References

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.
Cunningham, B., and Lowenstein, J. M. (1965), *Biochim. Biophys. Acta 96*, 535.
Dixon, M. (1953), *Biochem. J. 55*, 170.
Fairclough, G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.
Freundlich, M., and Umbarger, H. C. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 491.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.
Jensen, R. A., and Nester, E. W. (1966), *J. Biol. Chem. 241*, 3373.
LePage, G. A. (1948), *Cancer Res. 8*, 193.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, J. (1951), *J. Biol. Chem. 193*, 265.
Patte, J. C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128*, 440.
Rosen, O. M., and Rosen, S. M. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1156.
Seraydarian, K., Mommaerts, W. F. H. M., and Wallner, A. (1962), *Biochim. Biophys. Acta 65*, 443.
Setlow, B., Burger, R., and Lowenstein, J. M. (1966), *J. Biol. Chem. 241*, 1144.
Setlow, B., and Lowenstein, J. M. (1967), *J. Biol. Chem. 242*, 607.
Setlow, B., and Lowenstein, J. M. (1968a), *J. Biol. Chem. 243*, 3409.
Setlow, B., and Lowenstein, J. M. (1968b), *J. Biol. Chem. 243*, 6216.
Smiley, K. L., Berry, A. J., and Suelter, C. H. (1967), *J. Biol. Chem. 242*, 2502.
Smiley, K. L., and Suelter, C. H. (1967), *J. Biol. Chem. 242*, 1980.
Threlfall, C. J. (1957), *Biochem. J. 65*, 694.
Wolfenden, R., Sharpless, T. K., and Allan, R. (1967), *J. Biol. Chem. 242*, 1977.
Wolfenden, R., Tomozawa, Y., and Bamman, B. (1968), *Biochemistry 7*, 3965.

Binding of Substrate and Transition State Analogs to Triosephosphate Isomerase*

Richard Wolfenden

ABSTRACT: Triosephosphate isomerase from rabbit muscle is competitively inhibited by 2-phosphoglycollate, which is maximally bound below apparent $pK_a = 7.35$. K_m for the substrate glyceraldehyde 3-phosphate and K_i for the substrate analog α-glycerophosphate show little variation with pH.

V_{max} for isomerization of glyceraldehyde 3-phosphate falls off below apparent $pK_a = 7.35$. Binding of the transition state analog 2-phosphoglycollate (unlike binding of the substrate or the substrate analog) is thus sensitive to the state of ionization of a catalytic residue on the protein, its affinity changing in parallel with the changing rate enhancement produced by the enzyme as catalyst. Saturating concentrations of 2-phosphoglycollate produce virtually complete protection of the enzyme against heat inactivation under conditions where saturating α-glycerophosphate produces only partial protection. These findings suggest that in solution, as previously observed in crystals, progressive changes in enzyme structure are brought about by α-glycerophosphate and by 2-phosphoglycollate.

2-Phosphoglycollate has recently been shown to be an exceptionally powerful competitive inhibitor of triosephosphate isomerase (D-glyceraldehyde 3-phosphate keto isomerase, EC 5.3.1.1) (Wolfenden, 1969). The inhibitory power of this compound is believed to result from its resemblance to a high-energy ene–diolate intermediate earlier proposed for enzymatic isomerization of aldoses (Rose, 1962). Studies described in the present paper were undertaken in order to provide information concerning the pH dependence of the catalytic activity of triosephosphate isomerase and the pH dependence of inhibition by 2-phosphoglycollate. A variable which changes the rate enhancement produced by the enzyme as catalyst (relative to the rate of the nonenzymatic reaction under identical conditions) might be expected to produce parallel changes in the affinity of the enzyme for a transition state analog (relative to the affinity of the enzyme for substrate) (Wolfenden, 1969).

2-Phosphoglycollate brings about a reversible contraction of 5% in the unit cell volume of crystals of triosephosphate isomerase; a smaller contraction (1.7% occurs) in the presence of the substrate analog α-glycerophosphate (Johnson and

* From the Department of Biochemical Sciences, Frick Laboratory, Princeton University, Princeton, New Jersey 08540. *Received April 27, 1970.* Supported by Grant No. GM-12725 from the National Institutes of Health, U. S. Public Health Service. Inquiries should be addressed to the Department of Biochemistry, University of North Carolina, Chapel Hill, N. C. 27514.

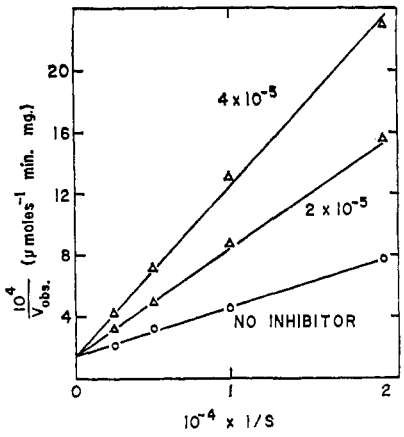

FIGURE 1: Double-reciprocal plot of observed reaction rate as a function of the concentration of the substrate glyceraldehyde 3-phosphate (expressed as molarity of the *dl* mixture) at 25° in imidazole–HCl buffer (0.05 mM, pH 8.30), in the absence and presence of 2-phosphoglycollate at concentrations of 2×10^{-5} and 4×10^{-5} M.

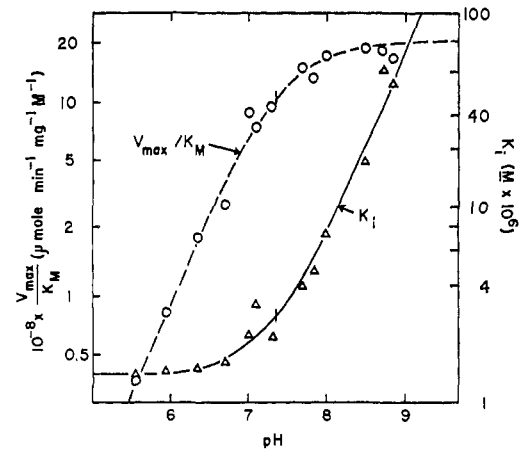

FIGURE 2: Effect of pH on observed V_{max}/K_m for glyceraldehyde 3-phosphate (scale on left ordinate), and on observed K_i for inhibition by 2-phosphoglycollate (scale on right ordinate).

Wolfenden, 1970). To determine whether structural changes also occur when inhibitors are bound to the enzyme in solution, the kinetics of heat inactivation in the presence and absence of inhibitors were also examined.

Experimental Section

Crystalline triosephosphate isomerase and α-glycerophosphate dehydrogenase were obtained from Boehringer Mannheim Corp. DL-α-Glycerophosphate, and DL-glyceraldehyde 3-phosphate and DPNH were obtained from Sigma Corp. 2-Phosphoglycollic acid and 2-phosphoglycolaldehyde were prepared by the method of Fleury and Courtois (1941). Methyl 2-phosphoglycollate (pK_a = 1.67 and 5.7) was prepared by treatment of 2-phosphoglycollic acid (pK_a = 1.7, 3.3, and 6.4) for 2 hr at room temperature with methanol saturated with HCl. After removal of methanolic HCl the product was shown to be quantitatively converted to the monomethyl ester by titration with KOH.

Assays of enzyme activity were routinely performed by measuring the decrease in absorbance at 340 mμ when enzyme was added to a solution containing DL-glyceraldehyde 3-phosphate (3×10^{-4} M), DPNH (1.5×10^{-4} M), and α-glycerophosphate dehydrogenase (5 μg/ml), at 25°, an assay similar to that described by Beisenherz (1655).

Initial rates of reaction were determined as a function of substrate concentration at various pH values, in the presence and absence of inhibitors, with other conditions as in the standard assay. Imidazole–HCl buffers, which were found not to be inhibitory, were used throughout the pH range examined at a concentration of 0.05 M. Preliminary experiments, such as that shown in Figure 1, showed that inhibition by 2-phosphoglycollate and by DL-α-glycerophosphate were strictly competitive with respect to glyceraldehyde 3-phosphate. Rate measurements were made with 1-cm quartz cuvets in a Zeiss PMQ II spectrophotometer equipped with a recorder capable of giving full-scale deflection for an absorbance change of 0.015. 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 ranging from 10^{-5} to 4×10^{-4} M, in the presence and absence of inhibitors. Slopes of these plots gave reproducible values for K_m/V_{max} even at the extremes of the imidazole buffer range employed. The intercepts at extreme pH values were less reproducible since the low buffering power of the solutions prevented measurements at very high substrate concentrations.

Heat inactivation of the enzyme was measured by incubating the enzyme (10 μg/ml) in Tris-HCl buffer (pH 8.1, 0.05 M) at 67° and removing 10-μl aliquots at intervals for the standard assay at 25°. Samples were removed over a span of at least five half-times, and the results plotted semilogarithmically against time, yielding good first-order kinetics in all cases (Figure 3). Heat inactivation was found to be irreversible; no recovery of activity was observed after 2 hr in the presence or absence of added factors. Control reactions at room temperatures showed no detectable loss of activity after 4 hr.

Results

Double-reciprocal plots of initial reaction rate as a function of substrate concentration were linear in the presence and absence of inhibitors, all of which were found to give competitive inhibition. The range of substrate concentration examined (0.01–0.40 mM) was far below that in which substrate inhibition has been found to occur for horse muscle isomerase (Snyder and Lee, 1967). Figure 1 shows an example of the results obtained in the absence and presence of 2-phosphoglycollate. Values for V_{max}/K_m, obtained from data collected in imidazole–HCl buffers, are plotted as a function of pH in Figure 2. V_{max}/K_m falls off below pH 8 in a manner consistent with titration of a group on the free enzyme with apparent pK_a = 7.35. The broken line is a theoretical curve calculated for titration of a group with this pK_a.

Observed K_i values for 2-phosphoglycollate were found to increase with increasing pH, in a manner consistent with titration of a group on the free enzyme with apparent pK_a = 7.35 (Figure 2). The solid line is a theoretical curve calculated for titration of a group with this pK_a. No substantial deviation was noted in the neighborhood of the

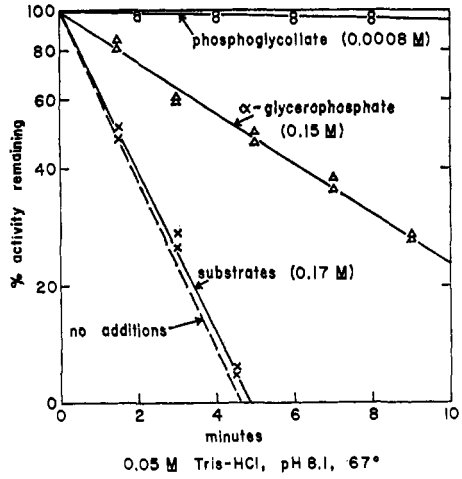

FIGURE 3: Loss of activity of isomerase (10 μg/ml) in Tris-HCl buffer (0.05 M, pH 8.1) at 67°, in the absence of additions (broken line), in the presence of 1.7×10^{-1} M *dl*-glyceraldehyde 3-phosphate (×), in the presence of 1.5×10^{-1} M *dl*-α-glycerophosphate (Δ), and in the presence of 8×10^{-4} M 2-phosphoglycollate (a).

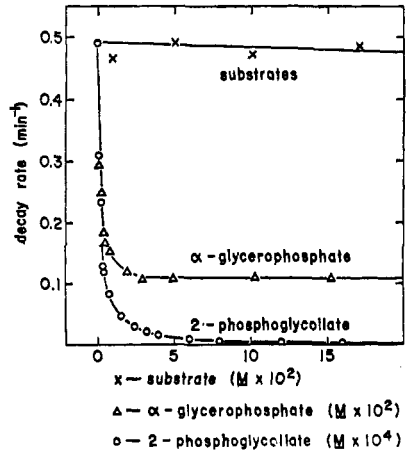

FIGURE 4: Effects of varying concentrations of added compounds on observed first-order rate constants for inactivation, other conditions as in Figure 3.

pK_a of the inhibitor (pK_3 = 6.35 measured titrimetrically under the same conditions).

In contrast, observed Michaelis constants for glyceraldehyde 3-phosphate and K_i values for α-glycerophosphate (Table I) were found to be relatively invariant through most of the pH range examined, increasing slightly below pH 6 near which secondary phosphate ionization occurs (Kiessling, 1934). Glycolaldehyde phosphate and methyl 2-phosphoglycollate were found to be relatively weak inhibitors (Table I).

Isomerase was rapidly inactivated at 67° in the presence of Tris-HCl buffer (0.05 M, pH 8.1). The disappearance of enzyme activity followed good first-order kinetics (Figure 3), with a half-time of approximately 1.5 min. Preliminary experiments (Figure 3) showed that α-glycerophosphate afforded some protection against heat inactivation, whereas protection was almost complete with 2-phosphoglycollate. When the observed rate constants (calculated from the relationship $k = 0.693/t^{1/2}$ were plotted as a function of the concentration of added substrates or inhibitors, results were obtained as shown in Figure 4. Substrates gave little protection. α-Glycerophosphate gave partial protection, such that the rate of inactivation of the enzyme in saturating concentrations of α-glycerophosphate was reduced by a factor of 4. 2-Phosphoglycollate gave virtually complete protection, such that the rate of inactivation was reduced by a factor of at least 100 in the presence of saturating concentrations of inhibitor. Approximate concentrations of α-glycerophosphate (2.5×10^{-3} M) and 2-phosphoglycollate (1.2×10^{-5} M) required to give half-maximal protection in these experiments were 3- to 10-fold higher than the corresponding K_i values determined under similar conditions at 25° (Wolfenden, 1969).

TABLE I: Michaelis and Inhibition Constants.[a]

	pH	K_m	K_i
dl-Glyceraldehyde 3-phosphate	8.50	3.6×10^{-4}	
	7.05	3.0×10^{-4}	
	5.55	5.4×10^{-4}	
2-Phosphoglycollate	8.50		2.1×10^{-5}
	7.05		2.2×10^{-6}
	5.55		1.5×10^{-6}
dl-α-Glycerophosphate	8.50		1.8×10^{-4}
	7.80[b]		2.3×10^{-4} [b]
	7.05		1.5×10^{-4}
	5.55		4.9×10^{-4}
Glycolaldehyde phosphate	7.05		1.2×10^{-4}
Methyl 2-phosphoglycollate	7.05		2.0×10^{-3}

[a] Measured in imidazole–HCl buffers (0.05 M) at 25°, other conditions as described in text. [b] In triethanolamine–HCl buffer (0.02 M, pH 7.8) (Burton and Waley, 1968).

Discussion

With increasing pH in the range from 6.5 to 9, V_{max}/K_m for isomerization rises to a plateau above apparent pK_a = 7.35 (Figure 2). K_m for the substrate glyceraldehyde phosphate (and K_i for the substrate analog α-glycerophosphate) are relatively invariant through this range (Table I). V_{max} is therefore the kinetic parameter responsible for the changing rate, and the observed behavior suggests titration of a basic group on the protein, pK_a = 7.35, required for catalysis; this pK_a is well above those of substrates and inhibitors. Phosphoglucose isomerase behaves comparably (Dyson and Noltmann, 1968).

2-Phosphoglycollate is maximally bound by the enzyme below apparent pK_a = 7.35, dissociating from the enzyme above this pH (Figure 2). Binding of this inhibitor, previously considered from its structure and inhibitory power to be a potential transition state analog (Wolfenden, 1969), is thus sensitive to the state of ionization of an enzyme residue which appears to be specifically involved in the catalytic event.

Theory predicts that an inhibitor, resembling in binding properties the substrate part of the enzyme–substrate complex

in the transition state, should be bound very much more tightly to the enzyme than the substrate itself. For an ideal case, this "binding ratio" should equal or exceed the ratio by which the limiting rate of the enzymatic reaction exceeds that of the nonenzymatic reaction under similar conditions (the "rate ratio") (Wolfenden, 1969). Conditions which produce a change in the rate ratio should produce a proportionate change in the binding ratio.

The nonenzymatic isomerization of triose phosphate is too slow to measure near neutrality, but as in other examples of the Lobry de Bruyn–van Ekenstein rearrangement (Michaelis and Rona, 1912; Speck, 1958) the rate of this reaction presumably rises with pH in proportion to the concentration of alkali. Figure 5 shows that, if this assumption is made, the rate ratio (k_{cat}/k_{nonenz}) reaches a plateau below apparent pK_a = 7.35. The ratio of the binding constant of 2-phosphoglycollate to the apparent binding constant of the substrate, (K_m/K_i), varies in a similar manner, reaching a plateau below apparent pK_a = 7.35 (Figure 5). The observed dependence of kinetic and binding constants on pH tend to confirm the hypothesis that 2-phosphoglycollate acts as an analog of an intermediate in the catalyzed reaction, rather than a substrate analog of the conventional type.

These results are consistent with our earlier interpretation (Wolfenden, 1969) that 2-phosphoglycollate serves as an analog of an ene–diolate intermediate formed by proton transfer to the enzyme (Rose, 1962). However the actual states of ionization of the species involved cannot be determined unambiguously by steady-state kinetics. An alternative possibility is that 2-phosphoglycollic acid with an undissociated carboxylic acid group (an analog of an undissociated ene–diol intermediate) is the true inhibitor. This appears unlikely, since glycolaldehyde phosphate and methyl phosphoglycollate, with no dissociable hydrogen, are two orders of magnitude less tightly bound (Table I), with K_i values comparable with P_i and many simple phosphate esters (Wolfenden, 1969). In addition, species of phosphoglycollic acid with undissociated carboxylic acid groups are present at very low concentrations in the pH range considered. If any of them were the true inhibitor, its K_i would be several orders of magnitude lower than the apparent K_i of approximately 10^{-6} M. Even if binding proceeded at diffusion-controlled rates, release would be expected to be a fairly slow process. However, inhibition by 2-phosphoglycollate has been found to fully reversed by dilution within the few seconds required for conventional kinetic measurements. Further experiments with rapid reaction techniques are in progress, but present results are consistent with inhibition by the fully ionized form of the inhibitor.

Protection of triosephosphate isomerase by inhibitors against heat inactivation suggests a change in the structure of the enzyme in solution from its native form in the presence of the substrate analog α-glycerophosphate resulting in limited protection, and a further change in the presence of 2-phosphoglycollate resulting in complete protection. The relative extents of heat protection by saturating inhibitors is positively correlated with the relative tightness of binding of these compounds in solution, and with the relative changes which they bring about in the unit cell dimensions and volume of the crystalline enzyme from chicken muscle (Johnson and Wolfenden, 1970). This is understandable if the transition state analog possesses additional points of interaction with the enzyme, not present in the substrate analog, which result in a further change in enzyme structure. Whether these structural changes are limited to the immediate neighborhood of the active site is a question which can only be answered by further physical studies.

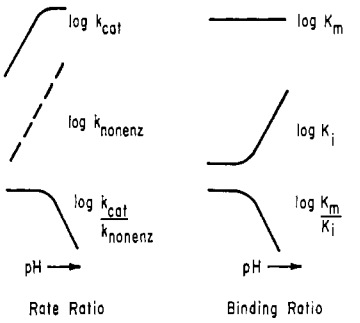

FIGURE 5: pH dependence of kinetic parameters (see Discussion).

References

Beisenherz, G. (1955), *Methods Enzymol. 1*, 387.

Burton, P. M., and Waley, S. G. (1968), *Biochim. Biophys. Acta 151*, 714.

Dyson, J. E. D., and Noltmann, E. A. (1968), *J. Biol. Chem. 243*, 1401.

Fleury, P., and Courtois, J. (1941), *Bull. Soc. Chim. Fr. 8*, 69.

Johnson, L. N., and Wolfenden, R. (1970), *J. Mol. Biol. 47*, 93.

Kiessling, W. (1934), *Biochem. Z. 273*, 103.

Michaelis, L., and Rona, P. (1912), *Biochem. Z. 47*, 447.

Rose, I. A. (1962), *Brookhaven Symp. Biol. 15*, 293.

Snyder, R., and Lee, C. W. (1967), *Arch. Biochem. Biophys. 117*, 587.

Speck, Jr., J. C. (1958), *Advan. Carbohydrate Chem. 13*, 63.

Wolfenden, R. (1969), *Nature (London) 223*, 704.