Rabbit Muscle Aldolase Catalyzed Proton Exchange of Hydroxyacetone Phosphate with Solvent†

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ABSTRACT: Rabbit muscle aldolase catalyzes the exchange with solvent of all three methyl hydrogens of hydroxyacetone phosphate. Under saturating conditions, rates of the following processes have been measured: deuteration of hydroxyacetone phosphate in ^2H_2O (by an NMR method), tritiation of hydroxyacetone phosphate in H_2O and ^2H_2O, and detritiation of tritiated hydroxyacetone phosphate in H_2O and ^2H_2O. It is clear from these measurements (1) that there is no primary kinetic isotope effect and hence that hydrogen abstraction is not rate determining to the exchange and (2) that only one (as the closest integer) methyl hydrogen exchanges per turnover. The argument is made that these observations are mutually exclusive in terms of the accepted aldolase mechanism in the absence of further restrictions imposed by the enzyme. Possible restrictions are discussed.

The mechanism of action of the class I aldolases and particularly that of Fru-1,6-P_2[1] aldolase has been extensively investigated. The currently accepted reaction pathway for the Fru-1,6-P_2 aldolase is shown in eq 1 (Horecker et al., 1972)

$$E + D \underset{K_D}{\rightleftarrows} E \cdot D \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\text{-}D \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E\text{-}D^- \underset{-G,K_G}{\overset{+G}{\rightleftarrows}} E\text{-}D^- \cdot G \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E\text{-}F \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} E \cdot F \underset{K_F}{\rightleftarrows} E + F \quad (1)$$

where D = DHAP, G = GAP, and F = Fru-1,6-P_2. E·D, E-D^-·G and E·F are the productive Michaelis complexes of aldolase with D, G, and F, respectively. E-D and E-F are the covalent (ketimine or Schiff base) complexes of aldolase with D and F, respectively, and E-D^- is the covalent enamine (carbanion) complex of aldolase with D. The Michaelis binding steps are here assumed to be fast with respect to the steps where chemical reaction occurs.

Elucidation of the mechanism was initiated by the discovery (Rose and Rieder, 1955; Bloom and Topper, 1956) that, in the absence of GAP, aldolase catalyzes the exchange of one of the C-3 (i.e., CH_2OH) hydrogens of DHAP with solvent water. This enabled an independent investigation of several steps of the aldolase reaction, viz., E + D $\rightleftarrows$ E-D^-. It was demonstrated that the reaction consists of an ordered sequence in the direction given in eq 1 (Rose et al., 1965) and sodium borohydride reduction established the existence of ketimine complexes of both DHAP (Grazi et al., 1962; Speck et al., 1963) and Fru-1,6-P_2 (Avigad and Englard, 1972). The presence of the enamine is indicated by the solvent-exchange reaction, described above, and by the reaction of the aldolase-DHAP complex with a variety of oxidants (Healy and Cristen, 1972, 1973). Thus, considerable progress has been made in defining the intermediates along the reaction pathway. However, because the ketimine-enamine interconversion is a fast kinetic step, the mechanism of proton transfer has been difficult to probe.

One approach which might be adopted would be to use a modified form of the enzyme where proton transfer has been shown to be rate determining (Rose et al., 1965; Pugh and Horecker, 1967; Midelfort and Mehler, 1972; Davis et al., 1970). Another possible approach stems from the observation that the native enzyme catalyzes tritium exchange at the methyl group of the substrate analogue, HAP (Richards and Rutter, 1961; Rose and O'Connell, 1969). Since the exchange rate with HAP was much smaller than with the normal substrate DHAP, it seemed not impossible that the observed rate with HAP might be limited by the ketimine-enamine interconversion. If so, then proton transfer could be studied using the native enzyme. This possibility has now been investigated and the results have implications for the aldolase mechanism with the natural substrate.

Materials and Methods

Reagents. Fru-1,6-P_2, deuterium oxide (99.8%), and tritiated water (5 Ci/mL) were commercial products and used without further purification. DHAP was obtained from Sigma Chemical Co. as the dimethyl acetal and converted to the free ketone in either H_2O or ^2H_2O as required, according to the manufacturers procedure.

Enzymes. The aldolase (10–12 units/mg at 25 °C) was prepared from frozen rabbit muscle (Eagles and Iqbal, 1973). It was stored prior to use either as an ammonium sulfate suspension or as a freeze-dried solid. It was shown by assay to be free of triosephosphate isomerase activity.

The assay enzymes glycerol-1-phosphate dehydrogenase and triosephosphate isomerase and the coenzyme NADH were purchased from Boehringer Corp. (London) Ltd.

Assay Methods. Aldolase activity was assayed by means of the usual triosephosphate isomerase/glycerol-1-phosphate dehydrogenase method (Rajkumar et al., 1966) in 0.1 M triethanolamine buffer at pH 7.5. DHAP was assayed by NADH oxidation in the presence of glycerol-1-phosphate dehydrogenase.

Since the classical method of preparation of HAP (Sellinger and Miller, 1958) is tedious and often troublesome, an alternative synthesis was sought. A method involving displacement of halide from a haloacetone by phosphate would seem the most direct. A satisfactory method for phosphate ester syn-

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[1] Abbreviations used are: HAP, hydroxyacetone phosphate; DHAP, dihydroxyacetone phosphate; GAP, D-glyceraldehyde 3-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; NADH, nicotinamide adenine dinucleotide; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

thesis by this approach has been developed (Zwierzak and Kluba, 1971).

Hydroxyacetone Phosphate. Dibenzyl phosphate (10 g, mp 75–77 °C), prepared by oxidation of dibenzyl phosphite (Coulson, 1969), was dissolved in acetone (100 mL) containing water (10 mL) and neutralized at 0 °C to an apparent pH 7 (glass electrode) with 30% (in water) tetramethylammonium hydroxide solution. The resulting solution was evaporated to dryness under reduced pressure.

The solid tetramethylammonium dibenzyl phosphate salt was dissolved in boiling 1,2-dimethoxyethane (150 mL) (dried by distillation from lithium aluminium hydride) and chloroacetone (3.5 g, 1.025 equiv) added. The resulting solution was heated under reflux for 3 h and the precipitate formed on cooling filtered off. Removal of the solvent from the filtrate under reduced pressure left an oil which was taken up into chloroform and the chloroform solution extracted with water several times. The dried ($MgSO_4$) chloroform solution was evaporated to dryness giving a pale yellow oil (7.6 g). Acidification of the aqueous washings gave unreacted dibenzyl phosphate (3.5 g). The oil (one spot on TLC) had ν_{max} ($CHCl_3$) 1730 (C=O), 1280 (P=O), and 1020 cm^{-1}(P-O-C); τ ($CDCl_3$) 7.93 (3 H, s, CH_3CO), 5.59 (2 H, d, J = 9 Hz, $COCH_2OP$), 4.93 (4 H, d, J = 9 Hz, $PhCH_2OP$), 2.66 (10 H, s, Ph).

The benzyl protecting groups were removed by hydrogenolysis (in ethanol over 10% Pd/C) at atmospheric pressure and at room temperature. Under these conditions, hydrogen uptake ceased after 2 equiv had been consumed. The solution was filtered and the ethanol removed under reduced pressure. The residual oil was taken up into water and the pH of the solution adjusted with sodium hydroxide to 7. A slight cloudiness of the solution was removed by chloroform extraction. The barium salt of HAP was precipitated by addition of a solution of barium chloride (2 equiv) in water followed by sufficient ethanol to bring the final concentration to 50% (v/v). The precipitate was isolated by centrifugation and washed with 50% aqueous ethanol. It was suspended in water at pH 4 and insoluble material removed by filtration. Again the pH was adjusted to 7 and the barium salt of the product precipitated with an equal volume of ethanol. The final precipitate was washed with 50% aqueous ethanol, ethanol and acetone and dried under reduced pressure to give barium HAP (3.9 g, 60% with respect to dibenzyl phosphate reacted).

The method of Briggs was used for phosphate analyses (Briggs, 1922). Inorganic phosphate was estimated directly after addition of Na_2SO_4 to a weighed amount of the barium salt in water. Total phosphate was estimated after hydrolysis of a sample in 1 M H_2SO_4 for 24 h at 100 °C. Such analyses on the material above showed that it contained 1.17% inorganic phosphorus (i.e., 8.8% of the material was $BaHPO_4$).

Because of this high inorganic phosphate content the HAP was loaded onto a column of Dowex-1 equilibrated with HCl at pH 3. After washing with several column volumes of 1 mM HCl, HAP was eluted from the column with 0.02 M HCl. The appearance of HAP was monitored by its absorption at 265 nm. The eluent fractions containing HAP were evaporated to a small volume under reduced pressure, a solution containing 2 equiv of barium chloride was added, and the pH was adjusted to 7. A small amount of precipitated material was removed by centrifugation and the barium salt of HAP isolated from the remaining solution by ethanol precipitation as described above. Anal. Calcd for $C_3H_5O_5PBa$: 10.7% total phosphorus. Found: 0.17% inorganic phosphorus, 10.7% total phosphorus. ν_{max} 1720 (C=O) and strong absorption in the 1050–1200 cm^{-1} range (P=O, P-O-C); τ(^2H_2O, p^{2}H 7.5) 7.81 (3 H, s, CH_3CO), and 5.54 (2 H, d, J = 6 Hz, $COCH_2OP$). This spectrum was identical to that of a sample of HAP prepared by the conventional method (Sellinger and Miller, 1958) (a gift from Dr. J. R. Knowles).

HAP isotopically substituted on the methyl group was prepared by incubation of solutions of HAP prepared as described above, in ^2H_2O or tritiated water with aldolase at pH 7.5 for 24 h at 35 °C. The pH was adjusted to 3, precipitated protein was removed by Millipore filtration, and the isotopically substituted products were isolated by chromatography on Dowex-1. The concentration of HAP in subsequent solutions was estimated with glycerol-1-phosphate dehydrogenase (Rose and O'Connell, 1969).

β-Phosphoglycerylaldolase was obtained from aldolase and DHAP by reduction with sodium borohydride (Lai et al., 1965). Its aldolase activity after reduction twice was ca. 0.5% of the original (cf. 92.5% for a control in the absence of DHAP).

Rate Measurements. Rates of deuteration of HAP in ^2H_2O were measured by NMR spectroscopy. The instrument used was a 270-MHz Brüker NMR spectrometer, fitted with an Oxford Instruments magnet, a Nicolet 1085 computer, and Fourier transform facilities. In a typical run a suitable quantity of solid aldolase (assayed later) was added to the HAP solution (0.5 mL) preequilibrated in the probe of the spectrometer. No additional buffer was used. The p^{2}H of the solution had been adjusted using small quantities of concentrated NaO^2H or ^2HCl solutions. The p^{2}H was measured before and after each kinetic run. At appropriate times after the addition, 64 scans (taking about 1.5 min) were taken and transformed separately. These gave a series of spectra which show the disappearance of the methyl protons as a function of time. This could be analyzed as a first-order rate process. Longitudinal relaxation (T_1) times and null points were obtained by the usual 180°–τ–90° pulse sequence.

Initial rates of tritiation and detritiation were measured according to the general method of Rose and O'Connell (1969). Tritiation of HAP was initiated by addition of an aldolase solution (0.01 mL) to a HAP solution (0.5 mL) in H_2O or ^2H_2O containing 0.01 mL of tritiated water (5 Ci/mL). At appropriate times, aliquots (0.05 mL) were withdrawn from the reaction mixture and the aldolase reaction was quenched by addition to aliquots (1 mL) of 50% aqueous ethanol, which denatures the enzyme. The tritiated water and HAP were then separated on a small (1 × 3 cm) Dowex-1 column equilibrated with 1 mM HCl. The tritiated water was eluted with 1 mM HCl and the HAP with 0.1 M HCl. Scintillation counting for tritium was carried out using a Beckman DPM-100 liquid scintillation counter and a scintillation fluid composed of naphthalene (100 g), PPO (5 g), and POPOP (0.1 g) per liter of dioxane (the latter freed from peroxides by passage through an alumina column). Initial rates of detritiation were measured similarly but with the water counted rather than the HAP.

p^{2}H values were obtained by adding 0.4 to the pH meter reading (glass electrode) (Fife and Bruice, 1961).

Results

Deuteration of Hydroxyacetone Phosphate. A series of typical NMR spectra (showing the methyl region) obtained during the deuteration of HAP in the presence of aldolase is given in Figure 1. No spectral change was observed in the absence of aldolase and no change to the CH_2OP group resonance occurred either in the absence or presence of aldolase (cf. dihydroxyacetone phosphate (Lowe and Pratt, 1976)). It is clear from the disappearance of the methyl-group resonance in the spectrum that aldolase catalyzes exchange of all three

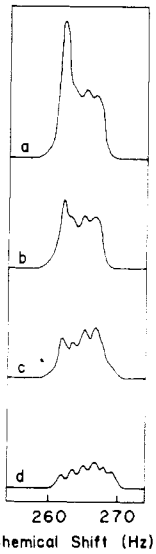

FIGURE 1: Changes in the methyl region of the NMR spectrum of hydroxyacetone phosphate (0.11 M) in ^2H_2O at p^{2}H 7.5 caused by the addition of aldolase (ca. 4.5 units/mL) at 25 °C. No buffer was added. These spectra were taken after (a) 0.94, (b) 1.96, (c) 2.90, and (d) 5.70 half-times for the disappearance of the original methyl singlet at 262 Hz upfield from the tetramethylammonium ion.

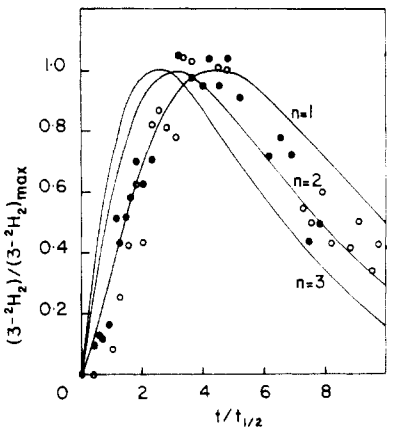

FIGURE 2: Plot of $(3\text{-}^2H_2)/(3\text{-}^2H_2)_{max}$ against $t/t_{1/2}$ for hydroxyacetone phosphate hydrogen exchange in ^2H_2O at p^{2}H 7.5 and 25 °C. No buffer was added. (3-^2H_2) represents the [3-$^2H_2$]HAP concentration at time t and $(3\text{-}^2H_2)_{max}$ the maximal concentration of [3-$^2H_2$]HAP achieved during the exchange; $t_{1/2}$ is the half-time for the original methyl resonance. ● and ○ represent experimental points from two separate runs (4.3 and 6.0 units/mL of aldolase, respectively) using the same hydroxyacetone phosphate concentrations (0.065 M). The solid lines represent theoretical curves for n = 1, 2, and 3 (see Appendix).

methyl protons with solvent. It is also clear that this exchange does not occur completely on a single turnover of the enzyme, since the appearance of the intermediates [3-^{2}H]- and [3-$^2H_2$]HAP is obvious from the spectra shown in Figure 1.

The intermediate spectra are well in accord with this interpretation and can be simulated by a combination of spectra of [3-$^2H_2$]-, [3-^{2}H]-, and unlabeled HAP, deuterium having a spin quantum number of 1. The apparent geminal coupling constant J_{H^2H} of about 2 Hz agrees well with literature values (Bernstein and Sheppard, 1962; Lewis and Norcross, 1965) as does the difference in chemical shift (4 Hz, about 0.015 ppm) between the protons of the methyl and [^{2}H]methyl groups (Tiers, 1958; Gutowsky, 1959). Further evidence comes from the observation that virtually identical intermediate spectra were obtained for hydroxide-ion-catalyzed deuteration of acetone in ^2H_2O. These observations are consistent with the proposal that only one hydrogen of the HAP methyl group is exchanged per turnover.

First-order rate constants for the disappearance of the methyl resonance were obtained from these spectra and converted to the initial rates reported in Table I. (Since, as seen in Figure 1, there is very little overlap between the downfield resonance of the [^{2}H]methyl triplet and the HAP methyl singlet at the chemical shift maximum of the latter, the height of the HAP methyl peak was taken from the spectra as a measure of the HAP concentrations in obtaining these rate constants; good first-order plots were obtained for at least three half-times.)

At p^{2}H 7.5 no change in rate was observed over the HAP concentration range indicated in Table I. This is as expected, since K_m is 6.7 mM (Rose and O'Connell, 1969). The maximal velocity showed a slight increase to higher p^{2}H and a considerable decrease to lower p^{2}H, which could be due to changes in K_m, V_{max}, or both. At all three p^{2}H examined, the succession of intermediates discussed above was observed and this also occurred at p^{2}H 7.5 and 50 °C.

Variation of the Concentration of [3-$^2H_2$]Hydroxyacetone Phosphate with Time during the Deuteration of Hydroxyacetone Phosphate. It is not easy from the NMR spectra to plot out with confidence the concentration of the intermediates [3-^{2}H]- and [3-$^2H_2$]HAP as a function of time. This is because of the overlap of the individual spectra and uncertainties as to the effects of the various T_1 and T_2 values on them. Because of these problems, the spectra were simplified by removal of the [^{2}H]methyl signal by using a 180°-τ-90° pulse sequence with τ set at the null point time for the [^{2}H]methyl resonance. The latter time was obtained by variation of τ in the above pulse sequence until the null point was obtained (using a previously prepared mixture of [3-^{2}H]- and unlabeled HAP). In this way, the variation of the concentration of [3-$^2H_2$]hydroxyacetone phosphate with time could be obtained. The results of this experiment are plotted in Figure 2. The solid lines are theoretical and are explained in the Discussion.

TABLE I: Initial Rates of Deuteration of Hydroxyacetone Phosphate (i.e., Disappearance of HAP) in ^2H_2O at 25 °C in the Presence of Aldolase.

p^{2}H	HAP (M)	v^a (μmol min^{-1} unit^{-1})
7.50	0.05-0.2	0.067 ± 0.005
8.46	0.13	0.080
6.28	0.13	0.023

[a] No buffer was added and the aldolase concentration was between 3 and 15 units/mL. A statistical correction has been made to allow for the fact that three hydrogens are available for exchange in a methyl group; i.e., the rates are quoted per hydrogen. At p^{2}H 7.50 six measurements were made, whereas at p^{2}H 8.46 and 6.28 the figures represent single determinations.

Rates of Tritiation and Detritiation. These are given in Table II. The concentrations of HAP used were in the range 0.1-0.15 M.

The rates of exchange observed are only about 3% of those observed for DHAP. These values agree more closely with those of Richards and Rutter (1961) than those of Rose and O'Connell (1969) who reported a value 17% that of DHAP.

An Attempt to Reduce Hydroxyacetone Phosphate onto Aldolase with Sodium Borohydride. This reaction was carried out exactly as for DHAP (Lai et al., 1965), using 0.017 M HAP and 10 mg/mL aldolase in 0.02 M sodium acetate buffer at pH 6.0 and 0 °C. Two sodium borohydride additions (each equivalent to the amount of HAP) reduced the aldolase activity

TABLE II: Initial Rates of Tritiation and Detritiation of Hydroxyacetone Phosphate at pH = p^{2}H = 7.5 and 25 °C in the Presence of Aldolase.

Reaction	Solvent	v^a (μmol min^{-1} unit^{-1})
Tritiation of HAP	H_2O	0.047
	^2H_2O	0.043
Detritiation of [3-^{3}H]HAP	H_2O	0.069
	^2H_2O	0.052
Detritiation of [3-$^3H^2H_2$]HAP	^2H_2O	0.070

[a] No buffer was added and the aldolase concentration was between 0.5 and 1 unit/mL. HAP concentrations were between 0.1 and 0.15 M. With the exception of the tritiation of HAP (which was done twice), these data represent single determinations. The estimated uncertainty is ±0.005 μmol min^{-1} unit^{-1} which includes the reproducibility of the method and the standard deviations from least-squares treatment of the initial rate measurements.

to 86% of the original. The activity of a control, in the absence of HAP, decreased to 92.5% of the original.

Deuteration of Hydroxyacetone Phosphate in the Presence of β-Phosphoglycerylaldolase. Under conditions where in the presence of aldolase the half-life of the methyl to [^{2}H]methyl exchange reaction was 5 min, no change in the height of the methyl signal in the NMR spectrum occurred in 1 h when β-phosphoglyceryl aldolase was used. Hence, the aldolase site catalyzing the HAP exchange is blocked by reduction of DHAP onto the normal aldolase active site (Lai et al., 1965).

Hydroxyacetone Phosphate as an Inhibitor of Fructose 1,6-Bisphosphate Cleavage. Under normal aldolase assay conditions with Fru-1,6-P_2 at 6 μM, the rate of Fru-1,6-P_2 cleavage was reduced to 68.5% in the presence of 2.46 mM HAP and to 61.0% in the presence of 2.57 mM [3-$^2H_3$]HAP. This indicates that isotopic substitution at the methyl group does not significantly change the binding constant of HAP to aldolase.

Discussion

Rabbit muscle Fru-1,6-P_2 aldolase catalyzes the exchange of all three methyl hydrogens of HAP with solvent but there is no unequivocal evidence that the mechanism of the HAP exchange is the same as that for DHAP. The hydrogen exchange reaction of DHAP would consist, of course, of the first three steps of eq 1. An attempt to inhibit aldolase with HAP and sodium borohydride did not lead to a large decrease in aldolase activity, as it does with DHAP and sodium borohydride under the same conditions. The binding constant of HAP to aldolase, as indicated by the K_m of detritiation and the $K_{Q/2}$ for quenching of protein fluorescence (Rose and O'Connell, 1969), is much smaller than that of DHAP, and is in fact very similar to the binding constants of simple non-imine-forming phosphates (Spolter et al., 1965). These observations suggest that, whereas in the case of DHAP the bound substrate exists mainly as an intermediate subsequent to the Michaelis complex, i.e., as the ketimine or enamine, in the case of HAP it probably exists substantially as the Michaelis complex. Such a difference in the relative stabilities of the Michaelis and subsequent covalent complexes (along with the implied kinetic differences) could explain the negligibly small inhibition caused by HAP and sodium borohydride.

No exchange of the HAP protons occurs in the presence of β-phosphoglycerylaldolase (where DHAP has been reduced onto the enzyme with sodium borohydride), which suggests that this process occurs at the same site on the enzyme as the

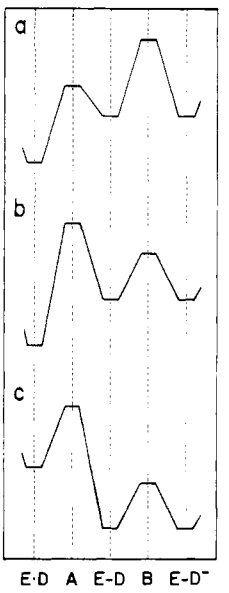

FIGURE 3: Free-energy profiles for the hydrogen-exchange reactions of DHAP and HAP in the presence of aldolase. E·D, E–D, and E–D$^-$ represent Michaelis complexes and covalent ketimine and covalent enamine complexes, respectively, and A and B are the transition states between them. See main text for discussion.

DHAP exchange and the Fru-1,6-P_2 cleavage. Moreover, a slow aldolase-catalyzed condensation of HAP with D-glyceraldehyde 3-phosphate has been reported (Richards and Rutter, 1961; Rose and O'Connell, 1969), and the rate of detritiation of [3-^{3}H]HAP was greatly reduced by prior treatment of the aldolase with carboxypeptidase A (Rose and O'Connell, 1969). This evidence on balance suggests that the path of the HAP proton exchange reaction is the same as that of the DHAP exchange as far as the nature of the intermediates is concerned; the rate-determining step will be discussed below.

It is clear from the data of Tables I and II that no primary kinetic hydrogen isotope effect is associated with the HAP exchange reaction: in particular we have

$$\frac{\text{rate of replacement of } ^1H \text{ by } ^2H \text{ in } ^2H_2O}{\text{rate of replacement of } ^3H \text{ by } ^2H \text{ in } ^2H_2O} = 1.3 \pm 0.2$$

This small isotope effect and the others apparent in the tables are probably attributable to solvent, equilibrium, and secondary isotope effects. This means that any free-energy profile for the exchange process that has the transition state, B, for deprotonation of the ketimine as the highest point, e.g., Figure 3a, cannot be correct. Consequently, the highest point on the profile must be the transition state, A, for imine formation as in Figure 3b,c. (Note that in these diagrams substrate binding and release is not considered to be the major barrier, as assumed in the original general scheme, and that ketimine and enamine are represented as having equal free energy. The latter assumption is just a convenience, of course, since the relative free energy of the ketimine and enamine is not known but its actual value is immaterial here.)

Since it seems likely, from the difficulty of reducing HAP onto the enzyme by borohydride, that for HAP the Michaelis complex is more stable than the ketimine, then Figure 3b will be the correct profile (cf. DHAP where also no primary kinetic isotope effect is observed (Pratt and Lowe, unpublished observations) but where reduction onto the enzyme is possible (Lai et al., 1965); here Figure 3c must be closer to correct).

Any profile, however, such as those of Figure 3b,c where A

is of higher energy than B, will allow equilibration of ketimine and enamine prior to release of the substrate from the enzyme, i.e., complete exchange of all three hydrogens of HAP on a single turnover. It is clear, however, from the NMR results that this is not observed. When HAP is deuterated in ^2H_2O in the presence of aldolase, species with intermediate degrees of deuteration, [3-^{2}H]- and [3-$^2H_2$]HAP, are released into solution prior to the final [3-$^2H_3$]HAP and in quantities consistent with the assumption that only one hydrogen can exchange per turnover (Figure 2).

These two observations, the absence of a primary kinetic isotope effect and the presence of partially deuterated intermediates in solution, appear from the above discussion mutually exclusive. This conclusion remains valid irrespective of the number of unimolecular enzyme/substrate rearrangement steps between the initial substrate binding and the proton removal and irrespective of which of them is rate determining. It also remains true even if release of the product from the Michaelis complex were rate determining.

In order to escape from this dilemma, it is necessary to question the assumptions implicit in the original scheme (i.e., in the first three steps of eq 1). Some of these which appear to bear on the present problem are as follows.

(*1*) *Rotation of the HAP Methyl Group in the Enzyme-Bound Ketimine Is Fast with Respect to Hydrolysis of the Ketimine.* If this were not so, then the presence of partially exchanged intermediates would be expected.

The actual rate of ketimine hydrolysis required for comparison is not, in fact, obtainable. If the rate-determining step of the HAP proton exchange reaction is formation of the ketimine, as seems likely from the above discussion, then all we can say about the hydrolysis is that it is faster than this, i.e., $k > 1\ s^{-1}$ or the activation free energy < 18 kcal/mol. For DHAP exchange, however, ketimine hydrolysis is probably rate determining; i.e., the rate of ketimine hydrolysis is probably about $25\ s^{-1}$; i.e., the activation free energy is about 16 kcal/mol. If restriction of methyl of [^{2}H]methyl rotation is the explanation of the dilemma, we probably need to envisage a barrier in the HAP/aldolase ketimine of at least 16 kcal/mol at room temperature.

Recently, examples have appeared in the literature where barriers to methyl-group rotation in certain organic molecules are much higher than hitherto observed. Thus, in 9-methyltriptycene derivatives (Anderson and Rawson, 1973; Nakamura et al., 1973) free-energy barriers up to 10.6 kcal/mol at room temperature have been found. In horse ferricyanomyoglobin, a rotational barrier of a hememethyl group with $\Delta H^* = 14.8 \pm 1.8$ kcal/mol and $\Delta S^* = -2.1 \pm 0.6$ eu at 25 °C has been observed (Morishima and Iizuka, 1974), and in sperm whale ferrimyoglobin the rotational barrier to a heme methyl group appears to be even higher (Wüthrich et al., 1968, 1970). Hence, a barrier of the order of 16 kcal/mol to methyl-group rotation must be regarded as at least a possibility.

(*2*) *The Energy Barrier to Formation of the Enamine from the Ketimine Mainly Involves the Energy Required to Remove the Proton.* It is possible to imagine circumstances where this need not be true; for example, if an enzymatic conformational change largely contributed to the energy barrier—made necessary perhaps by the change of geometry at C-3 of the substrate from tetrahedral to planar trigonal. Under these circumstances, no intermediates would be seen, although no primary kinetic isotope effect would be observed. In this case the conformational change would be associated with the normal aldolase reaction. Indeed, there is considerable evidence for conformational changes in aldolase on substrate binding (Adelman et al., 1968; Lehrer and Barker, 1970, 1971; Midelfort and Mehler, 1972). This explanation, however, suffers from the observation that the DHAP exchange reaction is much faster than that of HAP. There seems no obvious reason why the proposed conformational change should be so much slower with HAP than with DHAP.

(*3*) *Exchange of the Abstracted Proton with Solvent Is Fast with Respect to Hydrolysis of Ketimine.* The appearance of the intermediates would also be possible if this were not true. If, as seems likely, a proton is removed from the ketimine by an enzymatic functional group acting as a general base then, given free access of the base to the solvent, how fast should exchange occur? Should a histidine imidazole group, for example, function as the general base (as seems quite likely from the identification of an active-site histidine by affinity labeling (Hartman et al., 1973; Hartman and Welch, 1974)) then the exchange rate would be of the order of $10^3\ s^{-1}$ (Jencks, 1969) which is much faster than the observed HAP exchange rates. The rate of exchange of the proton on the protonated enzymatic general base with solvent could be smaller if a much stronger general base were involved, but use of such a base would seem inefficient at physiological pH.

Slow exchange with the solvent could probably be better explained by the presence of a slow protein conformational change opening the protonated general base to the solvent. Such a conformational change need not, of course, be involved in the normal enzymatic reaction and would mean in this case that turnover of HAP would be faster than the observed hydrogen exchange rates. Here again, however, there seems no obvious reason (although it is, of course, possible) that the slow conformational change limiting HAP exchange is much more rapid when DHAP is involved.

A final possibility in this category is the extreme case where no exchange with solvent hydrogens occurs when the substrate is on the enzyme. In order to explain the observed exchange process, we can imagine exchange with a limited number of protons occluded with the substrate at the active site—on adjacent enzymatic functional groups, for example, or on a small number of occluded water molecules. The amount of exchange on a single turnover would then be limited by the number of protons available for exchange (and possibly the rates of the various exchanges). Complete exchange of the enzyme-bound protons with solvent would occur on release of the substrate. A purely statistical analysis of the results (see Appendix where this possibility is referred to as the limited exchange mechanism) shows that the NMR data interpreted in this way imply exchange with only one (as the best-fitting integer, Figure 3) proton on the enzyme. Under these conditions also the turnover rate would be greater than the observed exchange rate.

If the limited exchange process does occur with HAP then it could also be true for DHAP exchange. This does not, however, remove the requirement for stereospecificity, since even if proton exchange is limited in the sense described above, in the absence of restricted rotation of the CH_2OH group both C-3 protons of DHAP would eventually exchange.

There is considerable precedent for the limited-exchange hypothesis. Transaldolase is an enzyme with a mechanism and function very closely related to aldolase and which forms a ketimine and probably an enamine with dihydroxyacetone. Here no exchange of the hydrogens of dihydroxyacetone occurs; i.e., the proton removed from the dihydroxyacetone ketimine is shielded from exchange with bulk solvent by the protein (Tsolas and Horecker, 1972). The absence of exchange is also seen in a variety of epimerases such as ribulose-5-phosphate 4-epimerase (McDonough and Wood, 1960), and lactate racemase (Shapiro and Dennis, 1965, 1966).

Although there seems to be no direct evidence at present to

distinguish the above possibilities, the author feels that the last discussed is the most likely. Irrespective of which possibility is correct, it is clear that another kinetic dimension to the aldolase active site has been uncovered.

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Appendix: The Limited Exchange Mechanism—Derivation of Rate Equations

Here it is assumed, as described in the main body of the text, that exchange of the substrate hydrogens occurs by way of fast exchange with a limited number of enzyme bound or occluded hydrogens in the enzyme-substrate complex and by fast exchange with solvent of the enzyme-bound hydrogens in the free enzyme. The situation for a single turnover of HAP in ^2H_2O can be represented as follows:

$$\begin{array}{ccccc} E(^2H_n) + HAP & \rightleftharpoons & E(^2H_n)\cdot HAP & \overset{\text{slow}}{\rightleftharpoons} & E(^2H_n)\text{–}HAP \\ \text{fast}\uparrow & & & & \downarrow\uparrow\ \text{fast} \\ E(H_x{}^2H_{n-x}) + [3\text{-}^2H_x]HAP & & & & E(H_x{}^2H_{n-x})\text{–}[3\text{-}^2H_x]HAP \\ & \nwarrow & & \swarrow\!\!\!\swarrow & \\ & & E(H_x{}^2H_{n-x})\cdot[3\text{-}^2H_x]HAP & & \end{array}$$

where $E(^2H_n)$ represents the enzyme in ^2H_2O with its n hydrogens available for exchange with HAP in the active site deuterated and x is the average number of ^2H in the HAP after one turnover. The enzyme-substrate complexes are represented as in eq 1.

If rapid exchange with only one hydrogen in the active site was possible then the probability of unlabeled HAP returning after one turnover is 0.25 and the probabilities of [3-^{2}H]-, [3-$^2H_2$]-, and [3-$^2H_3$]HAP appearing are 0.75, 0, and 0, respectively. Then

$$V/V_t = V^{^2H}/V_t = 0.75$$

and

$$V^{^2H_2}/V_t = V^{^2H_3}/V_t = 0$$

where V is the initial rate of disappearance of HAP, $V^{^2H}$, $V^{^2H_2}$, and $V^{^2H_3}$ are the initial rates of appearance of [3-^{2}H]-, [3-$^2H_2$]-, and [3-$^2H_3$]HAP, respectively, and V_t is the turnover rate.

In the general case, where exchange with n hydrogens on the enzyme occurs, on one turnover the probability of:

$$\text{HAP release} = \frac{6}{(n+1)(n+2)(n+3)}$$

$$[3\text{-}^2H]HAP \text{ release} = \frac{18n}{(n+1)(n+2)(n+3)}$$

$$[3\text{-}^2H_2]HAP \text{ release} = \frac{9n(n-1)}{(n+1)(n+2)(n+3)}$$

$$[3\text{-}^2H_3]HAP \text{ release} = \frac{n(n-1)(n-2)}{(n+1)(n+2)(n+3)}$$

and hence

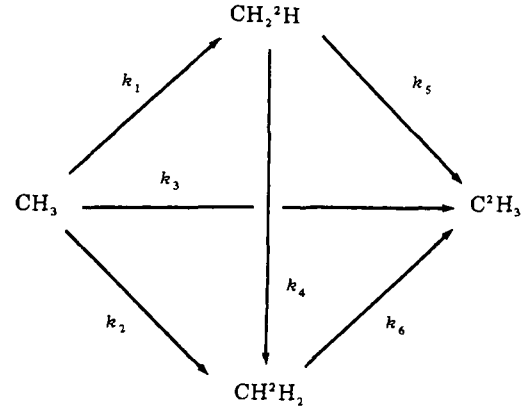
$$V/V_t = \frac{n(n^2+6n+11)}{(n+1)(n+2)(n+3)} \quad (2)$$

This is, of course, a purely statistical analysis and assumes no secondary isotope effects. It assumes then that V_t does not change with isotopic substitution. This seems close to being true, since, as seen in Table II, the rates of detritiation of tritiated HAP and tritiated [3-$^2H_3$]HAP are very similar. It also assumes that the K_m value is not changed by isotopic substitution. The experiment showing the almost identical effectiveness of HAP and [3-$^2H_3$]HAP as inhibitors of Fru-1,6-P_2 cleavage suggest that this too is justified.

A theoretical plot (not shown) of V/V_t vs. n from eq 2 indicates that the rate of turnover is only much greater than the measured rate of HAP disappearance at small ($\ll$1) values of n. A value of n <1 (and nonintegral values in general) could arise if hydrogen exchange occurred on only a fraction of the occasions that the enzyme was free or on a fraction of occasions on which the substrate was bound to the enzyme.

Clearly strong evidence for this limited-exchange mechanism would be obtained if it could be shown that turnover (measured by a method other than hydrogen exchange) was faster than the disappearance of HAP. Unfortunately, the background rate of ^{18}O exchange into HAP would probably be too rapid to allow the turnover rate to be estimated by the conventional method of ^{18}O exchange (Model et al., 1968).

Another approach is simply that of monitoring the concentration of the intermediates [3-^{2}H]- and [3-$^2H_2$]HAP with time. In particular, if $n > 1$ then the [3-$^2H_2$]methyl signal will build up far more rapidly than if $n \leq 1$, since only in the former case is direct conversion of HAP into [3-$^2H_2$]HAP possible. A formal scheme illustrating this is shown below:



Equations can be derived for the variation with time of the concentrations of the various species.

$$CH_3/C_0 = e^{-k_x t} \quad (3)$$

$$CH_2{}^2H/C_0 = \frac{k_1}{k_y - k_x}(e^{-k_x t} - e^{-k_y t}) \quad (4)$$

$$CH^2H_2/C_0 = Ae^{-k_x t} + Be^{-k_y t} + Ce^{-k_6 t} \quad (5)$$

where $k_x = k_1 + k_2 + k_3$; $k_y = k_4 + k_5$; $A = [k_2 + k_1k_4/(k_y - k_x)]/(k_6 - k_x)$; $B = k_1k_4/(k_y - k_x)(k_y - k_6)$; $C = [k_1k_4/(k_6 - k_y) - k_2]/(k_6 - k_x)$ and C_0 is the total HAP concentration. Now in terms of the limited-exchange mechanism outlined above, we can write for any given value of n expressions for $k_1 \ldots k_6$ in terms of the turnover rate constant k. Thus, for $n = 1$, we have $k_1 = 0.75k$, $k_2 = 0$, $k_3 = 0$, $k_4 = 0.5k$, $k_5 = 0$, and $k_6 = 0.25k$; for $n = 2$, $k_1 = 0.6k$, $k_2 = 0.3k$, $k_3 = 0$, $k_4 = 0.6k$, $k_5 = 0.1k$, and $k_6 = 0.4k$, etc.

From eq 3, 4, and 5 it is easy to obtain theoretical plots of the variation of the concentration of [3-$^2H_2$]-, [3-^{2}H]- and unlabeled HAP with time and as a function of n. From the NMR spectra accumulated from the direct pulse and free-induction decay it is not easy to accurately define the time course of the [3-^{2}H]- or [3-$^2H_2$]HAP concentrations because of partial overlap of their spectra and overlap with that of HAP itself. (It should be noted, however, that the form of the NMR

spectra and the apparent maximal concentrations of the intermediates for the hydroxyacetone phosphate exchange reaction are extremely similar to those observed during the exchange reaction of acetone in alkaline solution which, of course, is equivalent to the $n = 1$ case and for the acetoacetate decarboxylase catalyzed exchange reaction of acetone in ^2H_2O (Hammons et al., 1975).) However, because the relaxation times of the protons in [3-$^2H_2$]-, [3-^{2}H]- and unlabeled HAP decrease in that order, it is possible to remove the [^{2}H]methyl resonance from the spectrum by applying a 180°-τ-90° pulse sequence where τ, the delay time, corresponds to the time required for relaxation of the magnetization of the [^{2}H]methyl resonance to the null point. In this way it is possible to obtain the variation of the concentration of [3-$^2H_2$]hydroxyacetone phosphate with time. The experimental results, along with theoretical plots obtained from eq 3, 4, and 5 for $n = 1$, 2, and 3 are shown in Figure 2. It appears from this that the experimental data are best fitted when $n = 1$, rather than to any other integer. The fit to the $n = 1$ line is by no means perfect, however, even taking into account the experimental uncertainties indicated by the scatter of points. It should be noted though that the apparent deviations from the $n = 1$ line do not uniformly suggest that an integer greater than one would be more appropriate (i.e., that there is firm evidence against a sequential one-at-a-time deuteration), since this should have lead to a more rapid appearance as well as disappearance of [3-$^2H_2$]HAP; although the latter is observed, the former is not. Discrepancies between the experimental points and the theoretical line could be real, caused, for example, by the small isotope effects still allowed by the data of Tables I and II, or they could reflect some systematic error in data collection or treatment. The deviations, on the one hand, are not significant enough to warrant any change in the gross interpretation of the results but, on the other, do not allow either a firm definition of n as an integer (it need not, of course, be an integer), assuming the limited proton-exchange mechanism as described here, or even, since n is apparently so close to 1, a distinction between the limited exchange mechanism and the other possibilities mentioned in the main text.

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