial velocity assays were carried out. The kinetic parameters V and V/K for NADPH and 2′,3′-cyclic-NADPH were determined by varying the concentration of nucleotide between 5 and 20 μM at a saturating GSSG concentration (3 mM). The kinetic parameters V and V/K_{NADH} were determined by varying the concentration of NADH between 50 and 200 μM at a saturating GSSG concentration (3 mM). The kinetic parameters V and V/K_{GSSG} were determined by varying the concentrations of GSSG between 50 and 200 μM at a saturating NADPH concentration (200 μM). The pK_i values for inhibitors versus NADPH at pH 6–10 were determined at a saturating concentration of GSSG (3 mM), at varying concentrations of NADPH (5–20 μM), and at three fixed concentrations of inhibitor including zero.

Determination of the pK of the 2′-Phosphate of AADP and 2′,5′-ADP by ^{31}P NMR. The ^{31}P NMR titration of the phosphate moieties of AADP and 2′,5′-ADP was performed at 21 °C with a Varian XL-200 NMR spectrometer operating at 81 MHz. Spectra were obtained by using broad-band proton decoupling with a spectral width of 10 KHz (8000 points), 11-μs (90°) pulse widths, 0.4-s acquisition times, and repetition times of 2 s. Samples containing 30 mM MES (or BES), pH 4.5–8.5, 2 mM EDTA, 20% D_2O, and 1.9 mM AADP or 1 mM 2′,5′-ADP were prepared in 10-mm diameter NMR tubes in a final volume of 3 mL. Two hundred to five hundred transients were collected, and pH values were measured before and after taking the spectrum. The chemical shifts of the 2′-phosphate and the pyrophosphate moiety of AADP and the 2′- and 5′-phosphates of 2′,5′-ADP were plotted as a function of pH (with 85% phosphoric acid as an external standard).

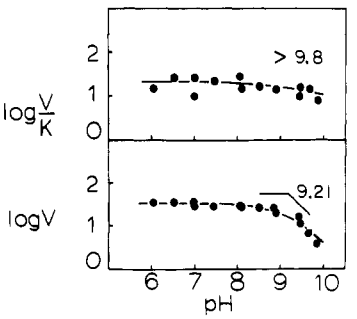

FIGURE 1: Effect of pH on kinetic parameters V and V/K with NADPH as the variable substrate at a saturating concentration of GSSG. The V profile shows a single enzymatic group with a pK_a of 9.21 ± 0.07 whose protonation abolishes activity. The V/K profile shows the beginning of a break whose pK_a is greater than 9.8.

Data Analysis. Reciprocal initial rates were plotted against reciprocal substrate concentrations, and the data were fitted to the appropriate equations by using the Fortran programs of Cleland (1979). The individual saturation curves used to obtain pH profiles were fitted to eq 1, where A is the variable substrate concentration. Data for pH profiles that showed a decrease in log V, log V/K, or pK_i with a slope of −1 as pH was increased were fitted to eq 2. In eq 2, y is the parameter whose pH dependence is being determined, and C is the pH-independent value of y. Data for linear competitive inhibition were fitted to eq 3, where K_{is} is the slope inhibition constant. Apparent pK values as a function of temperature were fitted to eq 4. The ^{31}P NMR titration curve of the 2′-phosphate of AADP and 2′,5′-ADP was fitted to eq 5, where y is the chemical shift value and A and B are the chemical shift value at low pH and the difference in chemical shifts at low and high pH, respectively.

$$v = VA/(K + A) \quad (1)$$

$$\log y = \log [C/(1 + K_1/[H^+])] \quad (2)$$

$$v = VA/[K(1 + I/K_{is}) + A] \quad (3)$$

$$pK = \Delta H_{ion}/(2.303RT) + B \quad (4)$$

$$y = A + B/(1 + [H^+]/K) \quad (5)$$

RESULTS

pH Profiles of the Reductive Half-Reaction. The pH dependence of the kinetic parameters using NADPH as the variable substrate is shown in Figure 1. The log V profile (lower panel) decreased with a slope of −1 above a pK of 9.21 ± 0.07. The log (V/K) profile (upper panel) shows no apparent break at low pH in the region where the 2′-phosphate of NADPH exhibits a solution pK of 6.52 ± 0.02 (Mas & Colman, 1984). At high pH, V/K_{NADPH} decreases as a result of the deprotonation of a group with a pK_a of greater than 9.8.

Human erythrocyte glutathione reductase uses 2′,3′-cyclic-NADPH as an excellent substrate, with V and V/K values identical with those for NADPH. The phosphate of 2′,3′-cyclic-NADPH is in a phosphodiester linkage and exists exclusively as the monoanionic species in the experimental pH region. The pH dependence of the kinetic parameters with 2′,3′-cyclic-NADPH as the variable substrate is shown in Figure 2. The log V and V/K pH profiles for 2′,3′-cyclic-

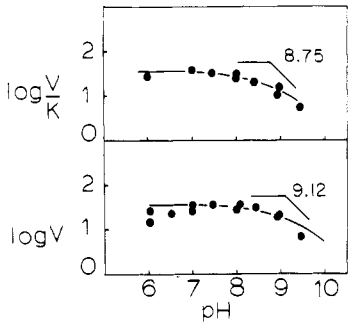

FIGURE 2: Effect of pH on kinetic parameters V and V/K with 2′,3′-cyclic-NADPH as the variable substrate at a saturating concentration of GSSG. The V profile shows a single enzymatic group with a pK_a of 9.12 ± 0.22 whose deprotonation abolishes activity. The V/K profile shows a single enzymatic group with a pK of 8.75 ± 0.1 whose deprotonation decreases binding.

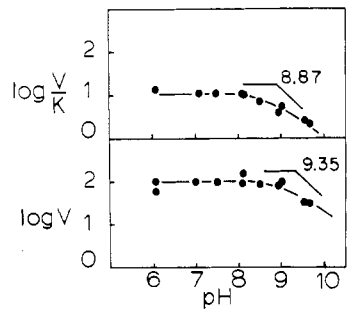

FIGURE 3: Effect of pH on kinetic parameters V and V/K with NADH as the variable substrate at a saturating concentration of GSSG. The V profile shows a single enzymatic group with a pK_a of 9.35 ± 0.16 whose deprotonation abolishes activity. The V/K profile shows a single group with a pK_a of 8.87 ± 0.08 whose deprotonation decreases binding.

NADPH decrease with slopes of −1 above pK_a's of 9.12 ± 0.22 and 8.75 ± 0.1, respectively. NADH, which lacks any 2′-phosphate, is a poor substrate for human erythrocyte glutathione reductase, exhibiting a V equal to 73% that of NADPH but a K_m in excess of 250 μM. The log V and V/K pH profiles for NADH decrease with slopes of −1 above pK_a's of 9.35 ± 0.16 and 8.87 ± 0.08, respectively (Figure 3).

pH Dependence of K_i's for Inhibitors. The NADP analogues AADP, ATP-ribose, and 2′,5′-ADP are linear competitive inhibitors versus NADPH (Figure 4, insert), exhibiting K_i's of 1.9 ± 0.2 μM, 1.2 ± 0.4 μM, and 0.6 ± 0.1 μM, respectively, at pH 8.1. The pH dependence of K_i for these three inhibitors is shown in Figure 4. The K_i of AADP is pH independent in the range examined. The K_i's of ATP-ribose and 2′,5′-ADP are pH independent in the low-pH region, but increase at high pH as a group with a pK_a of 8.87 ± 0.12 and 8.54 ± 0.09, respectively, is deprotonated.

The pK values of groups observed in pK_i profiles represent the ionization behavior of groups on free enzyme and free inhibitor. In order to distinguish between these two possibilities, it is essential that the pK's of the inhibitor be known. While the pK of the 2′-phosphate of ATP-ribose is known, the pK's of the 2′-phosphate of AADP and 2′,5′-ADP were not. The pH titration behavior of the 2′-phosphate of AADP and 2′,5′-ADP were determined by ^{31}P NMR (Figure 5). Between pH 4.5 and 9.0, the pyrophosphate moiety of AADP does not titrate, but the 2′-phosphate chemical shift is pH dependent. These data were fitted to eq 5 to yield a solution pK_a of 6.39 ± 0.02 for the 2′-phosphates of AADP. The 2′- and 5′-phosphates of 2′,5′-ADP show a similar pH titration between pH 4.8 and 8.0. The data for 2′,5′-ADP were fitted to eq 5 to yield solution pK's for the 2′-phosphate of 6.49 ± 0.02 and

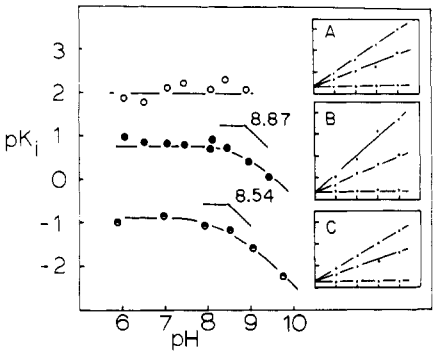

FIGURE 4: Effects of pH on K_i for AADP (A), ATP-ribose (B), and 2′,5′-ADP (C), which are competitive inhibitors versus NADPH. Representative double-reciprocal plots were at pH 8.1. The pK_i pH profile for AADP exhibits no pH dependence (○). The pK_i pH profile for ATP-ribose (●) and 2′,5′-ADP (⊖) shows a single enzymatic group with a pK_a of 8.87 ± 0.12 and 8.54 ± 0.09, respectively, whose deprotonation increases the slope inhibition constants. The data have been offset by 1 log order for clarity.

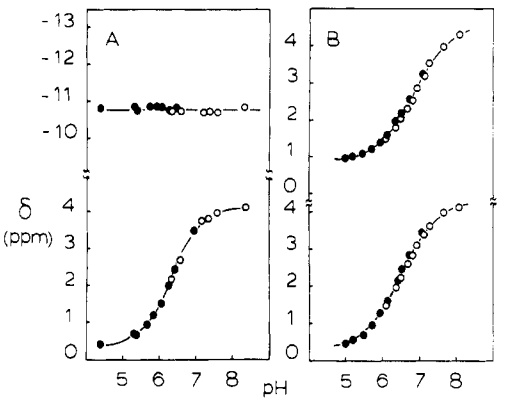

FIGURE 5: Effects of pH on the ^{31}P NMR chemical shifts of the 2′-phosphate and pyrophosphate of AADP (A) and 2′- and 5′-phosphates of 2′,5′-ADP (B) in MES (●) or BES (○) buffers. The 2′-phosphates of AADP and 2′,5′-ADP exhibit solution pK_a's of 6.39 ± 0.02 and 6.49 ± 0.02, respectively (bottom). The pyrophosphate resonance of AADP is pH independent in this pH range while the 5′-phosphate of 2′,5′-ADP exhibits a pK_a of 6.85 ± 0.02 (top).

6.85 ± 0.02 for the 5′-phosphate. The assignment of resonances to the 2′- or 5′-phosphates was accomplished by analyzing the phosphorus–hydrogen coupling patterns under heteronuclear undecoupled conditions. The 2′-phosphate resonance was split into a doublet, while the 5′-phosphate resonance was split into a poorly resolved triplet.

Temperature Dependence of pK Observed in the V Profile. To determine the chemical identity of the acidic group observed in the V pH profile, the temperature dependence of the pK observed in the V_{GSSG} pH profile was determined (Figure 6). Values obtained from the fits to eq 2 of the V_{GSSG} pH profile gave pK_a's of 8.83 ± 0.07, 8.98 ± 0.07, 8.97 ± 0.09, and 8.98 ± 0.23 at 35, 25, 20, and 15 °C, respectively. When the pK_a values were plotted versus reciprocal absolute temperature, a linear dependence on reciprocal temperature was observed and was fitted to eq 4 (Figure 6, right). The slope of this line is equal to $\Delta H_{ion}/2.303R$, allowing the calculation of a value for ΔH_{ion} of 3.2 ± 1.2 kcal/mol.

Discussion

The selective binding of NADPH by oxidoreductases requires the presence of binding sites that interact with the 2′-phosphate monoester. For example, *p*-hydroxybenzoate hydroxylase has a presumptive 2′-phosphate binding site for NADPH containing the side chains of His-162, Arg-166, and

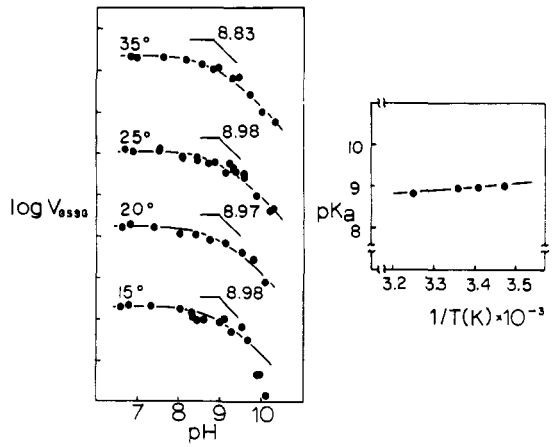

FIGURE 6: Temperature dependence of the pK_a exhibited by the enzymatic group observed in the V profile with GSSG as the variable substrate at a saturating concentration of NADPH (left). A plot (right) of reciprocal absolute temperature against pK_a yields a line whose slope equals $\Delta H_{ion}/2.303R$. The calculated enthalpy of ionization for this enzymatic group is 3.2 ± 1.2 kcal/mol.

Arg-269 (Wierenga et al., 1982). Dihydrofolate reductase from *Lactobacillus casei* has a 2′-phosphate binding site for NADPH composed of the side chains of Arg-43, Thr-63, and His-64 (Filman et al., 1982). The residues present in the 2′-phosphate site of the human erythrocyte glutathione reductase are Arg-218, His-219, and Arg-224 (Pai & Schulz, 1983; Pai et al., 1988). This positively charged pocket allows for discrimination between 2′-phosphorylated nucleotides and nucleotide analogues and nonphosphorylated nucleotides. Michaelis constants for NADPH and 2′,3′-cyclic-NADPH with human erythrocyte glutathione reductase are in the range of 5–10 μM, while the Michaelis constant for NADH is ~250 μM. The pH variation of the kinetic parameters has been defined for these three reduced pyridine nucleotide substrates and three inhibitors of human erythrocyte glutathione reductase. The details of the enzyme–ligand interaction for each of these compounds has been defined by X-ray crystallography (Pai & Schulz, 1982; Pai et al., 1988). In this report, we attempt to determine the nature of the binding interactions between the 2′-phosphate and the enzyme.

pH Variation of V and V/K for Nucleotide Substrates. The pH dependence of V_{NADPH} indicated that an acidic group with a pK_a of 9.21 ± 0.07 is deprotonated with the loss of catalytic activity. The same group was previously observed in the log V_{GSSG} pH profile for this enzyme (Wong et al., 1988). In the V/K_{NADPH} pH profile, which measures the ionization behavior of titratable groups on free substrate and free enzyme, no decrease in V/K is observed as the 2′-phosphate of NADPH is protonated. In the basic region of the V/K_{NADPH} pH profile, a group with a pK_a greater than 9.8 is observed whose deprotonation decreases the binding of NADPH to the enzyme. If only the dianionic form of the 2′-phosphate of NADPH were bound to the enzyme, V/K_{NADPH} would be expected to decrease as the 2′-phosphate was protonated below the solution pK_a of 6.52 (Mas & Colman, 1984). Two interpretations are consistent with these data. The first is that NADPH is kinetically sticky (Cleland, 1977), causing the 2′-phosphate pK_a to be observed at a pH below the experimentally accessible pH region. The second is that the enzyme does not discriminate between the dianionic and monoanionic forms of NADPH and binds both with similar affinity. To distinguish between these two possibilities (Cleland, 1977), the pH dependence of the kinetic parameters V and V/K was determined for two additional substrates, 2′,3′-cyclic-NADPH and NADH, and for the K_i of three linear competitive inhibitors, AADP, ATP-ribose, and 2′,5′-ADP.

The substrate 2′,3′-cyclic-NADPH has the same K_m and V_{max} as NADPH, suggesting that the enzyme accommodates the 2′,3′-cyclic phosphodiester moiety. The titration of an acidic group with a pK_a of 9.12 ± 0.22 is seen in the log $V_{2',3'cNADPH}$ profile, which corresponds closely to the pK_a of 9.21 for the acid seen in the V_{NADPH} pH profile. The log $V/K_{2',3'cNADPH}$ pH profile shows no pH dependence in the acid pH region, as the 2′,3′-phosphodiester is not protonated in this pH region. The basic pH region shows that deprotonation of an acid group with a pK_a of 8.75 ± 0.10 abolishes binding. It is likely that this is the same group seen in V/K_{NADPH}. The discreprency between the pK values observed by using 2′,3′-cyclic-NADPH and NADPH may be because 2′,3′-cyclic-NADPH is not as sticky as NADPH. To confirm this interpretation, pH studies using NADH as the variable substrate were performed.

The log V_{NADH} pH profile is dependent upon the ionization behavior of an acidic group with a pK_a of 9.35 ± 0.16. This corresponds closely to the pK_a's of 9.12 and 9.21 for a group seen in the $V_{2',3'cNADPH}$ and V_{NADPH} pH profiles, respectively, whose deprotonation abolishes catalytic activity. The kinetic parameter V/K_{NADH} is pH independent in the acidic region, since NADH has no titrable phosphoester, but decreases in the basic region as an acidic group with a pK_a of 8.87 ± 0.08 is deprotonated. On the basis of similarity in pK values, this behavior can be explained by deprotonation of the same group seen in V/K_{NADPH} and $V/K_{2',3'cNADPH}$ pH profiles.

pH Variation of K_i of Competitive Inhibitors. To identify the group or groups responsible for the pH behavior of V/K for reduced pyridine nucleotide substrates, the pH dependence of K_i for three linear competitive inhibitors ATP-ribose, AADP, and 2′,5′-ADP was determined. The pH dependence of K_i of a competitive inhibitor yields information on the correct pK's of groups in free substrate and free enzyme whose ionization state are important in binding (Cleland, 1977). The compound AADP is a redox-inactive analogue of NADPH (Anderson & Fisher, 1980), is a linear competitive inhibitor versus NADPH (K_i = 1.8 μM), and binds at the NADPH binding site (Pai et al., 1988). The ^{31}P NMR chemical shift titration of the 2′-phosphate yields a pK_a value for the 2′-phosphate of 6.39 ± 0.02, in excellent agreement with values for the 2′-phosphate pK of NADPH (6.52 ± 0.03) and ATP-ribose (6.36 ± 0.02) (Mas & Colman, 1984). The pK_i profile of AADP shows no dependence on pH, and thus, the inhibition is independent of the ionization state of the 2′-phosphate.

The compounds ATP-ribose and 2′,5′-ADP bind to the enzyme at the NADPH binding site (Pai & Schulz, 1982) and are linear competitive inhibitors versus NADPH (K_i = 1.2 μM and 0.6 μM, respectively). The solution pK_a of the 2′-phosphate of ATP-ribose has been determined to be 6.36 ± 0.02 (Mas & Colman, 1984). The pK_i pH profile for ATP-ribose shows no pH dependence in the acid pH region where the pK of the 2′-phosphate of ATP-ribose titrates. In the basic pH region, the deprotonation of an acidic group with a pK_a of 8.87 ± 0.12 results in an increase in the K_i of ATP-ribose. This acid has a similar pK_a to the enzyme group observed in the three V/K pH profiles for reduced pyridine nucleotide substrates.

The third inhibitor tested, 2′,5′-ADP, exhibits very similar pH dependence of its K_i compared with ATP-ribose. The solution pK_a of the 2′-phosphate was determined to be 6.49, and again the 2′-phosphate pK_a is not observed in the pK_i

profile. In the basic region, the deprotonation of an acidic group, exhibiting a pK_a of 8.54 ± 0.09, results in an increase in the K_i of 2′,5′-ADP.

Nature of the Interaction between Enzyme and Pyridine Nucleotide. The 2′-phosphate binding pocket of human erythrocyte glutathione reductase consists of three potentially cationic residues, Arg-218, Arg-224, and His-219, suggesting that the primary enzyme–substrate interactions were ionic in nature. The identity of the kinetic parameters measured by using NADPH, whose ribophosphate is dianionic, and 2′,3′-cyclic-NADPH, whose ribophosphate is monoanionic, suggested that this may not be the only interaction. Comparison of the V/K pH profiles determined by using NADPH or 2′,3′-cyclic-NADPH as reductant argues that the charge, and thus protonation state, of the ribophosphate moiety is unimportant. An important test of this would be the analysis of the pH dependence of the K_i's for inhibitors that are structural analogues containing a 2′-phosphate, since this type of analysis yields true pK values for the free enzyme or substrates. The pH independence of the K_i's for ATP-ribose, AADP, and 2′,5′-ADP rules out substrate stickiness as a cause for not observing the pK of the 2′-phosphate of NADPH in the V/K_{NADPH} profile and suggests that the enzyme does not preferentially bind the dianionic or monoanionic forms of the 2′-phosphate moiety of substrates or inhibitors. We suggest that a significant interaction between the enzyme and ribophosphate moiety is hydrogen bonding. The pH independence of V/K_{NADPH} and $pK_{i,ATP\text{-}ribose}$, in the pH region where their 2′-phosphates would titrate, has also been observed for dihydrofolate reductase (Morrison & Stone, 1988).

The V/K values of the reduced pyridine nucleotide substrates and the pK_i values of competitive inhibitors decrease in the basic pH region as a group on the enzyme is deprotonated. When NADPH is used as the variable substrate, this pK is observed at pH >9.8. When other substrates or competitive inhibitors are used, this pK is observed at pH ~8.8 (the pK_i profile for AADP was not performed at basic enough pH values to define any enzymatic pK). It is unlikely that this group is involved in 2′-phosphate binding, since deprotonation of the enzymic group also results in a decrease of V/K_{NADH}. One possibility considered was that this group is Tyr-197, which rotates away from the enzyme-bound FAD to allow binding of the reduced pyridine nucleotide or several fragments of NADPH (Pai et al., 1988). To test this possibility, we examined the pK_i profile for 2′,5′-ADP, which does not promote the movement of Tyr-197 (Pai et al., 1988). The observation that 2′,5′-ADP binding decreases as a result of the deprotonation of an enzymic group with a pK_a of 8.5 suggests that Tyr-197 is not the group being titrated. Another possibility is that the group is Arg-218, which makes contacts with the adenine and adenosylribose ring in the E·NADPH complex (Pai et al., 1988). The present data are insufficient to identify this enzymic group further.

Chemical Identity of Acidic Group Observed in V–pH Profiles. Pre-steady-state stopped-flow experiments with yeast glutathione reductase have demonstrated that the oxidative half-reaction is 1.8 times slower than the reductive half-reaction using NADPH as reductant (Williams, 1976). Solvent kinetic isotope effect studies, using the human erythrocyte enzyme, have identified a one-proton transfer step to the first glutathione thiolate anion as rate limiting in the oxidative half-reaction (Wong et al., 1988). The crystal structure of human erythrocyte glutathione reductase reveals a "pseudo-charge-relay" system composed of Cys-58, His-467′, and Glu-472′ present in the GSSG catalytic site (Karplus & Schulz, 1987). These three residues are arranged in a linear fashion and are within hydrogen bonding distances.

The temperature dependence of the pK observed in the V_{GSSG} profile allowed us to calculate a ΔH_{ion} of 3.2 ± 1.2 kcal/mol for the group whose deprotonation abolishes activity. On the basis of the kinetic and structural studies discussed above, it is likely that this group is an acid involved in reactions in the glutathione reducing half-reaction (Pai & Schulz, 1983; Wong et al., 1988). In two electron reduced yeast glutathione reductase, a group with a pK_a of about 9.2 has been asscribed to a histidine residue in the active site on the basis of spectroscopic titrations (Sahlman & Williams, 1987). Our value for the enthalpy of ionization is significantly lower than the expected value of ~7 kcal/mol for the enthalpy of ionization of an isolated imidazole side chain. However, since His-467′ and Glu-472′ are observed to be within hydrogen bonding distances at the GSSG active site of human erythrocyte glutathione reductase (Karplus & Schulz, 1987), we propose that these two residues function in an unified manner and exhibit a single pK_a of about 9 which is observed in all V pH profiles. We suggest that deprotonation of the His-467′–Glu-472′ ion pair prevents the protonation of the first product glutathione thiolate anion and thus abolishes activity.

Registry No. NADPH, 53-57-6; NADH, 58-68-4; 2′,3′-cyclic NADP, 62640-02-2; AADP, 54758-28-0; ATP-ribose, 53595-18-9; 2′,5′-ADP, 3805-37-6; glutathione reductase, 9001-48-3.

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