Active-Site Labelling of Triose Phosphate Isomerase

THE REACTION OF BROMOHYDROXYACETONE PHOSPHATE WITH A UNIQUE GLUTAMIC ACID RESIDUE AND THE MIGRATION OF THE LABEL TO TYROSINE

By SUSAN DE LA MARE, A. F. W. COULSON and J. R. KNOWLES
The Dyson Perrins Laboratory, University of Oxford, Oxford OX1 3QY, U.K.

and

J. D. PRIDDLE and R. E. OFFORD

Laboratory of Molecular Biophysics, Department of Zoology, University of Oxford, Oxford OX1 3PS, U.K.

(Received 16 March 1972)

Triose phosphate isomerase from chicken muscle reacts stoichiometrically with the active-site-directed irreversible inhibitor bromohydroxyacetone phosphate with concomitant loss of all catalytic activity. The primary site of attachment has been shown to be a unique glutamic acid residue in the sequence Ala-Tyr-Glu-Pro-Val-Trp. Unless the inhibitor-enzyme bond is stabilized by reduction of the C-2 carbonyl group with borohydride, the phosphate group is lost and the label migrates to the adjacent tyrosine residue. It is suggested that the γ -carboxylate group of the glutamic acid residue may be the base responsible for primary proton abstraction from substrate in the catalysis. The failure of this reagent specifically to inactivate either muscle or yeast aldolase, and the use of the reagent in preparing isomerase-free glycolytic enzymes, is discussed.

Most active-site-directed irreversible inhibitors (Shaw, 1970) of enzymes are designed to combine two requirements: specific binding at the active site and covalent attachment to a group on the enzyme in the site. The first is satisfied by building into the inhibitor many of the structural elements on which specific binding depends, and the second, by incorporating a reactive group that, being reasonably but not excessively reactive, will preferentially label a residue at the active site owing to the high local reagent concentration. In favourable cases, where the outline of the enzyme mechanism is known, it may also be possible to design the reagent to react with a particular postulated catalytic functionality. For triose phosphate isomerase, this approach has proved a valid one, resulting in the synthesis of a remarkably effective and specific irreversible inhibitor, and allowing the identification of what may well be a catalytically important enzyme group.

From the studies of Rieder & Rose (1956, 1959) and of Bloom & Topper (1956, 1958), a chemically attractive pathway for the triose phosphate isomerase-catalysed reaction was formulated by Rose (1962), involving the abstraction of a proton from C-3 of dihydroxyacetone phosphate to give an enediolate anion, which then picks up a proton at C-2 to yield D-glyceraldehyde 3-phosphate (Scheme 1). The identity of the basic group on the enzyme (B^-) was the target of the present work, and since most bases are reasonable nucleophiles, it was hoped that the α -bromoketone substrate analogue, bromohydroxyacetone phosphate, would act as an efficient, specific irreversible inhibitor for triose phosphate isomerase.

It is noteworthy that, although aldolase probably shares with triose phosphate isomerase a similar primary proton-abstraction step in the reaction pathway, aldolase is not inhibited by near-stoichiometric concentrations of bromohydroxyacetone phosphate.

Scheme 1. Triose phosphate isomerase reaction

A wide variety of conditions have been used, and possible reasons for the inertness of aldolase are discussed below.

While the present work was in progress, similar studies were reported by Hartman (1968, 1970a, 1971), and the conclusions from the present work broadly agree with those of Hartman, and also those of Waley *et al.* (1970), who have used a rather different inhibitor. Preliminary reports of parts of this work have appeared (Coulson *et al.*, 1970a,b).

Experimental

Materials

Triose phosphate isomerase. Crystalline triose phosphate isomerase was isolated from chicken muscle by the method outlined by Putman *et al.* (1972). The concentration of enzyme solutions was determined from E_{280} , assuming $E_{280}^{0.1\%}$ of 1.21 for a 10mm light-path [P. M. Burton & S. G. Waley (personal communication) give 1.21; R. Cecil (personal communication) gives 1.23; and Norton *et al.* (1970) give 1.31, for the rabbit muscle enzyme]. Enzyme activity was assayed with DL-glyceraldehyde 3-phosphate as substrate and α -glycerophosphate dehydrogenase as coupling enzyme (Beisenherz, 1955).

Aldolase. Crystalline aldolase from rabbit muscle was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. The crystalline enzyme from chicken muscle was isolated by Miss S. J. Putman by the method used for the rabbit muscle enzyme (Eagles *et al.*, 1969). For both enzymes, a value of $E_{280}^{0.1\%}$ of 0.91 for a 10mm light-path was assumed. Enzyme activity was assayed with fructose 1,6-diphosphate as substrate and α -glycerophosphate dehydrogenase as coupling enzyme (in the absence of triose phosphate isomerase) as described by Baranowski & Niederland (1949).

Crystalline aldolase from yeast was a gift from R. Jack and J. I. Harris, Laboratory of Molecular Biology, Cambridge, U.K.

α -Glycerophosphate dehydrogenase. This was obtained from Sigma (London) Chemical Co. Ltd.

Pepsin. This was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Acid phosphatase. This was supplied by Boehringer Corp. (London) Ltd., London W.5, U.K.

Amino-peptidase M. This was obtained from Röhm G.m.b.H. through Hexoran Co. Ltd., Belper, Derbyshire, U.K.

Trypsin. This, treated with L-1-chloro-4-phenyl-3-tosylamidobutan-2-one, was a gift from A. R. Rees, Department of Zoology, Oxford.

Substrates and coenzymes. DL-Glyceraldehyde 3-phosphate (as the diethylacetal monobarium salt), NADH (as the disodium salt), and fructose 1,6-

diphosphate (as the tetrasodium salt) were obtained from Sigma (London) Chemical Co. Ltd.

Orthophosphoric acid (crystalline). This was obtained from Fluka A.G., Buchs, Switzerland.

Radiochemicals. Bromo[1- 14 C]acetic acid (40mCi/mmol) and [32 P]phosphoric acid (67 Ci/mg) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Bromohydroxyacetone phosphate. Bromodiazoacetone (0.5g) (prepared as described by Husain & Lowe, 1968) was added to a solution of crystalline orthophosphoric acid (0.62g) in sodium-dried diethyl ether (25ml). A few drops of BF_3 -diethyl-etherate were added, and the mixture was left in the dark at room temperature overnight. The mixture was protected from atmospheric moisture with a drying tube. The solution became colourless, and no diazo compound could be detected by t.l.c. (on silica-gel plates eluted with ether). The solvent was removed under reduced pressure to yield a viscous yellow oil. Microanalysis of the crude oil gave a bromine/phosphorus ratio of 1:2, showing that no bromine was lost in the reaction (Found: C, 24; H, 6; Br, 16.2; P, 12.7%). Paper electrophoresis at pH 3.48 showed that there were only two phosphate-containing components (detected by using the reagent of Hanes & Isherwood, 1949), the faster-running of which was the excess of P_i . The n.m.r. spectrum in $^2\text{H}_2\text{O}$ showed a singlet at τ 6.3 ($\text{Br}-\text{CH}_2-\text{CO}-$), a doublet at τ 5.3 ($-\text{CO}-\text{CH}_2-\text{OPO}_3\text{H}_2$), and peaks due to residual ether.

The nature of the phosphate ester was further established by treating the crude reaction mixture (100mg) with an ethereal solution of diazomethane made in the usual way from *N*-methyl-*N*-nitrosourea (5g) (Arndt, 1943). There was a brisk effervescence. After 30min, a few drops of acetic acid were added, the solvent was removed under reduced pressure, and the mass spectrum was observed. The heaviest molecular ion present was that from the dimethyl ester, $\text{Br}-\text{CH}_2-\text{CO}-\text{CH}_2-\text{OPO}(\text{O}-\text{CH}_3)_2$, and the cracking pattern was consistent with this, though because of the presence of an excess of trimethyl phosphate arising from the excess of P_i originally present, the decomposition of the ion from the bromo compound cannot be traced very far. In later experiments, a fivefold molar excess of P_i over bromodiazoacetone was used, to minimize the possible formation of di- and tri-alkyl phosphates.

Attempts have been made to separate the bromohydroxyacetone phosphate from excess of phosphate in non-aqueous solvents (to avoid hydrolysis of the α -bromoketone), by chromatography, or by the isolation of salts. These approaches were unsuccessful, however, and the stability to hydrolysis of the bromine group and the phosphate group were investigated. Hydrolysis of the bromine group to give dihydroxyacetone phosphate was followed by the appearance

of material that could be assayed by using α -glycerophosphate dehydrogenase and NADH. It was found that bromohydroxyacetone phosphate is relatively stable in aqueous solution below pH 6.5, and the purification method finally used was electrophoresis on paper at pH 1.9. Good separation of P_i and bromo compound was obtained, with essentially no concomitant hydrolysis of the bromo compound to dihydroxyacetone phosphate. When radioactive bromohydroxyacetone phosphate was used, the electrophoretic purification was also followed by strip scanning. Elution from paper was effected with water and the material was then freeze-dried. The concentration of bromohydroxyacetone phosphate was determined either by enzymic assay of the dihydroxyacetone phosphate produced after hydrolysis at pH 10.5, or (in later experiments) by 'titration' with a known concentration of triose phosphate isomerase.

Bromohydroxyacetone [^{32}P]phosphate. A solution of 10 mCi of [^{32}P]orthophosphate in dilute HCl (1 ml) was evaporated at reduced pressure at 80°C in a silicone-treated vessel. A solution of crystalline orthophosphoric acid (0.5 g) in dry ether (10 ml) was allowed to achieve isotopic equilibrium with the radioactive residue overnight. Portions (25 μ l) of this solution were shaken with water (5 ml) and the ether was allowed to evaporate. To obtain the specific radioactivity, the aqueous solutions were analysed for orthophosphate (Briggs, 1922) and the radioactivity of 0.1 ml samples was measured under the same conditions as those used for the inhibited protein samples.

To the remainder of the ethereal solution was added bromodiazoacetone (0.16 g) followed by a few drops of BF_3 -diethyletherate. A colourless solution was obtained on leaving the mixture at room temperature overnight, and t.l.c. confirmed the disappearance of all the diazo compound.

Bromohydroxy[^{14}C]acetone phosphate. Bromodiazo[^{14}C]acetone was prepared by the method of Husain & Lowe (1968). Bromoacetic acid (82.8 mg) was dissolved in sodium-dried redistilled benzene (2 ml). This solution was used to wash out phials containing 0.2 mCi of bromo[1- ^{14}C]acetic acid. Oxalyl chloride (0.17 ml) was added and the mixture was refluxed for 2 h. The solvent was removed by evaporation in a rotary evaporator fitted with a short fractionating column. The column was rinsed and the residue dissolved with dry ether (2 $\times$ 5 ml). The solution was added slowly to a KOH-dried ethereal solution of diazomethane (4 ml, prepared from 4 g of *N*-methyl-*N*-nitrosourea). After 10 min, a drop of A.R. acetic acid was added, and the solvent removed in a rotary evaporator. The residue was dissolved in $CHCl_3$ (5 ml), filtered and applied as a narrow streak to a preparative t.l.c. plate (silica gel HF₂₅₄; 20 cm $\times$ 20 cm; 1 mm thick). The plate was developed

with sodium-dried benzene, and the silica gel containing the major band (R_f 0.1) was located by examination in u.v. light, scraped off the plate and eluted with dry ether (10 ml).

The solvent was removed *in vacuo*, and the residue (56 mg) collected. Comparison with an authentic sample by t.l.c. (HF₂₅₄+366 plates, eluted with ether) showed that the material contained one major component with the same R_f value as bromodiazoacetone, and a minor, slower-running impurity.

The material was redissolved in dry ether (2 ml) and the solution stored at -20°C.

Crystalline orthophosphoric acid (92 mg) was dissolved in dry ether (10 ml). Ethereal bromodiazo[^{14}C]acetone (1 ml) was added, followed by a drop of BF_3 -diethyletherate. A clear solution was obtained on leaving the mixture overnight, and t.l.c. confirmed the disappearance of the diazo compound. The specific radioactivity of the bromohydroxy[^{14}C]acetone phosphate was assessed from the specific radioactivity of the bromoacetic acid used in the preparation, on the basis of the amount of radioactive acid taken and the amount of non-radioactive acid used to dilute it. Counting efficiency for ^{14}C was determined by using [1- ^{14}C]hexadecane.

Chlorohydroxyacetone phosphate. Chlorohydroxyacetone phosphate was prepared exactly analogously to the bromo compound. As expected, the chloro compound was found to be rather more resistant to hydrolytic breakdown than the bromo derivative, and purification was effected by chromatography on Dowex 1 (Cl^- form).

Buffers. All buffers were prepared with deionized water and A.R.-grade reagents.

'Polyamide layers'. 'Polyamide layers' for t.l.c. of dansyl-amino acids (Gray, 1967) were obtained from the Cheng-Chin Trading Co. Ltd., Hankow Street, Taipei, Taiwan.

Solvents. All solvents for chromatography and other operations were redistilled before use.

Methods

Spectrophotometry. U.v. spectra were measured with a Unicam SP.800 recording spectrophotometer. Fixed-wavelength and kinetic measurements were made on a Unicam SP.1800 recording spectrophotometer. Difference spectra were measured on a Cary 14 recording spectrometer, by using divided cells and the 0-0.1 slide-wire.

pH measurements. These were determined with a Radiometer TTT1c instrument with a pH A 630 scale-expander attachment, standardized against standard buffer solutions from British Drug Houses Ltd., Poole, Dorset, U.K.

Scintillation counting. This was done with an automatic Beckman model DPM 100 instrument.

Radiochromatogram scanning. This was done with

a Packard 7201 gas-flow counter with a model 385 recording ratemeter.

Amino acid analyses. These were done with a Beckman-Spinco model 120C instrument.

Mass spectra. These were obtained by Dr. R. T. Aplin in the Dyson Perrins Laboratory on an A.E.I. MS 9 spectrometer.

N.m.r. spectra. These were obtained by Mrs. R. E. Richards in the Dyson Perrins Laboratory on Perkin-Elmer 60 and 100 MHz instruments.

Microanalyses. These were performed in the Dyson Perrins Laboratory by Dr. Strauss.

Peptide isolation and sequence determination methods. The techniques used in the peptide isolation and sequence determination were as described fully by Bayliss *et al.* (1969): paper electrophoresis, gel filtration, monitoring of column eluates, scintillation counting, radiochromatogram scanning, elution of peptides from paper, acid hydrolysis of peptides, and sequencing by the dansyl-Edman method of Gray (1967).

Results

Inactivation of triose phosphate isomerase

Bromohydroxyacetone phosphate is a very potent inactivator of this enzyme. Fig. 1 shows how the enzyme activity after a 5 min incubation with inactivator depends on the amount of bromo compound used, and it is essentially a titration curve. The inactivation reaction is too fast for convenient study of the inactivation kinetics, and even at micromolar concentrations of enzyme, a 1.2-fold molar excess of inhibitor inactivates the enzyme within 5 min. To establish the specificity of the inactivation further, the reaction was studied at very low enzyme concentrations in the presence and absence of the highly effective competitive inhibitor discovered by Wolfenden (1969), 2-phosphoglycollate. The K_i for this material is $6.8 \mu\text{M}$, and protection of the enzyme by phosphoglycollate against inactivation by bromohydroxyacetone phosphate was clearly demonstrated (Fig. 2).

The stoichiometry of the enzyme-inactivator reaction was studied by using both ^{32}P - and ^{14}C -labelled material. With ^{32}P -labelled inactivator, experiments similar to those of Fig. 1 were first performed to establish the amount necessary completely to inactivate the enzyme. The enzyme (2 mg) was then incubated with labelled inactivator, and subjected to gel filtration on a column (1 cm $\times$ 111 cm) of Sephadex G-25 (fine grade) in 50 mM-triethanolamine-HCl buffer, pH 7.8. On the basis of a molecular weight of 50000 and an $E_{280}^{0.1\%}$ of 1.21, the incorporation of ^{32}P label was 1.3 mol/mol of enzyme. Such a non-integral value may arise if the protein is impure, if there are errors in either the molecular weight or in the value of

Fig. 1. *Inactivation of triose phosphate isomerase*

Enzyme (200 μg) in 10 μl of 0.5 M- β , β -dimethylglutarate buffer, pH 6.45, was added at room temperature to a tube containing various volumes of bromohydroxyacetone phosphate solution (approx. 2 mM) from which the ether had been evaporated under reduced pressure. Samples (2 μl) were removed and the reaction was stopped by diluting to 25 ml, before assay of residual enzymic activity. Samples were removed for assay after 5 (●) and 30 (○) min.

$E_{280}^{0.1\%}$, if the labelling of protein is not irreversible, or if the reaction is non-stoichiometric. The first three possibilities are rendered unlikely by the following observations. First, the enzyme preparation used shows a single sharp band on polyacrylamide-gel electrophoresis, in the absence and presence of sodium dodecyl sulphate (Putman *et al.*, 1972). Secondly, the molecular weight is, by a number of methods, within 5% of 50000 (Putman *et al.*, 1972); the value of $E_{280}^{0.1\%}$ (see the experimental section) is not in doubt. However, the irreversibility of the labelling reaction was called into question when it was shown that on dialysis of inactivated enzyme overnight against aq. 0.5% (w/v) NH_4HCO_3 , essentially all the ^{32}P label was lost from the protein. No enzymic activity appeared.

Fig. 2. Inactivation of triose phosphate isomerase by bromohydroxyacetone phosphate in the presence (○) and absence (●) of 2-phosphoglycollate

Isomerase (300 μ g) in 2 ml of 0.5 M- β , β -dimethylglutarate buffer, pH 6.45, was added to 100 μ l of water (●) and to 100 μ l of 300 μ M-2-phosphoglycollate in water (○). To each solution was added 10 μ l of approx. 1.2 mM-bromohydroxyacetone phosphate in 0.5 M- β , β -dimethylglutarate buffer, pH 6.45. Portions (100 μ l) were removed from each solution at timed intervals, and diluted to 25 ml with water before assay of residual enzymic activity.

The fact that no enzymic activity reappeared on loss of 32 P suggested that the phosphate group might be lost from the inhibited enzyme more rapidly than the carbon skeleton. Accordingly, bromohydroxy-[14 C]acetone phosphate was synthesized from bromo-[2- 14 C]acetic acid of known specific radioactivity. It was found that enzyme inhibited with 14 C-labelled inactivator retained essentially all its radioactivity on dialysis at neutral pH, loss of label only becoming apparent at high pH values. After gel filtration at pH 7, the E_{280} and radioactivity of the protein fractions were measured. In a number of experiments, the stoichiometry of labelling of the enzyme by 14 C-labelled inactivator was found to be 2.1 ± 0.1 mol/50000 daltons of triose phosphate isomerase. These results provide further evidence for the view that triose phosphate isomerase contains two active sites and two identical subunits, of molecular weight 25000. No evidence for co-operation between subunits was obtained.

Further indication that bromohydroxyacetone phosphate reacts at the active site comes from the close similarity in u.v. difference spectra for the inactivated enzyme and the native enzyme with bound competitive inhibitor. Johnson & Wolfenden

Fig. 3. U.v. difference spectra

Curve (b) (taken from Johnson & Wolfenden, 1970) is of triose phosphate isomerase in triethanolamine-HCl buffer (0.02 M, pH 7.7) with 2-phosphoglycollate (0.25 mM) in double-compartment cuvettes. The mixed cuvette is in the sample beam. Curve (a) is of triose phosphate isomerase (0.772 mg/ml in β , β -dimethylglutarate buffer, 0.02 M, pH 6.5) with bromohydroxyacetone phosphate (1.5-fold molar excess) versus enzyme with P_i (equimolar with the inactivator concentration in the sample).

(1970) have determined the difference spectra for triose phosphate isomerase in the presence and absence of the inhibitors DL- α -glycerophosphate (K_i 0.15 mM) or 2-phosphoglycollate (K_i 6.8 μ M). These spectra are close to the difference spectrum of inactivated versus native enzyme, and are compared in Fig. 3. The evident similarity in the perturbation of the protein aromatic chromophores argues that the attachment of the $-\text{CH}_2-\text{CO}-\text{CH}_2-\text{OPO}_3^{2-}$ moiety is to a group at the enzyme's active site.

Isolation of labelled peptides

Triose phosphate isomerase inhibited with bromohydroxy[14 C]acetone phosphate was freed from excess of inhibitor by gel filtration in aq. 1% (w/v) NH_4HCO_3 . This material was reduced in guanidine hydrochloride (6 M) containing dithiothreitol (0.1 M) at 37°C for 3 h. Iodoacetic acid (to 0.08 M) was then added and the mixture left at room temperature for 30 min. The reduced, carboxymethylated protein was then dialysed exhaustively against aq. 0.5% (w/v) NH_4HCO_3 , and subjected to tryptic digestion (1%, w/v, trypsin) for 4 h at 37°C. The freeze-dried digest was then fractionated electrophoretically on paper at

pH 6.5. Most of the radioactivity was in the neutral band; traces that remained at the origin behaved on subsequent electrophoresis in a manner identical with the main (neutral) fraction. The paper containing the radioactivity was cut out and sewn into another sheet, for further electrophoresis at pH 8.9. The radioactive spot (of mobility 0.23 relative to 'Orange G') was eluted from the paper with aq. 0.25% (w/v) NH_4HCO_3 . No other radioactive areas were detected, so all the label in the original reduced, carboxy-methylated protein was contained in a single spot after two-dimensional paper electrophoresis. This tryptic peptide was too large for convenient characterization of the site of attachment of the radioactive label, and it was further digested with pepsin [1% (w/w) in aq. 5% (v/v) formic acid at 37°C overnight]. After electrophoresis at pH 6.5 and at pH 8.9 only one radioactive peptide was found. This material was Ehrlich-positive (Dawson *et al.*, 1969), showing the presence of tryptophan. Amino acid analysis yielded Glu_{1.1}, Pro_{1.0}, Ala_{1.0}, Val_{0.9}, Tyr_{0.9}. It is clear from the lack of other radioactive spots that this peptide uniquely contains the amino acid residue labelled by the inactivator.

The sequence of the peptide was determined by the dansyl-Edman method (Gray, 1967). Carboxypeptidase A digestion of the peptide yielded tryptophan as the only free amino acid. The sequence was: Ala-Tyr-Glu-Pro-Val-Trp [see Coulson *et al.* (1970b) for details].

Site of attachment of label

Since when the ^{32}P -labelled inhibitor is used, radioactivity is lost from the protein rather readily, ^{14}C -labelled inhibitor was used to identify the amino acid residue to which the label was attached. However, the fact that the phosphate moiety of the inhibitor was

lost from inactivated enzyme more readily than the carbon skeleton, whereas such phosphate groups are attached relatively firmly to the carbon skeleton in analogous small molecules (e.g. bromohydroxyacetone phosphate or dihydroxyacetone phosphate), makes it likely that the increased lability of the phosphate group in the inactivated enzymes was due to a displacement reaction by a neighbouring protein nucleophile rather than to some enhancement of hydrolytic lability. In this case, the position of the ^{14}C label (after phosphate loss) would be different from the primary site of attachment. To check this possibility, it was decided to 'lock' the phosphate group in place and prevent any migration of the label, by reduction of the carbonyl group of the inactivator immediately after incubation with the enzyme. Chemically, this carbonyl group will cause both α -substituents (that is, the phosphate group on C-1, and the enzyme on C-3) to be susceptible to nucleophilic displacement. After reduction, the lability of both groups will be very much less. The isolation of radioactive peptide from two different preparations of inactivated triose phosphate isomerase was carried out: with bromohydroxy[^{14}C]acetone phosphate, and with bromohydroxyacetone[^{32}P]phosphate followed immediately by the reduction of the carbonyl group at C-2 with NaBH_4 .

^{14}C -labelled peptide. The hexapeptide from protein inactivated with ^{14}C -labelled inhibitor has four possible sites of attachment: the two terminal groups, the phenolic group of tyrosine, and the carboxyl group of glutamic acid. Since the peptide was isolated by peptic digestion of a longer tryptic peptide, the C-terminus of which is lysine, clearly the α -carboxyl group of the C-terminal tryptophan in the hexapeptide cannot be the site of attachment. The α -amino group of N-terminal alanine in the hexapeptide is ruled out by the facts that (i) the

Table 1. Predicted and observed electrophoretic mobilities of active-site peptides from triose phosphate isomerase inactivated by bromohydroxyacetone phosphate

For experimental details see the text. Predicted values are by the method of Offord (1966). The predicted values for peptides containing phosphate groups are known to be less reliable than the others (Offord, 1966).

pH	Inhibitor attached to glutamic acid		Inhibitor attached to tyrosine		Observed mobility	
	With phosphate group	Without phosphate group	With phosphate group	Without phosphate group	^{14}C -labelled peptide	^{32}P -labelled peptide
6.5	0.4	0	0.66	0.31	0.31	0.31
6.5*	0.31; 0	0.31; 0	0.59; 0.31	0.59; 0.31	0.64	—
1.9	<0.2	0.57	<0.2	0.57	0.45	0
6.5†	—	0	—	0.31	—	0

* After performic acid oxidation [both possible products (Hassall, 1957) are shown].

† After phosphatase treatment.

N-terminal residue of the longer tryptic peptide is valine, (ii) from the hexapeptide, *N*-Dns-alanine is produced in good yield, and (iii) the hexapeptide is ninhydrin-positive. This leaves tyrosine and glutamic acid as possible sites of attachment. Table 1 shows the electrophoretic mobilities of the peptide, with the predicted mobilities (Offord, 1966) for the peptide with inhibitor (with or without the phosphate group) attached to either glutamic acid or tyrosine. Table 1 indicates that the most likely site of attachment of the ^{14}C label is tyrosine, with phosphate having been lost. Doubly labelled inhibitor was not prepared, but in parallel experiments, we know that phosphate is largely lost from enzyme under pH conditions less extreme than those used during the isolation and purification of the peptide. The electrophoretic mobility at pH 1.9 (see Table 1) confirms that phosphate has been lost. It can therefore be assumed that the ^{14}C -labelled hexapeptide contains no phosphate group, and the choice is between a mobility of 0 or 0.31. The actual mobility was 0.31, indicating that the γ -carboxylate group of glutamic acid is free, and the label is on the phenolic group of tyrosine. This assignment was confirmed by Baeyer–Villiger oxidation of the labelled peptide with performic acid [formic acid–100-vol. H_2O_2 , 19:1 (v/v), 6h, 0°C], which will convert some of the neutral hydroxy-acetonyl inhibitor into a carboxymethyl group (Hassall, 1957). This treatment roughly doubles the mobility of the peptide (see Table 1) and confirms the assignment of tyrosine as the site of covalent attachment.

^{32}P -labelled peptide. To effect reduction of the inhibitor carbonyl group after enzyme inactivation, solid NaBH_4 (equal to the weight of protein) was added to the enzyme after 5min incubation with bromohydroxyacetone [^{32}P]phosphate, and left for 10min. This treatment resulted in the ^{32}P label remaining attached to the protein throughout the digestion and purification of labelled peptide. Purification of the peptide followed the same course as for the ^{14}C -labelled material with an additional electrophoresis at pH 1.9. Amino acid analysis of the resulting radioactive peptide gave results identical with those obtained with ^{14}C -labelled material. That is, the two radioactive labels are found in the same peptide. If the tyrosine residue is not the primary site of attachment, then glutamic acid in this peptide remains as the only alternative (see the arguments above). Following the approach of Waley *et al.* (1970), the purified ^{32}P -labelled peptide was treated with acid phosphatase (2 μg , added to 10nmol of peptide in 0.2ml of 0.1M-pyridine–acetate buffer, pH 5.6, 37°C , 90min). If the glutamic acid residue were free, then removal of phosphate by phosphatase would leave the peptide negatively charged at pH 6.5, whereas if the label were attached to glutamic acid, the dephospho-peptide would be neutral at pH 6.5.

On electrophoresis at pH 6.5 of the phosphatase digest, two neutral, non-radioactive peptides were detected by ninhydrin. Each of them had the amino acid composition of the original peptide. Since only neutral material was produced, it is likely that glutamic acid is the site of attachment (via its γ -carboxyl group) of the inhibitor in the ^{32}P -labelled peptide. It is possible that the additional neutral spot was due to aminolysis of the glutamic γ -ester by the small amount of NH_3 present [phosphatase is supplied as an $(\text{NH}_4)_2\text{SO}_4$ suspension].

Confirmation that glutamic acid is the site of inhibitor attachment in this peptide was obtained by treatment with aminopeptidase M (1mg added to 100nmol of peptide in 0.4ml of 10mM-sodium phosphate buffer, pH 6.5, 4h, 37°C) followed by paper electrophoresis at pH 6.5. About 70% of the radioactivity was associated with a spot of electrophoretic mobility 0.59, whereas most of the ninhydrin-positive material remained near the origin. By staining the paper for tyrosine (with the α -nitroso- β -naphthol reagent: Dawson *et al.*, 1969), it was shown that this residue remained at the origin and was not associated with the radioactive spot of mobility 0.59. The same was found for tryptophan, by using the Ehrlich reagent (Dawson *et al.*, 1969). Amino acid analysis of this spot yielded amino acids as follows: glutamic acid (5.5nmol), proline (5.1nmol) with small amounts of contaminating serine and glycine (expected contaminants from the paper) and valine (0.80nmol) and tyrosine (0.27nmol). The radioactivity was estimated at 4–8nmol of ^{32}P . The predicted mobility (Offord, 1966) of a dipeptide containing proline and glutamic acid esterified with dihydroxyacetone phosphate is about 0.61. This experiment shows that a dipeptide (Glu, Pro) has the radioactivity associated with it, and confirms the attachment of the ^{32}P label to glutamic acid. The amount of tyrosine present is about one-twentieth of that required to account for the radioactivity associated with this spot.

It is evident, then, that when the inhibited enzyme is reduced with borohydride resulting in the firm association of the phosphate group to the enzyme, the glutamic acid residue in the peptide Ala-Tyr-Glu-Pro-Val-Trp is the site of attachment of the label. Chemical and hydrolytic lability considerations suggest the attachment as an ester to the γ -carbonyl group. If ^{14}C -labelled inhibitor is used without borohydride reduction, the phosphate group is lost, and the label is found on the tyrosine residue of the same peptide.

Inhibition of aldolase by bromohydroxyacetone phosphate

Attempts were made to inactivate aldolases both of class I and of class II (Rutter, 1964) with bromohydroxyacetone phosphate. Preliminary experiments showed that rabbit muscle and chicken muscle

aldolase activity is lost on incubation of the enzymes with bromohydroxyacetone phosphate, but the reaction is very much slower than that with triose phosphate isomerase. Even with a 2000-fold molar excess of inhibitor over enzyme, only 66% of the enzyme activity was lost in 1 h. The presence of 3 mM-D-arabinitol 1,5-diphosphate, a powerful competitive inhibitor with K_i 1.5 μM (Hartman & Barker, 1965), had no effect on the time-course of the inactivation. Conditions were searched for that would give both faster and more specific inhibition of the enzyme. At concentrations of enzyme between 6.8 and 12 μM , inhibitor concentration was varied over the range 2 to 22 mM. The pH of incubation varied from 5.2 to 7.2, being limited by the stability of enzyme and inhibitor respectively. Reactions were performed in the presence of 1 M-sodium phosphate, to check the effect of contaminating phosphate (K_i 5.5 mM) in the preparation of bromohydroxyacetone phosphate. Electrophoretically purified inhibitor (essentially phosphate-free) was also used. On the basis that bromohydroxyacetone phosphate is an analogue of substrate dihydroxyacetone phosphate, some incubations contained the other substrate also, 2 mM-D-glyceraldehyde 3-phosphate. In these experiments, the inactivation observed was no greater than that expected from inhibitor alone plus that effected by glyceraldehyde

3-phosphate (Lai *et al.*, 1968). The effect of added hexose substrate, 0.27 M-fructose 1,6-diphosphate (K_m 42 μM), was also checked. In no case did the inhibition become either significantly more rapid or, more importantly, active-site-specific.

Finally, in the hope of forcing reaction at the active site, attempts were made with the muscle enzyme to attach the inhibitor to the active-site lysine residue that is known to form a Schiff base with dihydroxyacetone phosphate (Morse & Horecker, 1968). In control experiments, aldolase was rapidly inactivated by incubation with dihydroxyacetone phosphate in the presence of NaBH_4 , but when substrate was replaced by chlorohydroxyacetone phosphate in an equimolar amount, only about 15% of the catalytic activity was lost.

Efforts to detect active-site-directed inhibition of yeast aldolase (a zinc-containing aldolase of class II) by bromohydroxyacetone phosphate were also frustrated, though Lin *et al.* (1971) have reported the relatively slow inactivation of yeast aldolase by chloro- or bromo-hydroxyacetone phosphate in 10- to 400-fold molar excess enzyme. Evidently aldolases of both classes are unaffected by anything except a large molar excess of haloacetone phosphate, which are so specific and very rapid inactivators of triose phosphate isomerase.

Scheme 2. Possible mechanism for the migration of active-site label from glutamic acid to tyrosine

Discussion

When triose phosphate isomerase is inactivated with bromohydroxyacetone [^{32}P]phosphate and the C-2 carbonyl group of the inhibitor moiety is immediately reduced with NaBH_4 , the lability of both the enzyme-inhibitor bond and the inhibitor-phosphate bond is decreased. The inhibitor is found to have esterified a unique glutamic acid residue in the sequence-Ala-Tyr-Glu-Pro-Val-Trp-. Catalytic activity is completely lost, and one inhibitor molecule per protein subunit (of approx. 25000 daltons) is incorporated. If ^{14}C -labelled inhibitor is used, the phosphate group is lost from the inactivated protein, and the carbon skeleton of the inhibitor is found attached to the tyrosine residue in the same peptide. This migration illustrates one of the less well-recognized dangers inherent in active-site-labelling work. Whenever an inactivated enzyme still contains sites open to nucleophilic attack, as is the case here, then migration is a real possibility. During the isolation of the peptide, pH variations will change the ionization state and nucleophilicity of neighbouring residues and may lead to intramolecular rearrangement reactions. Such migrations have been seen, for instance, in mono-acetylated glyceraldehyde 3-phosphate dehydrogenase, where an *S*-acylcysteine rearranges to an *N*-acyl-lysine derivative (Mathew *et al.*, 1967). In the present case, it appears that glutamic acid is the primary site of attachment of the label, and that subsequent migration to tyrosine occurs. This can be attractively rationalized as shown in Scheme 2. After primary reaction at the γ -carboxylate group of glutamic acid [(i) to (ii)], we have a system with two good leaving groups (phosphate and glutamic acid) α to the carbonyl group at C-2 of the inhibitor. Phosphate will be the more readily displaced by a nucleophile, and if the pH is raised (e.g. by dialysis against NH_4HCO_3 solution: see the Results section) the adjacent tyrosine residue may be well placed for displacement of phosphate, to give the bridged species (iii). This explains the fact that ^{32}P is lost from labelled protein more rapidly than is ^{14}C . At some point during peptide isolation this cyclic system hydrolyses, the better of the two leaving groups, glutamate, which has the lower $\text{p}K_a$, being displaced. This leaves the carbon skeleton of the inhibitor attached to tyrosine (iv).

Since glutamic acid is the primary site of attachment, it is pertinent to consider the evidence that this is actually at the active site, and that it is the identity of the enzyme base responsible for proton abstraction from substrate (see the introduction). Active-site modification is supported by the following facts: (a) the inactivation is a very fast reaction indeed, having a second-order rate constant around $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at room temperature; (b) the reaction is very specific: apparently no other sites in the protein are attacked; (c) the stoichiometry of labelling by ^{14}C -labelled

material agrees closely with independent evidence for an enzyme of two identical subunits each of mol. wt. 25000; (d) the powerful competitive inhibitor, 2-phosphoglycollate, protects the enzyme against inactivation (Fig. 2); and (e) the u.v. difference spectrum of inactivated enzyme (versus enzyme) is very similar to that of enzyme with competitive inhibitor (versus enzyme), as shown in Fig. 3. It is likely, then, that this particular glutamic acid residue is actually part of the active site of triose phosphate isomerase, and it is very possible that this residue acts as the postulated enzyme base in the catalytic act. The reagent bromohydroxyacetone phosphate was devised with this in mind, of course, but clarification of this point, and of the reasons for the extraordinarily high reactivity of this glutamic acid side chain, will have to await the solution of the crystal structure of the chicken enzyme and enzyme-substrate complex at high resolution (Banner *et al.*, 1971). It is assumed here that glutamic acid is the enzyme base, largely on the basis of structural economy, and the narrow specificities both of the enzyme and of the inhibitor (see below).

During the course of these studies, the work of other groups has pointed to this essential glutamic acid residue. Thus Hartman (1970a, 1971) has reported that chlorohydroxyacetone phosphate inactivates rabbit muscle triose phosphate isomerase with a stoichiometry of about 1.8 mol/53000 daltons of enzyme. After reduction with NaB^3H_4 , the label was attached to a glutamic acid residue in a peptide containing the same sequence as that found here. By using a different inhibitor, glycidol phosphate, that inactivates rabbit triose phosphate isomerase (Rose & O'Connell, 1969), it has been established (Waley *et al.*, 1970; Miller & Waley, 1971) that glutamic acid, in a hexapeptide of identical sequence to that found here, is the site of attachment. In this case, the epoxide-ring opening probably leads to the same product (chemically, if not stereochemically) as that from the halohydroxyacetone phosphates after borohydride reduction, though attack by the carboxylate group on the epoxide at C-2 of the inhibitor is a possibility.

The failure of either bromo- or chloro-hydroxyacetone phosphate to act as an active-site-directed inhibitor of class I or class II aldolases is surprising. The enzymes are inactivated by large molar excesses of reagent, but this is not affected by strong competitive inhibitors, and presumably is caused by non-specific reactions, very possibly with thiol groups. There is excellent evidence that aldolase binds dihydroxyacetone phosphate in the absence of the other substrate (glyceraldehyde 3-phosphate) and catalyses the exchange of one of the hydrogen atoms on C-3 with the solvent (Rose & Rieder, 1955). The most reasonable postulate is that an enediolate anion is formed (this is supported by the results of trapping

experiments with both classes of aldolases: Riordan & Christen, 1969), by the abstraction of carbon-bound hydrogen by an enzymic base just as is postulated for triose phosphate isomerase. The anions arising from the two enzymes are enantiomeric. From the failure even to label aldolase with chloro- or bromo-hydroxyacetone phosphate by reduction of the expected Schiff base with borohydride, it appears that these materials may not bind at the active site. Evidently aldolase is even more specific for dihydroxyacetone phosphate than is triose phosphate isomerase, though this view is not substantiated by experiments on the catalysed rate of exchange of the methyl hydrogen atoms of monohydroxyacetone phosphate (acetol phosphate) with solvent. Aldolase is considerably more effective in the catalysis of this exchange than is triose phosphate isomerase (Putman *et al.*, 1972). Nor is the problem clarified by the fact that whereas the aldolase-bound carbanion from dihydroxyacetone phosphate reacts rapidly with tetranitromethane (Riordan & Christen, 1969), the putative carbanion bound to triose phosphate isomerase cannot be efficiently trapped by this approach (A. Bissett, unpublished work). It may be that our views of the triose phosphate isomerase active site are oversimplified, or that aldolase (but not isomerase) requires the C-3 hydroxyl group for substrate binding. We must await the crystallographic results to resolve this enigma.

In partial agreement with the present work, Hartman (1970b) has found that muscle aldolase is inactivated by iodohydroxyacetone phosphate, and has pointed to the oxidation of thiol groups as a cause of the activity loss. He did not find significant inhibition by the bromo- or chloro-compounds. On the other hand, Meloche (1970) has successfully used bromopyruvate to inactivate an analogous aldolase, namely 3-deoxy-2-oxo-6-phosphogluconate aldolase, for which pyruvate is the first substrate. Evidently the specificity requirements of this last enzyme allow the successful application of the now accepted approach to active-site-directed inhibition.

The high specificity of bromohydroxyacetone phosphate for triose phosphate isomerase can be turned to good account, however. The isomerase is a remarkably effective catalyst ($k_{\text{cat.}}/K_m$ for glyceraldehyde 3-phosphate is $2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$; Knowles *et al.*, 1971) and a very small contamination of other glycolytic enzymes by isomerase can, on a weight basis, still result in unacceptably high isomerase activities. Many of the experiments reported elsewhere (Knowles *et al.*, 1971) required isomerase-free preparations of aldolase, glyceraldehyde 3-phosphate dehydrogenase and α -glycerophosphate dehydrogenase. Brief (10 min) incubation of these enzymes at pH 7 with $10 \mu\text{M}$ -bromohydroxyacetone phosphate, followed by gel filtration, allows the isolation of enzymes containing undetectably low isomerase activity.

We are grateful to the Science Research Council (for A. F. W. C.), the Medical Research Council (for S. de la M., R. E. O. and J. D. P.) and Merck, Sharp and Dohme (for R. E. O.) for support, and to Dr. S. G. Waley for helpful discussions. This is a contribution from the Oxford Enzyme Group.

References

- Arndt, F. (1943) *Organic Syntheses* Collective vol. 2, 165
- Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C. & Pogson, C. I. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 151
- Baranowski, T. & Niederland, T. R. (1949) *J. Biol. Chem.* **180**, 544
- Bayliss, R. S., Knowles, J. R. & Wybrandt, G. B. (1969) *Biochem. J.* **113**, 377
- Beisenherz, G. (1955) *Methods Enzymol.* **1**, 387
- Bloom, B. & Topper, Y. J. (1956) *Science* **124**, 982
- Bloom, B. & Topper, Y. J. (1958) *Nature (London)* **180**, 1128
- Briggs, A. P. (1922) *J. Biol. Chem.* **53**, 15
- Coulson, A. F. W., Knowles, J. R. & Offord, R. E. (1970a) *Chem. Commun.* **7**
- Coulson, A. F. W., Knowles, J. R., Priddle, J. D. & Offord, R. E. (1970b) *Nature (London)* **227**, 180
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd. edn., p. 529, Clarendon Press, Oxford
- Eagles, P. A. M., Johnson, L. N., Joynson, M. A., McMurray, C. H. & Gutfreund, H. (1969) *J. Mol. Biol.* **45**, 533
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469
- Hanes, C. S. & Isherwood, F. A. (1949) *Nature (London)* **164**, 1107
- Hartman, F. C. (1968) *Biochem. Biophys. Res. Commun.* **33**, 888
- Hartman, F. C. (1970a) *J. Amer. Chem. Soc.* **92**, 2170
- Hartman, F. C. (1970b) *Biochemistry* **9**, 1783
- Hartman, F. C. (1971) *Biochemistry* **10**, 146
- Hartman, F. C. & Barker, R. (1965) *Biochemistry* **4**, 1068
- Hassall, C. H. (1957) *Org. React.* **9**, 73
- Husain, S. S. & Lowe, G. (1968) *Biochem. J.* **108**, 855
- Johnson, L. N. & Wolfenden, R. (1970) *J. Mol. Biol.* **47**, 93
- Knowles, J. R., Leadlay, P. F. & Maister, S. G. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 157
- Lai, C. Y., de Dretz, G. M., Bacila, M., Marinello, E. & Horecker, B. L. (1968) *Biochem. Biophys. Res. Commun.* **30**, 665
- Lin, Y., Kobes, R. D., Norton, I. C. & Hartman, F. C. (1971) *Biochem. Biophys. Res. Commun.* **45**, 34
- Mathew, E., Meriwether, B. P. & Park, J. H. (1967) *J. Biol. Chem.* **242**, 5024
- Meloche, H. P. (1970) *Biochemistry* **9**, 5050
- Miller, J. C. & Waley, S. G. (1971) *Biochem. J.* **123**, 163
- Morse, D. E. & Horecker, B. L. (1968) *Arch. Biochem. Biophys.* **125**, 942
- Norton, I. L., Pfuderer, P., Stringer, C. D. & Hartman, F. C. (1970) *Biochemistry* **9**, 4952
- Offord, R. E. (1966) *Nature (London)* **211**, 591

- Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B. & Knowles, J. R. (1972) *Biochem. J.* **129**, 301
- Rieder, S. V. & Rose, I. A. (1956) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **15**, 337
- Rieder, S. V. & Rose, I. A. (1959) *J. Biol. Chem.* **234**, 1007
- Riordan, J. F. & Christen, P. (1969) *Biochemistry* **8**, 2381
- Rose, I. A. (1962) *Brookhaven Symp. Biol.* **15**, 293
- Rose, I. A. & O'Connell, E. L. (1969) *J. Biol. Chem.* **244**, 6548
- Rose, I. A. & Rieder, S. V. (1955) *J. Amer. Chem. Soc.* **77**, 5764
- Rutter, W. J. (1964) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **23**, 1248
- Shaw, E. (1970) *Physiol. Rev.* **50**, 244
- Waley, S. G., Miller, J. C., Rose, I. A. & O'Connell, E. L. (1970) *Nature (London)* **227**, 181
- Wolfenden, R. (1969) *Nature (London)* **223**, 704