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# Energetics of Triosephosphate Isomerase: Deuterium Isotope Effects in the Enzyme-Catalyzed Reaction†

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ABSTRACT: The effect of isotopic substitution of the specifically labilized hydrogen in the substrates of triosephosphate isomerase on the steady-state rates of the enzyme-catalyzed reaction has been examined. The $k_{cat}$ value for the enzyme-catalyzed transformation of [1(*R*)-$^2$H]dihydroxyacetone phosphate is 2.9 times smaller than that for the 1(*R*)-$^1$H compound. Because of the rapid loss of $^2$H to solvent from the enzyme–enediol complex, this factor represents the full kinetic isotope effect of the proton abstraction step. The values of $k_{cat}$ and of $K_m$ for D-[2-$^2$H]glyceraldehyde 3-phosphate are indistinguishable from those of the 2-$^1$H material. This arises from the rapid loss of $^2$H from the enzyme–enediol intermediate, which results in $^1$H rather than $^2$H transfer in the rate-limiting step. The steady-state kinetic results reported in this paper qualitatively confirm and quantitatively extend the results from the previous papers on the variation of the free energy along the reaction path.

The use of tritium labeling as a mechanistic probe for the reaction catalyzed by triosephosphate isomerase has been described in the previous three papers (Herlihy et al., 1976; Maister et al., 1976; Fletcher et al., 1976). The use of this hydrogen isotope is normally confined to competitive situations, in which isotope effects in product-forming steps become apparent from the discrimination between hydrogen and tritium in the product under scrutiny. This approach is particularly useful for the investigation of reaction steps that may not be rate limiting in the overall reaction. Deuterium can often be used in a similar manner, but in addition, the use of deuterium-labeled substrates provides information about the importance of the particular protonation and deprotonation steps in the actual rate of the overall process (see Figure 5 of Albery and Knowles, 1976a).

We present here the steady-state parameters for the reaction of the two specifically deuterated substrates, [1(*R*)-$^2$H]-dihydroxyacetone phosphate and D-[2-$^2$H]glyceraldehyde 3-phosphate, with triosephosphate isomerase.

## Experimental Section

*Materials.* Enzymes, substrates, cofactors, and other materials were as specified by Herlihy et al. (1976). Additionally, phosphoenolpyruvate (monopotassium salt) and pyruvate kinase (from rabbit muscle, as a crystalline suspension in ammonium sulfate) were obtained from the Sigma Chemical Co. (London, England). $^2H_2O$ (>99.8% isotopic purity) was obtained from Norsk Hydroelektrisk (through Rivan Chemicals, Southampton, England).

[*1(R)-$^2$H*]*Dihydroxyacetone phosphate* was prepared as follows. Dowex 50 ($H^+$ form, 100–200 mesh, 4% cross-linked), 3.5 g, was washed thoroughly with $^2H_2O$. The precursor of dihydroxyacetone phosphate (dicyclohexylammonium 2,2-dimethoxy-1,3-propanediol phosphate), 25 mg, was dissolved in $^2H_2O$ (2 ml) and swirled with the washed resin for 30 s, and the resin was then removed by rapid filtration. The resin was washed with small portions of $^2H_2O$ and the combined filtrates were incubated at 37 °C for 4.5 h. The "pH" of the solution was then adjusted to ca. 7.5 by the addition of anhydrous $K_2CO_3$. To this solution containing dihydroxyacetone phosphate (48 μmol) in $^2H_2O$ (3 ml) was added triosephosphate isomerase (5 μl of a solution of 16 mg/ml) and the mixture left at 20 °C for 1.5 h. All operations up to this point were performed in stoppered vessels with guard tubes to prevent the incursion of atmospheric moisture. After the incubation with isomerase, the solution was cooled to 0 °C and the "pH" lowered rapidly to about 4 by the addition of 1 M HCl. The mixture was then applied to a column (15 × 1.5 cm$^2$) of Dowex 1 ($Cl^-$ form, 200–400 mesh, 8% cross-linked) equilibrated at 4 °C with 0.16 mM HCl, pH 3.8. The column was washed with

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

ten column volumes of dilute HCl, pH 3.2, and the triose phosphates were eluted with 22 mM HCl, pH 1.66. Fractions of 2 ml were collected. Fractions containing the triose phosphates ([1(*R*)-$^2$H]dihydroxyacetone phosphate and D-[2-$^2$H]glyceraldehyde 3-phosphate) were pooled, and the pH was raised to ca. 7 with 1 M NaOH. To this solution (28 ml) was added isomerase-free aldolase (de la Mare et al., 1972) (50 $\mu$l of a solution of 0.5 mg/ml), and the mixture was left at 37 °C for 2 h. The remaining [1(*R*)-$^2$H]dihydroxyacetone phosphate was then purified on a second column of Dowex 1 ($Cl^-$), as above. Nondeuterated dihydroxyacetone phosphate for kinetic comparison was subjected to the final chromatographic column analogously to the deuterated material.

D-[2-$^2$H]Glyceraldehyde 3-phosphate was prepared from 3-phospho-D-[2-$^2$H]glycerate. In order to ensure complete deuteration, some $^3H_2O$ (20 $\mu$l) was added to the $^2H_2O$ (10 ml) and the specific radioactivity of the substrates used as a check on the exchange with the medium. 3-Phospho-D-[2-$^2$H]glycerate was prepared as follows. Exchangeable hydrogens in the buffer components were replaced by deuterium by freeze-drying a solution of EDTA (disodium salt: 3.7 mg), disodium hydrogen arsenate·7$H_2O$ (3.1 mg), Tris (0.5 ml of a 0.5 M solution in $^2H_2O$), and Tris-HCl (1.5 ml of a 0.5 M solution in $^2H_2O$), all in $^2H_2O$ (7.0 ml). The freeze-dried mixture of solids was redissolved in $^2H_2O$ (8.0 ml), and a solution of dihydroxyacetone phosphate in $^2H_2O$ (20.9 $\mu$mol in 0.9 ml) was added. $NAD^+$ (19.2 mg) was added as the solid. The "pH" (meter reading) was 7.75. To this mixture was added a solution of glyceraldehyde-phosphate dehydrogenase in $^2H_2O$, prepared by dissolution of 2.5 mg (spun down from ammonium sulfate suspension in $H_2O$) in $^2H_2O$ (0.45 ml). Tritiated water (20 $\mu$l of 5 Ci/ml) was then added, followed by triosephosphate isomerase (10 $\mu$l of a solution of 8 mg/ml). The reaction was followed spectrophotometrically in a cuvette of 1-mm path length until completion (ca. 5 h; room temperature). Duplicate samples (10 $\mu$l) for determination of the specific radioactivity of the medium were removed at this stage. The reaction mixture was freeze-dried and washed by successive additions of water (1 ml) followed by freeze-drying. The reaction mixture was now dissolved in 0.16 mM-HCl, pH 3.8, and applied to a column (13 cm × 1.8 $cm^2$) of Dowex 1 ($Cl^-$ form, 200–400 mesh, 8% cross-linked), equilibrated at 4 °C with 0.16 mM HCl at pH 3.8. The column was washed with about 20 column volumes of 0.16 mM HCl, until the radioactivity of the eluate was close to background, and then eluted with a nonlinear pH gradient (60 + 60 ml; 0.16 mM HCl to 0.1 M HCl), fractions of 1 ml being collected. Fractions containing 3-phosphoglycerate were pooled and assayed, and the specific radioactivity was determined to confirm the completeness of the equilibration with $^3H_2O$ (and therefore $^2H_2O$). The 3-phospho[2-$^2$H]glycerate (containing trace amounts of 3-phospho[2-$^3$H]glycerate) was freeze-dried.

This 3-phospho[2-$^2$H]glycerate was converted into D-[2-$^2$H]glyceraldehyde 3-phosphate using phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase. The equilibria are well over toward 3-phosphoglycerate, and they are normally perturbed (in a 3-phosphoglycerate assay, for instance; Maister et al., 1976) by the addition of hydrazine. But in the present case, free glyceraldehyde phosphate was required, and the equilibria were driven in the required direction by the generation of ATP from ADP with phosphoenolpyruvate and pyruvate kinase. This approach also avoids the danger that the hydrazone of glyceraldehyde phosphate may exchange its $\alpha$ protons (in this case, leading to loss of the $^2$H label) faster than the free aldehyde. The reaction mixture contained: 3-phospho[2-$^2$H]glycerate (19.2 $\mu$mol in 2.0 ml of deionized water); 50 mM triethanolamine-HCl buffer containing EDTA (1 mM), pH 7.6 (3.9 ml); 1 M KCl (0.9 ml); 0.5 M $MgCl_2$ (0.4 ml); 20 mM ADP in the above buffer (0.1 ml); 16 mM phosphoenolpyruvate in the above buffer (5.0 ml) with 10 M KOH (10 $\mu$l); 14 mM NADH in the above buffer (7.0 ml); glyceraldehyde-3-phosphate dehydrogenase [0.5 ml of a suspension of 10 mg/ml in ammonium sulfate was centrifuged, and the sedimented crystals dissolved in the above buffer (0.5 ml) containing bromohydroxyacetone phosphate (1.5 nmol) )de la Mare et al., 1972), and then dialyzed against the above buffer containing 2-mercaptoethanol (8 mM)] (0.5 ml of this solution was used); and pyruvate kinase [0.1 ml of a suspension of 7 mg/ml in ammonium sulfate was centrifuged and the precipitate dissolved in the above buffer (0.2 ml) containing 2-mercaptoethanol (8 mM)] (0.1 ml of this solution was used). The reaction, at 25 °C, was initiated by the addition of phosphoglycerate kinase [0.1 ml of a suspension of 2 mg/ml in ammonium sulfate was centrifuged and the precipitate taken up in the above buffer (0.2 ml) containing 2-mercaptoethanol (8 mM)] (0.1 ml of this solution was used). The progress of the reaction was followed spectrophotometrically at 366 nm, using a molar extinction coefficient for NADH at this wavelength of $3.3 \times 10^3$ $M^{-1}$ cm (Hohorst, 1956). The reaction was complete in approximately 30 min under these conditions. The reaction mixture was cooled to 0 °C and the pH adjusted to 4 with 1 M HCl. To ensure that the glyceraldehyde phosphate would be retained by the ion-exchange column, the mixture was diluted to ca. 800 ml with 0.16 mM HCl, pH 3.8. This was applied to a column of Dowex 1 and eluted with a nonlinear pH gradient exactly as described above in the preparation of 3-phospho[2-$^2$H]glycerate. Fractions containing D-glyceraldehyde 3-phosphate were pooled and freeze-concentrated to give a solution 37.8 mM in D-[2-$^2$H]glyceraldehyde 3-phosphate. It was found that the specific radioactivity of the product D-[2-$^2$H]glyceraldehyde 3-phosphate was, within experimental error, the same as that of the starting material, indicating that the label on C-2 had remained intact.

Nondeuterated D-glyceraldehyde 3-phosphate for kinetic comparison was prepared from dihydroxyacetone phosphate via 3-phosphoglycerate in an exactly analogous manner to the deuterated material.

*Methods.* Measurements of pH, enzyme, and substrate concentrations, etc., were done as described by Herlihy et al. (1976). Kinetic experiments were done at 30 °C using a Unicam SP 1800 recording spectrophotometer, according to the methods described by Plaut and Knowles (1972).

## Results

With dihydroxyacetone phosphate as substrate, two separate determinations of the effects of isotopic substitution were made on different preparations of the 1(*R*)-$^2$H substrate. Results from one of these determinations are shown in Figure 1. The average value for the ratio of $K_m^H/K_m^D$ was 1.0 ± 0.1, and for the ratio of $k_{cat}^H/k_{cat}^D$ was 2.9 ± 0.1; that is, there is no observable change in $K_m$ value on deuterium substitution, but there is a 2.9-fold decrease in $k_{cat}$. One important feature of the determination of the initial rates of reaction of [$^2$H]dihydroxyacetone phosphate is that very small extents of reaction must be studied. Since the 1(*R*)-$^2$H label will, during the course of the reaction, wash out into the medium of $^1H_2O$, only at the very beginning of the reaction will the substrate be fully deuterated. As soon as significant amounts of $^1$H substrate are formed, the observed reaction rate will increase and indicate a spuriously low kinetic isotope effect. To avoid this, the re-

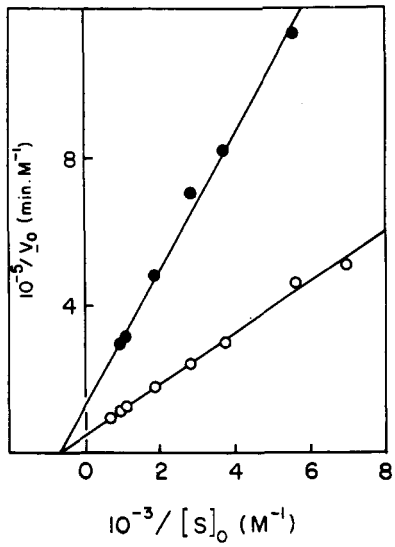


FIGURE 1: Lineweaver-Burk plots for the triosephosphate isomerase catalyzed reaction for [1(*R*)-$^2$H] (●) and [1(*R*)-$^1$H]dihydroxyacetone phosphate (○). Reactions were carried out in 0.1 M triethanolamine-HCl buffer at 30 °C, pH 7.5. $v_0$ is the initial velocity at initial concentration $[S]_0$.

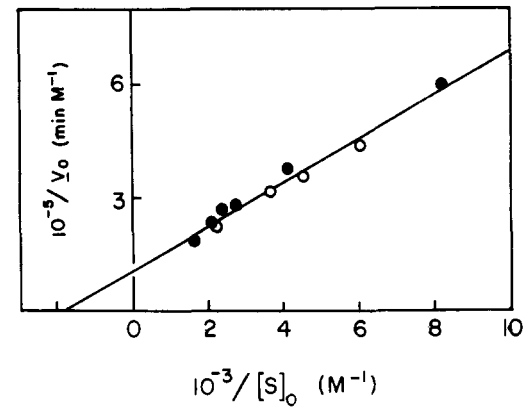


FIGURE 2: Lineweaver-Burk plots for the triosephosphate isomerase catalyzed reaction of D-[2-$^2$H]- (●) and D-[2-$^1$H]glyceraldehyde 3-phosphate (○). Reactions were carried out in 0.1 M triethanolamine-HCl buffer at 30 °C, pH 7.5. $v_0$ is the initial velocity at initial substrate concentration $[S]_0$.

action rates were measured under conditions where the extent of conversion of [1(*R*)-$^2$H]dihydroxyacetone phosphate to product was below 1.5%. No rate acceleration was detectable during the observed portion of the reaction.

With D-glyceraldehyde 3-phosphate as substrate, two separate determinations using two different preparations of the 2-$^2$H substrate were performed, and in neither case could a significant difference between the deuterated and nondeuterated material be observed. Results from one determination are shown in Figure 2.

## Discussion

The observed effects of isotopic substitution found in the present work compare reasonably with those reported earlier by Rose and his collaborators. Rieder and Rose (1959) performed experiments at a single concentration of [1(*R*)-$^2$H]- and [1(*R*)-$^1$H]dihydroxyacetone phosphate, and found that the observed isotope effect (at 23 °C) was about 2.2, and was invariant with pH between pH 6.5 and 8.5. In the present work, a more reliable figure of 2.9 has been derived from double-reciprocal plots of the velocities of the isomerase-catalyzed reactions over a range of substrate concentrations, at pH 7.5. The effect can be ascribed completely to an alteration in $k_{cat}$ arising from the deuterium substitution. Care was taken to ensure that labeled and unlabeled materials were subjected to the same purification and isolation procedures before use. Analogous experiments with D-[2-$^2$H]glyceraldehyde 3-phosphate and the unlabeled substrate did not show any difference in their isomerase-catalyzed reaction rates. The preparation and isolation procedures were, mutatis mutandis, identical. These findings agree with the observation reported in a footnote in a paper by Müllhofer and Rose (1965).

Are these observations consistent with the results on the mechanism of the isomerase-catalyzed reaction presented in the preceding papers? Consider the results with glyceraldehyde 3-phosphate first. We know from the results on the isotope discrimination against tritium in the reaction product reported by Maister et al. (1976) that the enzyme-bound enediol species that is in equilibrium with the solvent is also in equilibrium with the enzyme-bound forms of glyceraldehyde phosphate (i.e., species A, B, C, and D in Scheme II of Maister et al. (1976) are all in equilibrium). Glyceraldehyde phosphate that binds to the enzyme as substrate thus reaches very rapid isotopic equilibrium with the solvent, before being converted by a rate-limiting proton transfer ($k^H$ in Scheme II of Maister et al., 1976) into enzyme-bound dihydroxyacetone phosphate. There will be a negligible difference in the binding of protonated and deuterated glyceraldehyde phosphate to the enzyme, and this complex then rapidly equilibrates the C-2 hydrogen (or deuterium) with the solvent. In $^1H_2O$ as solvent, the deuterated substrate will lose all its label before the rate-limiting conversion to product dihydroxyacetone phosphate, and no isotope effect in either $k_{cat}$ or in $K_m$ is expected. None is observed. When deuterated dihydroxyacetone phosphate is substrate, binding to the enzyme is succeeded by a relatively slow abstraction of the 1(*R*) deuterium by the enzyme, giving the enediol intermediate species. As shown in Scheme II of Maister et al. (1976), this species then loses essentially all its isotope by equilibration with solvent $^1H_2O$. The observed rate of reaction is, of course, the rate of production of free glyceraldehyde phosphate, which is about one in four of the molecules arriving in the enediol-glyceraldehyde phosphate "pool". Since the isotopic content of the species in this pool is dependent only on the solvent, the partitioning of the molecules out of the pool will be the same, whichever isotope they started with. The relative rate of reaction is thus governed only by the flux of molecules *into* the pool. Because of the primary deuterium isotope effect in the abstraction of hydrogen isotope from dihydroxyacetone phosphate, this flux will be less for deuterated than for protonated substrate. In summary, although the flux of deuterated dihydroxyacetone phosphate into the A-B-C-D pool (Scheme II of Maister et al., 1976) is less than that of unlabeled substrate, the rapid isotopic equilibration of material in this pool ensures an identical partitioning out of it. This leads to the interesting state of affairs that one measures, in observing the differences in overall rate of product formation with labeled and unlabeled substrates, the full isotope effect on a process that is not cleanly rate limiting in the all-hydrogen system, but is the rate-determining process for the loss of the deuterium isotope.

In terms of the analysis presented by Albery and Knowles (1976a), the experiments reported here are HS$v$, DS'$v$, HP$v$, and DP'$v$. From Table III of Albery and Knowles (1976) the Lineweaver-Burk plots are described by:

TABLE I: Values of Ratios of Slopes and Intercepts of Double-Reciprocal Plots for $^2H$ and $^1H$ Substrates.[a,b]

| | |
|---|---|
| $A_3/A_1 = 2.9 \pm 0.25$ | $A_4/A_2 = 3.0 \pm 0.5$ |
| $B_3/B_1 = 1.15 \pm 0.14$ | $B_4/B_2 = 1.01 \pm 0.25$ |

[a]The experimental data of Figures 1 and 2 were subjected to least-squares treatment. [b]The errors quoted are standard errors of the mean.

$$\text{HS}v: \quad \frac{\Sigma e}{v_0} = \frac{A_1}{s_0} + A_2 \tag{1}$$

$$\text{DS}'v: \quad \frac{\Sigma e}{v_0} = \frac{A_3}{s_0'} + A_4 \tag{2}$$

$$\text{HP}v: \quad \frac{\Sigma e}{v_0} = \frac{B_1}{p_0} + B_2 \tag{3}$$

$$\text{DP}'v: \quad \frac{\Sigma e}{v_0} = \frac{B_3}{p_0'} + B_4 \tag{4}$$

where $\Sigma e$ is the total enzyme concentration, $v_0$ is the initial velocity of the reaction, $p_0$ and $s_0$ are the initial substrate concentrations (of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, respectively), and $A_n$ and $B_n$ are defined in Table IV of Albery and Knowles (1976a).

The main purpose of the present work was accurately to determine the effect of deuterium substitution on the slopes and intercepts of the Lineweaver–Burk plots. In the further analysis of the data (Albery and Knowles, 1976b) we require values for the ratios $A_3/A_1$, $A_4/A_2$, $B_3/B_1$, and $B_4/B_2$ (which correspond to intercept and gradient ratios for the $^2H$ and $^1H$ substrates, see eq 1–4). By carrying out the pairs of experiments HS$v$ and DS'$v$, and HP$v$ and DP'$v$ under the same conditions with the same enzyme solutions, we can obtain the required ratios with greater accuracy. The two pairs of experiments are presented graphically in Figures 1 and 2. Mean values of these ratios for two independent sets of experiments are given in Table I. It is clear that $A_3/A_1$ (which represents the ratio of $k_{cat}{}^{H}K_m{}^{D}/k_{cat}{}^{D}K_m{}^{H}$) and $A_4/A_2$ (which represents $k_{cat}{}^{H}/k_{cat}{}^{D}$) are each near 3 for dihydroxyacetone phosphate (see Figure 1). For D-glyceraldehyde 3-phosphate, there is almost no effect of deuterium substitution (see Table I and Figure 2).

From the work of Putman et al. (1972) we know accurately the values of $A_1$, $A_2$, $B_1$, and $B_2$, and from the ratios of Table I the parameters $A_3$, $A_4$, $B_3$, and $B_4$ are obtained.

Further analysis of the $A_n$ and $B_n$ parameters determined from this work is presented in a later paper (Albery and Knowles, 1976b).

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