The Influence of pH on the Interaction of Inhibitors with Triosephosphate Isomerase and Determination of the pK_a of the Active-Site Carboxyl Group†

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ABSTRACT: Ionization effects on the binding of the potential transition state analogues 2-phosphoglycolate and 2-phosphoglycolohydroxamate appear to be attributable to the changing state of ionization of the ligands themselves, therefore it is unnecessary to postulate the additional involvement of an ionizing residue at the active site of triosephosphate isomerase to explain the influence of changing pH on K_i in the neutral range. The binding of the competitive inhibitor inorganic sulfate is insensitive to changing pH in the neutral range. 3-Chloroacetol sulfate, synthesized as an active-site-specific reagent for triosephosphate isomerase, is used to provide an indication of the pK_a of the essential carboxyl group of this enzyme. Previously described active-site-specific reagents for the isomerase were phosphate esters, and their changing state of ionization (accompanied by possible changes in their affinity for the active site) may have complicated earlier attempts to determine the pK_a of the essential carboxyl group from the pH dependence of the rate of inactivation. Being a strong monoprotic acid, chloroacetol sulfate is better suited to the determination of the pK_a of the carboxyl group. Chloroacetol sulfate inactivates triosephosphate isomerase by the selective esterification of the same carboxyl group as that which is esterified by the phosphate esters described earlier. From the pH dependence of the rate of inactivation of yeast triosephosphate isomerase, the apparent pK_a of the active-site carboxyl group is estimated as 3.9 ± 0.1.

Triosephosphate isomerase from rabbit and chicken muscle exhibits highest V_{max} in the neighborhood of pH 7–8, and diminishing values of V_{max} are observed at lower pH values (Wolfenden, 1970; Plaut and Knowles, 1972). Consistent with these results is the possibility that there may be a change in the rate-determining step as the pH is lowered, or that catalysis requires the conjugate base of a group in the enzyme–substrate complex which undergoes ionization near neutrality. If an ionizing group of the enzyme is responsible for the observed pH dependence of V_{max}, the pK_a of this group is at least 6 (Wolfenden, 1970; Plaut and Knowles, 1972). It has been suggested that triosephosphate isomerase contains a basic residue which catalyzes proton abstraction from substrates (Rose, 1962), but that in order to account for the observed rates of catalyzed proton exchange into substrates, the pK_a of this basic residue should not exceed 5 (Rose, 1975).

To obtain further information about the phenomena which may be responsible for the observed ionization effects, we have examined the influence of pH on the binding of several reversible inhibitors of triosephosphate isomerase. We find that binding of the potential transition state analogues 2-phosphoglycolate (Wolfenden, 1969) and 2-phosphoglycolohydroxamate (Collins, 1974) is strongly pH dependent. The effect of pH on binding of these two strong inhibitors is not identical, but the difference can be understood in terms of the distinctive ionization properties of the inhibitors themselves in the pH range near neutrality. Each appears to be chiefly bound as a dianionic species, thus there appears to be no need to postulate an additional group on the enzyme, with a pK_a near neutrality, to explain the observed effect of pH on binding of these inhibitors. Whereas most ligands of triosephosphate isomerase contain phosphoryl groups and therefore undergo changes in their state of ionization in the pH range near neutrality, we find that competitive inhibition by inorganic sulfate is quite insensitive to changing pH, consistent with its unchanging state of ionization.

Glycidol phosphate and haloacetol phosphates inactivate triosephosphate isomerase by a highly selective esterification of an essential glutamyl residue (Hartman, 1971; Miller and Waley, 1971; De La Mare et al., 1972). In the enzyme from rabbit muscle, this residue has been identified as Glu-165 (Corran and Waley, 1973). The species invariance (from yeast to human) of the glutamyl residue and the homology of adjacent amino acid sequences indicate that the glutamyl residue serves an essential function in catalysis (Hartman, 1972; Norton and Hartman, 1972; Hartman and Gracy, 1973). It has been suggested (Hartman, 1970a) that the essential carboxyl group is the base which functions in proton abstraction from substrates.

In apparent agreement with this postulate was the assignment (Waley, 1972) of the pK_a of the carboxyl group as 6.0, based on the pH dependence of the observed rate of inactivation with glycidol phosphate. Interpretation of these results is complicated, however, by the changing state of ionization of this reagent over the pH range examined. Under somewhat different conditions, Schray et al. (1973) have shown that the maximal rate of inactivation of triose-

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phosphate isomerase by glycidol phosphate is virtually independent of pH in the pH range from 5.5 to 10, suggesting that the glutamic acid which is alkylated may not have a pK_a value in this range.

To circumvent the problem of phosphoryl group ionization, we have prepared chloroacetol sulfate, a new inactivating agent for triosephosphate isomerase. We find that this compound, which exists as a monoanion over the entire pH range in question, selectively esterifies the same glutamyl residue of triosephosphate isomerase as do the phosphorylated active-site-specific reagents which have been described previously. Unlike these reagents, chloroacetol sulfate inactivates the enzyme at a rate which is insensitive to changing pH at values above 5. The yeast enzyme is sufficiently stable to permit determination of the kinetics of inactivation by chloroacetol sulfate at lower pH values. The pH dependence of this reaction is consistent with a requirement for the conjugate base of a group on the enzyme with apparent $pK_a = 3.9$, presumably the γ-carboxyl group of the essential glutamyl residue at the active site.

Experimental Section

Materials

Commercially obtained materials and vendors were as follows: trimethylamine–sulfur trioxide complex, sodium *p*-hydroxymercuribenzoate, and 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.); NADH, NAD^+, DL-glyceraldehyde 3-phosphate, Pipes,[1] Hepes, Bicine, and glycerophosphate dehydrogenase (Sigma Chemical Co.); pepsin (Worthington Biochemical Corp.); sodium [^{3}H]borohydride (Amersham/Searle Corp.). 2-Phosphoglycolate was prepared by the method of Fleury and Courtois (1941). Chloroacetol and chloroacetol phosphate were synthesized as described previously (Hartman, 1970b). Rabbit muscle and yeast triosephosphate isomerases were purified to homogeneity using published procedures (Norton et al., 1970; Norton and Hartman, 1972).

2-Phosphoglycolohydroxamic acid was a gift from Dr. K. D. Collins of Yale Medical School, and glycidol phosphate was donated by Dr. I. A. Rose of the Institute for Cancer Research, Philadelphia, Pa.

Glycerophosphate dehydrogenase (Sigma Chemical Co.) was dialyzed against 0.01 *M* imidazole buffer (pH 5.8) (ionic strength adjusted to 0.05 with KCl), to remove inhibitory sulfate ions in experiments with competitive inhibitors. Concentrations of aqueous solutions of 2-phosphoglycolate and 2-phosphoglycolohydroxamate were determined by titration with KOH. Concentrations of aqueous solutions of DL-glyceraldehyde 3-phosphate were determined spectrophotometrically using glyceraldehyde-3-phosphate dehydrogenase, arsenate, and excess NAD^+ (Velick, 1955).

Methods

Elemental analyses were performed by Stewart Laboratories, Inc., Knoxville, Tenn.

Thin-layer chromatography was carried out on plastic sheets precoated with silica gel or cellulose (Sil N-HR and Cel 300, Brinkmann Instruments, Inc.) using a solvent composed of butanol–glacial acetic acid–water (7:2:5, v/v). Compounds were detected with 2,4-dinitrophenylhydrazine spray (Gray, 1952) followed by heating the chromatograms at 110° for 5 min.

Amino acid analyses were performed with a Beckman 120C amino acid analyzer according to the method of Spackman et al. (1958). Samples were hydrolyzed with 6 *N* HCl at 110° for 21 hr in sealed, evacuated (<50 μm of Hg) tubes.

Radioactivity was assayed with a Packard Model 3003 liquid scintillation spectrometer. The sample (0.1 ml) was dissolved in 1 ml of Beckman Bio-Solv (BBS-3), and to this mixture was added 10 ml of scintillation fluid composed of 4.6 g of 2,5-diphenyloxazole and 115 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene–ethanol (4:3, v/v).

Protein concentration was determined from $A_{280\,nm}$. The muscle and yeast isomerases have $\epsilon_{1\,cm}(1\%)$ of 13.1 and 10.0, respectively (Norton et al., 1970; Norton and Hartman, 1972), and subunit molecular weights of 26500 (Corran and Waley, 1973) and 28000 (Krietsch et al., 1970), respectively.

Enzyme activity at various pH values, in the presence and absence of reversible inhibitors, was assayed spectrophotometrically by measuring the decrease of absorbance at 340 nm in the presence of NADH and glycerophosphate dehydrogenase as described by Beisenherz (1955). To initiate the reaction triosephosphate isomerase (1.6–3.2 ng/ml of the enzyme from rabbit muscle) was added to a total volume of 1 ml which contained DL-glyceraldehyde 3-phosphate (5×10^{-5} to 5×10^{-4} *M*), NADH (1.5×10^{-4} *M*), α-glycerophosphate dehydrogenase (5 μg/ml; or 10–30 μg/ml at pH values above 9.0 or in the presence of 2-phosphoglycolohydroxamate), and imidazole-HCl or glycine-KOH buffer (0.01 *M*, with the ionic strength of the assay mixture adjusted to 0.05 with KCl). Rate measurements were performed with a Zeiss PMQ II spectrophotometer equipped with a recorder giving full-scale deflection for an absorbance change of 0.10. Initial rates of reaction were determined as a function of substrate concentration at various pH values in the absence or presence of inhibitors, and values for V_{max}/K_m and for K_i were obtained from double-reciprocal plots of reaction rate as a function of substrate concentration. The buffers used in the experiments exhibited negligible inhibition of isomerase activity at the concentrations employed, and losses of isomerase activity were negligible during the short time period required for rate determinations, which were carried out in a thermostated cuvette compartment at 25°. The ionic strength of assay solutions was held constant at 0.05 in the experiments, since the pK_a values of the phosphorylated ligands, as well as their apparent K_m and K_i values, were found to be strongly dependent on ionic strength (see Results). Determinations of the pH of assay mixtures were performed with the glass electrode of a Corning Model 12 pH meter, calibrated with several standard phosphate buffers.

Synthesis of 3-Chloroacetol Sulfate

3-Chloroacetol Sulfate Dimethyl Ketal (Ammonium Salt). To a solution of chloroacetol (250 mg, 16.2 mmol) in 1 ml of dry dimethylformamide was added sulfur trioxide–trimethylamine complex (360 mg, 25.9 mmol). The reaction mixture was stirred overnight, neutralized to pH 8.0 with 1 *N* NH_4OH, and then subjected to chromatography on a 1.2 × 22 cm column of DEAE-cellulose (Whatman DE-52). The column was eluted with a linear gradient composed of 200 ml each of 0.01 *M* (initial) and 0.2 *M* (limit)

[1] Abbrevations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Bicine, *N*,*N*′-bis(2-hydroxyethyl)glycine.

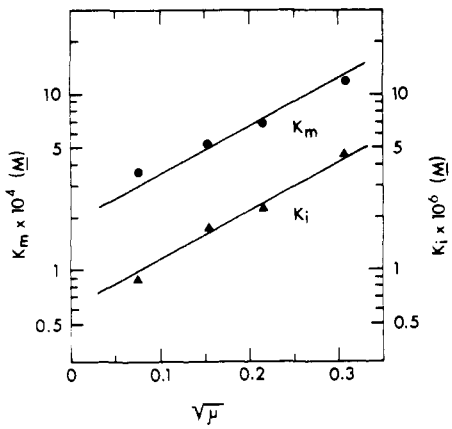

FIGURE 1: The effect of ionic strength (μ) on the apparent K_m for glyceraldehyde 3-phosphate and on K_i for 2-phosphoglycolate, in imidazole hydrochloride buffer (pH 7.0, 0.01 *M*) with ionic strength adjusted by appropriate addition of KCl, at 25°.

ammonium bicarbonate (pH 8.0). Fractions containing the ammonium salt of chloroacetol sulfate dimethyl ketal, which eluted around 0.03 *M* ammonium bicarbonate and was detected by spot tests with 2,4-dinitrophenylhydrazine (see Methods), were pooled and subjected to repeated lyophilization to remove completely the ammonium bicarbonate. The residual ketal (310 mg, 76%) appeared homogeneous (R_f 0.69) by thin-layer chromatography on silica gel. Crystallization of the salt from 5 ml of ethanol gave 220 mg of analytically pure material (mp 114–116° dec). Anal. Calcd for $C_5H_{14}ClNO_6S$ (251.70): C, 23.86; H, 5.61; Cl, 14.09; N, 5.57; S, 12.74; ($-OCH_3$), 24.66. Found: C, 23.77; H, 5.70; Cl, 13.96; N, 5.72; S, 12.90; ($-OCH_3$), 24.42.

3-Chloroacetol Sulfate. Dowex 50 (H^+) (600 mg) was added to 2.0 ml of 0.22 *M* chloroacetol sulfate dimethyl ketal ammonium salt, and the resulting mixture was incubated at 40° for 48 hr. During this time an approximate 90% conversion of ketal (R_f 0.75) to the ketone (R_f 0.62) occurred based on thin-layer chromatography (cellulose-coated sheets). The exact concentration of chloroacetol sulfate was determined by quantitating its reaction with the sulfhydryl group of glutathione, which was quantitated with Ellman's (1959) reagent. An aliquot (0.005 ml) of the stock chloroacetol sulfate solution was added to 1 ml of 2 m*M* glutathione in 0.1 *M* Bicine–1 m*M* EDTA (pH 8.0). At 20 and 60 min, 0.1 ml of the reaction mixture was added to 2.4 ml of 0.4 m*M* 5,5-dithiobis(2-nitrobenzoic acid) in the same Bicine buffer. The sulfhydryl concentration, determined from the increase in $A_{412\,nm}$, was found to be the same for the 20- and 60-min aliquots. Thus, the reaction between chloroacetol sulfate and glutathione was completed in less than 20 min, and from the observed decrease in sulfhydryl concentration, the chloroacetol sulfate concentration in the stock solution was calculated to be 0.21 *M*.

Kinetics of Enzyme Inactivation by Chloroacetol Sulfate. A variety of buffers were used, but in all cases their concentrations were 0.1 *M* and they contained 1 m*M* EDTA. The ionic strength of each buffer was adjusted with 1 *M* NaCl to equal the ionic strength of 0.1 *M* sodium formate (pH 5.0). To solutions (0.5 ml) of triosephosphate isomerase (78 μg/ml, 2.8 μM) at 24° was added 0.01 ml of 0.2 *M* chloroacetol sulfate. Periodically, 0.01-ml aliquots of the reaction mixtures were diluted into 1 ml of cold 0.05 *M* Hepes–1 m*M* EDTA (pH 7.0). Portions (0.01 ml) of the diluted samples were assayed immediately for isomerase activity. Controls, which were also monitored for enzyme activity, remained constant throughout the course of the inactivations.

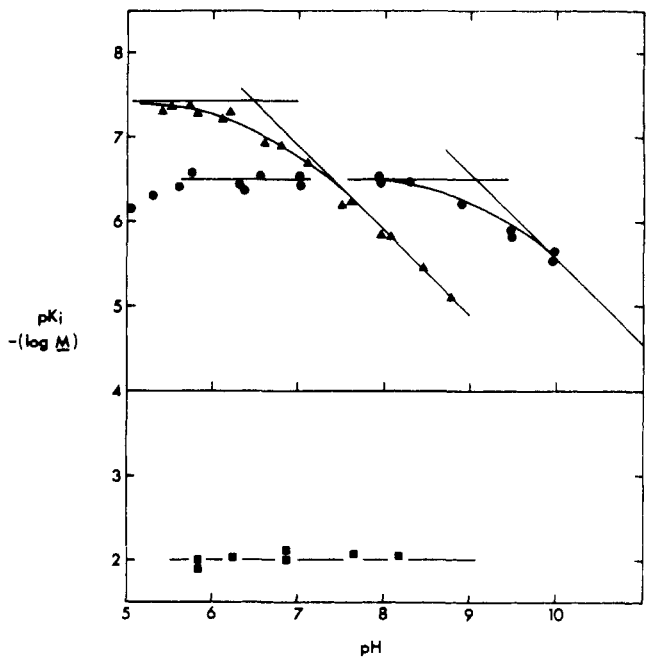

FIGURE 2: Variation in K_i values observed for 2-phosphoglycolic acid (▲), 2-phosphoglycolohydroxamic acid (●), and inorganic sulfate (■) as a function of changing pH at ionic strength 0.05, at 25°, for rabbit muscle triosephosphate isomerase.

Results

Effects of pH and Ionic Strength on Activity of the Rabbit Muscle Enzyme at 25°. The binding of substrates and inhibitors by rabbit muscle triosephosphate isomerase was found to be strongly dependent on ionic strength at constant pH (Figure 1). For this reason, experiments on the influence of changing pH on the kinetic parameters of this enzyme were performed at constant ionic strength, a precaution which was not observed in experiments reported earlier (Wolfenden, 1970). Values for V_{max}/K_m, obtained from double-reciprocal plots of reaction rate as a function of the concentration of glyceraldehyde 3-phosphate, rose with increasing pH to a maximum near pH 8, in a manner consistent with titration of a group with a $pK_a \sim 6.3$ at ionic strength 0.05, the conjugate base of which was required for activity. This value is markedly lower than that which was estimated in the absence of controlled ionic strength (Wolfenden, 1970), but slightly higher than the value (6.0) reported by Plaut and Knowles (1972) for the enzyme from chicken muscle at ionic strength 0.1. In agreement with results reported earlier (Wolfenden, 1970; Plaut and Knowles, 1972), K_m values exhibited little variation between pH 6 and 8.5, but increased at pH values above 9.

Binding of Competitive Inhibitors by the Rabbit Muscle Enzyme. In agreement with earlier results (Wolfenden, 1970; Collins, 1974), 2-phosphoglycolate and 2-phosphoglycolohydroxamate exhibited linear competitive inhibition of rabbit muscle triosephosphate isomerase at 25° and ionic strength 0.05 in various buffers. K_i values for 2-phosphoglycolate (Figure 2) varied with pH in a manner consistent with titration of a group with apparent $pK_a = 6.5$, the conjugate acid of which was required for inhibition. This apparent pK_a was also markedly lower than the value estimated earlier from experiments in the absence of controlled

Table I: pK_a Values of Ligands for Triosephosphate Isomerase.[a]

Compound	pK_a
Glyceraldehyde 3-phosphate	<2, 6.4
2-Phosphoglycolic acid	<2, 3.6, 6.8
2-Phosphoglycolohydroxamic acid	<2, 5.7, 9.5
Iodoacetol phosphate	<2, 6.4[b]
Glycidol phosphate	<2, 6.0[c]
Sulfuric acid	<2
Chloroacetol sulfate	<2

[a] Determined at 25° and μ = 0.05 except as noted. [b] Determined at μ = 0.1. [c] Determined at μ = 0.2.

ionic strength (Wolfenden, 1970). The minimal K_i for this inhibitor, in the neighborhood of pH 5 at ionic strength 0.05, was approximately 4×10^{-7} *M*, and considerably lower values could doubtless be obtained by reducing the ionic strength (Figure 1).

2-Phosphoglycolohydroxamate, in agreement with the findings of Collins (1974) and in contrast to 2-phosphoglycolate, exhibited little variation in binding affinity in the pH range near neutrality, attaining a minimal K_i value of approximately 3×10^{-6} *M* in the neighborhood of pH 7. Increasing values were observed for this inhibitor at high pH values (Figure 2). 2-Phosphoglycolohydroxamate inhibited α-glycerophosphate dehydrogenase, the coupling enzyme normally used in the assay with glyceraldehyde 3-phosphate as substrate. For this reason, it was necessary to increase the concentration of dehydrogenase in assays performed in the presence of this inhibitor (see Experimental Section).

Inorganic sulfate was found to be a competitive inhibitor, confirming a preliminary report (Wolfenden, 1969). The K_i value, slightly higher than that for the competitive inhibitor inorganic phosphate, was approximately 0.010 *M* at pH 6.8 and ionic strength 0.15. Inorganic sulfate exhibited negligible variation in K_i with changing pH in the range from pH 5.5 to 8.5, when the ionic strength was maintained constant on 0.15 (Figure 2).

The weak inhibition previously observed with dicarboxylic acids, under conditions in which ionic strength was not controlled (Wolfenden, 1969), appears to have been due entirely to salt effects on K_m. Malonate and succinate, at concentrations of 0.010 *M*, showed no evidence of inhibition at constant ionic strength, indicating that K_i values for these compounds (if they inhibit at all) are far in excess of 10^{-2} *M*.

pK_a Values of Ligands for Triosephosphate Isomerase. pK_a values at 25°, obtained potentiometrically at ionic strength 0.05 unless indicated otherwise, are shown in Table I. For comparison with published measurements of the kinetics of inactivation, pK_a values were determined at ionic strength 0.1 for iodoacetol phosphate (Hartman, 1968), and at ionic strength 0.2 for glycidol phosphate (Waley, 1972).

Rates of Reaction of Chloroacetol Sulfate and Chloroacetol Phosphate with GSH. Solutions containing glutathione (2 m*M*) and either chloroacetol sulfate (2 m*M*) or chloroacetol phosphate (2 m*M*) in 0.1 *M* Bicine–1 m*M* EDTA (pH 8.0) were monitored for sulfhydryl concentration by the method of Boyer (1954). Periodically, 0.1-ml aliquots of the reaction mixture were transferred to cuvettes containing 2.4 ml of 0.15 m*M* *p*-hydroxymercuribenzoate in 0.2 *M* ammonium acetate–2 *M* ammonium sulfate (pH 5.5), and the $A_{255\ nm}$ was determined. The calculated second-order rate constants for the reaction of chloroacetol sulfate and chloroacetol phosphate with glutathione were 8.0 and 1.2 M^{-1} sec^{-1}, respectively.

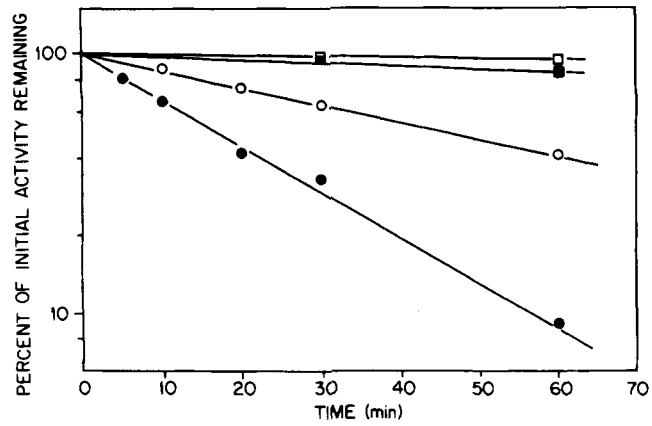

FIGURE 3: Inactivation of triosephosphate isomerase from yeast (●) and rabbit muscle (○) by chloroacetol sulfate (4 m*M*) in 0.1 *M* Bicine (pH 8.0) at 24°. Duplicate samples of the yeast (■) and muscle (□) enzymes were treated with the reagent in the presence of 2-phosphoglycolate (1 m*M*). Other details are given in Methods.

Interaction of Chloroacetol Sulfate with Triosephosphate Isomerase. Chloroacetol sulfate was tested as a competitive inhibitor of yeast triosephosphate isomerase. No inhibition of initial rates was observed at a reagent concentration of 10 m*M* in the presence of 0.4 m*M* DL-glyceraldehyde 3-phosphate [a concentration of substrate well below the K_m of 1.3 m*M* reported by Krietsch et al. (1970)]. In this comparison the ionic strength of the assay solution which did not contain chloroacetol sulfate was adjusted with NaCl so as to equal the ionic strength of the assay solution containing the reagent.

Both the yeast and the rabbit muscle enzymes were irreversibly inactivated by chloroacetol sulfate, and the competitive inhibitor phosphoglycolate protected both enzymes against inactivation (Figure 3). The apparent second-order rate constants for inactivation of the yeast and the muscle enzymes were 0.17 and 0.061 M^{-1} sec^{-1}, respectively. Consistent with the lack of affinity of the reagent for the enzyme, as indicated by the absence of competitive inhibition, is the observation that the rate of inactivation was directly proportional to the concentration of chloroacetol sulfate from 0.4 to 20 m*M*; there was no indication of a rate-saturation effect.

To determine the site of alkylation of triosephosphate isomerase by chloroacetol sulfate, a substantial quantity of the derivatized enzyme was prepared. To a solution of yeast triosephosphate isomerase (56 mg, 2 μmol of subunit) in 3.0 ml of 0.05 *M* Pipes–1 m*M* EDTA (pH 6.0) was added 0.06 ml of 0.2 *M* chloroacetol sulfate. After 1 hr, 10% of the initial activity remained; the protein was then reduced with sodium [^{3}H]borohydride as previously described for the enzyme inactivated by chloroacetol phosphate (Norton and Hartman, 1972). This treatment provides on acid-stable radioactive label in the protein-bound reagent moiety by the reduction of its carbonyl group to a hydroxyl group. A sample of the native enzyme was also subjected to reduction under the same conditions. The borohydride-treated proteins were dialyzed exhaustively against 0.02 *M* NaCl. After dialysis, the inactivated isomerase had a specific activity of 2.7×10^6 cpm/μmol of subunit (corrected for the incorporation of tritium into the native enzyme, which was 3% that of the inactivated sample). The modified isomerase

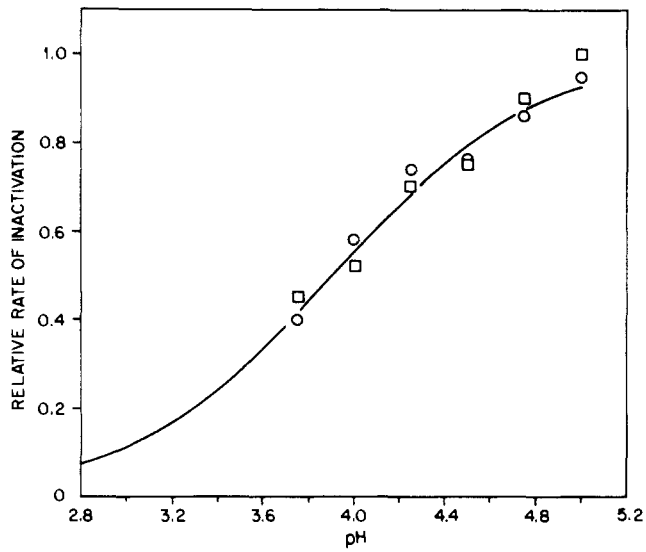

FIGURE 4: pH dependence of the rate of inactivation of yeast triosephosphate isomerase by chloroacetol sulfate (4 mM). The solid line is a theoretical titration curve for an acid with pK_a = 3.9. The rates of inactivation at the given pH are relative to the rate of inactivation at pH 8.0. Bicine (0.1 M) was used at pH 8.0, and sodium formate (0.1 M) was used at pH 3.75–5.0. Other experimental details are given in Methods.

was then carboxymethylated with iodoacetic acid and digested with pepsin as described earlier (Norton and Hartman, 1972). Chromatography of the peptic digest on Bio-Rad AG50W-X2 revealed one major radioactive component that contained 82% of the counts applied to the column. This component was purified further by successive chromatography on Sephadex G-25 (which resolved two radioactive peptides) and DEAE-cellulose. These three columns were run under conditions identical with those described earlier for the purification of active-site peptides from the yeast isomerase labeled with chloroacetol phosphate (Norton and Hartman, 1972). The amino acid compositions of the two purified peptides were Trp_1, Glu_1, Pro_1, Ala_1, $Val_{2.2}$, Tyr_1 (for the peptide which was eluted first from Sephadex G-25) and Trp_1, Glu_1, $Pro_{1.1}$, $Ala_{1.1}$, $Val_{0.9}$, Tyr_1 (for the peptide which was eluted last from Sephadex G-25). The combined overall yield of the two peptides was 39% (0.42 μmol of one and 0.36 μmol of the other). All of the radioactivity in both hydrolysates was eluted from the long column of the amino acid analyzer as a ninhydrin-negative peak at 28 min (coincident with the position of elution of glycerol), consistent with the derivatized residue in the peptides being a γ-glutamyl ester. Each peptide had a specific radioactivity of 3.2×10^6 cpm/μmol. From this number and the specific radioactivity of the undigested protein, the calculated extent of reagent incorporation was 0.84 molar equiv per mol of catalytic subunit.

Influence of pH on the Rate of Inactivation of Triosephosphate Isomerase by Chloroacetol Sulfate. The rate of inactivation of rabbit muscle triosephosphate isomerase was constant (±5%) between pH 5 and 8. In these experiments, formate and Pipes buffers were used at pH 5.0, Pipes and Hepes at pH 6.0, Hepes and Bicine at pH 7.0, and Bicine at pH 8.0. The instability of the rabbit muscle enzyme precluded experimentation below pH 5.

The yeast isomerase was sufficiently stable to permit the determination of its rate of inactivation by chloroacetol sulfate at pH values as low as 3.75. The pH dependence of the rate of inactivation between pH 3.75 and 5.0 (Figure 4) was consistent with the titration curve of a group with apparent pK_a = 3.9 ± 0.1, the conjugate base of this group being required for inactivation. Between pH 5 and 8 the rate of inactivation remained constant (±5%).

Discussion

Qualitatively, the reaction of triosephosphate isomerase with chloroacetol sulfate is strikingly similar to the previously described reaction with chloroacetol phosphate: (1) the enzyme is irreversibly inactivated, and competitive inhibitors protect against inactivation; (2) a stoichiometric amount of reagent is covalently incorporated during inactivation; (3) inactivation results from a highly selective esterification of the active-site glutamyl residue; (4) the relative rates of inactivation of the yeast and rabbit muscle enzymes (2.8-fold greater for yeast isomerase) are about the same as with chloroacetol phosphate (2.3-fold greater for yeast isomerase) (Hartman, 1972). The isolation of two peptides that contain an esterified glutamyl residue and that differ only in that one contains an additional valyl residue is not inconsistent with the stated selectivity. Two peptides with compositions identical with those seen here were isolated from the yeast enzyme inactivated by chloroacetol phosphate. The two peptides represent the same site of esterification, and arise because two adjacent peptide bonds are targets of the pepsin-catalyzed hydrolysis (Norton and Hartman, 1972).

In terms of absolute rates, the inactivation by chloroacetol sulfate is dramatically slower than by the corresponding phosphate ester. The second-order rate constant at 24° for the reaction of the rabbit muscle enzyme with chloroacetol sulfate was 0.061 M^{-1} sec^{-1} (see Results), as compared with a rate constant at 2° with chloroacetol phosphate of 2300 M^{-1} sec^{-1} (Hartman, 1972). When one considers the temperature differences and the fact that the sulfate reagent is inherently somewhat more reactive than the phosphate reagent on the basis of alkylation of the sulfhydryl group of glutathione (see Results), the rate enhancement (with respect to the inactivation of isomerase) produced by substitution of a phosphate group for sulfate is even more pronounced. The enhanced reactivity of the phosphate reagent as compared with the sulfate reagent toward the essential group of the isomerase is probably due at least in part to the enzyme's affinity for chloroacetol phosphate [K_D of approximately 5×10^{-6} M (Davis et al., 1973)], in contrast to the lack of detectable affinity for chloroacetol sulfate (see Results). Since α-chloro ketones are poor esterifying agents [e.g., under much harsher conditions than those used in the inactivation of isomerase, chloroacetol phosphate does not esterify carboxylic acid groups of model compounds (Hartman, 1970b)], esterification of the active-site carboxylate by chloroacetyl sulfate, a reagent without strong affinity for the active site, suggests that the carboxylate anion may possess unusually high nucleophilicity.

For the yeast enzyme, the apparent pK_a of the active-site carboxyl group calculated from the pH dependence of inactivation is approximately 3.9 (Figure 4). We could not determine the pK_a of the corresponding group in the enzyme from rabbit muscle because of its instability at low pH. However, since the inactivation rate was virtually unchanged between pH 5.0 and 8.0, the pK_a of the group in the rabbit muscle enzyme is presumably well below 5.0. This accords with the conclusion of Schray et al. (1973), based on the pH dependence of the inactivation rate of the rabbit muscle enzyme by glycidol phosphate, that the pK_a

of the carboxyl group is below 5.5. Earlier observations of Waley (1972) with the same reagent can be explained by attributing the inflection point observed near 6 to the ionization of glycidol phosphate. In the earlier experiments, in contrast to those of Schray et al., (1973), glycidol phosphate was present at a concentration well below its apparent K_i as a reversible inhibitor, and the change in inactivation rate with changing pH may have reflected changes in binding affinity. The potential difficulties encountered in measuring the pK_a of an essential residue with a reagent that has an affinity for the active site have been discussed (Schmidt and Westheimer, 1971).

We conclude that the pK_a of the active-site carboxyl group of triosephosphate isomerase is not abnormally high, and that changes in the apparent affinity of the enzyme for phosphorylated ligands near pH 6 are probably due, at least in part, to the changing state of ionization of these ligands. With a pK_a of about 4, the essential carboxyl group remains a likely possibility as the acid–base group that effects proton transfer.

The kinetic behavior of substrates, which changes as a function of changing pH, is a matter of continuing interest. V_{max} has been observed to reach maximal values above an apparent pK_a slightly above 6, whereas K_m values remain fairly constant throughout this range (see introduction). Further information will clearly be required to account for these effects, which may result from formation of nonproductive complexes with monoanionic forms of the substrates or from a change in the nature of the rate-determining step with changing pH. Although the present findings are not helpful in distinguishing between these alternatives, it is of interest that 2-phosphoglycolate and 2-phosphoglycolohydroxamate are tightly bound as dianionic species. In this form, both inhibitors exhibit K_i values several orders of magnitude lower than the apparent K_m values of substrates. It has been demonstrated (Reynolds et al., 1971) that the anhydrous triosephosphates are the true substrates of this enzyme, and that they are in unfavorable rapid equilibrium with their covalently hydrated *gem*-diols, which are not substrates. It is reasonable to suppose that these hydrates may be bound nonproductively at the active site (Collins, 1974; Fahey and Fischer, 1974), so that the apparent K_m values of the substrates may be higher or lower than those which would be observed for the anhydrous species which are the true substrates.

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References

Beisenherz, G. (1955), *Methods Enzymol. 1,* 387.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76,* 4331.
Collins, K. D. (1974), *J. Biol. Chem. 249,* 136.
Corran, P. H., and Waley, S. G. (1973), *FEBS Lett. 30,* 97.
Davis, R. H., Delaney, P., and Furfine, C. S. (1973), *Arch. Biochem. Biophys. 159,* 11.
De La Mare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D., and Offord, R. E. (1972), *Biochem. J. 129,* 321.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Fahey, R. C., and Fischer, E. F. (1974), *Anal. Biochem. 57,* 547.
Fleury, P., and Courtois, J. (1941), *Bull. Soc. Chim. Fr. 8,* 69.
Gray, R. A. (1952), *Science 115,* 129.
Hartman, F. C. (1968), *Biochem. Biophys. Res. Commun. 33,* 888.
Hartman, F. C. (1970a), *J. Am. Chem. Soc. 92,* 2170.
Hartman, F. C. (1970b), *Biochemistry 9,* 1776.
Hartman, F. C. (1971), *Biochemistry 10,* 146.
Hartman, F. C. (1972), *Methods Enzymol. 25B,* 661.
Hartman, F. C., and Gracy, R. W. (1973), *Biochem. Biophys. Res. Commun. 52,* 388.
Krietsch, W. K. G., Pentchev, P. G., Klingenbürg, H., Hofstätter, T., and Bücher, T. (1970), *Eur. J. Biochem. 14,* 289.
Miller, J. C., and Waley, S. G. (1971), *Biochem. J. 123,* 163.
Norton, I. L., and Hartman, F. C. (1972), *Biochemistry 11,* 4435.
Norton, I. L., Pfuderer, P., Stringer, C. S., and Hartman, F. C. (1970), *Biochemistry 9,* 4952.
Plaut, B., and Knowles, J. R. (1972), *Biochem. J. 129,* 311.
Reynolds, S. J., Yates, D. W., and Pogson, C. I. (1971), *Biochem. J. 122,* 285.
Rose, I. A. (1962), *Brookhaven Symp. Biol. 15,* 293.
Rose, I. A. (1975), Advances in Enzymology, Meister, A., Ed., New York, N.Y., Wiley (in press).
Schmidt, D. E., and Westheimer, F. H. (1971), *Biochemistry 10,* 1249.
Schray, K. J., O'Connell, E. L., and Rose, I. A. (1973), *J. Biol. Chem. 248,* 2214.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Velick, S. F. (1955), *Methods Enzymol. 1,* 401.
Waley, S. G. (1972), *Biochem. J. 126,* 255.
Wolfenden, R. (1969), *Nature (London) 223,* 704.
Wolfenden, R. (1970), *Biochemistry 9,* 3404.