AN ACTIVE-SITE PEPTIDE FROM HUMAN TRIOSE PHOSPHATE ISOMERASE*

F. C. Hartman and R. W. Gracy

Biology Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830 and Department of Chemistry,
North Texas State University, Denton, Texas 76203

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SUMMARY: Chloroacetol phosphate totally inactivates human triose phosphate isomerase by the selective modification of a single residue per catalytic subunit. The stability of the protein-reagent bond and the analogies of this active-site modification to those described previously for isomerases from other species indicate that inactivation results from the esterification of an essential glutamyl γ-carboxylate. From peptide maps and their autoradiograms, we conclude that the primary structure adjacent to the glutamyl residue is the same as or similar to that found in triose phosphate isomerases from rabbit and chicken muscle and from yeast.

Haloacetol phosphates are extremely potent, irreversible inhibitors of triose phosphate isomerase (EC 5.3.1.1) (1). By virtue of their structural similarity to the substrate dihydroxyacetone phosphate, these affinity-labeling reagents show absolute specificity for a single glutamyl γ-carboxylate at the active site. A variety of observations have suggested that this carboxyl group, which is esterified by haloacetol phosphates, is essential to catalysis, perhaps serving as the proton acceptor-donor that effects the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. These observations include the following: (i) The reaction between triose phosphate isomerases and chloroacetol phosphate is extremely rapid, whereas this reagent is unreactive toward carboxyl groups in model compounds (2). (ii) The pK of the active-site carboxyl group (3) is about the same as the pK of an essential residue determined from the pH-dependency of the kinetic parameters (4, 5). (iii) The active-site glutamyl residue seems to be species invariant, and there exist high degrees of homologies in the adjacent sequences; e.g., identical hexapeptides have been isolated from rabbit muscle (2, 6), chicken muscle (7), and yeast (8).

Triose phosphate isomerase from human red blood cells was recently isolated (9), and a number of its physical, chemical, and enzymatic properties were described (9, 10). A

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thorough characterization of this enzyme appears warranted in order to provide a basis for the elucidation of the molecular defect in a mutant enzyme present in patients with the genetic disease known as triose phosphate isomerase deficiency (11, 12, 13). To further characterize the human enzyme, we have studied its reaction with the site-specific reagent, chloroacetol phosphate.

MATERIALS AND METHODS

NADH, DL-glyceraldehyde 3-phosphate (as the dimethyl ketal), and glycerophosphate dehydrogenase were obtained from Sigma Chemical Company. $^{32}POCl_3$ and pepsin were products of Ammersham-Searle Corp. and Worthington Biochemical Corp., respectively. Chloroacetol phosphate was synthesized as described previously (14); the ^{32}P-labeled reagent (initial specific radioactivity of 1.64×10^6 cpm/μmole) was prepared by use of $^{32}POCl_3$ in the phosphorylation step, which was scaled down tenfold. Human triose phosphate isomerase was isolated as described by Rozacky et al. (9).

Triose phosphate isomerase was assayed by the glycerophosphate dehydrogenase-coupled method of Beisenherz (15) as modified by Norton et al. (16). Protein concentration was determined from the A at 280 nm using $\epsilon^{1\%}_{1\ cm}$ of 12.9 (9).

Radioactivity was assayed with a Packard Model 3003 liquid scintillation spectrometer. The sample (0.05-0.2 ml) was dissolved in 1 ml of Beckman Bio-Solv (BBS-3) and mixed with 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 liter of toluene-ethanol (4:3, v/v).

Peptide mapping was performed according to the method of Katz et al. (17) with slight modification (2). Autoradiograms of peptide maps were prepared by use of Kodax No-Screen Medical X-Ray film with a 2-day exposure time.

RESULTS AND DISCUSSION

Human triose phosphate isomerase is completely inactivated by a fivefold molar excess of chloroacetol phosphate during 1 min at 2° and pH 8.0. Inactivation is associated with a stoichiometric incorporation of reagent (Table 1). A sample of the enzyme that had been stored as a suspension in ammonium sulfate for several months and possessed only 30% of its initial enzymatic activity was also inactivated by the reagent, and the extent of incorporation was reduced proportionately. Thus, the reaction of isomerase with chloroacetol phosphate requires that the enzyme be catalytically functional, an observation entirely consistent with the supposition that chloroacetol is a true active-site-specific reagent (2).

Table 1. — Incorporation of Chloroacetol [^{32}P]Phosphate into Triose Phosphate Isomerase

Sample	Specific enzymatic activity (units/mg)		Specific radioactivity (cpm/μmole¶)		Molar equivalents of reagent per mole of triose phosphate isomerase subunit	
	Initial	After treatment with chloroacetol phosphate	Chloroacetol phosphate	Inactivated enzyme	Based on total protein	Based on initial quantity of active enzyme*
1	7900*	<0.1‡	3.10×10^5	2.81×10^5	0.91	0.93
2	2800†	<0.1§	1.64×10^6	6.30×10^5	0.38	1.10

*The specific activity of the pure enzyme has been reported to be 8100 units/mg (9).

†The activity of this sample had decreased during storage.

‡To a solution of the isomerase (0.5 mg, 0.018 μmoles) in 0.1 ml of 0.1 M sodium bicarbonate (pH 8.0) was added 0.09 μmoles of chloroacetol [^{32}P]phosphate. After 1 min, β-mercaptoethanol (0.01 M) was added to decompose the excess reagent. The sample was assayed for enzymatic activity and then dialyzed exhaustively against 0.05 M sodium chloride. The dialyzed sample was assayed for radioactivity.

§The sample (2.5 mg in 0.5 ml of buffer) was inactivated as described in footnote (‡). A 0.1-ml aliquot was dialyzed for the determination of incorporation; the remainder was used in the peptide mapping experiments [see Fig. 1].

¶In the case of human triose phosphate isomerase one μmole is 28 mg, since the enzyme has a molecular weight of 56,000 (9) and contains two subunits of the same size (10).

The product of the reaction is apparently an ester, since 95% of the incorporated radioactivity was released upon incubation of the inactivated enzyme (an aliquot of sample 2 [see Table 1]) for 12 hr at 25° with 1 M hydroxylamine in 6 M guanidine hydrochloride (pH 8.0). Preceding this incubation, the enzyme was reduced with sodium borohydride in order to stabilize the phosphate group of the incorporated reagent moiety [see Ref. 2].

The scarcity of purified human isomerase (the experiments reported were completed with a total of about 3 mg of the enzyme) precluded the isolation and sequencing of the active-site peptide. Thus, to evaluate the degree of homology between the active-site peptide from the human enzyme and those characterized previously, we resorted to a comparative study of peptide maps. The validity of this approach has been demonstrated in the characterization of the site in yeast triose phosphate isomerase that is modified by chloroacetol phosphate (8).

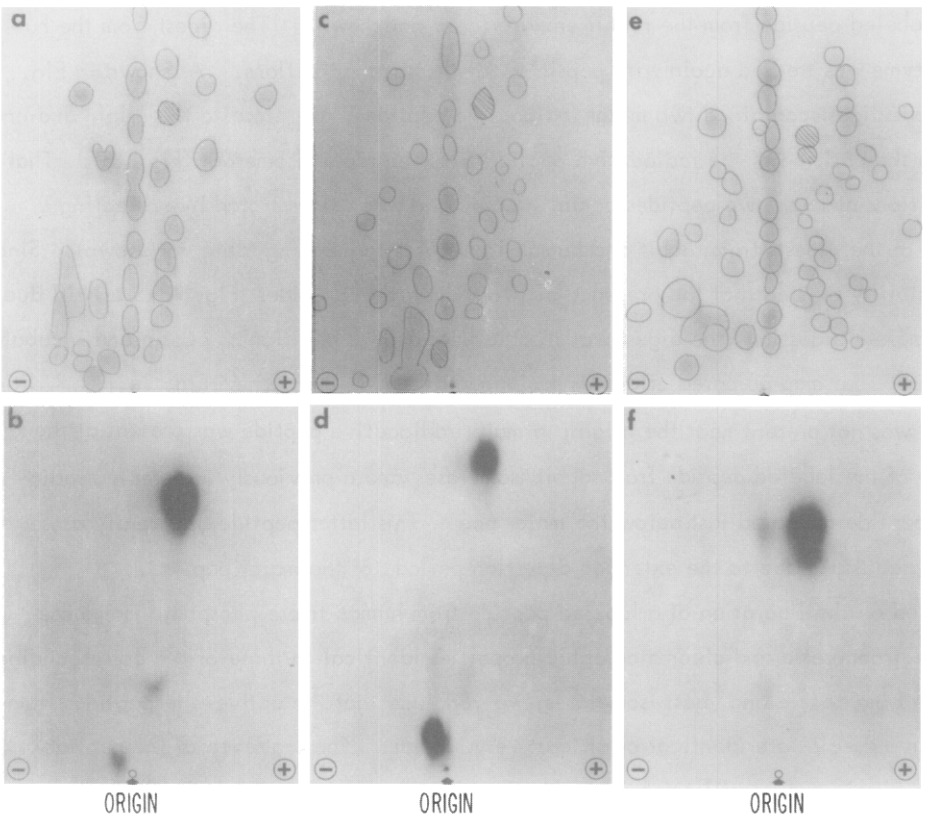

Figure 1. Peptide maps (upper) and autoradiograms (lower) of pepsin-digested rabbit (a,b) and human (c,d,e,f) triose phosphate isomerases after their inactivation with chloroacetol [^{32}P]phosphate. After inactivation as described in Footnote (†) of Table 1, the enzymes were reduced with sodium borohydride and carboxymethylated with iodoacetic acid [see Ref. 2 for experimental details]. The rabbit enzyme was digested with pepsin (1% by weight) in 0.05 M formic acid at 40° for 2 hr. The human enzyme was digested under the same conditions except additional pepsin (1% by weight) was added after 2 and 4 hr of digestion. The maps shown in c,d and e,f were obtained after digestion periods of 4 hr and 28 hr, respectively. In all cases, the equivalent of about 0.3 mg of protein was subjected to mapping.

Photographs of peptide maps and autoradiograms prepared from peptic digests of rabbit and human triose phosphate isomerase after inactivation with chloroacetol [^{32}P]phosphate are shown in Fig. 1. A single, major radioactive peptide was detected in the digest of the rabbit enzyme (Fig. 1a,b) in agreement with earlier observations (8). However, when the inactivated human enzyme was digested under the same conditions (2 hr at 40° with 1% by weight of pepsin) and subjected to mapping, most of the radioactivity was present in a peptide close to the origin; a very faint spot of radioactivity coincided with the position

of the labeled peptide from the rabbit enzyme (data not shown). The digest from the human enzyme was treated again with pepsin under the same conditions. As shown in Fig. 1c,d, this digest contained two major radioactive peptides, one close to the origin and one close to the position of the radioactive peptide from the rabbit isomerase (Fig. 1b). That the positions of these two peptides actually coincided was demonstrated by subjecting a mixture of the digests from rabbit and human isomerases to mapping (data not shown). Since the possibility existed that the presence of two radioactive peptides (Fig. 1d) was still due to incomplete digestion, the digest was treated with a third addition of pepsin and incubated for 24 hr. The map prepared after this prolonged digestion is shown in Fig. 1e,f. Radioactivity was not present near the origin; a major radioactive peptide was present at the position of the labeled peptide from rabbit isomerase, and a previously undetected radioactive peptide appeared just below the major one. This latter peptide apparently arose from degradation, due to the extended digestion period, of the major peptide.

Based on the liberation of a labeled peptide from human triose phosphate isomerase with electrophoretic and chromatographic properties identical to those of the corresponding peptides from muscle and yeast isomerases, we conclude that the active-site peptides from these three species are identical or at least very similar. The sequence of the peptide from the muscle and yeast isomerases is Ala-Tyr-Glu-Pro-Val-Trp (2, 6, 7, 9). Thus, there exists a remarkable degree of homology around the reactive glutamyl residue in triose phosphate isomerases found in species ranging from those as primitive as yeasts to humans. Such conservatism in primary structure is consistent with the proposed catalytic functionality of the glutamyl γ-carboxylate.

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