The Orientation and Accessibility of Substrates on the Active Site of Triosephosphate Isomerase†

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ABSTRACT: Tritiated sodium borohydride was used to reduce the substrates of triosephosphate isomerase in the presence of the enzyme, and the mixture of the four possible products (D-[1(*R*)-^{3}H]-; D-[1(*S*)-^{3}H]-; D-[2-^{3}H]-, and L-[2-^{3}H]glycerol 3-phosphate) was analyzed. While enzyme-bound dihydroxyacetone phosphate is reduced completely stereoselectively and at a rate eight times faster than in free solution, D-glyceraldehyde 3-phosphate is inaccessible to reduction by borohydride when bound to the active site of the enzyme.

The mechanism by which triosephosphate isomerase (EC 5.3.1.1) catalyzes the rapid interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate is well defined (Rose, 1962) and the energetics of the catalyzed reaction have been studied in detail (Knowles et al., 1971; S. J. Fletcher, J. M. Herlihy, P. F. Leadlay, S. G. Maister, C. Pett, W. J. Albery and J. R. Knowles, unpublished work). Proton transfer between C-1 and C-2 of the triosephosphate occurs on the *re-re* face of the proposed *cis*-enediol intermediate (Rose, 1958) and the transfer is apparently mediated by a single base in the active site (de la Mare et al., 1972; J. M. Herlihy, unpublished work). The unhydrated forms of each of the triose phosphates have been shown to be the forms handled by the enzyme (Reynolds et al., 1971; Trentham et al., 1969), the hydrates (of dihydroxyacetone phosphate, at least) binding very weakly if at all (H. B. Coates and K. A. McLauchlan, private communication).

Sodium borohydride has been used previously as a probe to investigate the orientation of substrate carbonyl groups in the active sites of oxaloacetate decarboxylase (Kosicki and Westheimer, 1968) and of pyruvate kinase (Phillips et al., 1973). The substrate, pyruvate in each case, was reduced stereoselectively to lactate at rates which showed that the enzyme was catalyzing the reduction. Because each of the substrates of triosephosphate isomerase has a carbonyl group that may be reduced, and because very detailed information about the enzyme-catalyzed reaction is available, the use of borohydride reduction is an attractive method of obtaining information about the orientation of bound substrate and the accessibility of each of the two substrate carbonyl groups. We report here on the analysis of the products of reduction of the enzyme-bound substrates by sodium boro[^{3}H]hydride. In a previous communication (Webb and Knowles, 1974) we described the analysis of the [^{3}H]glycerol 3-phosphate labeled at C-2 that derives from the reduction of bound dihydroxyacetone phosphate. The reduction of this substrate on the enzyme was found to be completely stereoselective and the rate of its reduction in free solution was shown to be about eight times less than that on the enzyme. This suggested the existence of an electrophilic group at the active site that polarizes the carbonyl group of bound dihydroxyacetone phosphate and thus increases the rate of reduction. Such an electrophilic group may be involved in the isomerase-catalyzed reaction effecting the more facile abstraction of the *pro-R* proton on C-1 (see Figure 1). In this paper, we report the methods of analysis for tritium in the glycerol phosphate product, and the relative extent of labeling of the 1-*pro-R* and 1-*pro-S* positions of D-[1-^{3}H]glycerol 3-phosphate resulting from reduction of D-glyceraldehyde 3-phosphate in the presence of large amounts of enzyme. The reactions involved in this analysis are outlined in Scheme I.

Experimental Section

Materials. Triosephosphate isomerase was prepared from chicken muscle by Mr. J. Law, by the method of Putman et al. (1972). Bromohydroxyacetone phosphate was prepared by Mr. L. M. Fisher, using the method of de la Mare et al. (1972), and was stored as a solution in ether at 4°C.

The following were obtained from Sigma (London) Chemical Co. Ltd., London SW6, U.K.: glycerol kinase, acid phosphatase, glycerolphosphate dehydrogenase, lactate dehydrogenase, aldolase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase; ATP, NAD^+, NADH, $NADP^+$, DL-glycerol 3-phosphate, sodium pyruvate, D-fructose 6-phosphate, and D-mannose 6-phosphate. 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. Fructose 1,6-bisphosphatase, mannose-6-phosphate isomerase, and D-glyceraldehyde 3-phosphate (as the cyclohexylammonium salt of the diethyl ketal) were obtained from Boehringer Corp. (London) Ltd., London W5, U.K.

Sodium boro[^{3}H]hydride (570 Ci/mol and 8.2 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K., and stored at −20°C as solutions in 1 *M* NaOH (50 mCi/ml and 200 mCi/ml, respectively). Tritiated water (5 Ci/ml) was also from the Radiochemical Centre and was stored 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 British Drug Houses Chemicals

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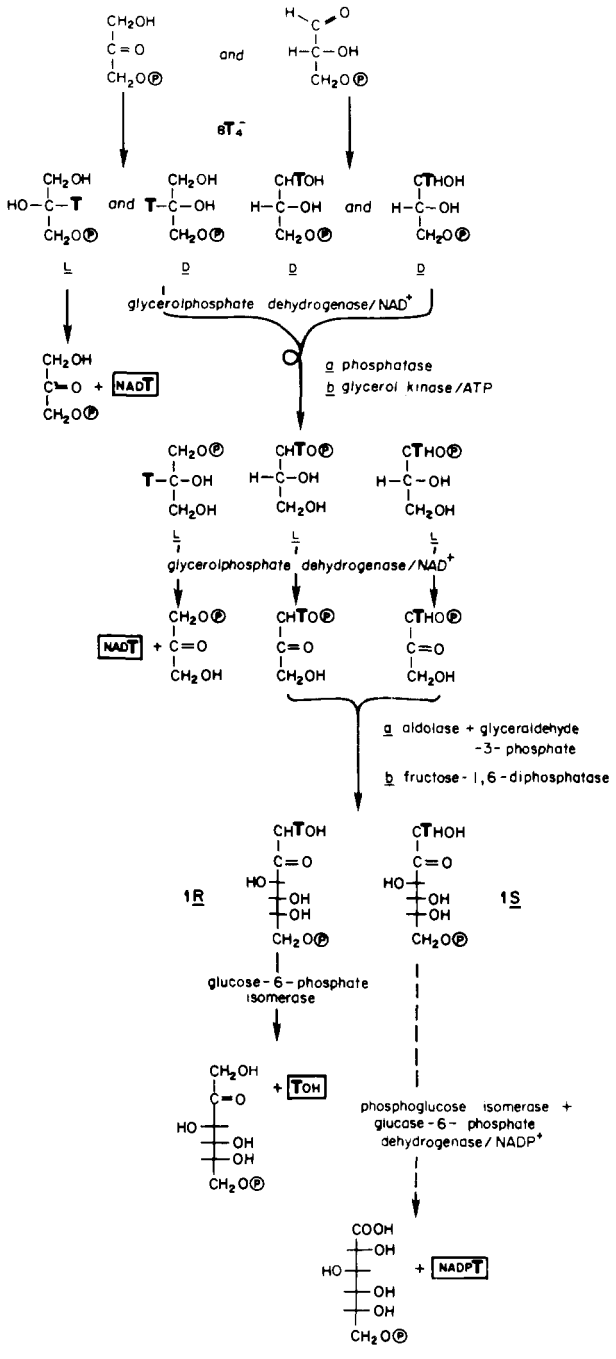
Scheme I: Outline of Reactions Involved in the Analysis of the Products Derived from Boro[^{3}H]hydride Reduction of Triose Phosphates.



Ltd., Poole, Dorset, U.K., and were of the highest grade available. All solutions were made up with deionized, distilled water.

Methods. The concentration of solutions of triosephosphate isomerase was calculated 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, slightly modified, the coupled-enzyme method of Beisenherz (1955) with glyceraldehyde 3-phosphate as substrate.

Dihydroxyacetone phosphate concentrations were assayed by measuring the conversion of NADH to NAD^+ during its reduction to L-glycerol 3-phosphate catalyzed by glycerolphosphate dehydrogenase. The assay reaction was performed in 200 m*M* triethanolamine-HCl buffer (pH 7.5) (1.3 ml) containing 0.4 m*M* NADH. The decrease in

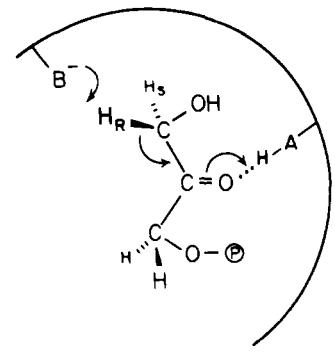


FIGURE 1: Postulated involvement of an electrophilic component (H-A) in the triosephosphate isomerase catalyzed reaction.

NADH concentration was measured by following the change in absorption at 340 nm using a Pye Unicam SP 1800 spectrophotometer. The concentration of D-glyceraldehyde 3-phosphate was measured by a similar method with triosephosphate isomerase (10 μg) in the reaction mixture to catalyze the formation of dihydroxyacetone phosphate.

The concentration of L-glycerol 3-phosphate was measured using the oxidation catalyzed by glycerolphosphate dehydrogenase. The reaction was performed in 1 *M* glycine–1 *M* hydrazine buffer (pH 9.3) (2.3 ml) containing NAD^+ (4 mg). The formation of NADH was followed spectrophotometrically.

D-Glucose 6-phosphate was assayed by its oxidation to D-glucono-δ-lactone 6-phosphate catalyzed by glucose-6-phosphate dehydrogenase/$NADP^+$. The reaction was performed in 200 m*M* triethanolamine-HCl buffer (pH 7.5) (2.3 ml) containing 10 m*M* $MgCl_2$ and 0.25 m*M* $NADP^+$. Addition of glucose-6-phosphate isomerase (0.05 mg) to the assay mixture enabled the concentration of D-fructose 6-phosphate to be measured separately from D-glucose 6-phosphate.

The activity of glucose-6 phosphate isomerase was measured by coupling the isomerization of fructose 6-phosphate to glucose 6-phosphate with the latter's oxidation to D-glucono-δ-lactone 6-phosphate catalyzed by glucose-6-phosphate dehydrogenase. The reaction was performed in 200 m*M* triethanolamine-HCl buffer (pH 7.5) (2.3 ml) containing 10 m*M* $MgCl_2$, 0.5 m*M* $NADP^+$, glucose-6-phosphate dehydrogenase (0.1 mg/ml), and 60 m*M* fructose 6-phosphate. Mannose-6-phosphate isomerase activity was assayed similarly using 60 m*M* mannose-6-phosphate and glucose-6-phosphate isomerase (0.02 mg/ml).

Preparation of [^{3}H]Glycerol 3-Phosphate from 3-Phosphohydroxyacetonyltriosephosphate Isomerase. Bromohydroxyacetone phosphate concentration was estimated from the rapid stoichiometric inactivation of known concentrations of triosephosphate isomerase. The enzymatic activity was measured 15 min after each addition of bromohydroxyacetone phosphate.

Triosephosphate isomerase (0.5 ml of a 14-mg/ml solution) previously dialyzed against 100 m*M* NH_4HCO_3 (pH 7.6) was assayed for enzymic activity and cooled to 0°C. A 1.2-fold molar excess of bromohydroxyacetone phosphate was added and the pH adjusted immediately to 7.5 by addition of 2 *N* NaOH. After 10 min at 0°C the solution was assayed to check that all the enzyme had been inactivated.

Sodium boro[^{3}H]hydride (2 mCi, 15.4 μmol) was added to this solution of 3-phosphohydroxyacetonyltriosephosphate isomerase, the pH was readjusted to 7.5 by addition

of 1 *N* HCl, and the solution was incubated for 3 hr at 20°C. The protein was isolated by gel filtration through a column (22 cm × 1.6 cm) of P-10 polyacrylamide gel. 2 *N* NaOH was added to the pooled fractions that contained protein until the pH was 10.2 and incubation at this pH for 2 hr at 60°C achieved hydrolysis of the 3-phospho-[2-^{3}H]glyceryltriosephosphate isomerase ester linkage. A control experiment showed that the product, [2-^{3}H]glycerol 3-phosphate, is stable under these conditions. The hydrolysate was then subjected to gel filtration as above. The eluate showed two radioactive peaks, the earlier, smaller one corresponding to protein and the later, larger one corresponding to the expected elution position of glycerol 3-phosphate. To the pooled fractions of this later peak was added DL-glycerol 3-phosphate as carrier and the material was purified on a column (12 cm × 0.9 cm) of DEAE-cellulose, equilibrated with 5 m*M* NH_4HCO_3. A linear gradient (50 ml) from 5 to 300 m*M* NH_4HCO_3 eluted the glycerol 3-phosphate, which had constant specific radioactivity across the peak. This product was freeze-dried and stored at 4°C.

Preparations of [^{3}H]Glycerol 3-Phosphate from Triose Phosphates. The formation of [^{3}H]glycerol 3-phosphate in the presence of triosephosphate isomerase for the analysis of the stereochemistry of tritium labeling at C-2 and the formation of DL-[2-^{3}H]glycerol 3-phosphate were as described earlier (Webb and Knowles, 1974).

DL-[1(*RS*)-^{3}H]Glycerol 3-phosphate was prepared by the reduction of DL-glyceraldehyde 3-phosphate (4 μmol) by sodium boro[^{3}H]hydride (1.25 mCi) which were incubated together in 200 m*M* triethanolamine-HCl buffer (pH 7.5) (0.7 ml) for 2 hr at room temperature. The glycerol 3-phosphate was purified by ion-exchange chromatography as described above. D-[1(*RS*)-^{3}H]Glycerol 3-phosphate was prepared by the reduction of D-glyceraldehyde 3-phosphate (2 μmol) by sodium boro[^{3}H]hydride (2.5 mCi), from which the product was obtained as above.

DL-[1(*S*)-^{3}H]Glycerol 3-phosphate was prepared as follows. Aldolase was treated with bromohydroxyacetone phosphate to inactivate any triosephosphate isomerase impurity and the enzyme solution was then dialyzed against 200 m*M* triethanolamine-HCl buffer (pH 7.5). A portion (0.1 mg) of this solution was added to dihydroxyacetone phosphate (0.5 μmol) in tritiated water (120 μl of a solution, 4 Ci/ml). Aldolase catalyzes the exchange of the 1-*pro*-*S*-hydrogen of dihydroxyacetone phosphate with the solvent. After incubation for 2 hr, the solution was freeze-dried to remove tritiated water. The residue was dissolved in 0.1 *M* HCl (2 ml) and kept at pH 1.5 for 5 min to inactivate the enzyme. The pH of the solution was raised to 8.0 with triethanolamine. Sodium borohydride (10 mg) was added and the solution was left for 3 hr at room temperature to reduce the [1(*S*)-^{3}H]dihydroxyacetone phosphate to DL-[1(*S*)-^{3}H]glycerol 3-phosphate. The product was then purified on DEAE-cellulose as described above.

For analysis of the stereochemistry of labeling by tritium at C-1, [^{3}H]glycerol 3-phosphate was prepared by incubating together in 100 m*M* NH_4HCO_3 buffer (pH 7.6) (3 ml) for 2 hr at room temperature, triosephosphate isomerase (7.2 mg/ml), dihydroxyacetone phosphate (0.4 μmol), and sodium boro[^{3}H]hydride (11.71 mCi of 1.6 Ci/mmol material). After this incubation, protein was removed by gel filtration through a column (22 cm × 1.6 cm) of P-10 polyacrylamide gel. The [^{3}H]glycerol 3-phosphate was further purified by chromatography on DEAE-cellulose as previously described.

The analysis of [^{3}H]glycerol 3-phosphate to obtain the amount of tritium at C-2 corresponding to the D and L enantiomers of [2-^{3}H]glycerol 3-phosphate was described earlier (Webb and Knowles, 1974).

Analysis of [^{3}H]Glycerol 3-Phosphate for the [1(R)-^{3}H] Isomer. The freeze-dried [^{3}H]glycerol 3-phosphate was dissolved in water (1 ml) to which was added acid phosphatase (10 μg) and then 1 *N* HCl until the pH of the solution was 5.6. Incubation for 12 hr at 37°C resulted in complete hydrolysis of the phosphate ester. The solution was passed through a small volume (4 ml) of DEAE-cellulose resin, equilibrated in 5 m*M* NH_4HCO_3 to remove any unreacted glycerol phosphate and enzyme. The glycerol was then phosphorylated by addition of ATP (10 mg), $MgCl_2$ (5 mg), and glycerol kinase (0.04 mg). After raising the pH to 8.8 by addition of 1 *N* NaOH, the solution was incubated for 2 hr at 25°C. L-[^{3}H]Glycerol 3-phosphate was purified from this solution by chromatography on DEAE-cellulose as described above. The fractions containing glycerol 3-phosphate were freeze-dried and then dissolved in water (3 ml). Portions of this solution were converted to D-fructose 1,6-bisphosphate as described below.

A solution (0.7 ml) containing aldolase (1.5 mg), glycerolphosphate dehydrogenase (0.5 mg), and lactate dehydrogenase (0.25 mg) in 200 m*M* triethanolamine-HCl buffer (pH 7.5) was treated with bromohydroxyacetone phosphate (0.1 μmol) and then dialyzed against 50 m*M* triethanolamine-HCl buffer (pH 7.5). An assay for triosephosphate isomerase activity showed that this treatment had inactivated all traces of isomerase.

To each portion (1 ml) of L-[^{3}H]glycerol 3-phosphate were added NAD^+ (15 mg), sodium pyruvate (5 mg), DL-glyceraldehyde 3-phosphate (0.2 ml of a 45 m*M* solution), and the isomerase-free enzyme mixture prepared above. After raising the pH to 9.6 by addition of 1 *N* NaOH, the solution was left for 10 min at room temperature. Under these conditions, dihydroxyacetone phosphate, an intermediate in this enzyme-catalyzed formation of D-fructose 1,6-bisphosphate, is stable. To terminate the reaction, the solution was rapidly mixed with Dowex-50 resin (4 ml, H^+ form). The solution was washed from the resin with approximately 10 ml of water and the pH of the combined washings was raised to 8.0 with 1 *N* NaOH. Purification on DEAE-cellulose, as above, gave three radioactive peaks, corresponding to glycerol 3-phosphate, NAD^+, and D-fructose 1,6-bisphosphate. The fractions containing D-fructose 1,6-bisphosphate from each portion of [^{3}H]glycerol phosphate were pooled and freeze-dried.

This preparation of D-fructose 1,6-bisphosphate was dissolved in water (1 ml), to which was added fructose 1,6-bisphosphatase (0.25 mg) and $MgCl_2$ (5 mg). The pH of the solution was raised to 8.8 with 1 *N* NaOH and, after incubation for 3 hr at 37°C, D-fructose 6-phosphate was isolated from the solution by ion-exchange chromatography as previously described. This radioactive material was freeze-dried and then redissolved in water (1 ml). Glucose-6-phosphate isomerase (0.1 mg) was added to labilize the 1-*pro*-*R*-hydrogen of D-fructose 6-phosphate and the solution in a sealed flask was incubated for 90 min at 37°C for this exchange to occur. The water was then isolated by bulb-to-bulb distillation in a closed system with the sample flask connected to the receiving flask by a short U-tube with a side arm. While the sample was immersed in liquid nitrogen, the system was evacuated and then isolated from the vacuum pump. To effect the distillation, the liquid nitrogen

bath was moved to the receiving flask. After the distillation was complete, the residue was dissolved in water (1 ml) and the total radioactivity of the residue and of the distillate was measured. More glucose-6-phosphate isomerase (0.1 mg) was added to the residue, which was again taken through the process of incubation and distillation to ensure that all exchangeable tritium had been removed from the residue. The proportion of tritium exchanged by the isomerase and removed by distillation gives the proportion of tritium in the 1-*pro-R* position of the original D-glycerol 3-phosphate (see Scheme I).

Analysis of [^{3}H]Glycerol 3-phosphate for the [1(S)-^{3}H] Isomer. The residue from the above exchange experiment is an equilibrium mixture of labeled D-fructose 6-phosphate and D-glucose 6-phosphate. D-Fructose 6-phosphate (1.5 mg) was added as carrier to this mixture, which was then purified on DEAE-cellulose as previously described. The [^{3}H]hexose 6-phosphate fractions had constant specific radioactivity across the peak and this pooled material was freeze-dried. This residue was redissolved in water (10 ml) to which was added $NADP^+$ (20 mg), $MgCl_2$ (5 mg), glucose-6-phosphate isomerase (0.05 mg), and glucose-6-phosphate dehydrogenase (0.2 mg). The increase in absorbance at 340 nm was followed as $NADP^+$ was reduced to NADPH during the oxidation of hexose 6-phosphate to D-glucono-δ-lactone 6-phosphate, catalyzed by the isomerase (to convert D-fructose 6-phosphate to D-glucose 6-phosphate) and the dehydrogenase. After the reaction was complete, NADPH was isolated on a DEAE-cellulose column (15 cm × 0.9 cm). Elution with a gradient (50 ml) from 150 to 500 m*M* NH_4HCO_3 gave NADPH with a constant specific radioactivity across the peak. From the specific radioactivity of NADPH and of the hexose 6-phosphate, the proportion of tritium on the 1-*pro-S* position of D-glycerol 3-phosphate can be calculated.

Results

Reduction of dihydroxyacetone phosphate produces [2-^{3}H]glycerol 3-phosphate, which can be analyzed by the method described earlier (Webb and Knowles, 1974), which involves the transfer of tritium at C-2 of L-glycerol 3-phosphate to NAD^+, catalyzed by glycerolphosphate dehydrogenase (see Scheme I). The oxidation of the D enantiomer does not occur and the glycerol 3-phosphate remaining after this treatment is solely the D enantiomer. Treatment of this material with acid phosphatase and then rephosphorylation of the glycerol using glycerol kinase/ATP results in L-glycerol 3-phosphate, with the phosphate, in effect, having been transferred from C-3 to C-1. The tritium at C-2 of what is now the L enantiomer of glycerol 3-phosphate can now be transferred to NAD^+ using glycerolphosphate dehydrogenase. The radioactivity of each batch of NADH gives the tritium content of the L- and the D-[2-^{3}H]glycerol 3-phosphate present originally at the 2 position.

Reduction of 3-Phosphohydroxyacetonyltriosephosphate Isomerase by Borohydride. Bromohydroxyacetone phosphate, a close structural analog of dihydroxyacetone phosphate, is an irreversible, active-site inhibitor of triosephosphate isomerase and reacts very rapidly and stoichiometrically with each enzyme subunit with complete loss of isomerase activity (de la Mare et al., 1972). After attachment to the enzyme, the phosphohydroxyacetonyl moiety may be reduced by sodium borohydride and this has been used to stabilize the phosphate ester linkage to hydrolysis (de la Mare et al., 1972).

In the present work, the validity of using boro[^{3}H]hydride to reduce substrate carbonyl groups of the enzyme-bound triose phosphates themselves was first checked by reduction of the carbonyl group of the 3-phosphohydroxyacetonyl moiety of the enzyme inactivated as described above. The successful reduction of this species, covalently bound in the active site, would augur well for the reduction of the noncovalently bound substrate molecules.

Reduction of the phosphohydroxyacetonyl moiety was achieved by sodium boro[^{3}H]hydride and this was followed by the alkaline hydrolysis of the ester link of the 3-phosphoglyceryl–enzyme, under conditions in which the glycerol-phosphate linkage is stable, to yield [2-^{3}H]glycerol 3-phosphate. This hydrolysis caused the release of approximately 90% of the radioactivity associated with protein after reduction.

The [^{3}H]glycerol 3-phosphate, with DL-glycerol 3-phosphate added as a carrier, was purified and analyzed for tritium at C-2 as outlined in Scheme I. The carrier glycerol phosphate is present in very large excess over that hydrolyzed from the enzyme, so that the concentrations of D and L enantiomer are equal. The specific radioactivity of the [^{3}H]glycerol 3-phosphate was 1.35×10^5 cpm/μmol. The NADH produced during the oxidation of the L enantiomer had a specific radioactivity of 2.09×10^5 cpm/μmol, while the specific radioactivity of the NADH from the oxidation of remaing (D-)glycerol 3-phosphate (after the latter's inversion by the phosphatase–glycerol kinase sequence) was less than 0.35×10^5 cpm/μmol. Thus at least 80% of the tritium on C-2 was in a position corresponding to L-glycerol 3-phosphate.

In a control experiment, triosephosphate isomerase was inactivated by bromohydroxyacetone phosphate and then dihydroxyacetone phosphate was added. The mixture was treated with sodium boro[^{3}H]hydride and [^{3}H]glycerol 3-phosphate was separated from the solution *without* hydrolysis of the phosphoglyceryl–enzyme bond. So the glycerol phosphate analyzed was that due only to the reduction of free dihydroxyacetone phosphate, in the presence of enzyme with every active site blocked. The analysis showed equal proportions of tritium (within 2%) on each enantiomer of glycerol 3-phosphate (Webb and Knowles, 1974).

Reduction of the Two Substrates in the Presence of Triosephosphate Isomerase. In principle, reduction of the two triose phosphates with sodium boro[^{3}H]hydride can produce four different tritiated isomers of glycerol phosphate. Glyceraldehyde 3-phosphate is reduced to the 1*R* and 1*S* isomers with tritium at C-1, and dihydroxyacetone phosphate gives the D and L enantiomers with tritium at C-2. The amount of each of these four species can be estimated by the transformations outlined in Scheme I.

(a) Analysis of the Products of Reduction at C-2. In free solution dihydroxyacetone phosphate exists predominantly in the hydrated and unhydrated forms in the ratio 45:55 at 20°C (Reynolds et al., 1971). The hydrate binds much more weakly (if at all) to triosephosphate isomerase than does the unhydrated form (H. B. Coates and K. A. McLauchlan, private communication), the latter being the species handled by the enzyme (Reynolds et al., 1971). The value of the dissociation constant (K_s) for the unhydrated form is 0.7 m*M* (W. J. Albery and J. R. Knowles, unpublished work), so that for given concentrations of enzyme and of dihydroxyacetone phosphate, the percentage of the unhydrated form that is enzyme bound can be calculated. [The percentage of enzyme that is complexed with other species

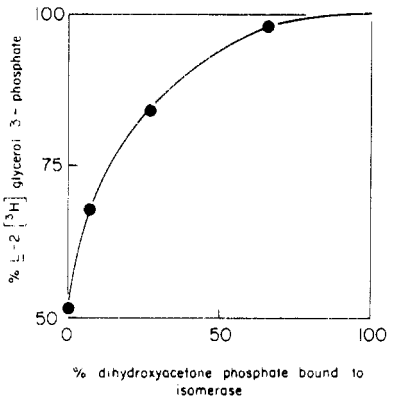


FIGURE 2: Plot of stereoselectivity of reduction of dihydroxyacetone phosphate by boro[^{3}H]hydride (measured by the percentage L enantiomer in the total [2-^{3}H]glycerol 3-phosphate) vs. the percentage of dihydroxyacetone phosphate bound to the isomerase.

of triose phosphate is negligible (W. J. Albery and J. R. Knowles, unpublished work).]

When reductions were performed at various concentrations of enzyme, the results illustrated in Figure 2 were obtained for analysis of the product of reduction of dihydroxyacetone phosphate. It is evident from this figure that the reduction of enzyme-bound dihydroxyacetone phosphate is completely stereoselective and that this reduction is accelerated relative to the reduction in free solution (for further discussion see Webb and Knowles, 1974). Assuming that all the triose phosphate species are in rapid equilibrium during the reduction (which they will be, considering the very high turnover number of the enzyme and the large enzyme concentration) analysis of the results indicates that the rate of reduction of dihydroxyacetone phosphate in the active site is eight times faster than that in free solution.

(b) Analysis of the Reduction at C-1. D-Glyceraldehyde 3-phosphate in free solution exists in the hydrated and the unhydrated forms in a ratio of 97:3 (Trentham et al., 1969). The latter form has been shown to be that which is handled by triosephosphate isomerase in the catalytic reaction (Trentham et al., 1969). The equilibrium constant of the unhydrated forms of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate is 420 in favor of dihydroxyacetone phosphate (at 37°C). So for the triose phosphates at equilibrium in the presence of the isomerase, the amount of reduction occurring at C-1 might be expected to be small, even allowing for the greater susceptibility of aldehydes, compared with ketones, to reduction by borohydride. Preliminary experiments indicated that the percentage of tritium at C-1 was indeed low, so in order to obtain enough tritiated glycerol 3-phosphate to analyze the tritium distribution at this position, sodium boro[^{3}H]hydride of higher specific radioactivity was used. The same large molar excess (17-fold) of boro[^{3}H]hydride over triose phosphate was used in all reductions of substrate in the presence of the isomerase to ensure that the reaction was pseudo-first-order. Sodium borohydride at this concentration has no effect on the activity of the isomerase in the presence or absence of substrate over the time period of the reduction.

The reactions involved in the analysis of tritium at C-1 of D-[^{3}H]glycerol 3-phosphate are shown in Scheme I. The transfer of the phosphate to the tritiated carbon atom by the phosphatase–glycerol kinase sequence not only produces the L enantiomer of glycerol 3-phosphate, the form handled by the dehydrogenase, but also protects against the adventitious exchange of these tritium atoms into the solvent. Only during the incubation with fructose 1,6-bisphosphatase is this phosphate removed from the tritiated carbon to give D-fructose 6-phosphate.

The two reactions involved in the conversion of L-glycerol 3-phosphate to D-fructose 1,6-bisphosphate were coupled and carried out at high pH in order to overcome the unfavorable equilibrium constant of the reaction catalyzed by glycerolphosphate dehydrogenase. To increase the rate of formation of dihydroxyacetone phosphate, the NADH also formed was rapidly reoxidized by coupling the glycerol phosphate oxidation to the reduction of pyruvate to lactate, catalyzed by lactate dehydrogenase. A high concentration of D-glyceraldehyde 3-phosphate was present to provide carbon atoms 4, 5, and 6 of the fructose 1,6-bisphosphate. However, as D-glyceraldehyde 3-phosphate in large concentrations inhibits aldolase irreversibly (Adelman et al., 1968), a large concentration of this enzyme was also required. During this two-reaction step it is crucial that no triosephosphate isomerase is present as it would rapidly form dihydroxyacetone phosphate from the D-glyceraldehyde 3-phosphate, so reversing the glycerol phosphate oxidation and preventing any tritiated material reaching the D-fructose 1,6-bisphosphate.

After removal of the 1-phosphate of D-fructose 1,6-bisphosphate with fructose 1,6-bisphosphatase, treatment of the resulting D-fructose 6-phosphate with glucose-6-phosphate isomerase labilizes the 1-*pro*-*R*-hydrogen (Rose and O'Connell, 1960) and all the 1*R*-tritium is exchanged into the solvent. Distillation of the solvent from the frozen solution under vacuum allows the measurement of both the radioactivity exchanged into the solvent and the radioactivity remaining associated with the residue (as hexose 6-phosphate). Addition of carrier D-fructose 6-phosphate to the residue and purification by ion-exchange chromatography allow the specific radioactivity of the hexose 6-phosphate to be calculated. The tritium from the [1(*S*)-^{3}H]glycerol 3-phosphate is now present in the aldehydic hydrogen of glucose 6-phosphate and in the 1-*pro*-*S*-hydrogen of fructose 6-phosphate. This hydrogen is transferred to $NADP^+$ during the reaction catalyzed by glucose-6-phosphate dehydrogenase. The resulting $NADP^3H$ was isolated by ion-exchange chromatography and the specific radioactivity of the purified material was determined.

The validity of the analytical scheme depends in part on the purity of the enzymes used. Thus, while glucose-6-phosphate isomerase labilized the 1-*pro*-*R*-hydrogen of D-fructose 6-phosphate, any contaminating mannose-6-phosphate isomerase would also labilize the 1-*pro*-*S*-hydrogen (i.e., the hydrogen to be transferred to $NADP^+$) (Topper, 1957; Rose and O'Connell, 1960). Accordingly, fructose 1,6-bisphosphatase, glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase were all tested for glucose-6-phosphate isomerase activity and for mannose-6-phosphate isomerase activity. No significant contaminating activity (i.e., less than 0.05%) was found. Had a mannose-6-phosphate isomerase contaminant been present, the 1-*S*-tritium would have been exchanged into the solvent. Glucose-6-phosphate isomerase activity in the fructose 1,6-bisphosphatase would have labilized the 1-*pro* *R*-hydrogen before the controlled "wash out" procedure.

Table I shows the results from the C-1 analysis of [^{3}H]glycerol 3-phosphate from reduction of D-glyceraldehyde 3-phosphate alone, of DL-glyceraldehyde 3-phosphate alone, and of dihydroxyacetone phosphate, with the equilib-

Table I: Radioactivity Measurements for the Analysis of Tritium in the 1*R* and 1*S* Positions of D-[^{3}H]Glycerol 3-Phosphate.

Reduction of	Triose phosphate (m*M*)	Triose-phosphate Isomerase (m*N*)[b]	Sodium Boro[^{3}H]-hydride (m*M*)	Radioactivity Measurement × 10^{-5} (cpm) after Exchange of 1 *R* Tritium into the solvent[c]		Ratio of Tritium in 1 *R* Position to Tritium in the Residue	Specific Radioactivities × 10^{-4} (cpm μmol^{-1}) for Determination of 1*S*-Tritium[d]		Ratio of Tritium in 1*S* Position, to Tritium in the D-Hexose 6-phosphate	Ratio of Tritium in Each Position [1-^{3}H]Glycerol 3-Phosphate *R*/*S*
				Residue (D-Hexose 6-Phosphate)	Solvent (Water)		D-Hexose 6-Phosphate	NADPH		
D-Glyceraldehyde 3-phosphate	1.95		1.2	2.473	1.303	0.53	6.76	3.45	0.51	1.04
DL-Glyceraldehyde 3-phosphate	2.10		0.6	0.821	0.437	0.53	1.19	0.67	0.56	0.95
Dihydroxyacetone phosphate[a]	0.13	0.29	2.2	1.070	0.375	0.35	4.14	1.44	0.35	1.00
Dihydroxyacetone phosphate[a]	0.13	0.38	2.2	0.340	0.195	0.57	0.85	0.48	0.57	1.00
	[1(*S*)-^{3}H]Glycerol 3-phosphate[e]			0.414	0.014	0.03	0.92	0.95	1.03	0.03

[a] Dihydroxyacetone phosphate, the triose phosphate added, rapidly equilibrates with D-glyceraldehyde 3-phosphate in the presence of the large concentration of triosephosphate isomerase, and both substrates are therefore available for reduction both on and off the enzyme. [b] The normality of the isomerase active sites was calculated assuming a subunit molecular weight of 25,000. [c] Incubation of labeled fructose 6-phosphate with glucose-6-phosphate isomerase results in the complete exchange of tritium from the 1*R* position into the solvent water. The amount of radioactivity removed by distillation and the amount of radioactivity remaining in the residue of D-hexose 6-phosphate were each measured. The radioactivity in the water represents the amount of tritium in the 1*R* position of the original fructose 6-phosphate. [d] The residue of D-hexose 6-phosphate (after removal of the 1*R* tritium) was diluted with a large excess of unlabeled fructose 6-phosphate and its specific radioactivity was determined. Oxidation of this material with glucose-6-phosphate dehydrogenase/$NADP^+$ in the presence of glucose-6-phosphate isomerase results in the transfer of the C-1 tritium of glucose 6-phosphate, to $NADP^+$. The specific radioactivity of the tritiated $NADP^3H$ measures the amount of tritium that derives from the 1*S* position of the original fructose 6-phosphate. [e] Control experiment in which authentic [1*S*-^{3}H]glycerol 3-phosphate was subjected to the analytical procedure of Scheme I. See the text.

rium proportion of D-glyceraldehyde 3-phosphate in the presence of triosephosphate isomerase. As a control on the analytical procedure for the tritium at C-1 of glycerol 3-phosphate (see Scheme I), DL-[1(*S*)-^{3}H]glycerol 3-phosphate was analyzed using this sequence. Only the tritium from the D enantiomer reaches the hexose phosphate stage and only this is therefore analyzed. The tritium in the L enantiomer stays in the 1-*pro*-*S*-hydrogen of dihydroxyacetone phosphate and this hydrogen is removed during the reaction with D-glyceraldehyde 3-phosphate catalyzed by aldolase. The analysis (see Table I) confirms that essentially all (ca. 97%) of the tritium of the D enantiomer is in the 1-*S* position and therefore the stereochemical integrity of the hydrogen atoms of C-1 of glycerol phosphate is unaffected by the reaction sequence involved in the analysis outlined in Scheme I.

Discussion

Bromohydroxyacetone phosphate rapidly and irreversibly esterifies a unique glutamic acid residue in the active site of triosephosphate isomerase (de la Mare et al., 1972) and the resulting phosphohydroxyacetonyl moiety can be reduced by borohydride stereoselectively from the *si* face. When dihydroxyacetone phosphate is reduced in the presence of the isomerase that has had all its active sites blocked by bromohydroxyacetone phosphate, there is no stereoselective reduction of the free substrate. These two results show that stereoselective reduction occurs specifically in the active site and requires the active site to be accessible to the carbonyl-containing species and to reducing agent.

Reduction of dihydroxyacetone phosphate on the surface of the enzyme has been shown to occur completely stereoselectively from its *si* face, and this reduction occurs at a rate approximately eight times faster than reduction in free solution (Webb and Knowles, 1974). On the basis of stereoselective reduction of both the covalently bound 3-phosphohydroxyacetonyl group and of the noncovalently bound dihydroxyacetone phosphate, it was expected that the carbonyl group of noncovalently bound D-glyceraldehyde 3-phosphate would also be reduced stereoselectively by borohydride. However, the results in Table I show that there is *no* stereoselectivity in the reduction of this aldehydic carbonyl, either in the presence or absence of the isomerase.

Before considering the implications of this result with regard to enzyme–substrate interactions, the lack of stereoselectivity observed for reduction in free solution deserves mention. D-Glyceraldehyde 3-phosphate, being a chiral molecule, would be expected to yield preferentially one or the other of the diastereoisomeric D-[1-^{3}H]glycerol 3-phosphates on boro[^{3}H]hydride reduction. In the reduction of free D-glyceraldehyde 3-phosphate in the absence of enzyme, however, no stereochemical preference was observed. Reductions of both D- and of DL-glyceraldehyde 3-phosphate were studied and in the case of the racemic mixture, it should be noted that the reduction of the L enantiomer will cause radioactivity to appear during the analysis in the 3 position of the hexose 6-phosphates, a position where it does not affect the analysis. Only reduction products from the D enantiomer are observed in this analytical method (Scheme I). The only observable effect that the presence of the L enantiomer might have could be in the generation of a reducing agent that is a complex between a chiral substrate (or product) molecule and borohydride. Such complexing evidently does not occur, and the results for reduction in free solution also preclude the more likely possibility of sig-

nificant asymmetry in the attack on the carbon atom next to the chiral center (Cram and Abd Elhafez, 1952).

Returning to the complete lack of stereoselectivity of reduction of D-glyceraldehyde 3-phosphate in the presence of triosephosphate isomerase, the result can be interpreted in three different ways. First, reduction on the surface of the enzyme could occur with complete lack of stereoselectivity. In view of the results from the reduction of bound dihydroxyacetone phosphate and of the 3-phosphohydroxyacetonyl moiety, this is unlikely. The enzyme is an extremely bulky, chiral molecule and is known to deliver a proton (in the catalytic reaction) solely to the *re* face of the carbonyl group (Rose, 1958). The base that transfers this proton must be in fairly close proximity to the carbonyl group, so at least some degree of stereoselectivity would be expected.

The second possible explanation is that the proportion of unhydrated D-glyceraldehyde 3-phosphate attached to the enzyme is so small that the reduction product derives exclusively from reduction in free solution. This explanation implies that the dissociation constant (K_s) for the enzyme–unhydrated substrate complex is greater than 1 m*M* (even assuming that the rate of reduction on the enzyme is only the same as that in free solution). However, it is known that the enediol and unhydrated D-glyceraldehyde 3-phosphate are in rapid equilibrium with each other on the surface of the isomerase (Knowles et al., 1971) and that the combined dissociation constant for these two species is approximately 5 μM (W. J. Albery and J. R. Knowles, unpublished work). A dissociation constant of 1 m*M* for D-glyceraldehyde 3-phosphate alone would then require an equilibrium constant between these two enzyme-bound species of at least 200 in favor of the enediol, which would in turn require a rate constant for enolization of enzyme-bound glyceraldehyde 3-phosphate to be at least 10^6 sec^{-1}. [The rate constant for conversion of enzyme-bound dihydroxyacetone phosphate to the enediol is known to be $\sim 2 \times 10^3$ sec^{-1} (W. J. Albery and J. R. Knowles, unpublished work).] This explanation, therefore assumes that the D-glyceraldehyde 3-phosphate binds very poorly to the enzyme and requires an improbably large rate of enolization of this enzyme-bound substrate.

The third and most attractive interpretation of the lack of stereoselectivity observed for reduction of D-glyceraldehyde 3-phosphate is simply that the rate constant for reduction of the enzyme-bound substrate is much less than that in free solution. In the case of dihydroxyacetone phosphate some parallels could be drawn between the enzyme-catalyzed enolization process and the enzyme-catalyzed reduction by borohydride. Both the proton abstraction of the enolization and the hydride attack on the carbonyl of the reduction process are expected to be similarly accelerated by polarization of the carbonyl group, when the substrate is bound to the enzyme. However, the steric requirements of the two reactions may be very different. In the reduction of enzyme-bound dihydroxyacetone phosphate the polarization of the carbonyl group that leads to more rapid reduction evidently outweighs any decrease in reactivity due to inaccessibility. For D-glyceraldehyde 3-phosphate, however, no acceleration of reduction and no stereoselective reaction by borohydride can be observed and the probable explanation is simply that the carbonyl group of D-glyceraldehyde 3-phosphate is, for steric reasons, inaccessible to borohydride. This prevents any reduction in the active site at C-1 of the substrate. The subtle difference in accessibility of C-1 and C-2 of the enzyme-bound substrates should be clarified by the promising high resolution crystallographic work of triosephosphate isomerase and of its complex with substrate (Banner et al., 1975).

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