Energetics of Triosephosphate Isomerase: The Nature of the Proton Transfer between the Catalytic Base and Solvent Water†

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ABSTRACT: The isomerization of specifically deuterium-labeled [1(*R*)-^{2}H]dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate, catalyzed by the enzyme triosephosphate isomerase, has been studied. It is shown that the extent of transfer of the ^{2}H label from the substrate to the product D-glyceraldehyde 3-phosphate is (after complete reaction) the same as that of the corresponding transfer of ^{3}H. The absence of an isotope effect shows that the exchange process of the isotopically labeled enzyme carboxyl group, -COOL + H_2O → -COOH + LOH, does not involve a rate-limiting transition state in which L is in flight. Possible modes for the nature of the ionization of -COOL in ^1H_2O are discussed.

There is considerable evidence that the reaction catalyzed by triosephosphate isomerase is mediated by a basic group at the active site, that is responsible for the shuttling of carbon-bound hydrogen between C-1 of dihydroxyacetone phosphate and C-2 of D-glyceraldehyde 3-phosphate (Rieder and Rose, 1959). Labeling studies with specific active-site directed inhibitors (Hartman, 1968, 1970, 1971; Waley et al., 1970; de la Mare et al., 1972) and crystallographic work (Banner et al., 1975) strongly indicate that the enzyme base involved is the γ-carboxyl group of a unique glutamate residue Glu^{165} (Corran and Waley, 1973).

The conjugate acid of this catalytic base may undergo partial proton exchange with solvent water during the course of the enzyme-catalyzed reaction (Rose, 1962; Herlihy et al., 1976) and this paper deals with the nature of this proton transfer reaction. The extent of deuterium transfer from [1(*R*)-^{2}H]dihydroxyacetone phosphate to C-2 of the final product 3-phosphoglycerate has been determined at two different extents of reaction under the conditions used for the tritium transfer experiments described by Herlihy et al. (1976). The enzyme-substrate systems in these experiments effectively provide -COO^{2}H and -COO^{3}H in solvent water. The isotopic label may undergo either of two fates: it may be transferred to the enediol intermediate to form labeled D-glyceraldehyde 3-phosphate, which may be lost from the isomerase and result in 3-phosphoglycerate labeled at C-2. Alternatively the carboxyl group may exchange the label for a proton from solvent water, ultimately giving rise to unlabeled 3-phosphoglycerate. Clearly the extent of transfer of label to the 3-phosphoglycerate reflects the competition between these two routes for the label on the carboxyl group and enables the transfer of label to solvent water to be investigated. Using the results from the transfer experiments and the generalized kinetic treatment (Albery and Knowles, 1976a), the isotope effect for the proton-transfer reaction between the carboxyl group and solvent water is derived and its mechanistic implications discussed.

A preliminary account of some of this work has appeared (Fisher et al., 1975).

Experimental Section

Materials. Enzymes, substrates, cofactors, and other materials were as specified by Herlihy et al. (1976). In addition 3-phospho-D-glycerate, *rac*-glycerol 3-phosphate, and *N,N*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane were obtained from the Sigma Chemical Co. Biorex 40 (H^+ form, 100–200 mesh) analytical grade cation exchange resin and ^2H_2O (99.84% isotopic purity) were purchased from Bio-Rad Laboratories.

Dehydrogenases were rendered free from triosephosphate isomerase by treatment with bromohydroxyacetone phosphate, prepared by the method of de la Mare et al. (1972). 3-Phospho[2-^{2}H]glycerate was prepared by the method of Leadlay et al. (1976). All other chemicals were obtained from Sigma or from Fisher and were the highest grade available. All solutions were made up with distilled deionized water.

Methods. Scintillation counting and measurements of ultraviolet absorbance, substrate concentrations, conductivity, and pH were done as described by Herlihy et al. (1976) and Fletcher et al. (1976). Molar extinction coefficients for NADH[1] of 6.22×10^3 M^{-1} cm at 340 nm (Horecker and Kornberg, 1948) and 3.3×10^3 M^{-1} cm at 366 nm (Hohorst, 1956) were assumed.

Mass spectrometry and high resolution mass spectrometry were done using an AEI-MS9 instrument. Gas chromatography–mass spectrometry was done using a Pye Series 104 analyzer coupled to an AEI-MS9 instrument.

[1(R)-^{2}H]Dihydroxyacetone phosphate was prepared using a modification of the method of Leadlay et al. (1976). The incubation of dihydroxyacetone phosphate (40 μmol) in ^2H_2O was done in the presence of tritiated water (5 μl of 5 Ci/ml) so that both the extent of proton equilibration and regio- and stereospecificity of the label could be checked. The labeled [1(*R*)-^{2}H]- and [1(*R*)-^{3}H]dihydroxyacetone phosphates were eluted from Dowex 1 by a nonlinear pH gradient (75 + 75 ml; 0.63 mM HCl to 0.1 M HCl). Fractions containing dihydroxyacetone phosphate (see Figure 1) were pooled, and the pH was adjusted to 4 with potassium carbonate prior to storage at −20 °C. The separation shown in Figure 1 obviated the need for the aldolase treatment used by Leadlay

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

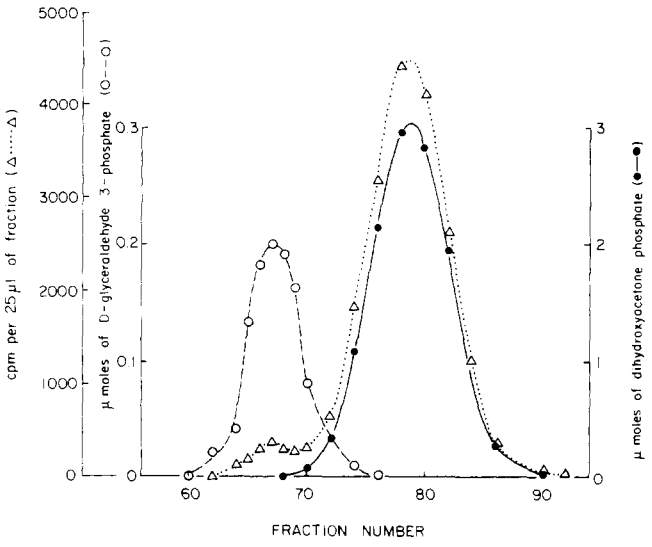


FIGURE 1: Separation of D-[2-^{2}H]glyceraldehyde 3-phosphate and [1(*R*)-^{2}H]dihydroxyacetone phosphate by anion-exchange chromatography. The ^2H_2O used in the preparation of labeled substrates also contained ^3H_2O. Radioactivity of fraction (cpm per 25 μl) (··· Δ ···); assay for D-glyceraldehyde 3-phosphate (μmol/ml) (- - O - -); assay for dihydroxyacetone phosphate (μmol/ml) (— ● —).

et al. (1976). The yield of [1(*R*)-^{2}H]dihydroxyacetone phosphate was 28 μmol. The specific radioactivity of the [1(*R*)-^{2}H]dihydroxyacetone phosphate (containing some 1(*R*)-^{3}H material) prepared by this method was about 50 000 cpm.μmol, which was within experimental error that expected for complete equilibration with the ^3H_2O (and therefore ^2H_2O) used.

The Extent and Location of Deuterium Labeling in [1(R)-^{2}H]Dihydroxyacetone Phosphate: Borohydride Reduction, Silylation, and Mass Spectrometry. The volatile derivative *rac*-[1(*R*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate was prepared as follows. Sodium borohydride, as a 0.8 M solution in 0.5 M sodium hydroxide (100 μl), was added to [1(*R*)-^{2}H]dihydroxyacetone phosphate (4 μmol) in 100 mM triethanolamine-HCl buffer, pH 7.6 (4 ml), and the solution left at room temperature for 1.5 h. The pH of the solution was raised to 8 with ammonium hydroxide, the conductivity lowered to <500 μS by dilution with distilled water, and the solution applied to a column (10 cm × 1.5 cm^2) of DEAE-cellulose (DE 52) equilibrated with 5 mM ammonium bicarbonate. A linear gradient (160 ml) of ammonium bicarbonate (5–300 mM) was applied to the column and fractions of 1.5 ml were collected. Fractions containing *rac*-[1(*R*)-^{2}H]glycerol 3-phosphate were pooled and ammonium bicarbonate was removed by repeated evaporation to dryness at room temperature. The residue was dissolved in distilled water and passed through Biorex 40 (H^+ form) cation-exchange resin to remove any remaining ammonium bicarbonate and to generate the free acid of *rac*-[1(*R*)-^{2}H]glycerol 3-phosphate, which was freeze-dried. Dry pyridine (40 μl) and bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (45 μl) were added and the mixture was left for 1 h. Mass spectrometry was carried out directly on this solution of *rac*-[1(*R*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate.

Mass spectra were obtained by direct insertion at a source temperature of 80 °C and 70 eV. The *m/e* 445–446 and 357–358 peaks were scanned slowly and repeatedly, and the extent of deuterium incorporation at C-1 and C-3 of the [1(*R*)-^{2}H]dihydroxyacetone phosphate was determined from the averaged *m/e* 446:445 and 358:357 intensity ratios for *rac*-[1(*R*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate, and *un*labeled material (prepared by silylation of *rac*-glycerol 3-phosphate) obtained similarly.

In one experiment gas chromatography–mass spectrometry was carried out on the labeled and unlabeled tetrakis(trimethylsilyl)glycerol 3-phosphates. Direct on-column injections of the silylation reaction mixture were employed using a 1% SE 30 on Gas Chrom Q (100–200 mesh) glass column (6 ft × 2 mm) with temperature programming from 150 to 250 °C at 6 °C/min and a helium flow rate of 40 ml/min. Spectra, recorded at 70 eV, were essentially identical in the *m/e* 445–446 and 357–358 regions with those obtained on the reaction mixture.

Isomerase-Catalyzed Reaction. The deuterium-transfer experiments were carried out analogously to the method used by Herlihy et al. (1976) in their study of the extent of tritium transfer. The solution contained: 100 mM triethanolamine-HCl buffer (pH 7.6), EDTA (5 mM), sodium arsenate (3 mM), NAD^+ (2.3 to 6.6 mM), [1(*R*)-^{2}H]dihydroxyacetone phosphate (0.7 to 1.68 mM), glyceraldehyde-3-phosphate dehydrogenase (300 μl of a solution of 5 mg/ml), and triosephosphate isomerase (25 μl of a solution of 5 μg/ml), in a total volume of 3.125 ml. The reaction was carried out at 30 °C. The relative concentrations of triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase required to ensure that the isomerase reaction was rate limiting were determined prior to each transfer experiment using a modification of the method of Plaut and Knowles (1972). The progress of the reaction was followed by monitoring the increase in absorbance at 366 nm. For the partial conversion experiments, the final absorbance change at 366 nm for complete conversion to 3-phosphoglycerate was obtained by monitoring a portion of the reaction mixture in an optical cuvette of 2-mm light path. The reaction in the 10-mm cuvette was quenched at the desired point by lowering the pH below 4.0 by the addition of 1 M HCl (120 μl) and cooling rapidly to 0 °C, conditions under which the isomerase is inactivated (Plaut and Knowles, 1972). The enzymes were then removed by passing the quenched reaction mixture through Biorex 40 (H^+ form) at pH 3.8: this quenching and enzyme removal procedure was omitted for experiments involving total conversion to 3-phosphoglycerate. The solution was adjusted to pH 8 with ammonium hydroxide, and NADH (which coelutes with 3-phosphoglycerate) was converted to NAD^+ by addition of sodium pyruvate and isomerase-free lactate dehydrogenase. This mixture was diluted with distilled water (conductivity <500 μS) and chromatographed at 4 °C as described above in the preparation of *rac*-[1(*R*)-^{2}H]glycerol 3-phosphate.

Determination of the Deuterium Content of the 3-Phosphoglycerate: Methylation with Diazomethane and Mass Spectrometry. Dry distilled ethereal diazomethane (Arndt, 1943) was added to a solution of the 3-phosphoglyceric acid in dry methanol (5–10 ml) at 0 °C until a persistent yellow coloration was observed. The solution was left at room temperature for a further 0.5 h and solvent removed to yield the methylated product.

Mass spectra were obtained at 45 °C and 70 eV by direct insertion. The *m/e* 169 and 170 peaks were scanned slowly and repeatedly and the extent of deuterium transfer in the isomerase reaction was determined from the averaged *m/e* 170:169 ratios of the labeled methylated 3-phosphoglycerate and of the unlabeled material obtained similarly.

TABLE I: Determination of the Extent of Deuterium Transfer to C-2 of 3-Phosphoglycerate by Mass Spectrometry.

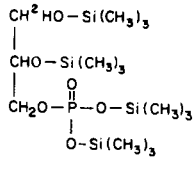
	Extent of Reaction 23%	100%
m/e 170:169 intensity ratios for samples of methylated 3-phosphoglycerate	25.3[a]	17.5[a]
	26.2	18.0
	24.6	18.4
	26.3	17.9
	24.9	17.9
	26.3	18.2
	26.1	17.4
	24.9	17.8
	24.5	17.4
		17.8
		17.8
		18.0
		17.8
Average *m/e* 170:169 intensity ratio (I_1)	25.5 ± 0.7[a]	17.8 ± 0.2[a]
Total % deuterium content of 3-phosphoglycerate[b]	17.1	11.4
% deuterium content at C-3 of 3-phosphoglycerate[c]	4.6	5.1
% deuterium content at C-2 of 3-phosphoglycerate[d]	12.1	6.3

[a] *m/e* 170 expressed as a percentage of *m/e* 169. [b] Calculated from $(I_1 - I_s)/[100 + (I_1 - I_s)]$ where $I_s = 4.9 \pm 0.1$, the averaged percent *m/e* 170:169 intensity ratio for unlabeled 3-phosphoglycerate. [c] Obtained from the *m/e* 358:357 intensity ratios of *rac*-[1(*R*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate and unlabeled *rac*-tetrakis(trimethylsilyl)glycerol 3-phosphate using an analogous equation to that in footnote *b*. [d] Corrected for 0.4% deuterium arising from D-[2-^{2}H]glyceraldehyde 3-phosphate.

Results

Equilibration of dihydroxyacetone phosphate in ^2H_2O in the presence of triosephosphate isomerase produces a mixture of [1(*R*)-^{2}H]dihydroxyacetone phosphate (96%) and D-[2-^{2}H]glyceraldehyde 3-phosphate (4%) which may be separated by ion-exchange chromatography (Figure 1) yielding [1(*R*)-^{2}H]dihydroxyacetone phosphate essentially free (≤0.1%) from D-[2-^{2}H]glyceraldehyde 3-phosphate.

To establish the extent and location of deuterium labeling in the substrate, the [1(*R*)-^{2}H]dihydroxyacetone phosphate was reduced with borohydride and silylated to form the volatile derivative *rac*-[1(*R*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate (I).



(I)

The mass spectrum of I, in agreement with earlier reports (Duncan et al., 1971; Curstedt, 1974), showed intense peaks at *m/e* 357 (M − 104), arising from loss of $CH^2HOSi(CH_3)_3$ by cleavage between C-1 and C-2 without hydrogen atom migration, and at *m/e* 446 arising from loss of a single CH_3 group.

The *m/e* 358:357 intensity ratio was used to determine the extent of deuterium incorporation at C-3 of the [1(*R*)-^{2}H]-dihydroxyacetone phosphate and this was typically found to be 4.5–5.1%. Since *m/e* 446 contains all three carbon atoms of the glycerol moiety, any ion intensity at *m/e* 445 would arise from dihydroxyacetone phosphate unlabeled at C-1. Negligible ion intensity at *m/e* 445 (compared with *m/e* 446) was observed indicating that the dihydroxyacetone phosphate contained >0.98 deuterium atom per molecule at C-1. Deuterium at C-3 does not interefere with the determination of deuterium incorporation at C-1 since this doubly labeled material will only increase the peak intensity at *m/e* 447.

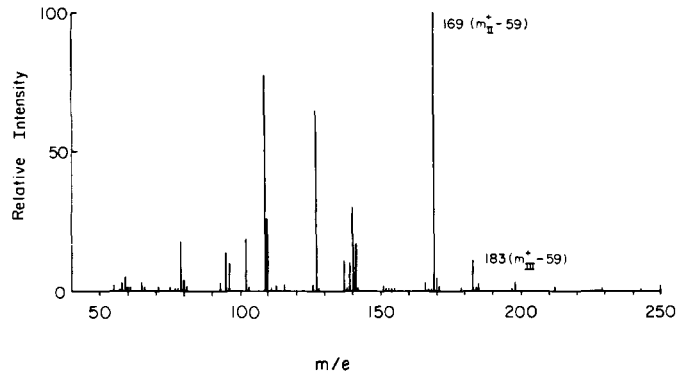
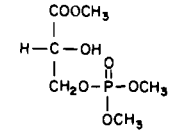


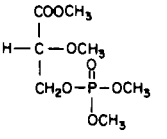
FIGURE 2: The 70-eV mass spectrum of methylated 3-phosphoglycerate.

The deuterium label at C-1 was shown to be specifically in the 1(*R*) position, as expected, by utilizing the tritium tracer simultaneously incorporated into the dihydroxyacetone phosphate. Incubation of the labeled substrate with triosephosphate isomerase followed by distillation resulted in an almost quantitative recovery of tritium as tritiated water (total cpm in tritiated [1(*R*)-^{2}H]dihydroxyacetone phosphate, 9200; total cpm recovered as tritiated water, 9207; cpm remaining in residue, 210).

The deuterium-transfer experiments were carried out under conditions essentially identical with those of Herlihy et al. (1976). The deuterium content of the resulting 3-phosphoglycerate was determined from the mass spectrum of methylated 3-phosphoglycerate (Figure 2). The intense ($M^+ - 59$) peaks at *m/e* 169 and 183 arise from loss of $-COOCH_3$ from II and III, respectively, the methylation products of 3-phosphoglycerate.



(II)

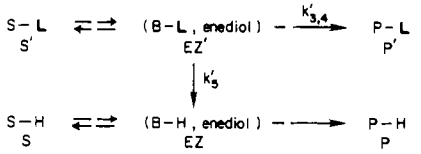


(III)

This assignment is consistent with high resolution studies (calcd for *m/e* 169: 169.0265; found 169.0248) and with the mass spectrum of methylated 3-phospho[2-^{2}H]glycerate which exhibited intense peaks at *m/e* 170 and 184.

The peak at *m/e* 169 was chosen for determinations of the deuterium content of 3-phosphoglycerate obtained from the transfer experiments. Incorporation of deuterium into the 3-phosphoglycerate increased the intensity at *m/e* 170 relative to that at *m/e* 169. Results from a typical experiment are shown in Table I. The extent of deuterium transfer to C-2 of the 3-phosphoglycerate arising from genuine transfer by isomerase was obtained from the deuterium contents determined mass spectrometrically by correcting for label at C-3 of the 3-phosphoglycerate originating from label at C-3 of the

SCHEME I: Partitioning Scheme for the Labeled Reaction Intermediate (B–**L**, Enediol). S–**L** and S–H Denote Labeled and Unlabeled Substrate Molecules; P–**L** and P–H Refer Analogously to Product. Species Are Identified (S, EZ, P, etc.) according to Albery and Knowles (1976a).



starting [1(*R*)-^{2}H]dihydroxyacetone phosphate. In some cases a small correction for any contribution from contaminating D-[2-^{2}H]glyceraldehyde 3-phosphate (in most experiments, <0.1%) was also made. The percent deuterium transfer was, for complete reaction, 6.5, 6.3, 5.0, and 5.9% (four separate experiments). For a reaction quenched after 23% conversion, the percentage of deuterium transfer was found to be 12.1%.

Quantitative Treatment. Measurement of the percent transfer of deuterium to 3-phosphoglycerate at different extents of reaction constitutes a DS′**p** experiment in terms of the theoretical treatment already presented (Albery and Knowles, 1976a). The relevant equation describing this type of experiment is eq 5.9 of Albery and Knowles (1976a):

$$\mathbf{p} = \frac{A_5(1 - \mathbf{s}r)}{(1 - r)} \tag{1}$$

where **p** is the fractional deuterium content of the 3-phosphoglycerate, **s** is the fractional deuterium content of the dihydroxyacetone phosphate, r is the fraction of substrate remaining, and A_5 is defined by eq 4.2 of Albery and Knowles (1976a). In accord with Scheme I:

$$A_5 = \frac{k_{3,4}'}{k_{3,4}' + k_5'} \text{ for L} = {}^2\text{H} \tag{2}$$

where k_5' describes the irreversible loss of label from EZ′, and $k_{3,4}'$ describes the rate of reaction of EZ′ to P′ through transition states 3 and 4:

$$k_{3,4}' = \frac{k_3'k_4'}{k_{-3}' + k_4'}$$

From eq 1, as $r \rightarrow 0$ and the reaction is complete, $\mathbf{p}_\infty \rightarrow A_5$. This is because P′ is formed by the $k_{3,4}'$ route, while all the EZ′ that exchanges by the k_5' route eventually forms unlabeled P.

From the work of Herlihy et al. (1976) for *tritium* transfer, we have $A_5' = 0.058 \pm 0.004$, i.e., after complete conversion of [1(*R*)-^{3}H]dihydroxyacetone phosphate to product, 5.8 ± 0.4% of the tritium label is transferred intramolecularly. In the present work, for *deuterium* transfer at $r = 0$ (complete reaction), $A_5 = 0.059 \pm 0.005$. From these experimental data we can calculate a value for the parameter A_{11}, where:

$$A_{11} = \frac{A_5'(1 - A_5)}{A_5(1 - A_5')} \tag{3}$$

From eq 2 and a similar expression for L = ^{3}H:

$$A_{11} = \left(\frac{k_5'}{k_{3,4}'}\right)_{2\text{H}} \left(\frac{k_{3,4}'}{k_5'}\right)_{3\text{H}} = \frac{\phi_5}{\Phi_5}\frac{\Phi_{3,4}}{\phi_{3,4}} \tag{4}$$

where ϕ_5 and Φ_5 are the deuterium and tritium fractionation factors for the exchange reaction, and $\phi_{3,4}$ and $\Phi_{3,4}$ are the mixed fractionation factors for the reaction of EZ′ to P′ (Albery and Knowles, 1976a). The Swain–Schaad relation (Swain et al., 1958) connects deuterium and tritium fractionation factors:

$$\Phi_n = (\phi_n)^{1.44} \tag{5}$$

This relation holds to within a few percent for most mixed fractionation factors (Albery and Knowles, 1976a) and from eq 4 and 5 we obtain the deuterium fractionation factor for the exchange reaction:

$$\phi_5 = (\Phi_{3,4})^{0.69(A_{11})^{-2.3}} \tag{6}$$

Now $\Phi_{3,4}$ is 0.83 ± 0.01 (Maister et al., 1976), and from eq 3 and 6 the values of ϕ_5 from our four experiments involving complete transfer are 1.1, 1.1, 0.61, and 0.92, i.e. $\phi_5 = 0.94 \pm 0.17$. This value is close enough to unity for it to be clear that the mechanism of exchange of the isotope from -COOL with the protons of the solvent does not involve a transition state in which L is in flight.

Confirmation of this finding is provided by the results from the experiments involving partial reaction. Using eq 1, a series of theoretical curves may be calculated describing the variation of **p** with r assuming that isotope effects of various magnitudes operate on the proton-transfer reaction between the carboxyl group and solvent water (ϕ_5).

From eq 3, 4, and 5 we may calculate values for A_5 corresponding to extreme values of ϕ_5 (0.1–1.0). Then from eq 1 and a knowledge of **s** in terms of r, we can calculate **p** as a function of r for various A_5 values. [The relationship between **s** and r depends on the magnitude of A_5, and for the sake of brevity is omitted from the present discussion (see eq 7.14, 5.4, and 5.7 of Albery and Knowles, 1976a). The required values of θ and A_3/A_1 were obtained from Albery and Knowles, 1976b.]

The calculated values for the fractional ^{2}H transfer (**p**) at 23% conversion ($r = 0.77$) are: for $\phi_5 = 1.0$, 0.112; for $\phi_5 = 0.50$ (i.e., a deuterium isotope effect of 2.0), 0.089; and for $\phi_5 = 0.143$ (i.e., a deuterium isotope effect of 7.0), 0.053. The experimental value (see Table I) is 0.124, which confirms the absence of an isotope effect in the exchange reaction of -COO^{2}H with ^1H_2O.

Discussion

We may conceive of three possible pathways for proton exchange between the carboxyl group and solvent water.

First, the pathway may involve ionization, solvent exchange, and reprotonation (Figure 3). This mechanism is consistent with our observed value for the fractionation factor, provided that either the diffusive step or the solvent reorganization step is rate limiting. The rate constant for solvent reorganization (k_3) is likely to be in the range of 10^8 to 10^{12} s^{-1} (Grunwald and Ralph, 1971; Leung and Safford, 1970), and k_{-2} will be about 10^9 M^{-1} s^{-1}. At neutral pH, therefore, k_3 will be much greater than $k_{-2}[H_3O^+]$. It is then clear that, in the overall isotope exchange process, the diffusive step will be rate limiting. [Only at low pH values does this diffusive step become faster than the solvent reorganization (cf. Grunwald and Ralph, 1971)]. Catalysis of the exchange process by buffer (100 mM triethanolamine-HCl) is very possible, and unless the ΔpK_a (of donor acid and acceptor buffer) is very small, the proton-transfer step is likely to be faster than the diffusive steps. So proton transfer to buffer (with or without the intervention of some bridging solvent molecules) will result in a small or negligible isotope effect.

A further possibility suggested by the stepwise scheme of Figure 3 would involve proton transfer followed by solvent reorganization from *within* the ion pair (Figure 4). However, this is unlikely to compete with the route laid out in Figure 3: it is improbable that the k_4 step (Figure 4) could be faster than

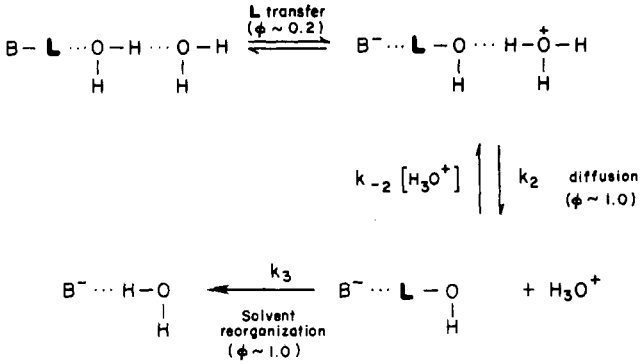


FIGURE 3: Stepwise proton exchange process between a labeled acid B-L, and H_2O.

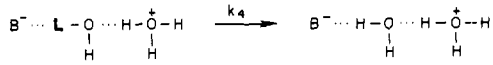
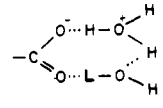


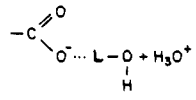
FIGURE 4: Solvent reorganization from a solvent-separated ion pair.

k_2 or k_3 (Figure 3) since the intervening LOH solvent molecule in the ion pair will be held more strongly than when it is associated just with the base B^-.

Secondly, the exchange of the isotopic label in -COOL may occur within a hydrogen-bonded complex as has been suggested to account for the ^1H NMR of acetic acid–acetate buffers (Luz and Meiboom, 1963) (see Figure 5). The absence of an isotope effect precludes the transfer of L being rate limiting so either of the two subsequent H transfers or the solvent reorganization step must be slow. Solvent reorganization, at 10^8 to 10^{12} s^{-1}, is probably faster than the known rate of proton transfer in such a cyclic system of $\sim 5 \times 10^7$ s^{-1} (Luz and Meiboom, 1963). Of the two H transfers, the first will be slower than the second since H_2O is a much weaker base than $RCOO^-$ (Albery, 1967). In the probable slow step, therefore (the vertical arrows of Figure 5), the effect of L is now secondary and, since the transition state is symmetrical, the fractionation factor for this step is $(l)^{1/2} = (0.69)^{1/2} = 0.83$ [where l is the fractionation factor for the process $L_2HO^+ + \frac{1}{2}D_2O \rightleftharpoons L_2DO^+ + \frac{1}{2}H_2O$, ignoring statistical factors (Gold, 1963; Kresge and Allred, 1963)]. Our experimental value for the fractionation factor is, therefore, consistent with this mechanism also. This route may be preferred over the normal dissociation path (Figure 3): it is some 10^2-fold faster (Luz and Meiboom, 1963) presumably because IV is of lower free energy than V, which is reflected in the transition states leading to these situations.



(IV)



(V)

Finally, it is possible that, during the lifetime of the enediol intermediate, L undergoes rapid exchange into a limited pool of water molecules sequestered from solution by the binding of the substrate at the active site. As soon as the product (or the substrate, after exchange of its L with the pool) leaves the active site, these water molecules could exchange into bulk

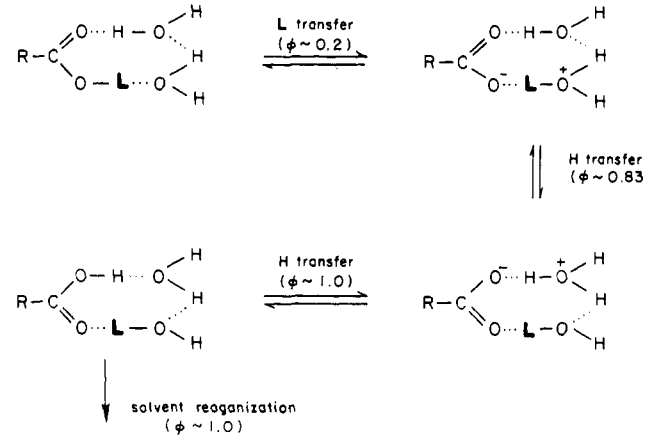
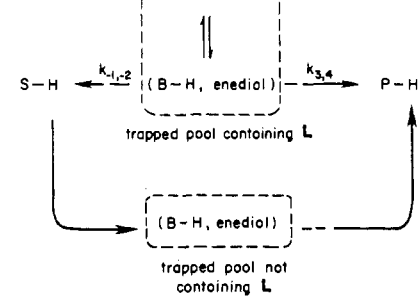


FIGURE 5: Proton exchange between a carboxyl group and H_2O via a cyclic hydrogen-bonded complex.

SCHEME II: Partitioning Scheme for the Labeled Reaction Intermediate (B–L, Enediol) That Includes a Trapped Pool of Solvent Molecules.



solution and be replaced by a fresh set of unlabeled water molecules that would be trapped by the binding of the next substrate molecule. For such a pathway, the extent of isotopic transfer to the product after complete reaction is independent of the identity of L (see below), being determined only by the number of exchangeable sites within the pool. This would result in there being no observable difference in the extent of transfer of a tritium or deuterium label.

Let there be n sites within the trapped pool, then making the reasonable assumption that the tritium and deuterium equilibrium fractionation factors for -COOH are unity

$$\frac{[\text{B-L, enediol}]}{[\text{B-H, enediol}]} = \frac{1}{(n-1)} \tag{7}$$

The extent of transfer of label to product after complete reaction will then depend on the partitioning of the intermediates (see Scheme II) and is given by

$$p_\infty = \frac{k_{3,4}'}{k_{3,4}' + (n-1)(k_{3,4} + k_{-1,-2})} \tag{8}$$

where $k_{-1,-2}$ describes the reaction of EZ to form S, through transition states 1 and 2:

$$k_{-1,-2} = \frac{k_{-1}k_{-2}}{k_{-1} + k_2}$$

This expression (eq 8) is similar to eq 2, except that k_5' is replaced by $(n-1)(k_{3,4} + k_{-1,-2})$. Since this quantity is the same for ^2H and ^3H, and since $k_{3,4}'$ is also much the same for ^2H and ^3H ($\Phi_{3,4} \simeq 1$), then the extent of isotopic transfer (eq

8) will be independent of the identity of the label. The $k_{-1,-2}$ term arises in eq 8 since any intermediate partitioning along this route releases S-H from the enzyme, and the label contained in the trapped pool is washed away. The S-H is then eventually converted to P-H (see Scheme II).

The partitioning of (B-H, enediol) between substrate and product, $k_{-1,-2}/k_{3,4}$, is given by θ which is approximately 2 (Albery and Knowles, 1976b). Then from eq 8

$$\frac{1}{A_5} \approx \frac{1}{A_5'} \approx 1 + 3(n-1) \qquad (9)$$

where we assume $k_{3,4} \simeq k_{3,4}'$ (since $\Phi_{3,4} \simeq 1$). Since $A_5 \approx A_5' = 0.06$, $n \simeq 6.2$. Hence we should require a pool containing about three water molecules for this third possibility to operate.

We do not favor this hypothesis since it does not conform to the nature of enzyme active sites in general nor to that of triosephosphate isomerase in particular (Banner et al., 1975). However, this point will be resolved unequivocally when the crystal structure of the isomerase-dihydroxyacetone phosphate complex has been solved at high resolution (Banner et al., 1975).

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