The Existence of an Electrophilic Component in the Reaction Catalysed by Triose Phosphate Isomerase

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In the presence of triose phosphate isomerase, the substrate dihydroxyacetone phosphate is reduced stereoselectively by NaBH_4 . The reduction of enzyme-bound substrate is almost completely or completely stereoselective and occurs about one order of magnitude faster than that in free solution. This acceleration implies a polarization of the carbonyl group when dihydroxyacetone phosphate is bound.

Triose phosphate isomerase (EC 5.3.1.1) catalyses the rapid interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. A chemically attractive pathway, proposed by Rose (1962), involves proton transfers between C-1 of dihydroxyacetone phosphate and C-2 of D-glyceraldehyde 3-phosphate mediated by a single base on the enzyme. The transfer occurs on the *re-re* face of the proposed *cis*-enediol intermediate (Rose, 1958). A hexapeptide containing a uniquely labelled glutamic acid residue has been isolated from the enzyme after inactivation with an active-site-directed inhibitor (de la Mare *et al.*, 1972) and it was suggested that the γ -carboxyl group of this glutamic acid is the base responsible for the proton-shuttling process.

To explain the very high catalytic activity of the enzyme and, more particularly, the fact that dihydroxyacetone phosphate enolizes some 10^7 -fold faster when bound to the isomerase than when in free solution (W. J. Albery & J. R. Knowles, unpublished work), the presence of an electrophilic function in the active site has been postulated. This function would take part in the reaction by interacting with the carbonyl groups of the substrates, so increasing the lability of the α -protons. The present paper provides the first evidence for the involvement of an electrophile in the isomerase-catalysed reaction.

Experimental

Triose phosphate isomerase was prepared from chicken muscle in this laboratory by Mr. J. Law, by the method of Putman *et al.* (1972). Glycerol phosphate dehydrogenase and acid phosphatase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Glycerokinase, adenosine 5'-triphosphate, NAD^+ , NADH , DL-glycerol 3-phosphate and triethanolamine hydrochloride were all from Sigma (London) Chemical Co. Ltd., London

S.W.6, U.K. DL-Glyceraldehyde 3-phosphate (as the monobarium salt of the diethyl ketal) and dihydroxyacetone phosphate (as the cyclohexylammonium salt of the dimethyl ketal) were also from Sigma. Sodium boro[^3H]hydride (570 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and stored as a solution in 1 M-NaOH (50 mCi/ml), frozen at -20°C . DEAE-cellulose anion-exchange resin (Whatman DE-52) was purchased from W and R. Balston Ltd., Maidstone, Kent, U.K. Bio-Gel P-10 polyacrylamide gel [100–200 mesh (wet)] was from Bio-Rad Laboratories, St. Albans, Herts., U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest grade available. All solutions were made up with deionized distilled water.

The concentration of triose phosphate isomerase solutions was calculated from the absorbance by assuming an $E_{280}^{0.1\%}$ value of 1.21 for a 10 mm light-path. The activity of the enzyme was measured by using a slightly modified version of the coupled-enzyme method of Beisenherz (1955) with glyceraldehyde 3-phosphate as substrate.

L-Glycerol 3-phosphate concentration was determined by measuring the formation of NADH from NAD^+ during the oxidation of L-glycerol 3-phosphate to dihydroxyacetone phosphate catalysed by glycerol phosphate dehydrogenase. The reaction was done in 1 M-glycine–1 M-hydrazine hydrate buffer (2.3 ml, pH 9.3) and NAD^+ (4 mg). The change in absorbance at 340 nm due to NADH formation was measured by using a Pye Unicam SP.1800 spectrophotometer.

DL-[2- ^3H]Glycerol 3-phosphate was prepared by reduction of dihydroxyacetone phosphate with sodium boro[^3H]hydride. Dihydroxyacetone phosphate (0.4 μmol) and sodium boro[^3H]hydride (0.5 mCi) were incubated in 200 mM-triethanolamine-HCl buffer (1.0 ml, pH 7.5) at room temperature for 2 h. DL-Glycerol 3-phosphate (4 mg) was added as

carrier and the glycerol phosphate was purified on a column (12cm $\times$ 0.9cm) of DEAE-cellulose, equilibrated with 5mM-NH₄HCO₃, pH 8.3, and eluted with a linear gradient (50ml) from 5mM- to 300mM-NH₄HCO₃. The glycerol phosphate (of constant specific radioactivity, 1.4×10^5 c.p.m./ μ mol, across the peak) was freeze-dried and stored at 4°C.

[³H]Glycerol 3-phosphate was prepared in the presence of triose phosphate isomerase by incubating together sodium boro[³H]hydride (3.75mCi), dihydroxyacetone phosphate (0.4 μ mol) and the isomerase (3ml of a solution of 1.5, 6.5 or 30mg/ml), previously dialysed against 100mM-NH₄HCO₃ containing 1mM-EDTA, at pH 7.3 to 7.5, 20°C, for 3h. The catalytic activity of the isomerase was always determined immediately before the incubation. After the incubation, protein was removed by gel filtration through P-10 polyacrylamide gel (22cm $\times$ 1.6cm). DL-Glycerol 3-phosphate (1mg) was added as carrier, and glycerol phosphate was isolated on a column of DEAE-cellulose as described above. In a control experiment, dihydroxyacetone phosphate was reduced by sodium boro[³H]hydride in the presence of triose phosphate isomerase that had been completely and specifically inactivated by bromohydroxyacetone phosphate (de la Mare *et al.*, 1972).

Analysis of [³H]glycerol 3-phosphate for the L-enantiomer. The [³H]glycerol phosphate was dissolved in 50mM-NH₄HCO₃ to give a solution, approx. 0.2mM in L-glycerol phosphate. To a portion (2ml) of glycerol phosphate solution was added NAD⁺ (8mg) and hydrazine hydrate (200 μ l), after which the pH was adjusted to 9.4 with 1M-HCl. The formation of NADH was measured spectrophotometrically after the addition of the dehydrogenase (200 μ l of a 2mg/ml solution, previously dialysed against 20mM-triethanolamine-HCl buffer, pH 7.45). The formation of NADH was quantitative under these conditions. NAD³H and the remaining [³H]glycerol phosphate (the D-enantiomer) were separated by chromatography on DEAE-cellulose (Whatman DE-52) as described above. The concentration of NADH in each fraction was calculated from the absorbance at 340nm by assuming an extinction coefficient of 6220M⁻¹.cm⁻¹ (Horecker & Kornberg, 1948). From the specific radioactivity of NAD³H, the proportion of L-[³H]glycerol phosphate present in the original mixture could be calculated. The D-[³H]glycerol 3-phosphate that remained was freeze-dried.

Analysis of [³H]glycerol 3-phosphate for the D-enantiomer. The D-glycerol 3-phosphate was dissolved in water (4ml) and the pH adjusted to 5.6 with 1M-HCl. Incubation with acid phosphatase (5 μ g) overnight at 37°C hydrolysed the ester to glycerol. Enzyme was removed by shaking the solution with DEAE-cellulose (Whatman DE-52) (4ml of slurry). To the filtered solution of glycerol

was added MgCl₂ (2mg), ATP (8mg) and glycerokinase (0.5ml of a 0.2mg/ml solution, previously dialysed against 20mM-triethanolamine-HCl buffer, pH 7.45). The pH was adjusted to 9.0 with 1M-NaOH and the solution incubated at room temperature for 2h. Under these conditions glycerokinase catalyses the formation of L-glycerol 3-phosphate from glycerol and ATP (Bublitz & Kennedy, 1954). The oxidation of L-glycerol 3-phosphate, formation of NADH and isolation of NAD³H proceeded as above. From the specific radioactivity of NAD³H, the amount of D-[³H]glycerol 3-phosphate present in the original mixture was calculated.

Results and discussion

In the presence of triose phosphate isomerase, the substrate species present are, for dihydroxyacetone phosphate: unhydrated form enzyme-bound, free unhydrated form and free hydrate [the last two in the ratio 55:45 (Reynolds *et al.*, 1971)]; and for D-glyceraldehyde 3-phosphate: unhydrated form enzyme-bound, free unhydrated form and free hydrate [the last two in the ratio 3:97 (Trentham *et al.*, 1969)]. The hydrated forms bind much more weakly (if at all) to the enzyme than do the unhydrated forms (H. B. Coates & K. A. McLauchlan, unpublished work), the latter having been shown to be the forms handled by the enzyme (Reynolds *et al.*, 1971; Trentham *et al.*, 1969). The values of the dissociation constant (*K_s*) for each of the unhydrated substrates is 0.7mM (dihydroxyacetone phosphate) and 5 μ M (D-glyceraldehyde 3-phosphate) (W. J. Albery & J. R. Knowles, unpublished work).

The four species present which are susceptible to reduction by sodium boro[³H]hydride are the free and enzyme-bound unhydrated forms of dihydroxyacetone phosphate and of D-glyceraldehyde 3-phosphate. The product in each case is [³H]glycerol phosphate and in the present paper we are concerned with the stereochemistry at C-2 resulting from reduction of dihydroxyacetone phosphate.

All ³H on C-2 of the L-glycerol 3-phosphate is transferred to NAD⁺ in the oxidation catalysed by glycerol phosphate dehydrogenase. The D-enantiomer is unaffected. After separation of the NAD³H and the D-glycerol 3-phosphate, the latter is treated in turn with acid phosphatase and then glycerokinase-Mg²⁺-ATP. These reactions convert the D-glycerol 3-phosphate into its L-enantiomer. The ³H that was on C-2 of the D-isomer is now measured by transfer to NAD⁺ in the presence of the dehydrogenase. Because a very large molar excess of unlabelled DL-glycerol 3-phosphate over the tritiated compound is added before analysis, there are equimolar amounts of the D and L-enantiomers and the ratio of specific radioactivities of the two batches of isolated NAD³H gives the ratio of ³H at C-2 of the L- and the D-glycerol

Table 1. Specific radioactivity of glycerol 3-phosphate and of NADH for the analyses of the reduction of dihydroxyacetone phosphate under various conditions

Total triose phosphate isomerase (mN)*	Dihydroxy-acetone phosphate (mM)	Sodium boro-hydro [3H]hydride (mCi/ml)	Unliganded triose phosphate isomerase (mN)*†	10 ⁻⁶ × Specific radio-activity of [3H]glycerol 3-phosphate from reduction of dihydroxyacetone phosphate (c.p.m./μmol)‡	10 ⁻⁶ × Specific radioactivity of NAD ³ H		R§
					From oxidation of L-glycerol 3-phosphate (c.p.m./μmol)	From oxidation of D-glycerol 3-phosphate after inversion of configuration (c.p.m./μmol)	
0	0.40	0.50	0	14.0	13.0	11.6	1.1
1.20	0.13	1.25	1.13	2.83	2.70	0.07	38.5
0.25	0.13	1.25	0.23	10.90	11.40	2.26	5.0
0.060	0.13	1.25	0.055	6.10	5.27	2.54	2.1
0.30	0.13	1.25	—	3.31	1.62	1.39	1.16

* The normality (N) of active sites was calculated by using a subunit molecular weight of 25000.

† Calculated by using the values of the equilibrium constants for each step of the triose phosphate isomerase-catalysed reaction (W. J. Albery & J. R. Knowles, unpublished work).

‡ After dilution with unlabelled material. This column lists the values of c.p.m./μmol of D-+L-glycerol-3-phosphate.

§ See the text.

|| Inactivated by bromohydroxyacetone phosphate; see the text.

3-phosphates. Table 1 gives the specific radioactivities of [3H]glycerol phosphate and NAD³H for reduction of dihydroxyacetone phosphate under various conditions. (Reduction of D-glyceraldehyde 3-phosphate gives D-glycerol 3-phosphate with ³H on C-1. This ³H is not transferred to NAD⁺ during the reactions described. The high specific radioactivity of the [3H]glycerol phosphate, compared with the sum of specific radioactivities of NAD³H for each experiment, shows that ³H is present on C-1 to 40–50% of the total ³H on glycerol 3-phosphate.)

If k_{+1} is the rate constant for the non-stereoselective reduction of unbound dihydroxyacetone phosphate and k_{+2} and k_{+3} are the rate constants for the reduction of enzyme-bound dihydroxyacetone phosphate to L- and to D-glycerol 3-phosphate respectively, then the rate ratio (R) for the production of L- and D-glycerol 3-phosphates is:

$$\frac{L}{D} = \frac{k_{+1} \cdot s/2 + k_{+2} \cdot es}{k_{+1} \cdot s/2 + k_{+3} \cdot es} = R$$

where s is the concentration of unbound unhydrated substrate and es is the concentration of the isomerase-dihydroxyacetone phosphate complex. Since $K_s = \frac{s \cdot e_{free}}{es}$, where e_{free} is the concentration of unliganded isomerase:

$$\frac{1}{R-1} = \frac{0.5k_{+1} \cdot K_s}{e_{free}(k_{+2} - k_{+3})} + \frac{k_{+3}}{k_{+2} - k_{+3}}$$

In Fig. 1 ($1/R-1$) is plotted against $1/e_{free}$. From the vertical intercept, k_{+2}/k_{+3} is approx. 130. This means that the reduction occurring at the enzyme active

Fig. 1. Plot of $1/(R-1)$ versus $1/e_{free}$

R is the ratio of the L/D enantiomers of glycerol 3-phosphate, and e_{free} is the concentration of unliganded isomerase (mN).

site is almost completely or completely stereoselective in favour of the L-enantiomer, which arises by the approach of borohydride on to the *si* face of the carbonyl group of bound dihydroxyacetone phosphate. On the basis that the borohydride approaches the more accessible face of the triose phosphate, the *re* face is the one shielded from the solvent. Since the product of dihydroxyacetone phosphate isomerization is the D-enantiomer of glyceraldehyde 3-phosphate, the proton is delivered by the enzyme to the *re* face of the dihydroxyacetone phosphate.

Scheme 1. Possible involvement of an electrophilic group (EH^+) in the triose phosphate isomerase-catalysed reaction

This face is, as expected, shielded from borohydride attack.

To confirm that the stereoselective reduction of dihydroxyacetone phosphate occurs in the active site and not elsewhere on the enzyme surface, the reduction was done in the presence of isomerase whose active site had been blocked by reaction with the specific inactivator bromohydroxyacetone phosphate (de la Mare *et al.*, 1972). Under these conditions the substrate is reduced with little or no stereoselectivity (Table 1). At the concentrations used and a fully accessible active site a stereoselectivity of about 6 would be expected.

From the gradient of the line in Fig. 1, $(k_{+2} - k_{+3})/k_{+1} = 10.1K_s$. Since $k_{+2}/k_{+3} > 100$, the ratio of rate constants $(k_{+2} - k_{+3})/k_{+1}$ gives the ratio of rates of reduction on the enzyme active site and in free solution [i.e. $(k_{+2} + k_{+3})/k_{+1}$]. The reduction occurs approx. seven times faster on the enzyme surface than in free solution, so that the enzyme must be catalysing the reduction. This catalysis suggests the existence of an electrophilic group that increases the

polarization of the substrate carbonyl group, and may well participate in the triose phosphate isomerization mechanism, increasing the lability of the α -protons. This electrophile may either be on the enzyme as shown in Scheme 1, or the substrate may be constrained at the active site in a cisoid conformation that allows the C-1 hydroxyl group to form a hydrogen bond with the carbonyl group. Such a possibility would attractively accommodate the fact that monohydroxyacetone phosphate suffers isomerase-catalysed proton exchange some 10^4 times slower than dihydroxyacetone phosphate (Putman *et al.*, 1972).

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