Critical Ionization States in the Reaction Catalyzed by Triosephosphate Isomerase†

Joel G. Belasco,‡ Julia M. Herlihy, and Jeremy R. Knowles*

ABSTRACT: To allow the detailed interpretation of the pH dependences of the steady-state parameters for the reaction catalyzed by triosephosphate isomerase, three kinds of experiments have been performed. First, the value of k_{cat}/K_m for enzyme-catalyzed isomerization of the phosphonate analogue of D-glyceraldehyde 3-phosphate (2-hydroxy-4-phosphonobutyraldehyde) has been shown to titrate with an apparent pK_a of 7.5, which is close to the phosphonate's second ionization constant. Secondly, the sulfate ester analogue of dihydroxyacetone phosphate (dihydroxyacetone sulfate), which exists only as a monoanion over the pH range of interest, has been shown not to bind detectably to the enzyme. Thirdly, an isotopic discrimination experiment at pH 5.2 has been compared with a similar investigation at pH 7.6. The results together demonstrate that both enzyme and substrate ionizations control the reaction rate in the pH range 5 to 8.

The pH dependence of enzyme reactions is a potentially powerful probe of the mechanisms and energetics of enzyme catalysis. However, the conclusions of such studies have often been of doubtful validity because unsubstantiated assumptions have been made about the kinetic significance of various elementary steps (Knowles, 1976).

Triosephosphate isomerase (EC 5.3.1.1) catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate via a *cis*-enediol intermediate (Rieder & Rose, 1959). The pH dependences of the steady-state parameters for the reaction catalyzed by the chicken muscle enzyme have been determined (Plaut & Knowles, 1972), and some controversy has ensued over whether the apparent pK_a values of 6 observed for k_{cat} and k_{cat}/K_m are due to substrate or enzyme ionizations. The simplicity of these pH dependences and the recent definition of the free-energy profile for the enzyme-catalyzed reaction (Albery & Knowles, 1976a) provide a unique opportunity to obtain a precise interpretation of the identity and catalytic importance of the observed ionizations. In this paper we describe experiments with substrate analogues and measurements of isotopic discrimination designed to delineate the origins of the pH dependence of the catalytic activity. The results demonstrate that *both* substrate and enzyme ionizations are kinetically important in the pH range 5 to 8 and that the enzyme utilizes the free energy of substrate binding to increase its catalytic power.

Experimental Section

Materials

Crystalline triosephosphate isomerase was prepared from chicken muscle by a modification of the methods of Putman et al. (1972) and McVittie et al. (1972). Aldolase (from rabbit muscle, as a sulfate-free, lyophilized powder) and α-glycerophosphate dehydrogenase (rabbit muscle), D-glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), and 3-phosphoglycerate kinase (yeast) (as crystalline suspensions in ammonium sulfate) were obtained from the Sigma Chemical Co., St. Louis, Mo. Before use, the two dehydrogenases and the kinase were treated with bromohydroxyacetone phosphate (de la Mare et al., 1972) to inactivate traces of triosephosphate isomerase and then dialyzed exhaustively to remove ammonium sulfate. NAD^+, NADH (disodium salt), ATP (disodium salt), dihydroxyacetone phosphate (as the dicyclohexylammonium salt of the dimethyl ketal), D-glyceraldehyde 3-phosphate (as the barium salt of the diethyl acetal), Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked), and Dowex 1 (Cl^- form, 200–400 mesh, 8% cross-linked) were obtained from Sigma. DEAE-cellulose (DE52) was obtained from Whatman, Ltd., Maidstone, U.K. Tritiated water (5 Ci/mL) was obtained from Amersham/Searle Corp., Arlington, Ill. Standard buffers were obtained from the Fisher Chemical Co., Medford, Mass. Buffer solutions were prepared with the highest grade components using distilled-deionized water.

DL-2-Hydroxy-4-phosphonobutyrate was prepared by the method of Dixon & Sparkes (1974, 1976) and was purified by chromatography on DEAE-cellulose. The dicyclohexylammonium salt was converted to the trisodium salt by treatment with Dowex 50 (H^+) followed by titration to pH 8 with NaOH. The concentration of the phosphonate was determined by enzymic reduction of the D-enantiomer to D-2-hydroxy-4-phosphonobutyraldehyde (Orr & Knowles, 1974).

D-2-Hydroxy-4-phosphonobutyraldehyde was prepared by enzymic reduction of 2-hydroxy-4-phosphonobutyrate. The reaction mixture (14.0 mL) contained 21 mM ammonium bicarbonate buffer, pH 7.8, $MgCl_2$ (2.1 mM), EDTA (0.09 mM), NADH (2.0 mM), ATP (80 mM, pH 7.8), DL-2-hydroxy-4-phosphonobutyrate (3.1 mM in the D enantiomer), glyceraldehyde phosphate dehydrogenase (0.75 mg/mL), and phosphoglycerate kinase (0.08 mg/mL). The course of the reaction was followed by observing the decrease in NADH absorbance at 367 nm. After the reduction was complete (approximately 4 min, 30 °C), the reaction mixture was immediately filtered through Dowex 50 (H^+) (4 g). The resulting solution was then treated with portions of prewashed charcoal until it had, after filtration, an absorbance of less than 0.01 at

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‡ National Science Foundation Predoctoral Fellow.

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both 259 nm and 340 nm. The mixture was diluted to 130 mL with water and the pH adjusted to 4.2 with 0.1 M NaOH. The solution was applied to a column (1.8 cm^2 × 5.0 cm) of DEAE-cellulose equilibrated at 3 °C with 5 mM sodium formate, pH 4.3. A linear gradient of sodium formate (40 + 40 mL, 5 to 125 mM, pH 4.3) was applied to the column, and fractions of 1.5 mL were collected. The fractions were assayed for D-2-hydroxy-4-phosphonobutyraldehyde (see below), 2-hydroxy-4-phosphonobutyrate (Orr & Knowles, 1974), and inorganic phosphate (Briggs, 1922). Fractions containing 2-hydroxy-4-phosphonobutyraldehyde that were free of inorganic phosphate and 2-hydroxy-4-phosphonobutyrate were pooled, and this solution was passed down a column (0.5 cm^2 × 3.3 cm) of Dowex 50 (H^+) and eluted with water (8 mL). The eluate was freeze-dried and the residue redissolved in water (1.0 mL) to give a solution of 4.85 mM D-2-hydroxy-4-phosphonobutyraldehyde (<1% contamination by 1-hydroxy-4-phosphono-2-butanone). In this form the product is stable for months at 3 °C.

Dihydroxyacetone sulfate was prepared by a modification of the method of Grazi et al. (1973). A mixture of dihydroxyacetone (0.45 g, 5.0 mmol) and trimethylamine–sulfur trioxide (0.21 g, 1.5 mmol) in dry dimethylformamide (2.25 mL) was stirred under N_2 at room temperature for 25 h. The solution was then diluted with water to 300 mL and applied to a column (4.9 cm^2 × 21 cm) of DEAE-cellulose equilibrated at 3 °C with 5 mM sodium benzoate, pH 4.7. The column was eluted with a linear gradient of sodium benzoate (400 + 400 mL, 5 to 100 mM, pH 4.7). Fractions containing dihydroxyacetone sulfate (assayed enzymically (Grazi et al. (1974)) were pooled, treated with Dowex 50 (H^+) (40 mL), and filtered. The filtrate (125 mL) was extracted with dichloromethane (3 × 125 mL). The pH was adjusted to 4.1 with sodium bicarbonate, and the solution was concentrated to 4 mL by rotary evaporation. The concentrate was then passed through a small column (0.13 cm^2 × 1.0 cm) of charcoal, giving 4.1 mL of 208 mM dihydroxyacetone sulfate (0.85 mmol), which was stored at 3 °C.

Methods

Ultraviolet measurements were made at 30 °C using a Perkin-Elmer 575 or Pye Unicam SP1800 spectrophotometer. An extinction coefficient for NADH of 6220 M^{-1} cm^{-1} at 340 nm was assumed (Horecker & Kornberg, 1948). pH measurements were made at 30 °C with a Radiometer PHM64 pH meter and a Radiometer GK2320C combination electrode. Scintillation counting was done with a Beckman LS233 or a Beckman LS100 automatic liquid scintillation counter. Samples (100 μL) for specific radioactivity determinations were added to scintillation fluid (7 mL), prepared from toluene (2460 mL), ethanol (1120 mL), naphthalene (210 g), 2,5-diphenyloxazole (10.5 g), and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.42 g).

2-Hydroxy-4-phosphonobutyraldehyde Isomerization. This reaction was monitored at 340 nm using a coupled-enzyme assay involving enzymic reduction (Stribling, 1974) of the product, 1-hydroxy-4-phosphono-2-butanone. The cuvette contained in 0.975 mL at 30 °C: 82 mM triethanolamine hydrochloride buffer, sodium chloride (15.4 mM), EDTA (0.82 mM), NADH (67 μM), D-2-hydroxy-4-phosphonobutyraldehyde (46 μM), α-glycerophosphate dehydrogenase (0.10 mg/mL), and triosephosphate isomerase (0.33 μg/mL). The ionic strength was 0.1. The rate of the uncatalyzed isomerization was determined before the addition of triosephosphate isomerase. pH measurements were made at the end of each experiment.

Tests of Dihydroxyacetone Sulfate as a Substrate for Triosephosphate Isomerase. Dihydroxyacetone sulfate (20 μmol) was incubated at 30 °C with tritiated water (10 μL of 5 Ci/mL) and triosephosphate isomerase (0.25 mg) in 200 mM triethanolamine hydrochloride, pH 7.4, containing EDTA (10 mM) (1 mL). Duplicate samples (25 μL) were removed for scintillation counting before and after the incubation. After 3 h, the reaction mixture was cooled in a dry ice–acetone bath, and 1 M HCl (200 μL) was added to inactivate the isomerase. After freeze-drying, the residue was dissolved in 0.16 mM HCl (40 mL) and applied to a column (1.5 cm^2 × 12 cm) of Dowex 1 (Cl^-) equilibrated with 0.16 mM HCl. The column was eluted with a nonlinear pH gradient (90 + 90 mL, 0.16 mM HCl to 1 M HCl). Fractions (1.5 mL) were collected and samples taken immediately for assay and scintillation counting.

Inhibition of Triosephosphate Isomerase by Dihydroxyacetone Sulfate. These experiments were performed under the conditions described by Plaut & Knowles (1972). The assay solution contained 80 mM triethanolamine hydrochloride, pH 7.6, EDTA (0.2 mM), sodium arsenate (1 mM), NAD^+ (1 mM), dihydroxyacetone phosphate (0.2–3.0 mM), glyceraldehyde phosphate dehydrogenase (0.3 mg/mL), and triosephosphate isomerase (10 ng/mL), in the presence or absence of dihydroxyacetone sulfate (20 mM). To compensate for the ionic strength contribution of the dihydroxyacetone sulfate, runs in the absence of the inhibitor contained 20 mM NaCl.

Incorporation of Solvent Tritium into Substrate and into Product during the Isomerase-Catalyzed Reaction. These determinations were made at pH 5.2, 30 °C using a modification of the method of Maister et al. (1976). The reaction mixture (3.61 mL) contained 70 mM pyridinium hydrochloride buffer, pH 5.2, EDTA (0.6 mM), monosodium arsenate (28 mM), NAD^+ (3 mM), glyceraldehydephosphate dehydrogenase (0.70 mg/mL), dihydroxyacetone phosphate (0.98 mM), and tritiated water (70 mCi/mL). The ionic strength was 0.1. Before the addition of triosephosphate isomerase (to give 15 ng/mL), two samples (10 μL) were withdrawn to determine the specific radioactivity of the solution. About 5 h after the addition of isomerase, a portion (100 μL) was removed and assayed for dihydroxyacetone phosphate. The reaction was then quenched by the addition of 3 M HCl (75 μL).

After treatment with charcoal to remove nucleotides, the product and the remaining substrate were separated by the method of Herlihy et al. (1976). The specific radioactivities of dihydroxyacetone phosphate and of 3-phosphoglycerate were determined by the method of Maister et al. (1976). To determine the percentage incorporation of tritium at each of the prochiral sites on C-1 of dihydroxyacetone phosphate, samples of the reisolated substrate (21.5 μM, 1–2 mL) were treated at room temperature either with triosephosphate isomerase (8 μg) for 11 h or with triosephosphate isomerase (8 μg) for 1.75 h followed by aldolase (700 μg) for 8.5 h. In each case the samples were freeze-dried after the incubation, and the radioactivities of the distillate and nonvolatile residue were determined.

Results

The Isomerization of D-2-Hydroxy-4-phosphonobutyraldehyde. A coupled-enzyme assay using glycerophosphate dehydrogenase was employed to determine the rates of both the uncatalyzed and the triosephosphate isomerase catalyzed isomerization of D-2-hydroxy-4-phosphonobutyraldehyde. Under the conditions of the experiment, the observed rates were limited by the rate of the isomerization step.

The pH profiles for these reactions are shown in Figure 1. The uncatalyzed rate was independent of buffer and coupling enzyme concentration. On the basis that the second pK_a value of a phosphonate is about 1.2 units above that of the corresponding phosphate ester, the pK_a of 2-hydroxy-4-phosphonobutyraldehyde is expected to be 7.5 (Plaut & Knowles, 1972; Orr & Knowles, 1974). In close agreement with this prediction, a kinetic pK_a of 7.6 is observed for its uncatalyzed rate of isomerization, which suggests that the phosphonate group acts as an intramolecular general base. The enzyme-catalyzed rate (which was eliminated when triosephosphate isomerase was pretreated with the irreversible inactivator bromohydroxyacetone phosphate) was in all cases first-order in substrate concentration and showed a similar pH dependence.

In passing, we should note that the phosphonate analogue of dihydroxyacetone phosphate has been reported *not* to be a substrate for triosephosphate isomerase (Dixon & Sparkes, 1974). Yet our observation of enzyme-catalyzed phosphonate isomerization in the opposite direction requires (by microscopic reversibility) that both phosphonates be substrates. This apparent discrepancy can be understood, however, in the light of the following: (a) the enzyme-catalyzed rate for the isomerization of the phosphonate analogue of D-glyceraldehyde 3-phosphate is some 800-fold slower than that of the natural substrate; (b) the equilibrium for the phosphonates lies dominantly (even more than for the phosphate esters themselves) toward the analogue of dihydroxyacetone phosphate (J. G. Belasco, unpublished results); and (c) the enzyme assays in the thermodynamically uphill direction (studied by Dixon & Sparkes, 1974) are comparatively more difficult to perform. The reason for the poor handling of the phosphonates by triosephosphate isomerase appears largely to be a binding problem (Dixon & Sparkes, 1974). This hypothesis is supported by recent x-ray crystallographic studies on the enzyme:substrate complex, which indicate that the amino group of Lys-13 may lie very close to the phosphate bridge oxygen of the substrate (Phillips et al., 1977; D. C. Phillips & I. A. Wilson, personal communication). Interaction of this lysine ammonium ion with the lone-pair electrons of the substrate's bridge oxygen, which is impossible with the phosphonate analogues, may be of critical importance to binding.

Dihydroxyacetone Sulfate as a Substrate for Triosephosphate Isomerase. Dihydroxyacetone sulfate was incubated with triosephosphate isomerase in tritiated water and then reisolated by ion-exchange chromatography. Under the conditions used, the *pro-R* hydrogen at C-1 of dihydroxyacetone phosphate would be completely equilibrated with the solvent protons within 30 s. With dihydroxyacetone sulfate, a small amount of tritium labeling (about 0.4%) was observed, which was comparable with a parallel incubation without enzyme.

Dihydroxyacetone sulfate is not, therefore, even a partial substrate for triosephosphate isomerase. To determine whether dihydroxyacetone sulfate binds to the enzyme, its action as an inhibitor was investigated. The apparent K_m for dihydroxyacetone phosphate was 0.99 ± 0.05 mM in the absence of dihydroxyacetone sulfate and 0.96 ± 0.03 mM in the presence of 20 mM dihydroxyacetone sulfate. It is evident that dihydroxyacetone sulfate binds undetectably to triosephosphate isomerase, and that the K_i for this material is at least 100 mM.

The Incorporation of Solvent Tritium into Substrate and into Product at pH 5.2. The discrimination against tritium in the enzyme-catalyzed formation of glyceraldehyde phosphate has been measured at pH 7.6 (Maister et al., 1976), and the relative heights of the transition states for the enolization and the dissociation of glyceraldehyde phosphate have been evaluated from the mixed fractionation factor $\Phi_{3,4}$ (Albery & Knowles, 1976b) at that pH. This discrimination experiment was repeated at pH 5.2. The enzyme-catalyzed isomerization was greater than 98% rate determining throughout the course of the reaction. Because the decomposition of NADH at low pH and the concomitant loss of its chromophore at 340 nm made it an unreliable indicator of the extent of reaction, this was determined by assaying a portion of the reaction mixture for remaining dihydroxyacetone phosphate. After quenching the reaction, dihydroxyacetone phosphate and 3-phosphoglycerate were isolated, and their specific radioactivities were determined (see Table I). The magnitude of $\Phi_{3,4}$ was evaluated from these specific radioactivities by combining eq 6.6 and 6.9 of Albery & Knowles (1976b).

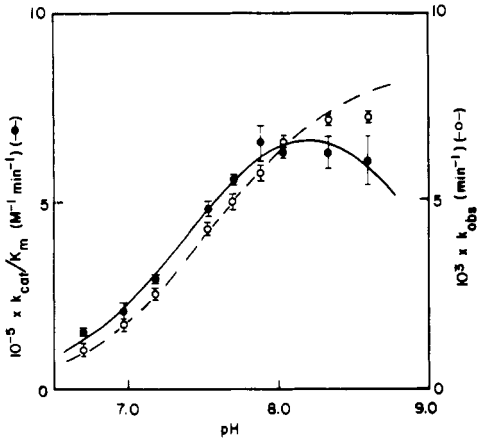


FIGURE 1: The pH dependence of the isomerization of 2-hydroxy-4-phosphonobutyraldehyde. (a) Spontaneous isomerization (○). The curve is theoretical for a pK_a value of 7.58. (b) Enzyme-catalyzed isomerization (●). The curve is theoretical for pK_a values of 7.47 and 8.93.

Discussion

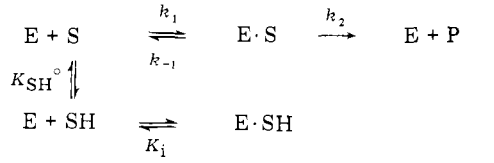
In the isomerization of each of the natural substrates by chicken muscle triosephosphate isomerase, k_{cat} falls at low pH with an apparent pK_a of 6, k_{cat}/K_m is bell-shaped with ionizations at pH 6 and 9, and K_m, which is pH independent below pH 8, then rises with an apparent pK_a of 9 (Plaut & Knowles, 1972). The identity of the apparent pK_a of 6 has been a subject of debate. Because dihydroxyacetone phosphate and glyceraldehyde phosphate have second ionization constants of 6.0 and 6.3, respectively, it has been variously suggested that the observed pK_a values of 6 are due to the substrates, that they arise from the protonation of a base on the enzyme (Plaut & Knowles, 1972), or that ionizations in enzyme and substrate are both involved (Cleland, 1977; Hartman & Ratrie, 1977).

We shall consider in turn these three general explanations for the observed pH dependences.[1]

I. *Only Substrate Ionization Is Important.* To accommodate the fall-off in k_{cat} at low pH in terms only of substrate ionization, Scheme I suffices. [The superscript (°) applies to parameters relating to the free enzyme or free substrate.] Both forms of the substrate bind, but only the dianionic form undergoes catalytic conversion to product. The effects of substrate hydration are neglected because it is known that the rates of hydration and dehydration are relatively fast (Plaut & Knowles, 1972), that the hydrated form of dihydroxyace-

[1] We shall not concern ourselves with the ionization at pH 9. This is evidently attributable to an enzyme residue that must be protonated in order for the substrate to bind.

SCHEME I: A Reaction That Responds Only to Substrate Ionization.[a]



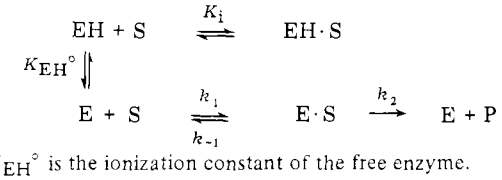
[a] K_{SH}° is the second ionization constant of the free substrate.

tone phosphate does not bind to the enzyme, and that at 30 °C the hydration equilibrium constant for dihydroxyacetone phosphate is essentially the same for the monoanionic and dianionic forms (Webb et al., 1977).

If the two protonation states of the enzyme:substrate complex (E·S and E·SH) can interconvert quickly, then the pH dependences observed for the steady-state parameters with the natural substrates are predicted only if the dissociation constants for the monoanionic and dianionic forms of the enzyme-bound substrate are equal and if $k_{-1} \gg k_2$. However, such a scheme is unacceptable because $k_{-1} < k_2$ with glyceraldehyde phosphate as the substrate (Albery & Knowles, 1976a). We must therefore assume in Scheme I that the interconversion of E·S and E·SH is slow enough to be neglected. In this case the pH dependences of the steady-state parameters are: $K_m^{obsd} = \tilde{K}_m/(f_S^{\circ} + \tilde{K}_m f_{SH}^{\circ}/K_i)$; $k_{cat}^{obsd} = k_2 f_S^{\circ}/(f_S^{\circ} + \tilde{K}_m f_{SH}^{\circ}/K_i)$; $(k_{cat}/K_m)^{obsd} = (k_2/\tilde{K}_m) f_S^{\circ}$ [where $f_S^{\circ} = K_{SH}^{\circ}/(H + K_{SH}^{\circ})$ is the fraction of free substrate that is dianionic, $f_{SH}^{\circ} = H/(H + K_{SH}^{\circ})$ is the fraction of free substrate that is monoanionic, K_i is the dissociation constant for the monoanionic form of the substrate, $\tilde{K}_m = (k_{-1} + k_2)/k_1$, and H is the hydrogen ion concentration]. These equations predict the observed pH dependences *only* if $K_i = \tilde{K}_m$, in which case: $K_m^{obsd} = \tilde{K}_m$; $k_{cat}^{obsd} = k_2 f_S^{\circ}$; $(k_{cat}/K_m)^{obsd} = (k_2/\tilde{K}_m) f_S^{\circ}$. Thus, if the dissociation constant for the substrate monoanion equals the Michaelis constant for the substrate dianion, the observed K_m will be pH independent, and k_{cat} and k_{cat}/K_m will reflect the pK_a of the free substrate.

II. *Only Enzyme Ionization is Important.* Scheme II depicts

SCHEME II: A Reaction That Responds Only to Enzyme Ionization.[a]



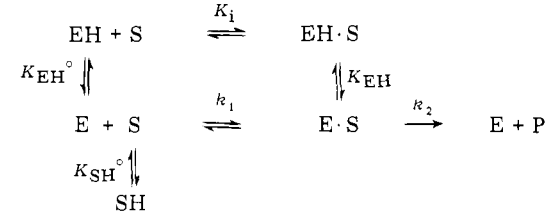
[a] K_{EH}° is the ionization constant of the free enzyme.

an enzyme which has a base of $pK_a = 6$ that is essential to catalysis. Substrate ionization is kinetically irrelevant. By reasoning similar to that above, the observed pH dependences are predicted *only* if $K_i = \tilde{K}_m$, in which case: $K_m^{obsd} = \tilde{K}_m$; $k_{cat}^{obsd} = k_2 f_E^{\circ}$; $(k_{cat}/K_m)^{obsd} = (k_2/\tilde{K}_m) f_E^{\circ}$ [where $f_E^{\circ} = K_{EH}^{\circ}/(H + K_{EH}^{\circ})$ is the fraction of unliganded enzyme that is deprotonated]. The observed K_m is pH independent, and k_{cat} and k_{cat}/K_m reflect the pK_a of the free enzyme.

III. *Both Enzyme and Substrate Ionizations Are Important.* This possibility requires that only the dianionic form of the substrate bind significantly to the enzyme and that the pK_a of a basic enzyme residue essential to catalysis be raised from below 5 up to 6 by substrate binding. In contrast to Schemes I and II, a treatment that ignores the enzyme:intermediate and enzyme:product complexes is an oversimplification, and a rigorous derivation is demanded (see Appendix). A simplified version is shown in Scheme III.

If the substrate binds at the diffusion-controlled rate at neutral pH (as suggested by Albery & Knowles, 1976a), then

SCHEME III: A Reaction That Responds to Both Enzyme and Substrate Ionizations.[a]



[a] K_{EH}° and K_{EH} are the ionization constants of the free and liganded enzymes respectively, and K_{SH}° is the second ionization constant of the free substrate.

the predicted pH dependences for the steady-state parameters above pH 5[2] are: $K_m^{obsd} = K_m f_E/f_S^{\circ}$; $k_{cat}^{obsd} = k_2 f_E$; $(k_{cat}/K_m)^{obsd} = (k_2/\tilde{K}_m) f_S^{\circ}$. From these equations it is evident that k_{cat} will reflect the pK_a of the liganded enzyme, while k_{cat}/K_m will reflect the pK_a of the free substrate. Interestingly, K_m will be pH independent even if the monoanionic form of the substrate cannot bind to the enzyme. This is because the elevation of the enzyme pK_a by the binding of substrate is thermodynamically equivalent to the substrate's having a higher affinity for the protonated form of the enzyme. If the pK_a values of the free substrate and of the liganded enzyme are the same, then the effect of protonating one will be exactly balanced by the protonation of the other, and no variation of K_m with pH will be observed.

Phosphate Analogues. Of the three possible schemes presented above, only two (I and III) predict that k_{cat}/K_m responds to the ionization state of the substrate. A substrate analogue with an altered pK_a is therefore an ideal probe to distinguish among the possibilities, and the phosphonate analogue of D-glyceraldehyde 3-phosphate was employed to this end. The rate of isomerization of this substrate analogue catalyzed by triosephosphate isomerase was measured under second-order conditions (i.e., $[S]_0 \ll K_m$) and shows an apparent pK_a of 7.5 (Figure 1). Since it is k_{cat}/K_m that is measured for enzyme catalysis under subsaturating conditions, we may conclude that the enzymic reaction is sensitive to the ionization state of the substrate, which has a pK_a near 7.5. This finding is inconsistent with Scheme II, but does not distinguish between Schemes I and III, for both of which the value of k_{cat}/K_m depends upon the substrate pK_a.

Although both Schemes I and III require that the monoanionic form of the substrate bind nonproductively, Scheme I demands that K_i for the monoanion equal K_m for the dianion, while Scheme III requires that the substrate monoanion bind negligibly to the enzyme. Because sulfate monoesters exist as monoanions over the whole of the neutral pH range, they are useful analogues for monoanionic phosphate esters.

Dihydroxyacetone sulfate is not even a partial substrate for triosephosphate isomerase, and its K_i is at least 100 mM, compared with a dissociation constant of 1.3 mM (Albery & Knowles, 1976a), and a K_m of 0.97 mM (Putman et al., 1972) for dianionic dihydroxyacetone phosphate. The lack of inhibition by dihydroxyacetone sulfate is not likely to be a consequence of steric factors. The x-ray crystallographic data for disodium glycerol 2-phosphate (Haque & Caughlan, 1966) and potassium ethanol sulfate (Truter, 1958) reveal very similar bond lengths and bond angles. On the basis of the NMR spectrum of the sodium salt of dihydroxyacetone sulfate (not shown), the extents of hydration of dihydroxyacetone

[2] This is the experimental limit for the chicken muscle enzyme. Consideration of the situation only above pH 5 explains the absence of terms involving pK_{EH}°, which is below 5.

phosphate and its sulfate analogue are also similar ([keto]/[hydrate] ≈ 1), and this too can be ruled out as an explanation for the large difference in binding free energy.

One must conclude that the sulfate analogue does not bind detectably to triosephosphate isomerase because its singly charged acid group cannot interact strongly with the anion-binding locus of the enzyme. It is therefore unlikely that monoanionic phosphate esters will bind as tightly as the corresponding dianions, a situation that is consistent with Scheme III but not with Schemes I and II. The low affinity of substrate monoanions for the enzyme is not surprising. It is thermodynamically equivalent to the downward perturbation of the second pK_a of the substrate on binding, which is a likely outcome of locating a phosphate group near a cationic lysine residue and several good hydrogen-bond donors (Phillips et al., 1977; D. C. Phillips & I. A. Wilson, personal communication).

The experimental results discussed are therefore consistent only with Scheme III, in which k_{cat}/K_m responds to the ionization of the substrate and k_{cat} responds to the ionization of the liganded enzyme. This scheme requires a basic enzyme residue essential for catalysis whose pK_a is raised from a value below 5 up to 6 by the binding of substrate. The prime candidate for this residue is Glu-165, which on the basis of both x-ray crystallographic and chemical modification studies appears to be the base responsible for abstracting the carbon-bound proton from the triosephosphate skeleton (Hartman, 1970; de la Mare et al., 1972; Phillips et al., 1977). Hartman et al. (1975) have in fact shown that this residue has a pK_a below 5 in the free enzymes from both rabbit and yeast.

An elevation in the pK_a of Glu-165 when the substrate binds is not unexpected. In the free enzyme this carboxylate group is well solvated both by water and by nearby polar side chains (e.g., Lys-13, His-95, Ser-96). But in the enzyme:substrate complex, the access of water to this residue is restricted, and the carboxylate group is confronted by the relatively non-polar surface of the substrate from which it must abstract a carbon-bound proton. It is probable that this reduction in solvation accounts for the free energy necessary to raise the pK_a of Glu-165 by two units or so. In the catalytic transition state, on the other hand, the negative charge of the carboxylate group emerges onto the incipient enolate oxygen, where it can once more be stabilized by interaction with the enzyme's electrophilic component (Webb & Knowles, 1975). In this manner the free energy of substrate binding is used to stabilize the transition and ground states differentially, thereby increasing the catalytic effectiveness of the enzyme.

There is, in fact, another possibility that cannot yet be ruled out. If His-95 was to act as a nucleophilic catalyst in an addition–elimination pathway analogous to that recently proposed by Bruice & Bruice (1976), then this residue could fulfill the requirements of the essential enzyme base. NMR studies of the unliganded enzyme interpreted on the basis of the crystal structure have suggested that this residue does not ionize over the pH range 5.4 to 9.0 due to its role as a hydrogen-bond acceptor from a backbone amide linkage (Browne et al., 1976). If this hydrogen bond is severed on substrate binding, the histidine residue might assume a more normal pK_a of 6, as required by Scheme III.

Hartman & Ratrie (1977) have recently reported that k_{cat} and k_{cat}/K_m for triosephosphate isomerase from yeast titrate at pH 4.6 and 5.9, respectively. These results are entirely consistent with the interpretation of the pH behavior of the chicken muscle enzyme presented above, except for a somewhat smaller elevation of the essential base pK_a in the case of the yeast enzyme (Hartman & Ratrie, 1977).

TABLE I: The Extent of Incorporation of Solvent Tritium into Substrate and Product at pH 5.2.[a]

fractional extent of reaction ($1 - r$)	0.39
specific radioactivity of solvent (**x**, cpm/μatom)	361×10^3
specific radioactivity of 3-phosphoglycerate (**p**, cpm/μmol)	288×10^3
specific radioactivity of remaining dihydroxyacetone phosphate at the *pro-R* site (**s**, cpm/μmol)[b]	61×10^3
mixed fractionation factor ($\Phi_{3,4}$) at pH 5.2[c]	0.81 ± 0.10

[a] Albery & Knowles (1976b). [b] Corrected for the adventitious incorporation of tritium at the *pro-S* site by contaminating aldolase. [c] This was calculated using the formula $\Phi_{3,4} = [(\mathbf{p}/\mathbf{x}) + (\mathbf{s}/\mathbf{x})(r/(1 - r))A_5']/(1 - A_5')$ (Albery & Knowles, 1976b), assuming a value of 0.01 for A_5' at pH 5.2.

Isotopic Discrimination. The weaker binding of substrate monoanions to triosephosphate isomerase could result from a lower "on" rate, a higher "off" rate, or both. We can distinguish among these possibilities by measuring the partition ratio of the enzyme:substrate complex between dissociation and enolization.

In the isomerase-catalyzed reaction, the enzyme-bound enediol intermediate rapidly exchanges protons with the solvent (Rieder & Rose, 1959; Herlihy et al., 1976). This intermediate can pick up a proton on C-2 to form glyceraldehyde phosphate, which is then lost from the enzyme. The tritium content of the free glyceraldehyde phosphate produced is a measure of the extent to which the proton transfer step is rate-limiting in the sequence: bound enediol → bound product → free product. A large isotopic discrimination (small mixed fractionation factor) is characteristic of relatively fast dissociation of the product, while a small isotope effect (large mixed fractionation factor) shows that the "off" step (which does not involve proton transfer) is relatively slow.

From the results in Table I it is clear that the mixed fractionation factor $\Phi_{3,4}$ is unaltered by lowering the pH from 7.6, where the free substrate is more than 95% in the dianionic form, to 5.2, where the free substrate is more than 90% in the monoanionic form ($\Phi_{3,4} = 0.83 \pm 0.01$ at pH 7.6 and 0.81 ± 0.10 at pH 5.2). Consequently, the relative heights of the transition states for the proton transfer step and the "off" step for glyceraldehyde phosphate are unchanged by this reduction in pH. Provided that prototropic equilibration of the phosphate group of the bound substrate is fast, we can conclude that the rate of association of the monoanion with the enzyme is at least ten times slower than that of the dianion (see Appendix).

The binding of substrate is probably diffusion-limited at neutral pH (Albery & Knowles, 1976a). Simple diffusion theory for the collision of uniformly charged spheres requires that dianions diffuse toward cationic loci at a rate no more than two times faster than that for monoanions (Alberty & Hammes, 1958). Diffusion alone cannot therefore account for a more than tenfold difference in the rates of binding of the two forms of the substrate. Instead, it is likely that the substrate binds in two steps, involving the diffusive formation of an encounter complex followed by a first-order "capture" process to form the productive enzyme:substrate complex, and that the "capture" step is rate determining for the binding of substrate monoanions.

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Appendix

In this appendix we derive the equations for the pH dependences of the kinetic parameters for the isomerase-catalyzed reaction. This reaction may be represented by:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot Z \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P \quad (1)$$

where Z represents the enediol intermediate.

On the basis of chemical and kinetic studies (Rieder & Rose, 1959; Albery & Knowles, 1976a; Webb & Knowles, 1975), the isomerization appears to involve an enzymic base that abstracts a carbon-bound proton from the substrate and an enzymic electrophile that facilitates this abstraction. The consequences of ionizations of these enzyme residues on the enolization steps are readily apparent. Their effect on the binding and dissociation steps is less obvious, however, and these must be considered separately.

The assignment of pH dependences to the elementary steps of the enzyme-catalyzed reaction requires assumptions about the rates of prototropic equilibration of the catalytically important ionizable groups. We first examine the case in which all prototropic equilibria are fast compared with the enolization and on-off steps. We shall then consider the possibility that some prototropic equilibria are slow and show that essentially the same conclusions can be drawn.

The Effect of Substrate Ionization. Scheme IV depicts the

SCHEME IV: The Binding of an Ionizable Substrate to an Enzyme.

$$\begin{array}{ccc} E + S & \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} & E\cdot S \\ {\scriptstyle K_{SH}^{\circ}}\Big\downarrow\Big\uparrow & & \Big\downarrow\Big\uparrow{\scriptstyle K_{SH}} \\ E + SH & \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} & E\cdot SH \end{array}$$

binding of a substrate that can assume either of two ionization states. The observed dissociation rate constant at any pH is:

$$k_{off} = k_{-a}f_S + k_{-b}f_{SH} \quad (2)$$

where $f_S = K_{SH}/(H + K_{SH})$ is the fraction of bound substrate that is deprotonated and $f_{SH} = H/(H + K_{SH})$ is the fraction of bound substrate that is protonated. Thermodynamics requires that:

$$K_{SH}^{\circ}k_{-b}/k_b = K_{SH}k_{-a}/k_a \quad (3)$$

so:

$$k_{off} = k_{-a}f_S\left(\frac{k_aK_{SH}^{\circ}/k_b + H}{k_aK_{SH}^{\circ}/k_b}\right)$$

Let $K_{SH}^{app} = k_aK_{SH}^{\circ}/k_b$. Then:

$$k_{off} = k_{-a}f_S/f_S^{app} \quad (4)$$

where $f_S^{app} = K_{SH}^{app}/(H + K_{SH}^{app})$ is the *apparent* fraction of bound substrate that is kinetically observed to be deprotonated when the pH is above pK_{SH} (i.e., when f_s approaches unity). The term f_S^{app} derives from an apparent pK_a:

$$pK_{SH}^{app} = pK_{SH}^{\circ} - \log(k_a/k_b) \quad (5)$$

The reason for the form of eq 4 is that, even though f_{SH} may be quite small, the contribution of the protonated form of the bound substrate to the observed dissociation rate can be significant.

The Effect of Enzyme Ionization. When an enzyme that can exist in either of two ionization states binds substrate (Scheme

SCHEME V: The Binding of a Substrate to an Ionizable Enzyme.

$$\begin{array}{ccc} E + S & \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} & E\cdot S \\ {\scriptstyle K_{EH}^{\circ}}\Big\downarrow\Big\uparrow & & \Big\downarrow\Big\uparrow{\scriptstyle K_{EH}} \\ EH + S & \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} & EH\cdot S \end{array}$$

V), the pH dependence of the dissociation rate constant is analogous to that derived above:

$$k_{off} = k_{-c}f_E/f_E^{app} \quad (6)$$

where $f_E = K_{EH}/(H + K_{EH})$ is the fraction of liganded enzyme that is deprotonated and $f_E^{app} = K_{EH}^{app}/(H + K_{EH}^{app})$ depends on an apparent pK_a of the liganded enzyme:

$$pK_{EH}^{app} = pK_{EH}^{\circ} - \log(k_c/k_d) \quad (7)$$

Alternatively, k_{off} can be expressed as a function of the dissociation rate constant for the protonated form of the liganded enzyme:

$$k_{off} = k_{-d}f_{EH}/f_{EH}^{app} \quad (8)$$

where $f_{EH} = H/(H + K_{EH})$ is the fraction of liganded enzyme that is protonated and $f_{EH}^{app} = H/(H + K_{EH}^{app})$ derives from the same apparent pK_a.

Throughout the pH range 5 to 10 the essential base of unliganded triosephosphate isomerase is deprotonated, while the essential acid of the free enzyme is likely to be protonated (see below). Since the substrate association rate is diffusion limited at pH 7.6 (Albery & Knowles, 1976a), pK_{EH}^{app} will be less than 5 for the essential base (eq 7, $k_d \leq k_c$) and greater than 10 for the essential acid (eq 7, $k_c \leq k_d$). Combining eq 6 and 8 gives the effect of the ionizations of these two enzyme residues on the dissociation rate constant between pH 5 and 10:

$$k_{off} = \tilde{k}_{off}f_Bf_{AH} \quad (9)$$

where f_B is the fraction of the essential base in the liganded enzyme that is deprotonated and f_{AH} is the fraction of the essential acid in the liganded enzyme that is protonated.

From the isotopic discrimination experiment we know that $\Phi_{3,4}$ is unchanged on going from pH 7.6 to pH 5.2; that is, the partition ratio k_{-3}/k_4 (eq 1) is not altered by the protonations that accompany this reduction in pH. Because enzyme ionizations affect the enolization (k_{-3}) and dissociation (k_4) barriers equally (eq 9), the same must be true for substrate ionization in this pH range. At pH 5.2, therefore, phosphate protonation neither accelerates dissociation nor slows enolization, and we must attribute its effect on binding at this pH to a diminished substrate *association* rate. In quantitative terms, since the experiments with dihydroxyacetone sulfate suggest that monoanions bind at least 100 times more weakly than dianions, we can deduce (eq 3) that the pK_a value of the bound substrate (pK_{SH}) is less than 4. On the basis of eq 4, then, pK_{SH}^{app} must be less than 5.2 and the association rate of monoanions must be at least ten times slower than that for dianions (eq 5, $k_a/k_b > 10$).

The pH Dependence of the Kinetic Parameters. The pH dependences of the elementary steps of eq 1 can now be formulated on the basis of the following assumptions:

(1) *Prototropic equilibria are fast relative to the enolization and on–off steps.*

(2) *Binding is diffusion limited when the substrate is dianionic but is much slower when the substrate is monoanionic.* (See the first part of this appendix).

(3) *The bound substrate and the essential base on the free enzyme titrate below pH 5, and the essential acid on the free enzyme titrates above pH 10.* Such behavior is expected from the likely candidates for the essential acid: His-95 (ionization to imidazolate), Lys-13, Ser-96, and water. Arguments concerning the pK_a values of the substrate and the essential base have already been presented.

(4) *Substrate enolization requires a deprotonated enzyme base and a protonated enzyme acid.*

(5) *The* pK_a *values of the enzyme residues are similar in all three of the bound complexes.*

(6) *The protonation states of the ionizable groups are mutually independent.*

These statements lead to the following pH dependences for the elementary steps of eq 1 between pH 5 and 10: $k_1 = \tilde{k}_1 f_S°$; $k_{-1} = \tilde{k}_{-1} f_B f_{AH}$; $k_2 = \tilde{k}_2 f_B f_{AH}$; $k_{-2} = \tilde{k}_{-2} f_{BH} f_A$; $k_3 = \tilde{k}_3 f_{BH} f_A$; $k_{-3} = \tilde{k}_{-3} f_B f_{AH}$; $k_4 = \tilde{k}_4 f_B f_{AH}$; $k_{-4} = \tilde{k}_{-4} f_S°$. These functions can be substituted into the expressions for k_{cat} and K_m that relate to eq 1 (for these expressions see, for example, Albery & Knowles, 1976b). If two conditions are met, that the pK_a of the essential acid in the liganded enzyme is above 10 and that the pK_a of the essential base in the liganded enzyme is the same as that of the free substrate, then the pH dependences reduce to:

$$K_m^{obsd} = \tilde{K}_m f_B / f_S° = \tilde{K}_m$$

$$k_{cat}^{obsd} = \tilde{k}_{ca} f_B$$

$$(k_{cat}/K_m)^{obsd} = (\tilde{k}_{cat}/\tilde{K}_m) f_S°$$

These relationships correctly predict the observed behavior of triosephosphate isomerase.

We have assumed to this point that all prototropic equilibria are fast. It is important to consider the consequences if this is not so. One possibility is that the rate of protonation of the phosphate group of the bound substrate is slower than substrate dissociation. In this case the relative rates of binding of substrate monoanions and dianions become kinetically irrelevant and cannot be evaluated from the isotopic discrimination data. The kinetic effect of substrate protonation still appears in the observed association rate constants, but is now simply due to the depletion at low pH of the available pool of productive dianionic substrate.

Incomplete prototropic equilibration of the liganded enzyme's catalytic groups must also be considered.[3] For example, the local environment in the active site of the enzyme:enediol intermediate may in principle prevent its catalytic residues from freely equilibrating with the solvent protons before collapse of the enediol to substrate or product. In this case, k_{-2} and k_3 would be pH independent, and the pK_a values of the essential base and essential acid in the E·Z intermediate would be indeterminate. As long as this intermediate is kinetically insignificant, the pH dependences of the steady-state parameters will be the same as those derived above.

A more extreme possibility is that the catalytic side chains undergo prototropic equilibration very slowly in all three of the liganded enzyme complexes. [This case resembles Scheme III of the main paper, except that interconversion of EH·S and E·S is slow enough to be ignored.] In the observable pH range (above the pK_a of the essential base in the unliganded enzyme and below the pK_a of the essential acid) the pH-dependences of the steady-state parameters would be: $K_m^{obsd} = \tilde{K}_m f_B^{app} / f_S°$; $k_{cat}^{obsd} = \tilde{k}_{ca} f_B^{app}$; $(k_{cat}/K_m)^{obsd} = (\tilde{k}_{cat}/\tilde{K}_m) f_S°$. Here, $f_B^{app} = K_{BH}^{app}/(H + K_{BH}^{app})$ is the apparent fraction of liganded enzyme whose essential base is kinetically observed to be deprotonated. This depends on an apparent pK_a: $pK_{BH}^{app} = pK_{BH}° + \log(\tilde{K}_m/\tilde{K}_i)$. [$\tilde{K}_i$ is the dissociation constant of the enzyme:substrate complex in which the essential base is protonated.] If $\tilde{K}_m$ is similar to the thermodynamic dissociation constant of the productive E·S complex (see Albery & Knowles, 1976a), this kinetic pK_a will resemble the thermodynamic pK_a of the essential base in the liganded enzyme. Even in this case, therefore, the observed pH dependences can only be accounted for by a substantial elevation of the pK_a of the essential base upon the binding of substrate.

[3] This possibility is not incompatible with the observed wash-out of 94% of the label from specifically tritiated dihydroxyacetone phosphate in the course of its enzyme-catalyzed isomerization. There is no evidence that the label is actually lost to bulk solution, and it may simply be exchanged into a small local pool of protons (see Fisher et al., 1976).

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Purification and Characterization of Cytoplasmic Thioltransferase (Glutathione:Disulfide Oxidoreductase) from Rat Liver†

Kent Axelsson, Stellan Eriksson, and Bengt Mannervik*

ABSTRACT: An enzyme catalyzing thiol-disulfide interchange of glutathione and disulfides and the reaction between glutathione and thiosulfate esters has been purified 40 000-fold from rat liver cytosol. The enzyme, named thioltransferase (Askelöf, P., Axelsson, K., Eriksson, S., & Mannervik, B. (1974) *FEBS Lett. 38,* 263-267), was homogeneous in several electrophoretic systems, had an isoelectric point at pH 9.6, and contained 8.6% (w/w) carbohydrate. The catalytic activity had a distinct optimum at pH 7.5. A series of substrates was tested at a constant glutathione level; the k_{cat} values (at 4 mM glutathione) were all in the range of about 10^4 min^{-1}. The substrates included mixed disulfides of glutathione, other low-molecular-weight disulfides, *S*-sulfocysteine and *S*-sulfoglutathione, and peptide disulfides such as insulin, oxytocin, ribonuclease, and the mixed disulfide of glutathione and egg-white lysozyme. The enzymatic reaction was inhibited by an excess of glutathione (>4 mM).

Glutathione is involved in a large variety of chemical reactions in the living cell, which include detoxification of oxidative and electrophilic reagents, maintenance of the proper redox state of sulfhydryl/disulfide groups, and specific reactions in cellular metabolism (e.g., isomerizations) in which glutathione serves as a coenzyme (see Flohé et al., 1974; Arias & Jakoby, 1976). However, the function of glutathione as a reductant in the scission of sulfur-sulfur bonds (in disulfides, RSSR, and thiosulfate esters, $RSSO_3^-$)

$$RSSR + 2GSH \rightleftharpoons 2RSH + GSSG$$

$$RSSO_3^- + 2GSH \rightleftharpoons RSH + HSO_3^- + GSSG$$

has received relatively little attention, in spite of the fact that this is the simplest process known for general reduction of sulfur-sulfur bonds (Mannervik & Eriksson, 1974). The biological importance of such reactions is related to the fact that low-molecular-weight thiols such as cysteine, homocysteine, and coenzyme A are used in reduced form, and several reactions occurring in aerobic cells will rapidly oxidize these thiols to disulfides. Also sulfhydryl groups in polypeptide chains may undergo oxidation to disulfides with concurrent change of their biological activities. A substantial fraction of disulfides formed by intracellular oxidation of sulfhydryl groups may be expected to be mixed disulfides of glutathione, because this compound is usually the most abundant thiol available in the cell. Even if the reduction of disulfides may result in a low steady-state level in normal cells, such mixed disulfides of low-molecular-weight thiols as well as proteins have been identified (see Mannervik & Eriksson, 1974). The maintenance of thiols in the reduced form may consequently to a large extent be related to the reduction of mixed disulfides of glutathione. The metabolism of naturally occurring thiosulfate esters, such as *S*-sulfoglutathione and *S*-sulfocysteine (Mannervik et al., 1974), and disulfide-containing peptides, such as insulin, may also be expected to require glutathione for reduction of sulfur-sulfur bonds.

An enzyme catalyzing thiol-disulfide interchange was first described by Racker (1955). Such enzyme activities have subsequently been found by several investigators (see Mannervik & Eriksson, 1974). Of the activities studied with low-molecular-weight substrates, an enzyme from bovine kidney (Chang & Wilken, 1966) and an enzyme from yeast (Nagai & Black, 1968) have been characterized in some detail. The most important reductant for the reaction in vivo is probably glutathione owing to its high concentration, but other *S*-nucleophiles can also serve as substrates for the enzyme from rat liver (Eriksson & Mannervik, 1970b). The essence of the reaction catalyzed is transfer of a thiol group (alkane sulfenyl group), and the name of "thioltransferase" has therefore been suggested for this class of enzymes (Askelöf et al., 1974) instead of the erroneous name of transhydrogenase commonly used.

In the present investigation the cytoplasmic thioltransferase found in rat liver (Eriksson & Mannervik, 1970a) has been purified extensively and characterized. Some results of this study have been reported previously (Axelsson et al., 1976).

Materials and Methods

Materials. Coenzyme A, L-cystine, cystamine, GSH, L-homocystine, NADPH, oxytocin, bovine pancreatic ribonu-

† From the Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden. *Received February 27, 1978.* This work was supported by a grant (to B.M.) from the Swedish Natural Science Research Council.

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