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Haloacetol Phosphates. A Comparative Study of the Active Sites of Yeast and Muscle Triose Phosphate Isomerase†

I. L. Norton and F. C. Hartman*

ABSTRACT: A procedure is described for purifying triose phosphate isomerase from Bakers' yeast to homogeneity in 35–40% yields. This enzyme, like the corresponding enzyme from rabbit muscle, is inactivated by the substrate analog, 3-chloroacetol phosphate, *via* selective esterification of a single glutamyl γ-carboxylate. The amino acid sequence around this reactive residue (Ala-Tyr-Glu-Pro-Val-Trp) is identical with that found in the rabbit muscle enzyme. Sequence homologies between the segments containing the active-site glutamyl residue in these two diverse species, rabbit and yeast, suggest that the carboxyl group is functional in catalysis.

Haloacetol phosphates are active-site-specific reagents for triose phosphate isomerase. In the case of the isomerase from both rabbit and chicken muscle, inactivation results from a highly selective esterification of a single glutamyl γ-carboxylate (Hartman, 1970a; Coulson *et al.*, 1970). The unusual reactivity of this carboxylate and the observation that chloroacetol phosphate inactivates triose phosphate isomerase from diverse species led Hartman (1971) to postulate that the carboxylate is probably functional in catalysis. Comparative studies on enzymes with the same function but from different species can be pertinent to ascertaining whether a given amino acid residue is catalytically essential. The validity of this statement resides in numerous examples supporting the concept that active-site regions of enzymes have been conserved during evolution; *i.e.*, residues involved in catalysis are species invariant, and the primary sequences adjacent to these residues are highly homologous (Smith, 1970). Therefore, as a further test of the functional significance of the active-site glutamyl residue, we have characterized the site of yeast triose phosphate isomerase that is modified by 3-chloroacetol phosphate.

Experimental Section

Materials. NADH, DL-glyceraldehyde 3-phosphate (as the dimethyl ketal), and glycerophosphate dehydrogenase were obtained from Boehringer Mannheim Corp. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, DFP-treated carboxypeptidase, and pepsin were products of Worthington Biochemical Corp. $^{32}POCl_3$ and NaB^3H_4 were purchased from Ammersham–Searle Corp. Ultra-Pure biological grades of ammonium sulfate and guanidine hydrochloride were purchased from Schwarz–Mann. Phenyl isothiocyanate and trifluoroacetic acid were obtained from Aldrich Chemical Co. Chloroacetol phosphate was synthesized as described previously (Hartman, 1970b); the ^{32}P-labeled reagent (initial specific radioactivity of 1.28×10^6 cpm/μmole) was prepared

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TABLE I: Purification of Yeast Triose Phosphate Isomerase.

Fraction	Vol (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Yield (%)
Extract	2650	53,000[a]	11,900,000	230	100
60–80% ammonium sulfate precipitate	375	22,500[a]	10,600,000	410	89
DEAE-cellulose chromatography (pH 8.0)	140	3,800[b]	7,600,000	2,000	63
CM-cellulose chromatography	37.5	587[b]	5,500,000	9,300	46
DEAE-cellulose chromatography (pH 7.5)	84	428[b]	4,500,000	10,500	38

[a] Based on Lowry determinations. [b] Based on A at 280 nm (see Experimental Section).

by use of $^{32}POCl_3$ in the phosphorylation step, which was scaled down tenfold.

Protein Determinations. The method of Lowry *et al.* (1951), with human serum albumin as the standard, or spectrophotometric measurements at 280 nm were used to determine protein concentrations. The $\epsilon_{1\text{ cm}}^{1\%}$ at 280 nm of purified yeast triose phosphate isomerase is 10.0 (see below).

Extinction Coefficient. Two samples of yeast triose phosphate isomerase at about 8 mg/ml were dialyzed exhaustively (three changes during 48 hr) against 0.01 M ammonium bicarbonate (pH 8.1). Aliquots of the dialyzed samples were diluted 20-fold into 0.1 M Tris-hydrochloride (pH 8.0 and 7.4), 0.02 M triethanolamine hydrochloride (pH 7.9), and 0.01 M sodium phosphate (pH 8.0); the absorbancies at 280 nm of these solutions were then determined and found to be identical. Samples (2 ml) of the dialyzed, undiluted protein solutions were lyophilized to dryness in tared vials, dissolved in 3 ml of water, and again lyophilized. The vials were then heated overnight at 110° and cooled in a vacuum desiccator to constant weight. The two determinations yielded $\epsilon_{1\text{ cm}}^{1\%}$ at 280 nm of 9.99 and 10.0.

Enzyme Assays. 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. A Beckman Acta V recording spectrophotometer was used in these determinations.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out with the instrument from Canal Industrial Corp. according to the instructions provided. The acrylamide concentration used was 7%. After electrophoresis, protein was detected with Amido Schwarz, and excess stain was removed electrophoretically.

Peptide Mapping and Autoradiography. Peptide maps on Whatman No. 3MM were prepared essentially by the method of Katz *et al.* (1959) except that electrophoresis preceded chromatography. The experimental conditions used were identical to those published previously (Hartman, 1971). Autoradiograms were prepared with Kodak No-Screen Medical X-Ray film; exposure times varied from 1 to 5 days depending upon the quantity of radioactivity present.

Amino Acid Analyses. Samples were 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).

Sequence Determinations. Subtractive Edman degradations as described by Konigsberg (1967) were used for sequence determinations. After each cycle, a sample of the peptide (0.01–0.02 μmole) was subjected to amino acid analysis.

Modification of Triose Phosphate Isomerase by Chloroacetol Phosphate. FOR ACTIVE-SITE CHARACTERIZATION. Triose phosphate isomerase (130 mg, 4.64 μmoles of catalytic subunit based on a subunit molecular weight of 28,000 (Krietsch *et al.*, 1970)) in 10 ml of 0.1 M sodium bicarbonate–1 mM EDTA (pH 8.0) at 2° was treated with 0.5 ml of 0.04 M chloroacetol phosphate (20 μmoles). Inactivation was completed within 1 min; the derivatized enzyme was then reduced with [^{3}H]sodium borohydride (0.2 mCi/μmole) and carboxymethylated with iodoacetate as described for rabbit muscle triose phosphate isomerase (Hartman, 1971). After exhaustive dialysis against 0.01 M potassium chloride followed by water, the protein solution was made 0.05 M in formic acid. Pepsin (1.3 mg) was added to the solution, which was then incubated for 2 hr at 40°. The digested protein had a specific radioactivity of 32×10^6 cpm/μmole of subunit; native enzyme that was subjected to the same series of procedures (inactivation with chloroacetol phosphate excluded) contained 2.1×10^6 cpm/μmole.

Modification of Triose Phosphate Isomerase by Chloroacetol Phosphate. FOR DETERMINATION OF STOICHIOMETRY AND FOR PEPTIDE MAPPING AND AUTORADIOGRAPHY. A 10-mg sample of the enzyme was inactivated with chloroacetol [^{32}P]phosphate under conditions described in the preceding paragraph. One-half of the sample was dialyzed exhaustively against 0.01 M potassium chloride, and aliquots were then assayed for radioactivity. The other half was reduced (unlabeled borohydride), carboxymethylated, and digested with pepsin as described above. The digest was divided into portions equivalent to 0.5 mg of protein and lyophilized to dryness for use in peptide-mapping experiments.

Radioactivity Measurements. 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-phenyloxazoly)]benzene in 1 l. of toluene–ethanol (4:3, v/v).

Purification of Yeast Triose Phosphate Isomerase. Unless stated otherwise, all the following operations were carried out at 4°. Buffers were prepared with glass-distilled water and in all cases contained 1 mM EDTA and 1 mM β-mercaptoethanol. Centrifugations were for 1 hr at 10,400g. The purification procedure is summarized in Table I.

AUTOLYSIS AND EXTRACTION. To 5 lb of crumbled, fresh *Saccharomyces cerevisiae* Hansen (Federal Bakeries) was added 600 ml of toluene. The mixture was maintained at 45° and stirred occasionally until the yeast liquefied (about 90 min), after which the autolysate was left undisturbed at 24° for 2 hr. After the addition, with stirring, of 2400 ml of cold 0.05 M potassium phosphate (pH 7.5) to the autolysate, the resulting mixture was left at 4° for 36 hr, during which time the toluene and aqueous phases separated. The aqueous

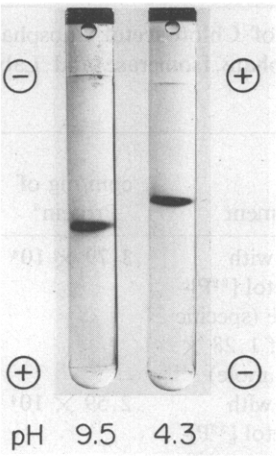

FIGURE 1: Polyacrylamide gel electrophoresis of triose phosphate isomerase from yeast. The experimental details are described in the Experimental Section.

layer was siphoned off and centrifuged. The dark, clear middle layer (the aqueous phase) was removed by siphoning and strained through glass wool.

AMMONIUM SULFATE FRACTIONATION. The aqueous extract was brought to 60% saturation with solid ammonium sulfate (390 g/l., based on solubility at 25°). Precipitated protein was removed by centrifugation. The supernatant was then brought to 80% ammonium sulfate (143 g/l. of supernatant), and the precipitate was again collected by centrifugation.

DEAE-CELLULOSE CHROMATOGRAPHY. The 60–80% ammonium sulfate precipitate was dissolved in about 200 ml of 0.01 M Tris-hydrochloride (pH 8.0), dialyzed against the same buffer, and placed on a 2.5 × 141 cm column of Whatman DEAE-cellulose (DE-52) equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride (0–0.1 M) in the Tris-hydrochloride buffer (the volumes of both the initial and limit buffer were 2 l.). Fractions (1730–2200 ml) containing isomerase with a specific activity of at least 1000 units/mg were pooled and brought to 90% in ammonium sulfate. Precipitated protein was collected by centrifugation.

CM-CELLULOSE CHROMATOGRAPHY. The protein from the previous step was dissolved in about 100 ml of 0.01 M sodium acetate (pH 5.0), dialyzed against the same buffer, and applied to a 2.5 × 41 cm column of Whatman CM-cellulose (CM-52) equilibrated with the same buffer. The column was eluted with a gradient composed of 1 l. of 0.01 M sodium acetate (pH 5.0) (initial) and 1 l. of 0.01 M sodium acetate–0.25 M sodium chloride (pH 5.5) (limit). Fractions (900–1500 ml) containing isomerase with a specific activity of at least 5000 units/mg were pooled and brought to 90% ammonium sulfate. Precipitated protein was collected by centrifugation.

DEAE-CELLULOSE CHROMATOGRAPHY. The protein from the previous step was dissolved in 0.01 M potassium phosphate (pH 7.5), dialyzed against the same buffer, and placed on a 2.5 × 25 cm column of Whatman DEAE-cellulose (DE-52) equilibrated with the same phosphate buffer. The column was eluted with a gradient composed of 500 ml of 0.01 M potassium phosphate (pH 7.5) (initial) and 500 ml of 0.05 M potassium phosphate (pH 7.5) (limit). Fractions comprising the major peak of isomerase activity (eluting between 160 and 340 ml) were pooled and brought to 90% ammonium sulfate. The precipitated enzyme (specific activity of 10,500 units/mg) appeared homogeneous by analytical acrylamide electrophoresis (Figure 1) and has been crystallized in a form suitable for preliminary crystallographic studies (Hawkinson *et al.*, 1972).

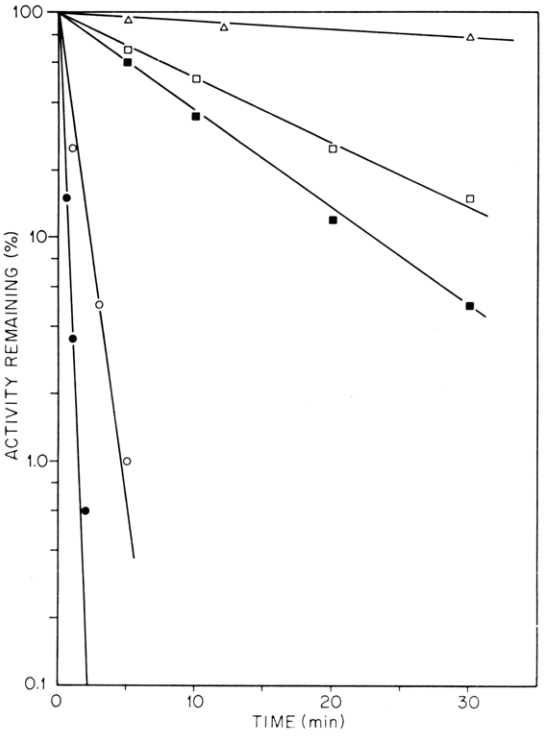

FIGURE 2: Inactivation of yeast triose phosphate isomerase by chloroacetol phosphate at 2°. All reaction mixtures contained 25 μg of enzyme/ml and 10 μM reagent in 0.1 M imidazolium hydrochloride (pH 6.5) with no other additions (●), 1 mM (○) or 10 mM (■) DL-α-glycerophosphate, or 0.1 mM (□) or 1 mM (△) glycolic acid phosphate. Periodically 0.1-ml samples of the reaction mixtures were diluted into 4.9 ml of cold 0.1 M ammonium acetate containing 10 mM β-mercaptoethanol, which reacts with the excess chloroacetol phosphate. Aliquots of these diluted samples were assayed immediately for isomerase activity as described in the Experimental Section.

Results

Kinetics and Stoichiometry of the Reaction between Chloroacetol Phosphate and Triose Phosphate Isomerase. Purified yeast triose phosphate isomerase is rapidly and totally (>99.99%) inactivated by chloroacetol phosphate (Figure 2). With the assumption that inactivation follows pseudo-first-order kinetics, the second-order rate constant for this reaction —calculated from eq 1 (Aldridge, 1950) in which $t_{1/2}$ is the

$$t_{1/2} = \frac{0.693}{[\mathrm{I}]k_{2\mathrm{nd}}} \qquad (1)$$

half-time of inactivation and [I] is the molar concentration of inhibitor—is 5700 M^{-1} sec^{-1}, in fair agreement with the value obtained previously using a crude yeast extract (Hartman, 1971). Glycolic acid phosphate and glycerol phosphate, competitive inhibitors of the enzyme (Wolfenden, 1970; Burton and Waley, 1968), reduce the rate of inactivation (Figure 2). In the cases of these reduced rates, pseudo-first-order loss of activity is more apparent.

Inactivated enzyme contains about 2 molar equiv of reagent/mole of enzyme. Radioactivity associated with the protein is only slowly removed by treatment with guanidine

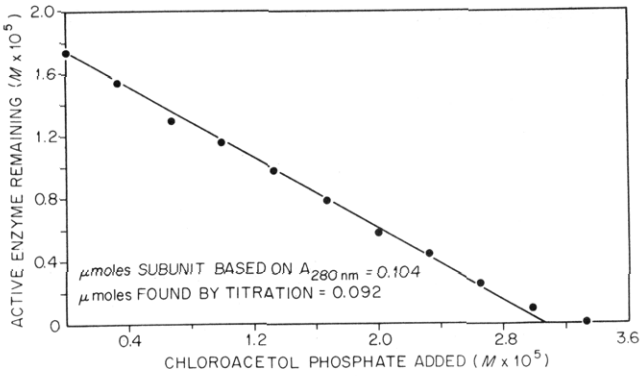

FIGURE 3: Titration of yeast triose phosphate isomerase with chloroacetol phosphate. Ten 10-μl samples of 1 mM reagent were added in succession to a solution (3 ml) containing 2.9 mg of enzyme in 0.01 M Tris-hydrochloride (pH 8.0). Fifteen minutes after each addition, 10-μl aliquots were diluted into 10 ml of Tris buffer (pH 8.0) containing 0.01 M β-mercaptoethanol and assayed for activity (see Experimental Section).

hydrochloride but is labile toward base and hydroxylamine (Table II). Approximately the same stoichiometry is obtained by direct titration of isomerase activity with chloroacetol phosphate (Figure 3).

Number of Active Sites. Yeast triose phosphate isomerase contains two subunits of equivalent size (Kreitsch *et al.*, 1970). If each subunit contains an active site, the extent of chloroacetol phosphate incorporation (2 moles of reagent/mole of enzyme) is indicative of a selective active-site modification. The labeling pattern, as observed by gel electrophoresis when the isomerase is treated with limiting quantities of reagent, suggests that each enzyme molecule contains two active sites. Whereas native and totally inactivated isomerase migrate as single bands (the inactive species is more negative because of the phosphate group on the incorporated acetol moiety), partially inactivated preparations contain a third species with an intermediate electrophoretic mobility (Figure 4). The ratios of the three components are approximately those predicted on the basis of the remaining enzymic activity and the assumption that the species of intermediate mobility is

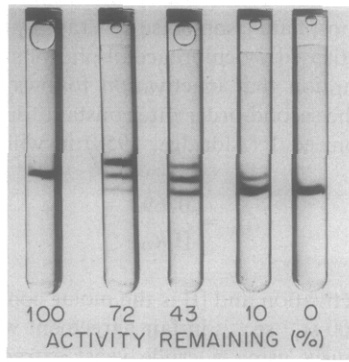

FIGURE 4: Polyacrylamide gel electrophoretic patterns of yeast triose phosphate isomerase that was treated with varying limiting quantities of chloroacetol phosphate. A solution of the enzyme (1.0 mg/ml; 0.036 mM in catalytic subunit) in 0.05 M ammonium acetate (pH 6.8) at 4° was treated sequentially with 0.01, 0.02, 0.03, and 0.06 mM chloroacetol phosphate. Ten minutes after each addition, an aliquot was diluted with an equal volume of cold 0.05 M ammonium acetate (pH 6.8) containing 0.01 M β-mercaptoethanol and subjected to electrophoresis at pH 9.5 as described in the Experimental Section.

TABLE II: Extent of Chloroacetol Phosphate Incorporation into Triose Phosphate Isomerase and Lability of Protein–Reagent Linkage.

Expt No.	Treatment	cpm/mg of Protein[a]	Moles of Reagent/Mole of Protein
1	Inactivated with chloroacetol [^{32}P]-phosphate (specific activity of 1.28×10^6 cpm/μmole)	3.79×10^4	1.66
2	Inactivated with chloroacetol [^{32}P]-phosphate (specific activity of 8.30×10^5 cpm/μmole)	2.59×10^4	1.75
3	Inactivated with chloroacetol phosphate (unlabeled) and reduced with NaB^3H_4	1.14×10^6	Not determined
4	Sample 3 incubated in 6 M guanidine hydrochloride (pH 8.0)[b]	0.78×10^6	Not determined
5	Sample 3 incubated in 6 M guanidine hydrochloride + 1 M hydroxylamine (pH 8.0)[b]	2.1×10^5	Not determined
6	Sample 3 incubated in 0.1 N sodium hydroxide[b]	1.2×10^5	Not determined

[a] Radioactivity was assayed after the protein solutions were dialyzed exhaustively against 0.01 M potassium chloride. [b] Incubations were carried out at 25° for 12 hr.

one in which only one active site per molecule is modified and therefore is 50% as active as the native enzyme.

Peptide Mapping. Yeast triose phosphate isomerase, after inactivation with chloroacetol [^{32}P]phosphate, was subjected to peptide mapping to confirm the specificity of the modification suggested by the stoichiometry and to provide an indication as to whether the primary structure at the modification site is the same as the corresponding site in the rabbit muscle enzyme. Peptide maps, and their autoradiograms, of tryptic and peptic digests of both yeast and rabbit muscle isomerase are shown in Figure 5. Although the two inactivated enzymes give rise to structurally different radioactive peptides upon tryptic digestion, seemingly identical peptides are present in peptic digests.

Purification and Amino Acid Composition of Active-Site Peptide. A peptic digest (120 mg, 4.28 μmoles with a specific radioactivity of 32×10^6 cpm/μmole) of triose phosphate isomerase that had been inactivated with chloroacetol phosphate, reduced with NaB^3H_4 to label the incorporated acetol moiety, and carboxymethylated (see Experimental Section) was chromatographed successively on Bio-Rad AG 50W-X2, Bio-Gel P-4, and DEAE-cellulose (Figure 6). After each chromatographic separation, the radioactive fractions were

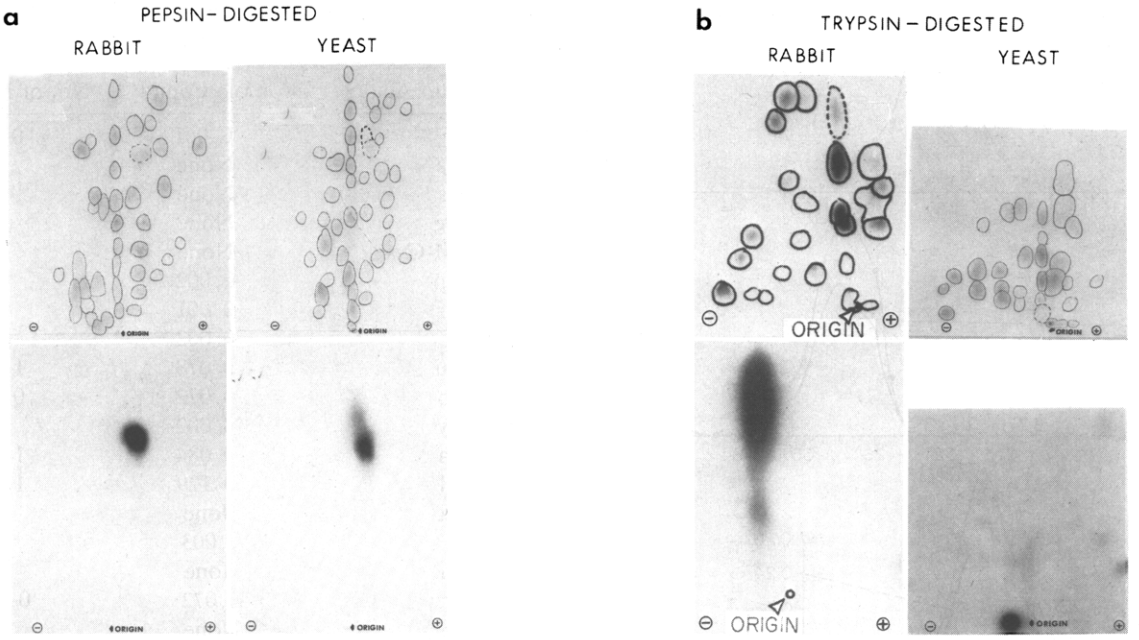

FIGURE 5: Peptide maps and autoradiograms of yeast and rabbit muscle triose phosphate isomerase that had been inactivated by chloroacetol [^{32}P]phosphate and digested with either pepsin (a) or trypsin (b). Radioactive peptides are circled with broken lines. The experimental details are described in the Experimental Section.

pooled and lyophilized to dryness. The residue was then redissolved in the equilibration buffer used for the next column. The recovery of radioactivity from each of the three columns was 110 × 10^6, 92 × 10^6, and 76 × 10^6 cpm (58% overall recovery), respectively. Elution profiles from both the Bio-Gel and DEAE columns indicated the presence of two radioactive peptides, which sequence analyses showed was a result of nonselectivity of the peptic digestion (see below).

An amino acid analysis of the material obtained from the DEAE column is presented in Table III. Except for the nonintegral quantity of valine, the amino acid composition (Glu_1,Pro_1,Ala_1,$Val_{1.7}$,Tyr_1,Trp_1) is identical with that of the hexapeptide isolated from chicken muscle triose phosphate isomerase labeled with bromoacetol phosphate (Coulson *et al.*, 1970) and a region of the pentadecapeptide isolated from the rabbit muscle enzyme labeled with chloroacetol phosphate (Hartman, 1971). The peptide material has a specific radioactivity (29 × 10^6 and 31 × 10^6 cpm per μmole in two separate determinations) equivalent to that of the modified enzyme before peptic digestion, thereby confirming the specificity of the reagent for a single residue.

Sequence of Active-Site Peptide. Before Edman degradations were initiated, the peptide (0.3 μmole) was treated with 0.01 N ammonium hydroxide for 24 hr at 25° to remove the reagent moiety. The sample was then dried by lyophilization. In the data that follow, the number after each amino acid is its molar ratio relative to the amino acid given in italics, which is arbitrarily set at 1.0. The amino acids removed at each cycle are given in boldface. Tryptophan was determined only before the first degradative cycle.

BEFORE FIRST DEGRADATION. Trp, 0.80; *Glu, 1,0;* Pro, 0.92; Ala, 1.1; Val, 1.7; Tyr, 0.93.

FIRST DEGRADATION. *Glu, 1.0;* Pro, 0.93; **Ala, 0.75**; **Val, 1.2**; Tyr, 0.91.

SECOND DEGRADATION. *Glu, 1.0;* Pro, 0.96; **Ala, 0.32**; Val, 1.1; **Tyr, 0.50**.

THIRD DEGRADATION. **Glu, 0.69**; Pro, 0.90; Ala, 0.25; *Val, 1.0;* **Tyr, 0.21**.

FOURTH DEGRADATION. **Glu, 0.41**; **Pro, 0.62**; Ala, 0.19; *Val, 1.0;* Tyr, 0.10.

These data are consistent with the presence of two peptides that have the following N-terminal sequences:

Val-Ala-Tyr-Glu-
Ala-Tyr-Glu-Pro-

To establish the C-terminal sequence, the peptide mixture was digested with carboxypeptidase. A sample of peptide material (0.2 μmole) in 0.9 ml of 0.1 M *N*-ethylmorpholine hydrochloride (pH 8.0) at 25° was treated with 0.10 mg of carboxypeptidase A. At 5 min, 1 hr, and 24 hr, 0.3-ml aliquots were withdrawn, diluted into 0.2 M sodium citrate (pH 2.2), and assayed on the amino acid analyzer. Only tryptophan (0.18 μmole/0.9 ml of digest) was found in the 5-min and 1-hr samples, and, in addition, valine (0.04 μmole/0.9 ml of digest) was found in the 24-hr sample. Thus, the probable sequences of the two peptides are Val-Ala-Tyr-Glu-Pro-Val-Trp and Ala-Tyr-Glu-Pro-Val-Trp.

Discussion

The prime objective of this study was to determine whether an active-site glutamyl residue, detected in rabbit and chicken muscle triose phosphate isomerase with haloacetol phosphates as affinity-labeling reagents, is an invariant feature among triose phosphate isomerases. Such should be the case if the glutamyl carboxylate is functional in catalysis as suggested previously (Hartman, 1971). The earlier findings of identical six-residue sequences around the reactive glutamyl carboxylate in the rabbit and chicken species (Coulson *et al.*, 1970; Waley *et al.*, 1970; Hartman, 1970c, 1971) are certainly consistent with the suggestion that this portion of the enzyme has been conserved during evolution and therefore is probably critical to function, but the rabbit and chicken are too closely related to provide a stringent test case. For example, of the 104 residues in cytochrome *c*, only 8 are variant in the chicken

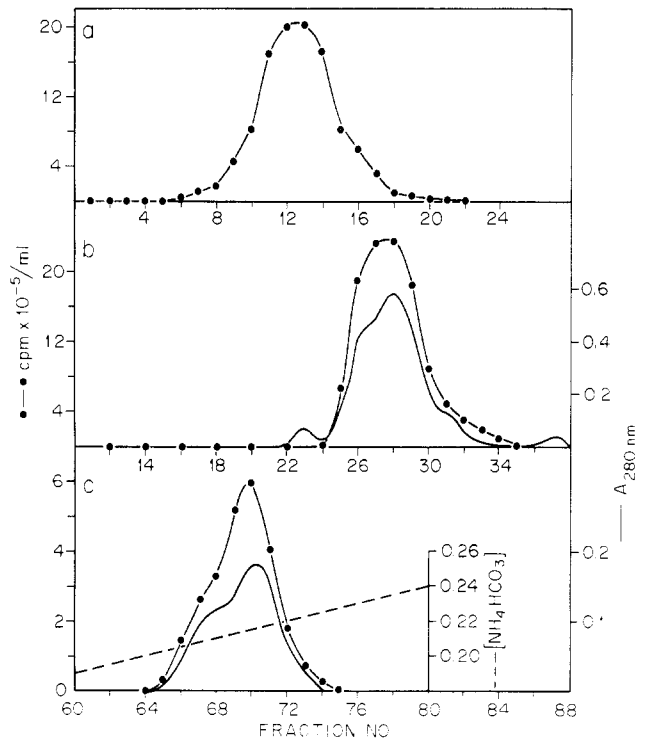

FIGURE 6: Purification of active-site peptide from peptic digest of yeast triose phosphate isomerase inactivated with chloroacetol phosphate and subsequently reduced with NaB^3H_4. (a) Ion-exchange chromatography of the digest on a 2.5 × 20 cm column of Bio-Rad AG 50W-X2. The column was eluted with a linear gradient composed of 750 ml each of 0.2 M pyridine–4.6 M acetic acid (pH 3.1) and 2.0 M pyridine–2.4 M acetic acid (pH 5.0). (b) Gel filtration of the radioactive pool (tubes 9–16) from the ion-exchange column on a 1.8 × 220 cm column of Bio-Gel P-4. The column was equilibrated and eluted with 0.01 M ammonium bicarbonate (pH 8.1). (c) Ion-exchange chromatography of the radioactive pool (tubes 25–31) from the gel filtration column on a 1.2 × 25 cm column of Whatman DEAE-cellulose (DE-52). The column was eluted with a linear gradient (250 ml each) of ammonium bicarbonate (pH 8.1, 0.02–0.3 M).

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

Amino Acid	μmole Found	No. of Residues
Trp[b]	0.061	0.78
Lys	None	
His	None	
Arg	None	
CM-Cys	None	
Asp	0.002	
Thr	0.001	
Ser	0.002	
Glu	0.078	1.0
Pro	0.072	0.92
Gly	0.002	
Ala	0.084	1.08
Val	0.129	1.65
Met	None	
Ile	0.003	
Leu	None	
Tyr	0.072	0.92
Phe	None	

[a] Samples equivalent to 0.072 μmole of peptide based on the specific radioactivity of the inactivated enzyme (32×10^6 cpm/μmole) were placed on each column of the amino acid analyzer. Of the radioactivity applied to the long column, 8% emerged at 18 min (the elution position of glycerol phosphate) and 88% emerged at 30 min (the elution position of glycerol). [b] Trp was determined by the method of Liu and Chang (1971).

protein as compared to the rabbit, whereas 44 are variant in the phylogenetically more distant yeast (Margoliash and Schejter, 1966). Therefore, we chose a comparison of yeast and rabbit triose phosphate isomerase as an indicator of the constancy of the active-site glutamyl residue and the adjacent amino acid sequence.

Triose phosphate isomerase from Brewers' yeast was isolated previously, and some of its physical, chemical, and enzymic properties were compared to those of the rabbit muscle enzyme (Krietsch *et al.*, 1970). The molecular and subunit weights of the two enzymes are similar, but substantial differences in their amino acid compositions probably reflect extensive differences in primary sequences. Such differences are also suggested by the dissimilarities seen in peptide maps of the two proteins (Figure 5). Thus, for defining regions of importance to function, comparative studies of yeast and rabbit muscle triose phosphate isomerase appear justifiable. The enzyme used in our studies was isolated from Bakers' yeast by extensive modifications of the procedure described by Krietsch *et al.* (1970). Although we have not thoroughly characterized our preparation, except for demonstrating homogeneity, we assume that it is virtually identical with that from Brewers' yeast.

The reaction of chloroacetol phosphate with yeast triose phosphate isomerase is quite analogous to the corresponding reaction with isomerase from rabbit muscle. (1) With high molar ratios of reagent to enzyme, loss of activity is pseudo-first-order (Figure 2). (2) Competitive inhibitors protect against inactivation (Figure 2). (3) One mole of reagent per mole of catalytic subunit is covalently incorporated (Table II and Figure 3). (4) Autoradiograms of peptide maps confirm a high degree of selectivity in the modification (Figure 5). (5) The stability (labile toward base, acid, and hydroxylamine) of the reagent–protein bond is that of an ester (Tables II and III). (6) The reagent can be used to demonstrate the presence of two active sites per molecule of enzyme (Figure 4). These observations, especially their role in establishing the occurrence of a selective active-site modification, have been adequately discussed in the case of the rabbit muscle enzyme and require no further elaboration here (Hartman, 1971).

Except for the determination of the extent of reagent incorporation, the characterization of the inactivated isomerase was preceded by the reduction, with sodium borohydride, of the carbonyl group of incorporated acetol phosphate moiety to a hydroxyl group. In addition to providing a means whereby a stable radioisotopic label can be introduced, the reduction stabilizes both the phosphate group and the ester linkage between reagent and protein (Hartman, 1971).

A comparison of autoradiograms prepared from peptide maps of chloroacetol phosphate labeled, pepsin-digested yeast, and rabbit muscle triose phosphate isomerase revealed coincident, radioactive peptides (Figure 5), thereby suggesting that the modification sites and adjacent sequences are identi-

cal. Two radioactive peptides were present in the digest from yeast isomerase, in contrast to only one from rabbit isomerase, but subsequent characterization showed this to be a result of the pepsin digestion rather than of the modification of two amino acid residues. The labeled peptides released upon tryptic digestion of the two modified enzymes are obviously quite different (Figure 5), but in this case larger segments are being compared, as trypsin releases a pentadecapeptide from the active site of rabbit muscle isomerase (Hartman, 1971) in contrast to the hexapeptide resulting from pepsin digestion (Coulson *et al.*, 1970; Waley *et al.*, 1970). Also, if the lysyl (or arginyl) residues (sites of trypsin attack) closest to the active-site glutamyl residue of rabbit muscle triose phosphate isomerase are variant, one may be comparing peptides with an unequal number of residues.

The two radioactive peptides from a peptic digest of yeast isomerase labeled with chloroacetol phosphate were isolated as a mixture. Sequence determinations on the unresolved mixture indicate that the only difference in the two is an additional valyl residue as the N-terminal of one of them. The probable sequences are Val-Ala-Tyr-Glu-Pro-Val-Trp and Ala-Tyr-Glu-Pro-Val-Trp; the latter peptide is identical with (or contained within) the active-site segments isolated previously from the muscle enzymes. One can account for the presence of these two peptides in a peptic digest by assuming that pepsin attacks adjacent peptide bonds at similar rates—in this case, Val-Ala and X-Val. Residue X in rabbit muscle isomerase is valine. An alternative explanation for the presence of two labeled peptides in the peptic digest is that the two subunits of yeast triose phosphate isomerase have slight differences in their primary structures; however, the apparent lack of isoenzymic forms argues against this possibility (Krietsch *et al.*, 1970). The position corresponding to the N-terminal valyl residue of the isolated peptide is occupied by a leucyl residue in the rabbit muscle enzyme. Thus, upon comparing yeast and muscle triose phosphate isomerase, one detects a conservative variation three residues removed from the active-site glutamyl.

The present findings that both yeast and muscle triose phosphate isomerases are inactivated by chloroacetol phosphate *via* esterification of a glutamyl γ-carboxylate and that the amino acid sequences adjacent to the reactive residues are identical provide additional evidence that a carboxyl group is functional in catalysis.

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