IRREVERSIBLE INACTIVATION OF TRIOSE PHOSPHATE ISOMERASE BY 1-HYDROXY-3-IODO-2-PROPANONE PHOSPHATE*

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SUMMARY

I have synthesized 1-hydroxy-3-iodo-2-propanone phosphate, a compound structurally similar to dihydroxyacetone phosphate, as a potential active-site specific reagent for triose phosphate isomerase. Under mild conditions, the reagent irreversibly inactivates the enzyme. The kinetics of the inactivation in the absence or presence of the competitive inhibitor α-glycerophosphate, the stoichimetric incorporation of the reagent, and the similarities of the pH dependencies of enzymic activity and of the inactivation rate are indicative of a specific modification at the active site of triose phosphate isomerase.

The active sites of a number of enzymes have been selectively modified by employing reagents that possess certain structural features essential to the affinity of the substrate for the active site (for reviews see Baker, 1967 and Singer, 1967). A reversible enzyme-inhibitor complex is initially formed, thereby greatly increasing the reagent concentration near the active site and enhancing the possibility of modification of residues within this region as compared to the same type of residues located elsewhere in the protein molecule. In an attempt to obtain an active-site specific reagent for triose phosphate isomerase (TPI) (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1), I have synthesized 1-hydroxy-3-iodo-2-propanone phosphate (HIPP), a

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compound resembling dihydroxyacetone phosphate, except for an iodine atom instead of a hydroxyl group at C-3. The inactivation of TPI by HIPP is described in this communication. In addition, such a potentially reactive derivative of dihydroxyacetone phosphate may be useful in labeling the active sites of aldolase (Hartman, 1968) and α-glycerophosphate dehydrogenase, enzymes which also catalyze reactions involving dihydroxyacetone phosphate.

MATERIALS AND METHODS

TPI, α-glycerophosphate dehydrogenase, DPNH, DL-glyceraldehyde-3-phosphate, and DL-α-glycerophosphate were obtained from the Sigma Chemical Company and used without further purification. ^{32}P-phosphorous oxychloride was purchased from New England Nuclear Corp. and 3-chloro-1,2-propanediol from Aldrich Chemical Company.

TPI was assayed by the α-glycerophosphate dehydrogenase-coupled method of Beisenherz (1955). The final concentrations of the constituents in the assay mixture (3 ml) were 0.15 mM DPNH, 1 mM DL-glyceraldehyde-3-phosphate, 8 μg/ml of α-glycerophosphate dehydrogenase, 0.3 mM EDTA, and 0.1 M imidazole, pH 6.5. Protein concentration was measured spectrophotometrically by use of the experimentally determined value of $E^{0.1\%}_{1\ cm} = 1.10$ at 280 mμ. The molecular weight of TPI was assumed to be 60,000 (Johnson and Waley, 1967).

Unless otherwise indicated, all reactions of HIPP with TPI were conducted in 0.1 M imidazole buffer, pH 6.5, containing 0.3 mM EDTA. In kinetic experiments on the inactivation of TPI by HIPP, aliquots from the reaction mixture were withdrawn periodically, diluted into buffer containing 0.01 M β-mercaptoethanol, which rapidly decomposes HIPP, and then assayed for enzymic activity.

HIPP (III) was synthesized from 3-chloro-1,2-propanediol (I) via the series of reactions depicted in Scheme I, the details of which will be published elsewhere. ^{32}P-labeled HIPP was prepared by employing ^{32}P-phosphorous oxychloride in the

phosphorylation step. The free acid of the dimethyl acetal (II), whose crystalline dicyclohexylammonium salt had the correct elemental analysis for the proposed structure, hydrolyzed spontaneously to HIPP. Although no attempts were made to isolate HIPP from the solution in which it was formed, its presence was substantiated by the appearance of 1.0 molar equivalent of base-labile phosphate and iodide during hydrolysis of II.

RESULTS AND DISCUSSION

At pH 6.5 and at room temperature, HIPP (0.01 mM) rapidly inactivates TPI (0.41 μM), as illustrated in Fig. 1. The loss of enzymic activity is clearly pseudo first-order, with a half-life of 2.5 minutes, indicating a single site of modification or the modification of several residues at identical rates. No detectable TPI activity remains after one hour. The presence of substrate or the competitive inhibitor α-glycerophosphate (Burton and Waley, 1968) in the reaction mixture reduces the rate of inactivation (Fig. 1); such a reduction in rate suggests that HIPP is reacting with the active site of TPI.

If an irreversible inhibitor forms a dissociable complex at the active site before covalent bond formation, a rate saturation will be observed as the inhibitor concentration is increased; i.e., at infinite inhibitor concentration the half-life of inactivation will reach a minimum finite value[1]. This effect is analogous to Michaelis-Menten type kinetics observed in double-reciprocal plots of velocity vs. substrate concentration. Because of the extreme rapidity of the TPI inactivation, attempts to demonstrate such a rate saturation effect and thus verify the formation of a reversible complex have been inconclusive. For example, at 4° and 1 mM concentration of HIPP, which is the approximate K_m for glyceraldehyde-3-PO_4 at pH 6.5 and may therefore be considerably less than the dissociation constant for a TPI-HIPP complex, the inactivation half-life

[1] For the derivation and discussion of pertinent equations see Meloche (1967).

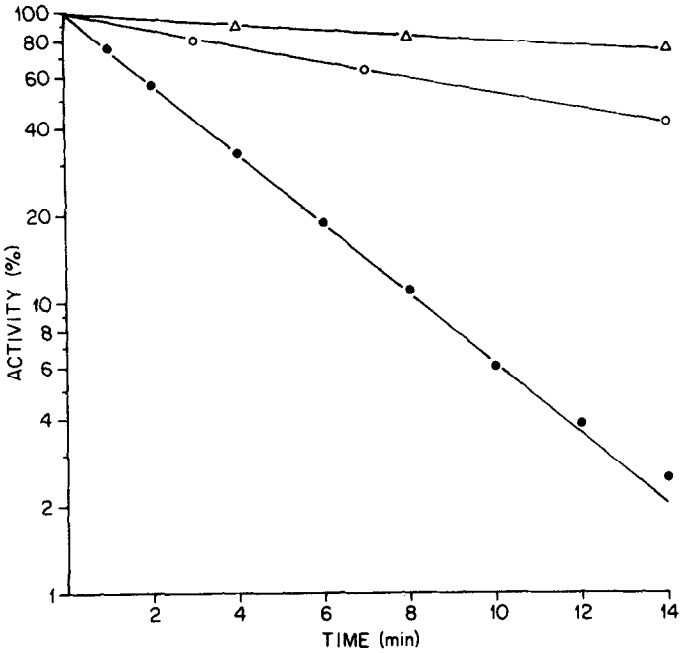


Fig. 1. Kinetics of TPI inactivation by HIPP. Reaction mixtures at room temperature contained TPI (25 μg/ml, 0.41 μM) and HIPP (10 μM) in 0.1 M imidazole -0.3 mM EDTA, pH 6.5, with no other additions (●—●), 10 mM DL-α-glycerophosphate (△ — △), or 5 mM DL-glyceraldehyde-3-phosphate (○—○).

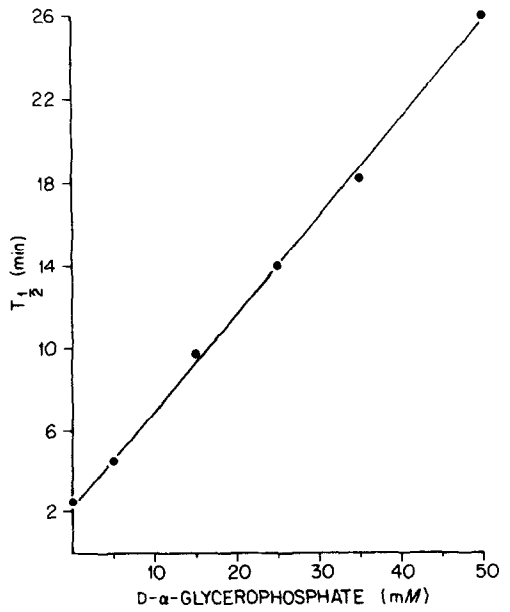


Fig. 2. Inactivation half-life as a function of DL-α-glycerophosphate concentration. Conditions were like those described in Fig. 1.

is only 5-10 seconds and difficult to measure accurately. However, evidence that HIPP does have an affinity for the active site of TPI was obtained by studying the effect of varying the concentration of α-glycerophosphate at a fixed HIPP concentration on the inactivation rate. When the inactivation half-life was plotted as a function of α-glycerophosphate concentration (Fig. 2), a straight line was obtained. The extrapolated y-intercept agreed well with the half-life found in the absence of α-glycerophosphate. These data are consistent with competition between α-glycerophosphate and HIPP for the same site[1].

To substantiate the kinetic implication of the alteration of a limited number of amino acid residues and thus strengthen the assumption of active-site modification, TPI was treated with ^{32}P-HIPP so that the extent of incorporation could be determined. A 10 ml solution of TPI (1 mg/ml) was treated with 1 mM HIPP (325,000 cpm/μmole); after 5 minutes, at which time inactivation was virtually completed, the reaction mixture was passed through a column of Sephadex G-25. The eluted protein fraction was then exhaustively dialyzed and the radioactivity assayed by scintillation counting. The modified TPI had a specific radioactivity of 9070 cpm/mg, which corresponds to 1.7 moles of ^{32}P per mole of protein. If the molecular weight (60,000) used in the calculation is correct and if there are two active sites per molecule as is possible in view of the proposal for two identical subunits (Burton and Waley, 1966; Johnson and Waley, 1967), the somewhat low incorporation may indicate that the TPI employed was only 85% active enzyme. Nevertheless, the experiment clearly shows that inactivation is accompanied by an amount of incorporation that may correspond precisely to the number of active sites.

It is not necessary to employ a large molar excess of HIPP, in order to obtain substantial inactivation of TPI. At equivalent concentrations (0.033 mM) of the enzyme's active sites and inhibitor, 75% inactivation occurs during 60 minutes.

The pH dependency of the inactivation is indirect evidence for a selective

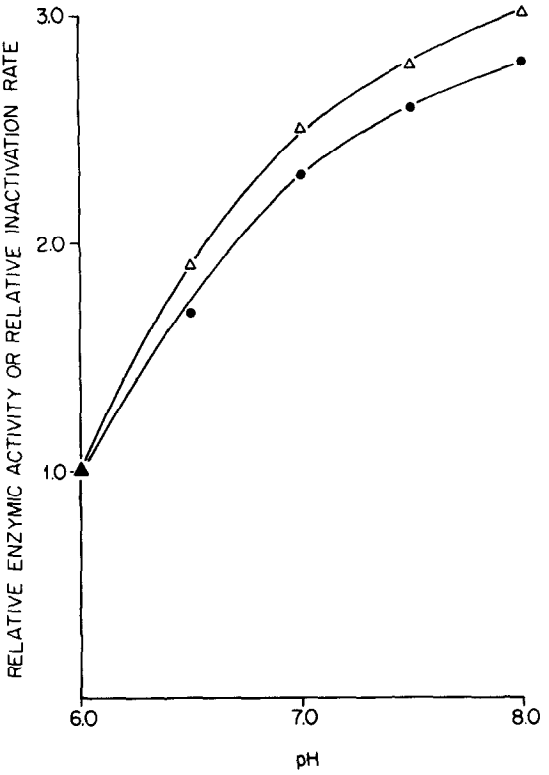
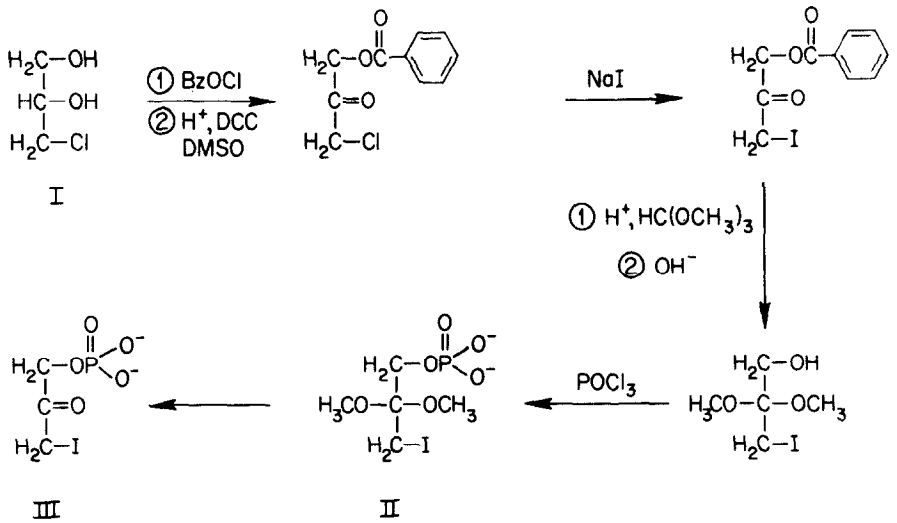


Fig. 3. Relative enzymic activity (●—●) and relative rate of TPI inactivation by HIPP (△— △) as a function of pH. TPI activity was measured as described in Materials and Methods, except for varying the pH of the imidazole buffer. Inactivations of TPI at various pH's were conducted as described in Fig. 1.

SCHEME I



modification of a catalytically functional residue within TPI. As seen in Fig. 3, a striking similarity exists between the pH-profiles of the relative enzymic activity toward glyceraldehyde-3-phosphate and the relative rate of inactivation by HIPP.

Thus, observations which strongly suggest that HIPP reacts specifically with the active site of TPI are as follows: (1) The reagent rapidly and totally inactivates TPI concomitantly with the incorporation of a minimum of 1.7 molar equivalents of reagent per mole of protein. (2) The kinetics of inactivation in the presence of α-glycerophosphate, a competitive inhibitor of TPI, indicate that HIPP is interacting with the enzyme's active site. (3) The pH dependencies of enzymic activity and of inactivation rate bear a close resemblance.

Although an alkylation of the active site is considered a likely type of reaction since the reagent used is an α-haloketone, direct proof awaits identification and characterization of the altered amino acid residues. Such experiments are now in progress, as are efforts to isolate labeled peptides from proteolytic digests of the modified TPI, so that sequence analyses can be performed.

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