The Active Centre of Rabbit Muscle Triose Phosphate Isomerase

THE SITE THAT IS LABELLED BY GLYCIDOL PHOSPHATE

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1. Glycidol (2,3-epoxypropanol) phosphate is a specific irreversible inhibitor of rabbit muscle triose phosphate isomerase (EC 5.3.1.1); the site of attachment has now been studied. 2. The labelled enzyme was digested with pepsin and a modified peptide isolated. The sequence of the peptide is: Ala-Tyr-Glu-Pro-Val-Trp. 3. It is the glutamic acid residue in this peptide that is labelled: the peptide is thus a γ-glutamyl ester derived from glycerol phosphoric acid. The same site is labelled by a mixture of glycidol and inorganic phosphate. 4. Kinetic and stereochemical features of these reactions are discussed.

Affinity labelling is now the favoured method for chemical study of the active centre of an enzyme (Singer, 1967; Shaw, 1970). Rose & O'Connell (1969) found that rabbit muscle triose phosphate isomerase (EC 5.3.1.1) was inactivated by glycidol (2,3-epoxypropanol) phosphate. This reagent was designed to mimic the *cis*-enediol that is the postulated intermediate in the reaction catalysed by triose phosphate isomerase (Rose, 1962). The reaction of glycidol phosphate with triose phosphate isomerase was inhibited by α-glycerophosphate, a competitive inhibitor of the enzyme (Burton & Waley, 1968). The reaction showed saturation kinetics, and introduction of one group/subunit led to inactivation. Thus, by the usual criteria, reaction was occurring at the active centre of the enzyme. The label was lost on treatment with alkali, and Rose & O'Connell (1969) suggested that inactivation could be attributed to formation of an ester of glycerophosphate. The results described below confirm this supposition, and identify the part of the sequence in which the reactive carboxylate is to be found. The preliminary account of this work (Waley, Miller, Rose & O'Connell, 1970) also described the inactivation of triose phosphate isomerase by a mixture of glycidol and P_i; here it appears that P_i, a competitive inhibitor, may combine first with the enzyme and the complex then reacts with glycidol. The results described

below show that the same carboxyl group reacts with either glycidol and P_i or glycidol phosphate.

The only other glycolytic enzyme that was inactivated by glycidol phosphate was enolase (EC 4.2.1.11) (Rose & O'Connell, 1969). There appears to be some resemblance between the active centres of triose phosphate isomerase and enolase, since the most powerful competitive inhibitor of triose phosphate isomerase, namely phosphoglycollate (Johnson & Wolfenden, 1970; Wolfenden, 1970), also inhibits enolase (Cardenas & Wold, 1968), and one of the substrates of enolase, phosphoenolpyruvate, is a competitive inhibitor of triose phosphate isomerase (Krietsch, Pentchev, Klingenbürg, Hofstätter & Bücher, 1970). Such a resemblance could have some bearing on the evolution of glycolytic enzymes (Waley, 1969).

MATERIALS AND METHODS

Enzymes. Rabbit muscle triose phosphate isomerase was from Boehringer Corp. (London) Ltd., London W.5., U.K. The enzyme labelled with glycidol [^{32}P]phosphate was a kind gift from Dr I. A. Rose and Dr E. L. O'Connell; the preparation and specific radioactivity are given by Rose & O'Connell (1969; Expt. 2 of Table 1). Pepsin, carboxypeptidase A treated with di-isopropyl phosphorofluoridate, and leucine aminopeptidase were from Worthington Biochemical Corp., Freehold 2, N.J., U.S.A. Aminopeptidase M (Rohm and Haas) was from The Hexoran Co. Ltd., Belper, Derbys., U.K. The enzymic activity of triose phosphate isomerase was assayed as described by Burton & Waley (1966) except that the concentration of NADH was $83\,\mu$M.

Other materials. Glycidol phosphate (Rose & O'Connell, 1969) was kindly given to us by Dr I. A. Rose. Glycidol and *N*-bromosuccinimide were from BDH Chemicals Ltd., Poole, Dorset, U.K.

CH₂
| ⟩O
CH
|
CH₂—O—PO₃²⁻

Glycidol phosphate

CH₂
| ⟩O
CH
|
CH₂—OH
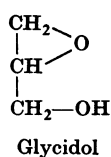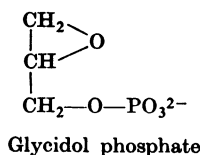
Glycidol

Reaction of triose phosphate isomerase with glycidol phosphate. Glycidol phosphate (10.6 mg) was added to triose phosphate isomerase (80 mg) in 9 ml of 50 mm-*N*-tris(hydroxymethyl)methyl - 1 - aminoethanesulphonate, pH 7.5, containing 2 mm-EDTA. After 25 min at 37°C, less than 0.03% of the enzymic activity remained. The inactivated enzyme was recovered by dialysis, first against 0.1 m-NaCl and then against 1% acetic acid, and freeze-drying.

Isolation and analysis of peptides. Paper electrophoresis at pH 1.9 or 6.5 used the buffers described by Ambler (1963), and a Locarte or Camag flat-bed high-voltage apparatus. Mobilities at pH 6.5 were calculated as described by Offord (1966). Peptides on paper were detected by the ninhydrin–cadmium reagent (Heilmann, Barollier & Watzke, 1957); the Ehrlich reagent for tryptophan was as described by Smith (1953), and the Pauly reagent was as described by Burton & Waley (1966) except that the pH 11 buffer was replaced by 0.1 m-Na$_2$CO$_3$–NaHCO$_3$ buffer, pH 9.5, tyrosine can be detected at pH 9.5. Peptides containing ester bonds were eluted with 1% acetic acid. Amino acid analyses and sequence studies were carried out as described by Miller & Waley (1971).

Cleavage with *N*-bromosuccinimide was done by treating the peptide (0.1 μmol) in 0.1 ml of 0.5% formic acid with 10 μl of acetonitrile containing 0.8 mg of *N*-bromosuccinimide; after 1 h at room temperature the mixture was fractionated by electrophoresis at pH 1.9. The use of formic acid as solvent cuts down oxidative side reactions (Wilchek, Spande & Witkop, 1968).

RESULTS

Isolation of labelled peptide. In the first experiment the enzyme was inactivated with glycidol [^{32}P]phosphate; the radioactivity was split off from the protein under alkaline conditions so that the digestion and fractionations were carried out in neutral or acidic solutions. The labelled triose phosphate isomerase (40 mg) in 2 ml of 5% (v/v) formic acid was treated with 0.8 mg of pepsin for 22 h at 25°C, and the digest fractionated on Sephadex G-25 (Fig. 1). The peptides were recovered from the appropriate peaks by rotary evaporation. The radioactive peptide was then fractionated by electrophoresis at pH 1.9; it had no net charge. The carboxylic acid groups of peptides are largely un-ionized, so that all ordinary peptides migrate. The final step was electrophoresis at pH 6.5. The peptide was Pauly-positive. The amino acid composition (expressed as molar proportions) was: Glu (0.99), Pro (1.12), Ala (0.91), Val (1.05), Tyr (0.93). Tryptophan was present (Ehrlich reaction). There was 1.03 mol of ^{32}P/mol of peptide.

In the second experiment there was no need to use radioactive glycidol phosphate, as the required phosphorylglyceryl-peptide could be recognized from its properties and composition: the late elution from Sephadex G-25 (often observed with peptides containing tryptophan) was helpful, as

was the zero net charge at pH 1.9. The inactivated enzyme (see the Materials and Methods section) (69 mg) was digested with 1 mg of pepsin in 3.2 ml of 5% (v/v) formic acid for 17 h at 37°C. The digest was fractionated as in the first experiment: the yield of phosphorylglyceryl-peptide was 1 μmol (36%) and the composition was: Glu (1.05), Pro (0.98), Ala (0.96), Val (1.06), Tyr (0.95); tryptophan was present.

The isolation of labelled peptide from the reaction of glycidol with triose phosphate isomerase was carried out as follows. Triose phosphate isomerase (1 mg/ml in the buffer described above) was treated with 0.27 m-glycidol in 20 mm-potassium phosphate buffer, pH 7.5, for 6 h at 37°C; the control (lacking glycidol) had retained 84% of its activity, and the activity of the treated sample was 40% of the control. The protein was isolated and treated with pepsin and the digest was fractionated on Sephadex G-25 (Fig. 1); electrophoresis of the material from tubes 59–65 at pH 1.9 and pH 3.5 gave a neutral glyceryl-peptide (Table 1) containing tryptophan whose composition was: Glu (1.36), Pro (0.95), Ala (1.05), Val (1.0), Tyr (0.57). The hydrolysate contained glycerol: 5.2 nmol of peptide gave 8.8 nmol of glycerol, estimated enzymically (Wieland, 1963); the assay is not very precise when such small amounts of material are used.

The isolation of unlabelled peptide from peptic digests of native triose phosphate isomerase was carried out similarly; the peptide was acidic (Table 1). The reaction of the enzyme with glycidol described above was incomplete, and unlabelled peptide was isolated from tubes 66–72 (Fig. 1).

These isolations were guided by knowing that the peptide is eluted late from Sephadex G-25, and that it is the only peptic peptide to contain both tyrosine and tryptophan (S. G. Waley, unpublished work).

Structure of labelled peptide. One of the amino acids in the peptide from triose phosphate isomerase treated with glycidol phosphate will be combined with the moiety from glycerophosphoric acid, and so the main question is which amino acid. The most likely candidates are an ester derived from glutamic acid or an ether derived from tyrosine. Enzymic cleavage into smaller fragments was the first approach tried. Digestion of the peptide (120 nmol) with carboxypeptidase A (12 μg, brought into solution as described by Smillie & Hartley, 1966) for 15–45 min released tryptophan rapidly, followed by valine; the residual peptide remained uncharged at pH 1.9. Digestion with leucine aminopeptidase released alanine rapidly. These results suggest the partial sequence:
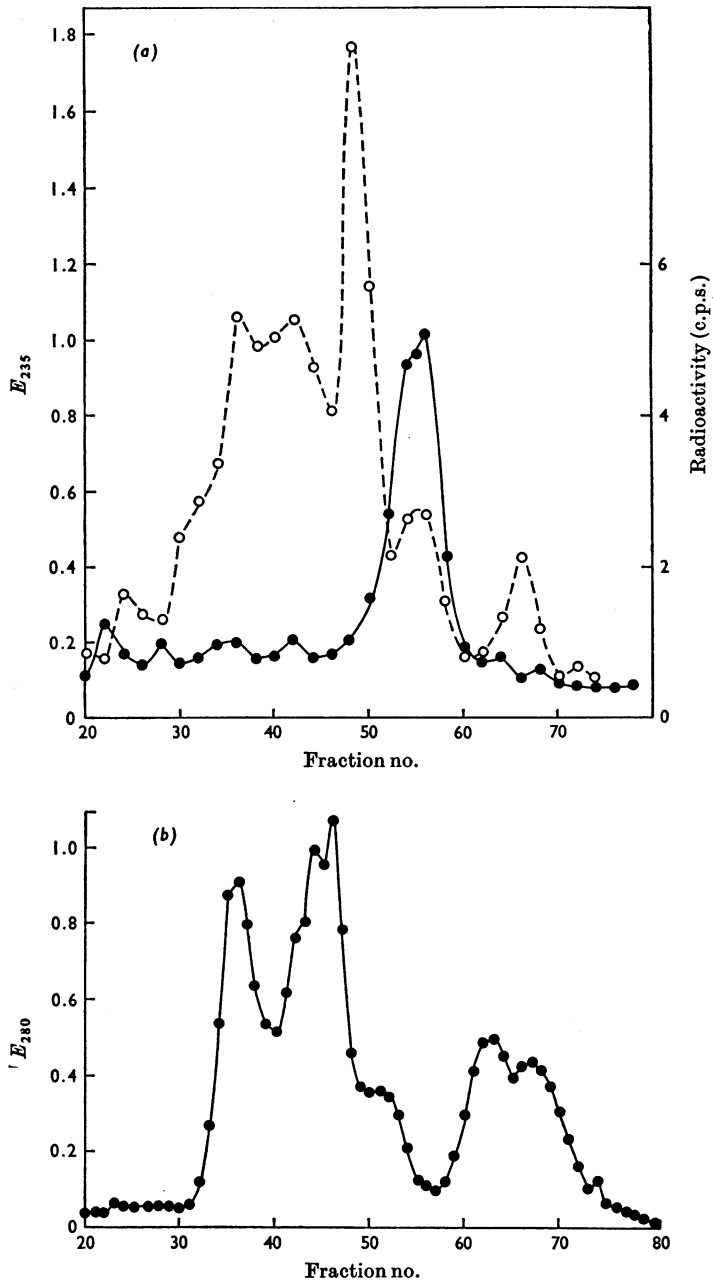
Ala(Tyr,Glu,Pro)Val-Trp

Fig. 1. Chromatography of peptic digests of (a) triose phosphate isomerase treated with glycidol [^{32}P]phosphate and (b) triose phosphate isomerase treated with glycidol and P$_i$. The column (140 cm long × 0.9 cm diam.) of Sephadex G-25 was eluted with 0.5% formic acid, and 1.8 ml fractions were collected in (a) and 1.6 ml fractions in (b). In (a): ○, E_{235}; ●, radioactivity of 2 μl portion. In (b) ●, E_{280}.

with either the glutamic acid or the tyrosine residue modified. Digestion of 20 nmol of peptide with 4 μg of aminopeptidase M for 24 h at 37°C released four amino acids; these were (molar proportions in parentheses): Ala (0.95), Val (0.97), Tyr (1.14), Trp (0.84). The two missing amino

Table 1. *Mobilities of labelled and unlabelled peptic peptide from triose phosphate isomerase*

Triose phosphate isomerase was treated with the reagent specified in the first column, and the peptic peptide isolated as described in the text. Electrophoretic mobilities at pH 6.5 are relative to aspartic acid $= -1$, and at pH 1.9 relative to 1-dimethylaminonaphthalene-5-sulphonamide, which has approximately the same mobility as serine.

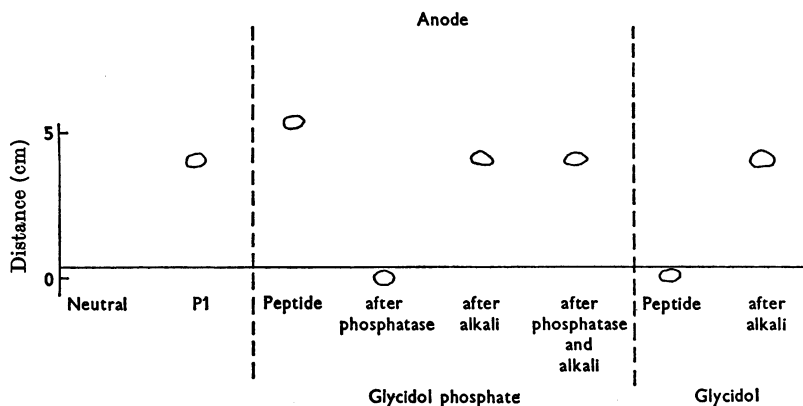|		Electrophoretic mobility	
Reagent	Product	At pH 6.5	At pH 1.9
None	Unlabelled peptide	−0.21	0.43
Glycidol phosphate	Phosphorylglyceryl-peptide	−0.27	0
Glycidol + P$_i$	Glyceryl-peptide	0	0.42

Fig. 2. Comparison of mobilities of peptic peptides obtained from triose phosphate isomerase. Paper electro-phoresis at pH 6.5 was carried out for 1 h at 50 V/cm. The panel on the left shows the unlabelled peptide (P1); the centre peptide shows the phosphoryl glyceryl-peptide obtained from the reaction of triose phosphate isomerase with glycidol phosphate, and the panel on the right shows the glyceryl-peptide obtained from the reaction with glycidol. Treatment with alkali was carried out in 1 M-NH$_3$ for 16 h at 20°C. Treatment of 80 nmol of the phosphorylglyceryl-peptide with 1 µg of acid phosphatase (potato acid phosphatase) was carried out in 0.1 M-sodium citrate buffer, pH 5.6, for 1 h at 25°C, and the peptide separated from salts on Sephadex G-10 in 0.5% formic acid.

acids are glutamic acid and proline. As the absence of proline together with the amino acid preceding it from aminopeptidase M digests has been observed on several occasions (Takahashi, Stein & Moore, 1967; Plummer, 1969), this result suggests a Glu-Pro bond, as well as showing that there is one residue of tryptophan in the peptide. Another digestion with aminopeptidase M released the same four amino acids and left a peptide uncharged at pH 1.9, which suggests that the label might be present in the dipeptide Glu-Pro. More definite evidence is given below, but further experiments on the sequence of the peptide are now described.

Cleavage of the unlabelled peptide with *N*-bromo-succinimide (as described in the Materials and Methods section) gave two main products: the first had the same mobility at pH 1.9 as authentic alanyltyrosine similarly treated, had *N*-terminal alanine and gave alanine on acid hydrolysis; the

second product gave glutamic acid, proline and valine on acid hydrolysis and had *N*-terminal glutamic acid. *N*-Bromosuccinimide cleaves at the *C*-terminal side of tyrosine and tryptophan residues (Ramachandran & Witkop, 1967), and although both these amino acids are present the tryptophan is at the *C*-terminus. Hence the cleavage is ascribed to reaction at the tyrosine residue, and these results, together with those already given, lead to the sequence:

Ala-Tyr-Glu-Pro-Val-Trp

This sequence was also obtained by use of the dansyl–Edman method.

The site of attachment in the modified peptides was clearly shown by their electrophoretic mobilities (Table 1). If the phosphorylglyceryl-peptide is the ester derived from glutamic acid, its net charge at pH 6.5 will be $-(1+x)$, where x is the fractional

charge arising from the second ionization of the phosphate group; the charge of the unlabelled peptide will be -1, and if we ignore the small differences in molecular weights the mobilities will be approximately proportional to the net charges (Offord, 1966). Hence, from the values in Table 1, $1+x=0.27/0.21$, and $x=0.3$; this is a reasonable value; Datta & Grzybowski (1958) give pK_2 6.65 for glycerophosphoric acid (corresponding to $x=0.4$). If the phosphorylglyceryl-peptide were an ether derived from tyrosine the net charge would be $-(2+x)$, and this would give a meaningless (negative) value for x. Further evidence was obtained by treatment of the phosphorylglyceryl-peptide with acid phosphatase: the product, the glyceryl-peptide, was neutral (Fig. 2). Saponification of the glyceryl-peptide restored its acidic character and gave the unlabelled peptide. Similarly the sample of the glyceryl-peptide obtained from the reaction of glycidol with triose phosphate isomerase was neutral and was converted into the unlabelled peptide by saponification (Fig. 2). These results confirm that the labelled peptides are esters derived from glutamic acid.

There are two other points to be discussed about the structure of the labelled peptides: one is structural and the other stereochemical. We have no direct evidence on whether it is C-1 or C-2 of the epoxides that reacts with the glutamate, but we favour C-1 by analogy with the reaction of triose phosphate isomerase with 1-halohydroxyacetone phosphates, which is discussed below. Nor is it certain which enantiomer of the DL-glycidol phosphate reacts, but it seems likely that the enantiomer configurationally related to D-glyceraldehyde phosphate will react preferentially.

DISCUSSION

Comparison of inhibitors. During the last few years two types of inhibitor have been used on triose phosphate isomerase from two species (Table 2). The main conclusion is that the same site is labelled in all these reactions (Coulson, Knowles, Priddle & Offord, 1970b; Hartman, 1970a,b; Waley, *et al.*

1970). The most noteworthy difference between the reaction of bromohydroxyacetone phosphate and that of glycidol phosphate with the enzyme is that the former gives a keto ester, which is not stable. The lability can be overcome by prompt reduction of the keto ester (Coulson *et al.* 1970b). If this is not done the group migrates to the tyrosine residue that is adjacent in the primary structure and the product is an ether. Moreover, migration is accompanied by loss of the phosphate group. This suggests that the first stage in the migration is that the hydroxyl group of the tyrosine residue displaces the phosphate group from C-3 of the inhibitor moiety to form an ether; then the carboxylic ester at C-1 of the inhibitor moiety is hydrolysed. This idea can be tested by labelling C-1 or C-3 of bromohydroxyacetone phosphate and, after reaction with triose phosphate isomerase, oxidizing the inhibitor moiety to a carboxymethyl group: if migration takes place as suggested, hydrolysis would give an O-carboxymethyltyrosine derived from C-3 of the original inhibitor.

As far as is known, then, the primary site attacked in both enzymes by both inhibitors is the glutamic acid residue. These reactions seem quite specific (Table 2), and there was only one radioactive spot in the peptide 'map' of the tryptic digest of triose phosphate isomerase labelled by glycidol phosphate. The precise location of the reactive glutamate residue in the primary structure is not yet known, but it is somewhere in the C-terminal half of the chain (J. C. Miller & S. G. Waley, unpublished work).

Stereochemical aspects of inhibition. As glycidol phosphate and halohydroxyacetone phosphates are effective and specific inhibitors of triose phosphate isomerase it is worthwhile to compare the stereochemistry of these reactions, which are nucleophilic substitutions, with the stereochemistry of the enzymic reaction. This is an aldose–ketone interconversion, consisting of two successive keto–enol isomerizations; the *cis*-enediol [structure (III) in Fig. 3] is a likely intermediate (Rose, 1962), and the proton removed from the prochiral C-1 of dihydroxyacetone phosphate is the one labelled H_R [structure (I) in Fig. 3] (Rose, 1958; Bentley, 1969a,b). It is

Table 2. *Inhibitors used to label triose phosphate isomerase*

| Source of enzyme	Inhibitor	Incorporation (mol of inhibitor/ subunit of mol.wt. 25000)	Reference
Rabbit muscle	Bromohydroxyacetone phosphate	1.0	Coulson, Knowles & Offord (1970a)
Rabbit muscle	Chlorohydroxyacetone phosphate		Hartman (1970b)
Chicken muscle	Bromohydroxyacetone phosphate		Coulson *et al.* (1970b)
Rabbit muscle	Glycidol phosphate	0.8	Rose & O'Connell (1969)
Rabbit muscle	Glycidol + P_i		Waley *et al.* (1970)
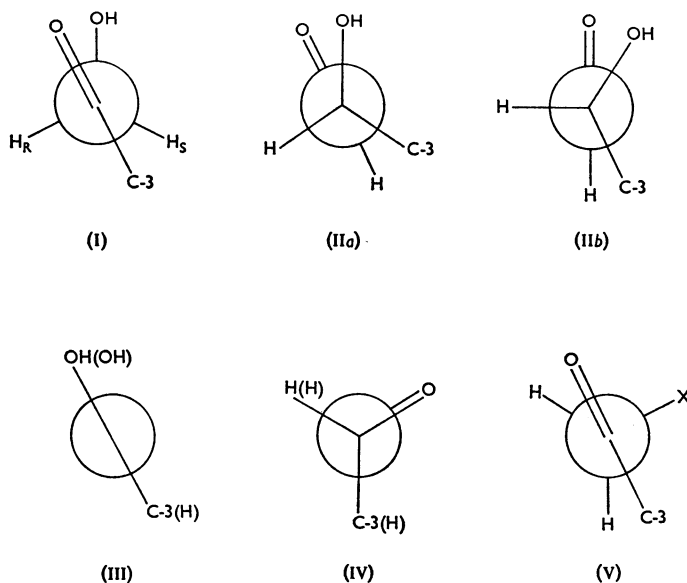
Fig. 3. Newman projections of substrates and inhibitors of triose phosphate isomerase. The view is along the C-2–C-1 bond, with C-2 nearer to the viewer: bonds to C-2 are shown by lines from the centre of the circle, and bonds to C-1 by lines from the circumference. Substituents on C-1 are shown in parentheses when they are eclipsed by those on C-2. Substituents attached to C-3 are omitted. (I) is dihydroxyacetone phosphate, (II) is glyceraldehyde phosphate, (III) is the *cis*-enediol, (IV) is glycidol phosphate and (V) is halohydroxy-acetone phosphate (X = Cl or Br.) The reactive group in triose phosphate isomerase is on the left, lower down ('8 o'clock'), near H_R in structure (I). Structures (IIa) and (IIb) show different orientations of the same conformation: in (IIb) C-3 is in the same position as in (I), but in (IIa) the bond to the hydrogen atom to be removed is parallel to the corresponding bond in (I).

the free aldehyde form of D-glyceraldehyde 3-phosphate that interacts with triose phosphate isomerase (Trentham, McMurray & Pogson, 1969), and presumably the same is true for dihydroxy-acetone phosphate. The high rate of the reaction catalysed by triose phosphate isomerase makes it a reasonable assumption that the substrates bind to the enzyme in the most favourable conformation for reaction. If this is the one in which the C–H bond being broken is perpendicular to the plane of the carbonyl group (Corey & Sneen, 1956; Hine, Houston, Jensen & Mulders, 1965) then the conformations leading to the *cis*-enediol will be as shown [structures (I) and (II) in Fig. 3]. The basis for this choice of conformation is none too firmly established, however (Feather & Gold, 1965; Bordwell & Scamehorn, 1968), and indeed it is possible that the preferred conformation for enolization may turn out to be most reliably inferred from crystallographic studies on triose phosphate isomerase and other isomerases. In the enzymic reaction one (or both) of the oxygen atoms, or C-3, or both [structures (I), (II) and (III) in Fig. 3] must move. Movement or 'judder' during the catalytic cycle is suggested by the perturbations

that are brought about by competitive inhibitors (Johnson & Wolfenden, 1970).

If the glutamic acid residue that reacts with the inhibitors is in fact the base that abstracts a proton from C-1 of dihydroxyacetone phosphate or C-2 of D-glyceraldehyde 3-phosphate, then the carboxylate will be positioned close to H_R in structure (I) and the inhibitors will be oriented as in structures (IV) and (V) (Fig. 3). Wolfenden (1970) finds that the maximum velocity of the enzymic reaction falls as the pH decreases, as if a basic group of pK 7.35 were being titrated; it may or may not be correct to assign this apparent pK to the glutamic acid residue that reacts with the inhibitors. The strict steric requirements for inactivation are shown by the fact that the epoxide phosphonomycin [(1R,2S)-1,2-epoxypropylphosphonic acid] (Christensen et al. 1969) is simply a competitive inhibitor but does not irreversibly inactivate triose phosphate isomerase even on prolonged incubation (S. G. Waley, unpublished work).

Kinetic aspects of inhibition. The reaction of triose phosphate isomerase with glycidol phosphate is much faster than the reaction of a low-molecular-weight carboxylate. The second-order rate constant
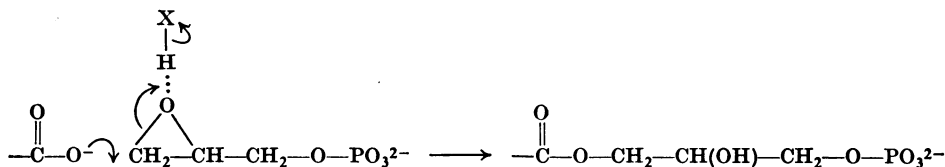
Table 3. *Rates of reaction of epoxides with triose phosphate isomerase*

The value for reaction (1) is from Rose & O'Connell (1969), and for reaction (2) from the inactivation of triose phosphate isomerase by glycidol in the presence of P_i described in the text. The values for reactions (3) and (4) are from Ross (1950). The values are given to one significant figure, and the reactions were carried out in aqueous solution at 15–40°C.

Reaction	$10^4 \times$ Rate constant	
	(s^{-1})	$(M^{-1} \cdot s^{-1})$
(1) Triose phosphate isomerase + glycidol phosphate	50	50000
(2) Triose phosphate isomerase $\cdots$ P_i + glycidol		2
(3) Sodium acetate + glycidol		0.001
(4) H_3O^+ + glycidol		100

for reaction (1) in Table 3 is derived from the overall rate of inactivation (about 3%/min) when the concentration of glycidol phosphate is 0.1 mM; the first-order rate constant is obtained from the maximum rate of inactivation. The second entry in Table 3 gives the rate of reaction of glycidol with triose phosphate isomerase in the presence of P_i expressed as a second-order rate constant; its magnitude suggests that the reaction with glycidol may be confined to a single site, but we have no direct evidence about this. The concentration of P_i is sufficient for nearly all of the triose phosphate isomerase to be present as a complex, and the kinetics suggest that this complex reacts with the glycidol (Waley *et al.* 1970), either in a bimolecular reaction or via a relatively weak complex. The role of P_i in this reaction is not clear, but there are apparently similar reactions with other enzymes: for example, methylguanidine enhances the inactivation of trypsin by iodoacetamide (Inagami & Hatano, 1969).

The high rate of reaction of glycidol with acids (Table 3) raises the question whether the epoxide oxygen atom in glycidol phosphate (or glycidol) is hydrogen-bonded to some group in the enzyme during reaction:

late from solvent when glycidol phosphate is present at the active site.

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Another possibility is that the high reactivity of the glutamic acid residue in triose phosphate isomerase might be due to its being an unsolvated anion: the water that normally interacts with the carboxylate may be displaced by the glycidol phosphate, and hence the reactivity of the glutamate enhanced. This idea is based on the assumption that groups in the enzyme shield the carboxy-

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