Haloacetol Phosphates. Characterization of the Active Site of Rabbit Muscle Triose Phosphate Isomerase*

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ABSTRACT: 3-Haloacetol phosphates, reactive analogs of the substrate dihydroxyacetone phosphate, inactivate triose phosphate isomerase by a selective modification of the active site as demonstrated by the following. (1) The loss of enzymic activity is pseudo first order. (2) Competitive inhibitors protect against inactivation. (3) One mole of reagent is covalently incorporated per mole of catalytic subunit inactivated. (4) Autoradiograms of peptide maps for enzyme inactivated with [^{32}P]haloacetol phosphates show most of the radioactivity in a single peptide. (5) Chloroacetol phosphate inactivates triose phosphate isomerase from all species that have been tested—rabbit muscle, mouse liver, human whole blood, yeast, *Escherichia coli*, and spinach.

A pentadecapeptide containing the incorporated reagent has been isolated from a tryptic digest of rabbit muscle triose phosphate isomerase inactivated by chloroacetol phosphate. Quantitative ester assays with hydroxylamine and subsequent conversion of the resulting hydroxamate into an amine identify glutamic acid as the essential residue esterified by chloroacetol phosphate. The sequence of the active-site peptide is Trp-Val-Leu-Ala-Tyr-Glu-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Lys.

The postulate that haloacetol phosphates (3-halo-1-hydroxy-2-propanone phosphates) might be active-site-specific reagents for triose phosphate isomerase (Hartman, 1968a) was confirmed in preliminary communications (Hartman, 1968b, 1970a,b; Coulson *et al.*, 1970a,b). In this manuscript, the kinetics of the inactivation of triose phosphate isomerase by haloacetol phosphates, the identification of the modification site, and the elucidation of the amino acid sequence adjacent to the labeled residue are described in detail.

Experimental Section

Materials. Triose phosphate isomerase was isolated from rabbit muscle and was shown to be homogeneous on disc gel electrophoresis and by analytical ultracentrifugation (Norton *et al.*, 1970). Haloacetol phosphates were synthesized as described earlier (Hartman, 1970c); the ^{32}P-labeled reagents (initial specific radioactivity of 320,000 cpm/μmole) were obtained by use of [^{32}P]$POCl_3$ (New England Nuclear Corp.) in the phosphorylation step which was scaled down tenfold. The following materials were purchased from Sigma Chemical Co.: NADH, DL-glyceraldehyde 3-phosphate, DL-α-glycerophosphate, and glycerophosphate dehydrogenase. Trypsin and chymotrypsin were obtained from Schwarz BioResearch, Inc. Tritium-labeled sodium borohydride was a product of New England Nuclear Corp. Phenyl isothiocyanate and trifluoroacetic acid were purchased from Aldrich Chemical Co.

Assays. Triose phosphate isomerase concentration was determined by its absorbancy at 280 nm using an $\epsilon^{1\%}_{1\ cm}$ value of 13.1 (Norton *et al.*, 1970). Triose phosphate isomerase activity was measured by the method of Beisenherz (1955) as modified by Norton *et al.* (1970) in which the glycerophosphate dehydrogenase catalyzed reduction of dihydroxyacetone phosphate, formed from D-glyceraldehyde 3-phosphate in the isomerase reaction, by NADH is monitored at 340 nm. Radioactivity was assayed with a Packard Model 3003 liquid scintillation spectrometer. The sample (0.1–0.3 ml) was mixed with 1 ml of Hyamine hydroxide and 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).

Amino Acid Analyses. Protein was hydrolyzed with 6 N HCl at 110° for 21 hr in sealed, evacuated (<50 μ of Hg) tubes. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed with a Beckman 120C amino acid analyzer according to the method of Spackman *et al.* (1958). Tryptophan and free sulfhydryl groups were separately analyzed in guanidine-denatured, unhydrolyzed protein with the methods of Edelhoch (1967) and Ellman (1959), respectively.

Sequence Determinations. Subtractive Edman degradations as described by Konigsberg (1967) were used for sequence determinations. After each cycle, a sample (0.02 μmole) of the peptide was subjected to amino acid analysis.

Peptide Mapping and Autoradiography. Peptide mapping was performed essentially by the method of Katz *et al.* (1959), but electrophoresis preceded chromatography. A salt-free sample of tryptic digest (representing 1–2 mg of protein) was applied as a single spot to a 350 × 400 mm sheet of Whatman No. 3MM chromatography paper and subjected to electrophoresis (1300 V for 1.5 hr) with a Brinkman Mini-Pherograph high-voltage apparatus; the buffer (pH 6.5) used was glacial acetic acid–pyridine–water (1:10:100, v/v). After electrophoresis, the paper was air-dried for 4 hr and subjected to descending chromatography (perpendicular to the direction of electrophoresis) for 12 hr using the top layer obtained by partitioning 1-butanol–water–glacial acetic acid (4:5:1, v/v). The peptides were visualized by dipping the dried chromatogram into 0.5% ninhydrin in acetone and heating at 80°. Autoradiograms of peptide maps were prepared using Kodak No-Screen Medical X-Ray film with a 5-day exposure time.

Modification of Triose Phosphate Isomerase by Haloacetol Phosphates. A. FOR ACTIVE-SITE CHARACTERIZATION. Triose

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phosphate isomerase (150 mg, 5.66 μmoles of catalytic subunit) in 10 ml of 0.1 M sodium bicarbonate (pH 8.0) containing 1 mM EDTA was treated with 0.5 ml of 0.04 M chloroacetol phosphate (20 μmoles). After 5 min, inactivation was completed, and the reaction mixture was made 0.01 M in β-mercaptoethanol to react with the excess chloroacetol phosphate. After cooling the solution to 4°, the carbonyl group of the incorporated reagent was reduced by a 30-min treatment with [^{3}H]sodium borohydride (4 mg, 0.3 mCi/μmole). To the reaction mixture was then added 10 ml of 8 M guanidine hydrochloride containing 0.1 M sodium phosphate (pH 8.0) followed immediately by 0.7 ml of 1 M sodium iodoacetate (pH 8.0) to carboxymethylate protein sulfhydryl groups. Twenty minutes later, arabinose (100 mg) was added to the solution to oxidize the excess borohydride; the reaction mixture was then made 0.1 M in β-mercaptoethanol and dialyzed exhaustively against water followed by 0.1 M ammonium bicarbonate (pH 8.0). The dialyzed solution (34 ml) contained 143 mg of protein with a specific radioactivity of 40.3×10^6 cpm/μmole. Native triose phosphate isomerase, treated with [^{3}H]sodium borohydride under the same conditions, contained only 53×10^3 cpm/μmole.

Modification of Triose Phosphate Isomerase by Haloacetol Phosphates. B. FOR DETERMINATION OF STOICHIOMETRY AND FOR PEPTIDE MAPPING AND AUTORADIOGRAPHY. Three 20-mg samples of triose phosphate isomerase were inactivated with ^{32}P-labeled chloro-, bromo-, and iodoacetol phosphate, respectively, under conditions described in the preceding paragraph. The inactivation with the iodo reagent was allowed to proceed for 1 hr. Half of each sample was dialyzed exhaustively at 4° against 0.1 M sodium phosphate (pH 6.0) and then assayed for radioactivity. The remainder of each sample was treated as described, except unlabeled sodium borohydride was used in the reduction. The three samples were individually digested for 2 hr at 40° with trypsin (1% by weight of the triose phosphate isomerase) in 0.1 M ammonium bicarbonate (pH 8.0). The digested samples were lyophilized to dryness and used in peptide mapping experiments.

Results

Kinetic Studies. Preliminary experiments showed that haloacetol phosphates rapidly inactivate purified rabbit muscle triose phosphate isomerase. To obtain accurate kinetic data, the rates of inactivation were measured at 2°. With tenfold molar excesses of the reagents, the loss of activity is pseudo first order (Figure 1). Second-order rate constants at 2° and pH 6.5—calculated from eq 1 (Aldridge, 1950)

$$\tau = \frac{\ln 2}{[\mathrm{I}]k_{2\mathrm{nd}}} \qquad (1)$$

in which τ is the half-time of inactivation, and I is the molar concentration of inhibitor—are 260, 2300, and 2600 M^{-1} sec^{-1} for the iodo, chloro, and bromo reagents, respectively.

If the inactivation is a simple bimolecular reaction

$$\mathrm{I} + \mathrm{E} \xrightarrow{k_{2\mathrm{nd}}} \mathrm{I\text{-}E} \qquad (2)$$

with the rate equation

$$\frac{-\mathrm{d}E}{\mathrm{d}t} = k_{2\mathrm{nd}}(E - x)(I - x) \qquad (3)$$

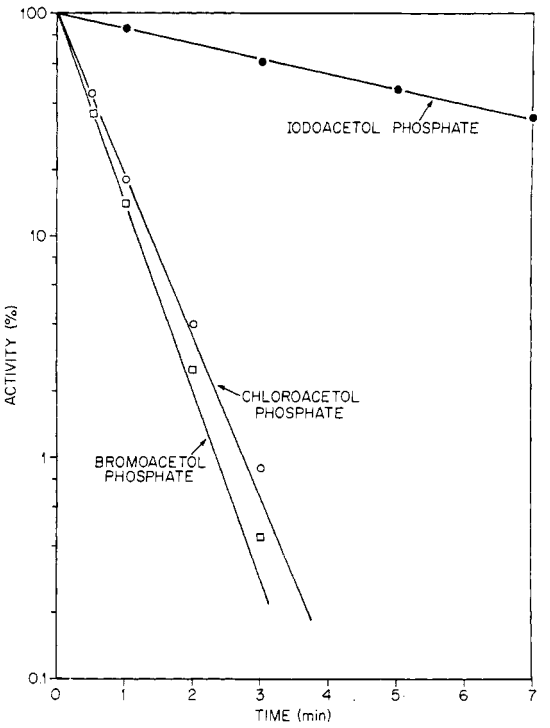

FIGURE 1: Inactivation of rabbit muscle triose phosphate isomerase by haloacetol phosphate at 2°. The reaction mixtures contained 25 μg of enzyme/ml and 10 μM reagent in 0.1 M imidazolium hydrochloride (pH 6.5). Periodically 0.1-ml samples of the reaction mixtures were diluted into 4.9 ml of 0.02 M triethanolamine hydrochloride (pH 7.9) containing 1 mM EDTA and 10 mM β-mercaptoethanol, which reacts with the excess haloacetol phosphate. Aliquots of these diluted samples were assayed for isomerase activity as described in the Experimental Section.

in which E and I are initial concentrations of the enzyme and inhibitor and x is the concentration of inactivated enzyme at time t, the observed pseudo-first order rate constant should be directly proportional to I. If the inhibitor combines reversibly with the active site before covalent modification (eq 4), as anticipated for a protein reagent resembling the substrate, the pseudo-first-order rate constant for inactivation

$$\mathrm{E} + \mathrm{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{-}I} \xrightarrow{k_2} \mathrm{E\text{-}I} \qquad (4)$$

should approach a maximum value as the inhibitor concentration is increased. This "rate saturation" effect results from the rate of formation of inactive enzyme (E-I) being dependent upon the concentration of the dissociable complex (E—I) whose maximal concentration is fixed by the initial enzyme concentration.

Applying steady-state kinetics, Meloche (1967) has derived a linear rate expression corresponding to eq 4 which clearly shows the dependence of the inactivation rate on inhibitor concentration.

$$\tau = \frac{1}{[\mathrm{I}]}(TK_{\mathrm{inact}}) + T \qquad (5)$$

K_{inact} is $(k_{-1} + k_2)/k_1$ and comparable to K_M in the Michaelis–Menten expression, and T is the minimal inactivation half-time. Since a plot of τ *vs.* 1/[I] should intercept the τ axis at

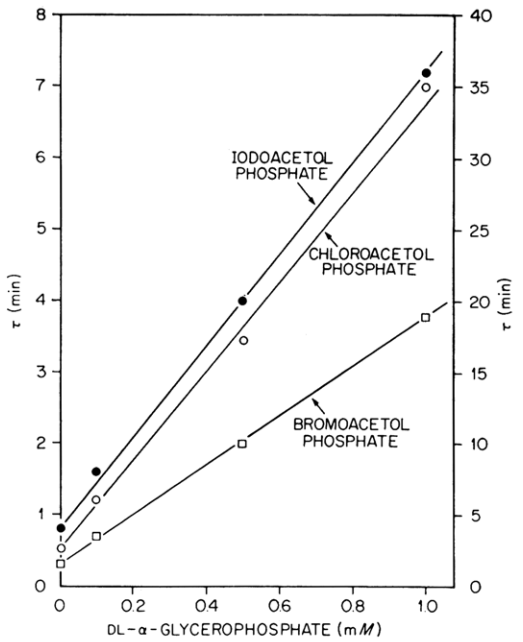

FIGURE 2: Protection by DL-α-glycerophosphate of triose phosphate isomerase against inactivation by haloacetol phosphates. The right-hand ordinate corresponds to the data obtained with iodoacetol phosphate. Reaction conditions were identical with those described in the legend to Figure 1.

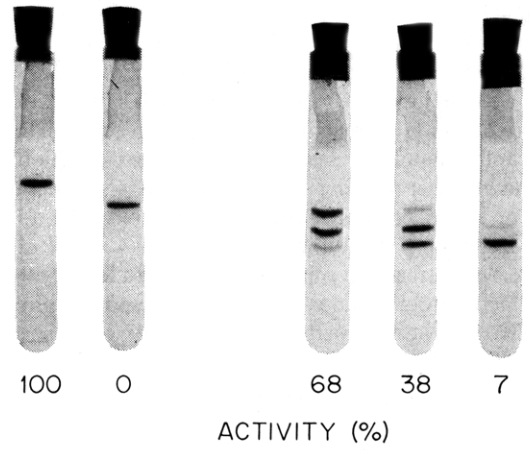

FIGURE 3: Disc gel electrophoretic patterns of triose phosphate isomerase that was treated with varying limiting quantities of chloroacetol phosphate. A solution of the enzyme (5.3 mg/ml; 0.2 mM) in 0.01 M Tris hydrochloride (pH 8.0) was treated sequentially with 0.06, 0.13, 0.19, and 0.3 mM chloroacetol phosphate. Thirty minutes after each addition, a sample was removed for electrophoresis. The number under each sample represents the percentage of the initial enzymic activity remaining. The electrophoresis was conducted on standard 7% gels at pH 9.5 according to the manufacturer's (Canal Industrial Corp.) instructions. The gels were stained with Amido Schwarz.

zero if eq 2 is operative, and at a finite value (T) if eq 4 is operative, the two mechanisms are kinetically distinguishable. However, when I is much smaller than K_{inact}, the rate of inactivation becomes directly proportional to I, and eq 4, even if operative, appears identical with eq 2.

The extremely rapid rates of inactivation of triose phosphate isomerase by haloacetol phosphates precluded using the kinetic test described to determine whether reversible complex formation between the enzyme and reagent is an obligatory step preceding covalent modification. The rate saturation effect predicted by eq 4 and 5 will not be apparent until the reagent concentration approaches K_{inact}, which is probably not less than 1 mM (K_M at pH 7.8 for dihydroxyacetone phosphate is 0.87 mM and for D-glyceraldehyde 3-phosphate is 0.46 mM (Burton and Waley, 1968a)). Since the half-time for inactivation with 0.01 mM iodoacetol phosphate, the slowest reacting of the three reagents, is only 5 min, the half-time will be in the order of 3 sec as the reagent concentration approaches K_{inact}. At present, such rapid reactions cannot be measured because the excess reagent cannot be decomposed instantaneously.

Another method to determine whether the irreversible inhibitor has an affinity for the substrate binding site is to observe the effect of substrate or competitive inhibitor on the inactivation rate. In the presence of substrate or a competitive inhibitor, S, with a dissociation constant, K_s, for enzyme–S complex, eq 5 becomes

$$\tau = [S]T \frac{1}{[I]} \frac{K_{inact}}{K_s} + \left(T + T \frac{K_{inact}}{[I]}\right) \qquad (6)$$

(Meloche, 1967). α-Glycerophosphate, a competitive inhibitor of triose phosphate isomerase (Burton and Waley, 1968a), decreases the rate of inactivation of the enzyme by haloacetol phosphates, and plots of τ *vs.* glycerophosphate concentration give straight lines as predicted by eq 6 (Figure 2).

Chloroacetol phosphate inactivates triose phosphate isomerase from all species that have been tested, and the second-order rate constants for these reactions are given in Table I.

Extent of Inactivation. The inactivation of triose phosphate isomerase by haloacetol phosphates is complete. The enzyme (5 mg/ml) in 0.1 M sodium bicarbonate (pH 8.0) was treated with 1 mM chloroacetol phosphate. At zero time, 0.02 ml (0.01 μg of enzyme) of a 1:10,000 dilution of the enzyme solution was assayed and gave a change in $A_{340\ nm}$ of 0.155. After the enzyme was incubated with the reagent for 30 min, 0.1 ml (500 μg of enzyme) of the undiluted reaction mixture was assayed and gave a change in $A_{340\ nm}$ of <0.001/min, corresponding to <0.00002% of the initial enzymic activity.

Stoichiometry and Number of Active Sites. Triose phosphate isomerase was inactivated with ^{32}P-labeled haloacetol phos-

TABLE I: Biomolecular Rate Constants for the Inactivation of Various Species of Triose Phosphate Isomerase by Chloroacetol Phosphate.[a]

Species	$k_{2nd} \times 10^{-3}$ (M^{-1} sec^{-1})
Escherichia coli	1.1
Spinach	1.6
Mouse liver	1.9
Rabbit muscle	2.3
Human whole blood	2.7
Bakers' yeast	5.3

[a] The conditions were identical with those described in the legend of Figure 1. Except for the rabbit muscle enzyme, crude extracts were used. In all cases the initial triose phosphate isomerase activity was equivalent to 0.025 mg of rabbit muscle enzyme/ml.

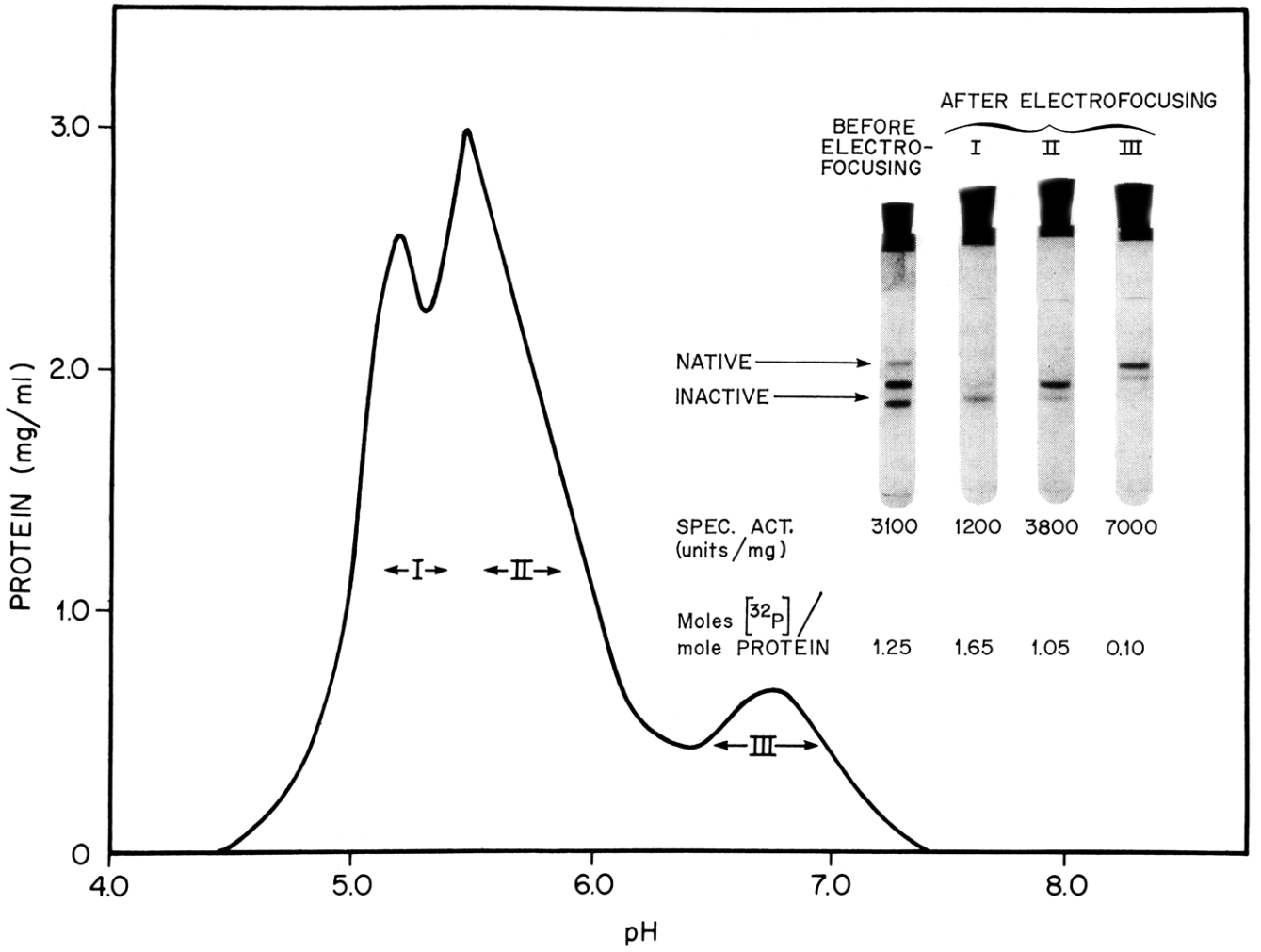

FIGURE 4: Isoelectric focusing of triose phosphate isomerase (35 mg) that was treated with [^{32}P]chloroacetol phosphate until only 40% of the enzymic activity remained; the initial specific activity was 7500 units/mg. The fractions indicated were assayed for enzymic activity, protein concentration, and radioactivity. These fractions were then dialyzed against 0.01 M ammonium acetate and subjected to disc gel electrophoresis (inserted photograph) as described in the legend to Figure 3. Electrofocusing was carried out on an Ampholine column (110 ml according to the instructions provided by LKB Instruments. A wide-range (pH 3–10) carrier ampholyte at a 1% concentration was used; focusing was continued at 600 V for 72 hr.

phates so that the extent of incorporation could be determined. With each of the three reagents 1.78–1.84 moles of phosphorus/mole (53,000 g) of protein was incorporated, suggesting two active sites per enzyme molecule. The inactivated enzyme was homogeneous by the criterion of disc gel electrophoresis. If triose phosphate isomerase was treated with limiting molar quantities of reagent, a species with an electrophoretic mobility intermediate between that of native and inactivated enzyme was detected (Figure 3). The species were present at about the ratios predicted from the percentage of enzymic activity remaining and the assumption that the species of intermediate mobility, due to only one of its active sites being modified, has a specific activity equal to 50% that of native enzyme. To verify this assumption, the three species were separated preparatively by electrofocusing (Figure 4). The species of intermediate electrophoretic mobility contained only 1 mole of phosphorus/mole of protein and had one-half the specific activity of native triose phosphate isomerase. This observation is in complete accord with triose phosphate isomerase containing two active sites per molecule.

Specificity of Modification. To substantiate the specificity of haloacetol phosphates for a single amino acid in triose phosphate isomerase that was inferred from the stoichiometry of the reaction, peptide mapping and autoradiography were carried out. Before the inactivated enzyme was digested with trypsin, the ketone group of the incorporated reagent was reduced to a hydroxyl group with sodium borohydride. This reduction stabilized the phosphate group, which was otherwise liberated as inorganic phosphate during lyophilization of the inactivated enzyme. The peptide map and its autoradiogram shown in Figure 5 was obtained from three separate enzyme samples that had been inactivated with ^{32}P-labeled chloro, bromo, and iodo reagent, respectively. After inactivation and tryptic digestion, equal portions of the three samples were mixed, and the mixture was subjected to peptide mapping. One peptide (no. 1) on the map from the native enzyme is absent from the map of the inactivated enzyme, and a peptide (no. 2) is of increased relative intensity on the map from the inactivated enzyme as compared to the corresponding peptide from the native enzyme. The finding of most of the radioactivity in one region, coinciding with the peptide of increased intensity, confirms a high degree of specificity of the reagent for a single site and also indicates that all three reagents react with the same site. The position of the minor

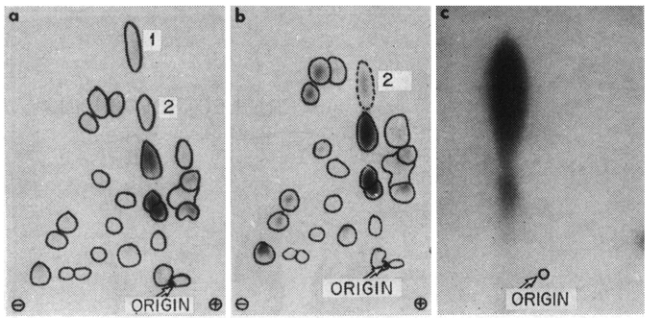

FIGURE 5: Peptide mapping of native (a) and [^{32}P]haloacetol phosphate inactivated (b) triose phosphate isomerase. The autoradiogram (c) was prepared from b. The experimental details are described in the Experimental Section.

radioactive component that migrates toward the anode coincides with α-glycerophosphate. Other minor radioactive components can be explained by incomplete tryptic digestion.

Amino Acid Compositions. Attempts to identify the type of amino acid residue modified by haloacetol phosphates by comparing amino acid contents in acid hydrolysates of native and inactivated triose phosphate isomerase were unsuccessful, as no differences were detectable. Tryptophan and free sulfhydryl groups were analyzed in unhydrolyzed samples and were also found to be identical. If the inactivated enzyme is treated with ^{3}H-labeled sodium borohydride, thereby incorporating tritium into the reagent moiety and permitting its detection, acid hydrolysis liberates two ninhydrin-negative compounds that emerge from the long column of the amino acid analyzer at the positions of α-glycerophosphate (21 min) and glycerol (28 min), respectively. Ninety per cent of the radioactivity is recovered in the glycerol peak.

Isolation and Amino Acid Composition of Active-Site Peptide. Triose phosphate isomerase (143 mg in 34 ml of 0.1 M ammonium carbonate, pH 8.0; specific radioactivity of 40.3 $\times$ 10^{6} cpm/μmole of subunit) that had been inactivated with chloroacetol phosphate, reduced with ^{3}H-labeled sodium borohydride, and carboxymethylated (see Experimental Section) was digested with trypsin (1.5 mg) for 1.5 hr at 40°. After lyophilization, the digest was dissolved in 0.25 M pyridine–3.8 M acetic acid (pH 3.2) and was chromatographed on a 2.5 $\times$ 15 cm column of Bio-Rad Aminex AG 50W-X2 resin, equilibrated, and eluted with the same pyridine–acetic acid buffer (Figure 6a). Most of the peptides (about 90% by weight) were retained by the resin. Fractions containing the major radioactive component were pooled and lyophilized. The residue (165 $\times$ 10^{6} cpm) was dissolved in 2.0 ml of 0.1 M ammonium bicarbonate (pH 8.0) and subjected to gel filtration on a 1.5 $\times$ 230 cm column of Bio-Gel P-4 in 0.01 M ammonium acetate (Figure 6b). The radioactive fraction (158 $\times$ 10^{6} cpm, 73% overall recovery) was lyophilized to dryness. Amino acid analyses showed that the isolated peptide was essentially homogeneous and was composed of Trp$_2$,Lys,-Thr,Glu,Pro,Gly$_2$,Ala$_2$,Val$_2$,Ile,Leu,Tyr (Table II). This pentadecaptide also contained 1 molar equiv of phosphate. The quantity of peptide subjected to amino acid analysis was 0.046 μmole on the basis of the specific radioactivity of the inactivated enzyme before tryptic digestion, corresponding closely to the amount of amino acids found (0.043–0.046). The specific radioactivity of the isolated, active-site peptide is thus the same as that of the modified protein from which it came, confirming the high degree of specificity of haloacetol phosphates for a single amino acid residue in triose phosphate isomerase.

Identification of Modified Residue. The active-site peptide reacts with hydroxylamine to form a hydroxamate (Table III). After treatment with hydroxylamine, the peptide emerges from Bio-Gel P-4 columns at the same position as the un-

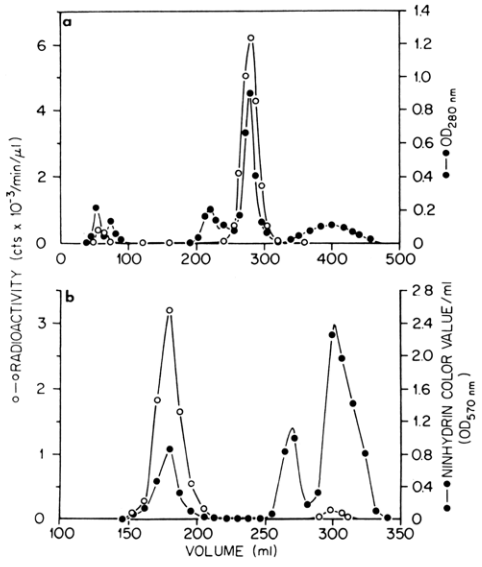

FIGURE 6: Purification of active-site peptide. (a) Ion-exchange chromatography of a tryptic digest from chloroacetol phosphate inactivated triose phosphate isomerase. (b) Gel filtration of radioactive peak obtained from ion-exchange chromatography. The experimental details are described in the Results section.

TABLE II: Amino Acid Composition of Active-Site Peptide.[a]

Amino Acid	μmole Found	No. of Residues
Trp[b]	0.089	2
Lys	0.046	1
His	None	0
Arg	None	0
Asp	0.001	0
Thr[c]	0.046	1
Ser	0.001	0
Glu	0.046	1
Pro	0.044	1
Gly	0.090	2
Ala	0.089	2
Cys	None	0
Val	0.095	2
Met	Trace	0
Ile	0.043	1
Leu	0.044	1
Tyr	0.043	1
Phe	Trace	0

[a] Samples equivalent to 0.046 μmole of peptide based on a specific radioactivity of 40.3 $\times$ 10^{6} cpm/μmole were placed on each column of the amino acid analyzer. [b] Trp was determined in the unhydrolyzed peptide by the method of Edelhoch (1967). [c] Corrected assuming a 5% destruction during hydrolysis.

TABLE III: Ester Content of Active-Site Peptide as Determined with Hydroxylamine–Ferric Chloride.[a]

Sample	Quantity Assayed (mμmoles)	Hydroxamate Found	
		OD_{540nm}	mμmoles
Acetylhydroxamate	50	0.31	50
	100	0.60	100
Glycine methyl ester	50	0.29	48
	100	0.58	96
Glutamine	50	0.015	2.5
	100	0.035	6.0
Active-site peptide	50	0.14	23
	100	0.28	46

[a] The assay was performed as described by Hestrin (1949) but was scaled down tenfold.

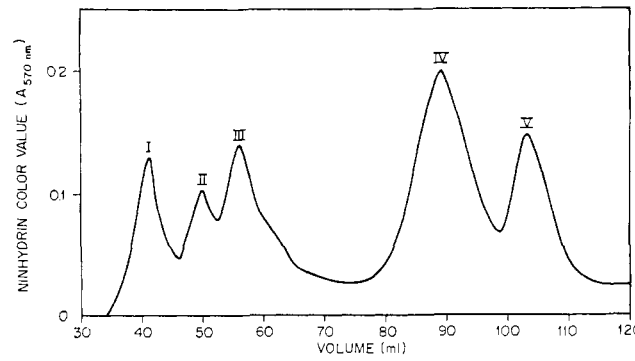

FIGURE 7: Ion-exchange chromatography of a chymotryptic digest of the active-site peptide. The active-site peptide (0.5 μmole), from which the glycerophosphate moiety was removed by treating with 0.01 N sodium hydroxide for 12 hr, was digested with 0.05 mg of chymotrypsin in 0.1 M ammonium bicarbonate (pH 8.0) for 8 hr at 40°. After lyophilization, the digest was placed on a 0.9 × 14 cm column of Bio-Rad Aminex AG 50W-X2 resin that was eluted with a pyridinium acetate gradient (initial buffer, 150 ml of 0.2 M pyridine, 4.6 M acetic acid (pH 3.1); limit buffer, 150 ml of 2.0 M pyridine, 2.3 M acetic acid (pH 5.0)). Fraction IV contained the desired peptide.

treated peptide, but the radioactivity appears in the salt fraction. Thus, the hydroxamate is formed by cleavage of the bond between the peptide and reagent moiety suggesting an ester linkage. A sample of the hydroxamate (from 0.5 μmole of peptide) was dinitrophenylated and treated with base as described by Gallop *et al.* (1960) to effect Lossen rearrangement. The amino acid composition of the resulting peptide was identical with the untreated peptide with two exceptions: glutamic acid was decreased from 1.0 to 0.59 molar equiv and an amino acid was present (0.40 molar equiv) that was eluted between lysine and histidine, the position at which authentic 2,4-diaminobutyric acid appeared. These experiments show that a glutamyl residue in triose phosphate isomerase is esterified by haloacetol phosphates.

Sequence of Active-Site Peptide. Subtractive Edman degradations established the sequence of the first nine residues in the active-site peptide. Before the sequential degradations were initiated, the peptide was treated with 0.01 N sodium hydroxide for 12 hr at 25° to convert the esterified glutamyl carboxyl group into the free acid. In the data that follow the number after each amino acid is its molar ratio relative to isoleucine, which was arbitrarily set at 1.0. The amino acid removed at each degradative cycle is given by boldface. Tryptophan and lysine were determined only in the initial peptide, whose composition is given in Table II.

FIRST DEGRADATION. The analysis was identical with that of the initial peptide.

SECOND DEGRADATION. Thr, 1.0; Glu, 1.0; Pro, 1.1; Gly, 2.2; Ala, 2.1; **Val, 1.2**; Ile, 1.0; Leu, 0.95; Tyr, 0.88.

THIRD DEGRADATION. Thr, 0.95; Glu, 1.1; Pro, 1.1; Gly, 2.1; Ala, 2.1; Val, 1.1; Ile, 1.0; **Leu, 0.33**; Tyr, 0.84.

FOURTH DEGRADATION. Thr, 0.97; Glu, 1.1; Pro, 1.1; Gly, 2.1; **Ala, 1.4**; Val, 1.1; Ile, 1.0; Leu, 0.30; Tyr, 0.82.

FIFTH DEGRADATION. Thr, 0.95; Glu, 1.0; Pro, 1.0; Gly, 2.1; Ala, 1.4; Val, 1.2; Ile, 1.0; Leu, 0.31; **Tyr, 0.42.**

SIXTH DEGRADATION. Thr, 0.95; **Glu, 0.57**; Pro, 1.1; Gly, 1.9; Ala, 1.4; Val, 1.1; Ile, 1.0; Leu, 0.28; Tyr, 0.35.

SEVENTH DEGRADATION. Thr, 1.0; Glu, 0.55; **Pro, 0.65**; Gly, 2.1; Ala, 1.4; Val, 1.1; Ile, 1.0; Leu, 0.28; Tyr, 0.31.

EIGHTH DEGRADATION. Thr, 0.95; Glu, 0.56; Pro, 0.65; Gly, 2.2; Ala, 1.4; **Val, 0.7**; Ile, 1.0; Leu, 0.26; Tyr, 0.30.

NINTH DEGRADATION. The analysis was identical with that after the eighth degradation.

To establish the sequence of the remaining six residues, the pentadecapeptide was digested with chymotrypsin, and a peptide composed of Ala,Thr,Ile,Gly_2,Lys was isolated (Figure 7, fraction IV). Edman degradations gave the following results.

FIRST DEGRADATION. **Ala, 0.3**; Thr, 1.1; Ile, 1.0; Gly, 2.2.

SECOND DEGRADATION. Ala, 0.2; Thr, 1.0 (arbitrary setting); **Ile, 0.4**; Gly, 2.1.

THIRD DEGRADATION. Ala, 0.2; Thr, 1.0; Ile, 0.4; **Gly, 1.5.**

FOURTH DEGRADATION. Ala, 0.2; **Thr, 0.6**; Ile, 0.4; Gly, 1.5 (arbitrary setting).

The assumption that lysine occupies the C-terminal position as the pentadecapeptide was obtained from a tryptic digest was confirmed by carboxypeptidase B digestion. The complete sequence is thus Trp-Val-Leu-Ala-Tyr-Glu-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Lys.

Discussion

Chemical modification has been instrumental in the characterization of many enzymic active sites, but often a reagent's lack of specificity negates interpretation with respect to the essentiality of any given modified residue. To overcome this disadvantage the procedure of "affinity labeling" was developed in which a protein reagent is designed to resemble the substrate and thus have an affinity for the substrate binding site (for detailed discussions, see Baker (1967), Singer (1967), and Shaw (1970)). The inactivation of triose phosphate isomerase by haloacetol phosphates, reactive analogs of the substrate dihydroxyacetone phosphate, appears to be a successful example of affinity labeling.

All data indicate a highly selective modification by haloacetol phosphates of the active site of triose phosphate isomerase. At high reagent to enzyme molar ratios, loss of activity is pseudo first order. This is anticipated if modification of only one residue is responsible for inactivation. Enzymic activity is completely abolished, likewise consistent with modification of an essential residue. α-Glycerophosphate, a competitive inhibitor of triose phosphate isomerase, protects the enzyme against inactivation, and kinetic data indicate

competition of haloacetol phosphates and glycerophosphate for the same site on the protein. The inactivated enzyme contains about 1 mole of covalently bound reagent/mole of catalytic subunit. Peptide maps shown that most of the label is associated with a single peptide. Triose phosphate isomerases from a wide variety of species are inactivated by haloacetol phosphates, increasing the likelihood that a common, essential region is the site of modification.

Although iodo-, bromo-, and chloroacetol phosphate react with the same residue of triose phosphate isomerase, the second-order rate constants, k_{2nd}, for the reactions differ significantly. Usually relative rates for displacement of halogen by an SN2 mechanism are I > Br > Cl (Hine, 1962). The finding that k_{2nd} for iodoacetol phosphate is only one-tenth that of the chloro and bromo reagents can nevertheless be explained. As $k_{2nd} = k_2/K_{inact}$ (see eq 4 and 5), the reagent with the greatest inherent reactivity can be the slowest reacting (on the basis of k_{2nd}) due to a large K_{inact}. The dissociation constant, K_{inact}, for the presumed reagent–protein complex probably increases upon replacing chlorine by iodine due to steric factors introduced by the larger iodine atom.

Triose phosphate isomerase contains two, apparently identical, subunits each with a molecular mass of 26,500 g mole^{-1} (Burton and Waley, 1968b; Norton *et al.*, 1970), but the number of active sites has not been determined previously. Based on a molecular weight of 53,000 and $\epsilon_{1\,cm}^{1\%}$ of 13.1, 1.8 moles of haloacetol phosphate/mole of enzyme is incorporated instead of 2.0 as predicted for a molecule with two active sites. This discrepancy may be due to using either an incorrect molecular weight or ϵ value. In a preliminary report, Coulson *et al.* (1970a), using a molecular weight of 50,000 and $\epsilon_{1\,cm}^{1\%}$ value of 12.1, reported the incorporation of 1.3 moles of phosphorus (from bromoacetol phosphate) per mole of enzyme. The low value was attributed to partial loss of the labile phosphate group from the enzyme during gel filtration at pH 7.9 subsequent to inactivation. With [^{14}C]bromoacetol phosphate the extent of incorporation was 2.1 moles/mole of protein. The phosphate group is labile; it is completely removed from the inactivated protein by the mild treatment of lyophilization. The bond between the reagent and protein is not broken, since after lyophilization tritium is still incorporated by reduction with [^{3}H]sodium borohydride. This demonstrates that the ketone group of the reagent is still associated with the protein.

The slightly low levels of incorporation reported here do not seem to result from loss of the incorporated [^{32}P]phosphate group. Excess reagent is removed by dialysis against 0.01 M phosphate (pH 6.0) at 4° to minimize hydrolysis of the phosphate ester. If hydrolysis occurred, it should have been detected by electrophoresis, since loss of phosphate from the inactivated enzyme would result in the formation of a more positive species. The totally inactivated enzyme, well separated from native enzyme, does not contain any trace of a slower moving component (Figure 3).

The labeling pattern observed when triose phosphate isomerase is treated with limiting amounts of chloroacetol phosphate is difficult to explain unless the enzyme contains two active sites per molecule. At varying levels of enzymic activity remaining, disc gel electrophoresis reveals a protein migrating between native and fully inactivated enzyme. This species contains only 1 mole of reagent per mole of protein and is 50% active.

It seemed desirable to stabilize the phosphate group of the incorporated reagent because after proteolytic digestion of the inactivated enzyme a peptide containing the highly acidic phosphate group should be readily separable from most other peptides in the mixture. The phosphate group was stabilized by reducing the adjacent ketone group to a hydroxyl group with [^{3}H]sodium borohydride. Effects of borohydride other than reduction of the ketone have been excluded. Insignificant amounts of radioactivity are incorporated into native triose phosphate isomerase when incubated with [^{3}H]sodium borohydride. Cross-link formation between the ketone and an amino acid side chain (*e.g.*, Schiff-base formation) does not occur since the position of the labeled peptide on peptide maps is not altered by carrying out the reduction of the inactivated enzyme in the presence of 7 M guanidine hydrochloride (conditions under which a specific cross-linking reaction is unlikely). Also, after reduction the reagent moiety is released during acid hydrolysis of the modified protein as glycerophosphate.

A peptide containing the glycerophosphate moiety was isolated from a tryptic digest of chloroacetol phosphate inactivated triose phosphate isomerase. The purified peptide was obtained in high yield (73% of the total radioactivity associated with the modified protein) and had a specific radioactivity identical with that of the catalytic subunit before proteolytic digestion. These data demonstrate conclusively that triose phosphate isomerase has two identical active sites and that haloacetol phosphates have an absolute specificity for a single residue in each of the two subunits.

Hydroxylamine liberated glycerophosphate from the peptide and formed a hydroxamate derivation in 50% yield, suggesting that the modified residue is an ester of glutamic acid. Confirmation was the finding of 2,4-diaminobutyric acid in hydrolysates after the hydroxamate had been subjected to Lossen rearrangement. The possibility of the hydroxamate arising from a glutaminyl residue was ruled out since glutamine reacted only slightly with hydroxylamine. Also, the active-site peptide contains neither asparagine nor glutamine (no aspartic acid and less than 1 molar equiv of ammonia in acid hydrolysates). The low yield of hydroxamate is thought to be due to partial hydrolysis of the ester by the basic hydroxylamine solution. Neutral hydroxylamine did not cleave the ester bond. I conclude that inactivation of triose phosphate isomerase by haloacetol phosphates results from an esterification of a single glutamyl γ-carboxyl group in the active-site region.

The sequence adjacent to the modified glutamyl residue is Trp-Val-Leu-Ala-Tyr-Glu-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Lys. In a preliminary note (Hartman, 1970b), I incorrectly placed alanine at position 9 and tryptophan at position 10. Nine cycles of the Edman degradation have been repeated three times, and in each case a gap was found at position 9, placing tryptophan in this position. The previous placement of tryptophan at position 10 was based on isolating, from a chymotryptic digest, a peptide containing Ala,Tyr,Glu,Pro,-Val,Trp and assuming alanine occupied position 9. Clearly, splits at -Leu-Ala- and -Trp-Ala- will give a peptide of the observed composition. Also, in the previous report, data obtained from carboxypeptidase digestion were incorrectly interpreted as indicative of glycine at positions 13 and 14; the positions of isoleucine and threonine were not specified.

Investigations in two other laboratories are consistent with the identification of glutamic acid at the active site and the adjacent primary sequence reported in this paper. A hexapeptide with the sequence Ala-Tyr-Glu-Pro-Val-Trp has been isolated from chicken muscle triose phosphate isomerase after inactivation with bromoacetol phosphate (Coulson

et al., 1970b). Although the label in the isolated peptide is found associated with tyrosine, the authors conclude that the initial modification is an esterification of glutamic acid, and that migration of the acetol moiety occurs during subsequent treatments. Waley *et al.* (1970) have isolated an identical hexapeptide from the rabbit muscle enzyme after inactivation with glycidol phosphate, an active-site-specific epoxide (Rose and O'Connell, 1969). This reagent also esterifies the essential glutamyl residue.

A difficult question to answer, even when highly selective modification is achieved, is whether the inactivation results from modifying a catalytically functional residue or from preventing substrate binding. Of course, modification of a residue involved in catalysis, particularly by a substrate analog, is also likely to prevent substrate binding. Several observations suggest that the glutamyl residue, esterified by haloacetol phosphate, is functional in catalysis. (1) The only functional group in model compounds for proteins which reacts with haloacetol phosphates is sulfhydryl (Hartman, 1970c), but the group in triose phosphate isomerase that reacts is a carboxyl. The rate of esterification of this glutamyl carboxyl group is extremely rapid (k_{2nd} at 2° and pH 6.5 is 2300 M^{-1} sec^{-1} for chloroacetol phosphate), whereas haloacetol phosphates do not esterify free glutamic acid. The reactivity of haloacetol phosphates toward glutamic acid was checked under conditions of 1000-fold higher reagent concentrations, higher temperature, and higher pH, but reaction was not observed. (2) One of the three reagents, iodoacetol phosphate, is not an alkylating reagent; it oxidizes sulfhydryl groups to disulfides (Hartman, 1970c). This reagent, however, appears to esterify a glutamyl residue in triose phosphate isomerase as does chloroacetol phosphate. The unusual reactivity, demonstrated by observations 1 and 2, of a single γ-carboxyl group in triose phosphate isomerase may reflect its functionality in catalysis. (3) If the glutamyl residue of rabbit muscle triose phosphate isomerase which becomes esterified is not functional but merely located in the vicinity of the active site, in some species residues not susceptible to esterification may occupy the corresponding position. The finding that triose phosphate isomerase from a wide variety of species are not only inactivated by chloroacetol phosphates, but inactivated at similar rates, is certainly consistent with modification of a catalytically functional, invariant residue.

Rose (1962) has shown that the probable mechanism of the triose phosphate isomerase catalyzed reaction is one of proton transfer. A proton α to the substrate carbonyl group is abstracted by a basic residue of the enzyme to form an intermediate *cis*-enediol and then transferred to the carbon atom originally bearing the carbonyl group. Based on the dependence of V_{max} on pH the catalytic groups have apparent pK_a values of 6.5 and 9.5 (Rose, 1962). Histidine's pK of 6.5 and results from the inactivation of triose phosphate isomerase by iodoacetate and photooxidation prompted Burton and Waley (1966) to suggest that a histidyl residue at the active site promotes proton transfer. The data are consistent with the involvement of histidine but inconclusive. Elevated temperatures (40°) and long reaction periods (10–24 hr) were required for iodoacetate to inactivate the enzyme. Sulfhydryl groups, and to a lesser extent methionyl and lysyl side chains, were carboxymethylated before inactivation resulted, making it difficult to exclude the possibility of conformational changes preceding histidine alkylation. Esterification of carboxyl groups would have escaped detection by the analytical procedures used. Photoinactivation data are also difficult to interpret due to oxidation of multiple residues. The loss of both tryptophan and histidine paralleled inactivation.

Any conjugate base, including a glutamyl γ-carboxylate, could function in proton transfer. Carboxylates can promote enolizations *via* general base catalysis (Hine, 1962; Jencks, 1969). In view of the rapid, selective esterification by haloacetol phosphates of a single glutamyl carboxylate at the active site of triose phosphate isomerase, it seems likely that this residue, rather than histidine, is the essential group with $pK = 6.5$ identified in kinetic studies. The pK, abnormally high for a carboxyl group, can be explained by assuming a nonpolar environment. Such an assumption seems valid, since of the 15 residues in the isolated active-site peptide, ten are hydrophobic. Lysozyme contains an essential glutamyl residue that is located in a nonpolar environment and has a pK of 6.3 (Donovan *et al.*, 1960; Blake *et al.*, 1967).

Haloacetol phosphates are ideal for inactivating triose phosphate isomerase present as a contaminant in commercial preparations of certain enzymes. Glycerophosphate dehydrogenase, the coupling enzyme in triose phosphate isomerase assays, can be freed of isomerase activity with no loss in dehydrogenase activity by a brief incubation period with chloroacetol phosphate. Similarly, aldolase can be freed of isomerase activity. Chloroacetol phosphate has also been used by Wykle and Snyder (1969) and by Snyder *et al.* (1970) to inactivate triose phosphate isomerase in microsomal preparations and thus permit the identification of dihydroxyacetone phosphate as the precursor of alkylglyceryl ethers.

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Studies with Aspartate Transcarbamylase*

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ABSTRACT: Aspartate transcarbamylase (ATCase) is shown to have more than two active sites and therefore at least four and probably six by an alalysis of activation of the enzyme at low concentration of L-aspartate by an aspartate analog, maleate. Two groups of dicarboxylate dianions are shown to differ in their effect on the kinetic behavior of ATCase. At low L-aspartate concentration, one group activates ATCase at low concentrations and inhibits at high concentrations whereas the other group only inhibits the enzyme. Inhibition of both groups is offset by L-aspartate; however, inhibition by the second group, but not the first, is also offset by carbamyl phosphate. The difference in behavior of the two groups can be a consequence of a positive cooperative effect in the kinetic binding of L-aspartate and carbamyl phosphate. Bromosuccinate inactivates ATCase by alkylation; enzyme activity is protected by ureidosuccinate, maleate, succinate, and carbamyl phosphate (but not L-aspartate). In the presence of maleate and carbamyl phosphate reaction of ATCase with bromosuccinate causes activation of the enzyme and loss of CTP inhibition and maleate activation. Activation by bromosuccinate does not involve dissociation of the enzyme into subunits although interactions among the subunits evidently are disrupted. Two reversible treatments which result in loss of interactions among subunits of ATCase, high pH and 1 M urea, are also shown not to dissociate the enzyme.

Aspartate transcarbamylase (ATCase),[1] the first enzyme unique to pyrimidine biosynthesis in *Escherichia coli*, is inhibited in a partially competitive fashion by the end product of the pathway, CTP (Gerhart and Pardee, 1962–1964; Bethell *et al.*, 1968). Inhibition by CTP is thought to play an important role in regulation of pyrimidine biosynthesis in *Escherichia coli* (Yates and Pardee, 1956b; Gerhart and Pardee, 1962–1964). Although CTP inhibits competitively with both substrates, the CTP binding sites are distinct from the active sites (Gerhart and Pardee, 1962) and are located on different subunits (Gerhart and Schachman, 1965). The native enzyme is readily dissociated into subunits by heat treatment or reaction with PMB (Gerhart and Pardee, 1962–1964; Gerhart and Schachman, 1965) and two types of subunits can be separated (Gerhart and Schachman, 1965; Gerhart and Holoubek, 1967). The native enzyme has a molecular weight of 3.1×10^5 (Gerhart and Schachman, 1965) and consists of two active catalytic subunits, $\bar{M} = 1.0 \times 10^5$, and three regulatory (CTP binding) subunits, $\bar{M} = 3.4 \times 10^4$. Each catalytic subunit is composed of three polypeptide chains and each regulatory subunit of two polypeptide chains (Weber, 1968; Wiley and Lipscomb, 1968; Meighen *et al.*, 1970).

Gerhart and Pardee have inferred from kinetic studies that binding of one molecule of L-aspartate to ATCase enhances binding of successive L-aspartate molecules, and that CTP inhibits the enzyme at saturating concentrations of carbamyl phosphate by decreasing the affinity of the enzyme for L-aspartate (Gerhart and Pardee, 1963, 1964). This interpretation has been supported by binding sites with succinate and CTP (Changeux *et al.*, 1968). Changeux *et al.* have also shown that in the presence of a saturating concentration of carbamyl phosphate ATCase binds four molecules of succinate suggesting that the enzyme has four binding sites for L-aspartate.

In the first part of this paper we have examined effects of various dicarboxylate analogs of L-aspartate on the activity of ATCase at low concentrations of L-aspartate. In the second part of this paper we describe the effect of the reaction of bromosuccinate and ATCase on enzyme activity and the

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† NASA Fellowship, 1966–1968. This work forms part of the dissertation submitted to the Department of Biochemistry, Columbia University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] Abbreviations used are: ATCase, aspartate transcarbamylase; PMB, *p*-mercuribenzoate; BDEAE, benzyldiethylaminoethyl; CAP, carbamyl phosphate.