Proton Diffusion in the Active Site of Triosephosphate Isomerase†

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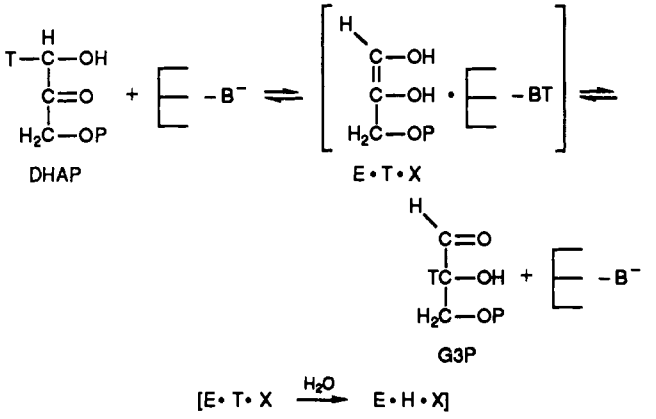
ABSTRACT: The current model for hydrogen flow in the aldose–ketose isomerases is probably incorrect. Enzymes of this class are characterized by both hydrogen transfer and proton exchange in the interconversion of substrate and product. The transer is believed to be due to the action of a unique basic residue in the active site. Exchange is presumed to occur by dissociation of the abstracted proton and reassociation from the medium prior to its transfer to the intermediate enediol on the way to product. Dissociation of a necessary proton from the intermediate state imposes limits on the overall catalytic rate depending on the pK_a of the protonated base and the pH of the medium. A case in point is triose-P isomerase (TIM), where k_{cat} is $\sim 10^4$ s^{-1}. T-Labeled substrate is found to lose ~95% of its T to the medium when totally converted to product. Although the active site base is believed to be a glutamate of pK_a = 3.9, the pH dependence of maximum velocity is known to be flat up to pH 10. The loss of hydrogen required to form product as indicated by isotope exchange must be restored completely at this high pH, requiring a base of very high pK_a, or there must be some other explanation for the loss of isotope. The present study demonstrates the existence of a single proton on human and rabbit TIM and three protons on yeast TIM that rapidly exchange with the abstracted proton at the E·enediol state *internal exchange*. Exchange with the medium external exchange occurs from the enzyme *after* substrate or product has dissociated. The evidence supporting internal exchange is that T, which is exchanged into triose-P isomerase by brief exposure to TOH, is found in substrate after the enzyme is diluted into a large volume of unlabeled water containing substrate. The evidence for external exchange is that loss of T introduced in this way is competitive with substrate concentration. It is suggested that a major factor in the evolution of some enzymes is the need to keep the intermediate state and its associated amino acids isolated from the medium to prevent the loss of functional protons. The extent to which DHAP becomes labeled in a chase is not greatly altered if phosphoglycolate is also present at concentrations that inhibit the steady-state rate ~90%. The T present on the E·inhibitor complex must be protected from exchange with the medium so that it will be trapped by DHAP after the inhibitor dissociates.

The triose-P isomerase catalyzed reaction is generally believed to proceed through a *cis*-enediol intermediate as shown in Scheme I,[1] i.e., a simple proton abstraction and transfer by a *single base* believed to be a glutamate. Proton, rather than hydride, transfer is strongly inferred in the TIM reaction from the observation that the substrate-derived tritium is largely exchanged for a medium proton (Reider & Rose, 1959; Simon et al., 1968; Maister et al., 1976) and by the slow formation of P_i and methylglyoxal, indicative of an enediol-P on the enzyme (Browne et al., 1976; Richard, 1984).

Knowledge of TIM structure began with the sequence determination of the chicken enzyme (Corran & Waley, 1973) followed by a 2.5-Å crystal structure (Banner et al., 1975). The protein was found to be a homodimer of 25-kDa subunits, each in a globular $(\alpha\beta)_8$ barrel configuration with an alternating helix–strand–helix arrangement. The active site is described as being made up of residues arising from the carboxy end of five of the eight parallel strands and a pocket that is made up of polar residues. Glu_{165} is characterized as the base, B^- in Scheme I, from its reactivity with alkylating substrate analogues (Waley et al., 1970; Coulson et al., 1970; Hartman et al., 1975) and its location relative to the competitive inhibitor phosphoglycolate, introduced into a crystal of yeast TIM by soaking (unpublished data available from G. Petsko). Although a direct test of the one-base mechanism has not been done with triose-P isomerase and is made difficult by the dominance of exchange over transfer in the reaction, a single-base mechanism has been assumed from the observation of transfer and by analogy with phosphoglucose isomerase (Rose & O'Connell, 1961) where net proton transfer was shown to occur within the same substrate molecule.

Scheme I[a]



[a] X = enediol of triose-P.

The present study began with the following question: What is the basis for the high T exchange rate in the isomerization of T-DHAP that allows only ~4% of the T label to be found

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[1] Abbreviations: DHAP, dehydroxyacetone phosphate; G3P, (2*R*)-glyceraldehyde 3-phosphate; 3-PGA, D-3-phosphoglyceric acid; T-water, tritiated water.

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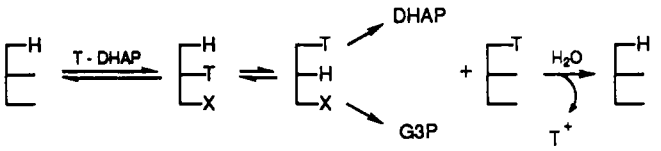
Scheme II



in G3P? One *apparently* good reason for proposing exchange at the intermediate state was the observation that in T-water the exchange labeling of substrate and labeling of product were approximately equal and did not depend on the direction in which the reaction was studied, implying a kinetically symmetrical process. However, it seems unlikely that this explanation of exchange could possibly result in an efficient catalyst. Considering that the intermediate from which T is presumed to exchange may be present at less than 1% of total enzyme (Rose, 1984), the rate constant for dissociation would have to be greater than $k_{cat} \times 100/0.04$ or $>2 \times 10^6$ s^{-1} in the slower forward reaction and 10 times greater in the G3P → DHAP conversion where only ~2% T transfer was found (unpublished). If indeed 4% transfer represents the fraction of E·T·X that has not dissociated, the state of ionization of the remaining 96% and therefore its contribution to the steady-state rate will depend on the pK_a of the donor and the pH of the medium. In the free enzyme form Glu_{165} has a pK_a of 3.9 on the basis of the pH dependence of its alkylation by substrate analogues (Hartman et al., 1978). Yeast and rabbit muscle TIMs are within their maximum velocities at pHs of 8 and 10, respectively (Krietsch et al., 1970), and chicken TIM is optimally active even at pH 10 in both directions of reaction (Plaut & Knowles, 1972). Therefore, both because Scheme I requires the nonfunctional exchange to be so much more rapid than the reaction and because it imposes such a heavy restraint on catalytic efficiency, some explanation other than dissociative exchange seems necessary.

An alternative mechanism, Scheme II, that avoids the inefficient use of the enzyme intermediate in the moderate to high-pH range is one in which the enzyme itself, not the medium, provides a hydrogen, or a pool of hydrogens, with which of the intermediate E^T·enediol-P can exchange. In this model, exchange with the medium occurs *after* product is released. To test this model, we have used the isotope trapping method to label the readily exchangeable hydrogens of TIM by brief incubation in T-water. The enzyme was then rapidly stirred into a solution containing substrate such that the T-water is diluted several hundred fold at the same time that substrate is provided for catalysis with an enzyme that has exchangeable hydrogens of high specific activity. Counts incorporated within 3 s, enough for ~100 turnovers, were compared with those of a control in which substrate was added after the dilution. This control was needed to determine the amount of T incorporated from the medium during the reaction period. The experiments were usually done in the cold to limit the size of this control. Counts recovered in the triose phosphates in excess of those of the control are evidence for internal exchange between the hydrogen abstracted from the substrate and hydrogen(s) of the enzyme.

Materials and Methods

Procedure. The application and analysis of the *pulse/chase* procedure to E·T has been reported previously (Kuo & Rose, 1987; Rose & Kuo, 1989). A pulse solution containing TIM in T-water and buffer (generally triethanolamine hydrochloride, pH 7.5, 50 mM) was prepared. After at least 5 min in the cold, 3 μL of this solution containing 0.3–1 nmol of TIM was transferred with an Eppendorf pipet to 2 mL of magnetically mixed chase solution containing substrate, usually DHAP and buffer, at ice-bath temperature. Care was taken to introduce the pulse solution below the surface of the chase solution, which was being continuously stirred at ~5000 cycles/min. TCA (220 μmol) was added at 3 s. Addition of the alternative substrate, usually G3P, was made at this time to be used to determine recovery. An aliquot was taken for total counts, from which the specific activities of the pulse and the chase media are calculated. The sample was diluted and placed on Dowex-1 acetate (0.5 × 2 cm) without neutralization to avoid reactivation of the enzyme. The column was washed with water until the counting rate reached background level. These steps are conducted in a very good fume hood. DHAP and G3P were eluted together with 50 mM HCl, and the whole region containing counts was taken to dryness in vacuo at 30 °C without neutralization. Generally, all of the counts recovered in this way were labilized by reaction with TIM. Recovery of triose-Ps was often determined and found to be >95% for DHAP and ~50–70% for G3P.

Enzymes. TIM from yeast and from rabbit muscle were obtained from Boehringer. Both preparations were >90% homogeneous on SDS gel electrophoresis. They were freed of all significant contaminants on DE-Sephacryl following the procedure of Lu et al. (1984) for preparing human TIM. Human placental TIM was prepared following Lu et al. (1984). Protein was determined by amino acid analysis for the yeast and from $A_{280}^{1\%} = 12.1$ for the rabbit and human enzymes [based on Miller and Waley (1971) and the amino acid homology of the two proteins (Lu et al., 1984)]. The three enzymes had the catalytic activity expected under standard conditions. They were used after being well dialyzed and concentrated to at least 1 mM. Enzymes used for analyses were obtained commercially (Sigma or Boehringer): α-glycerol-P dehydrogenase, glyceraldehyde-P dehydrogenase, phosphoglycerate mutase, enolase, and lactate dehydrogenase.

Reagents. DHAP, D-G3P, and D,L-G3P were obtained from Sigma Chemical Co. as their diethyl ketals and acetals and were hydrolyzed with mild acid shortly before use. They were assayed by standard methods, coupling to a dehydrogenase and following the change in absorbance at 340 nm, and were determined not to be cross contaminated. T-DHAP was prepared by incubation of DHAP with TIM in T-water, pH 8.0, with 100 mM triethanolamine. The reaction was terminated with TCA, neutralized, and freed of TOH by passage through Dowex-1 acetate. The combined triose-Ps were eluted with 50 mM HCl. Labeled G3P was removed by reaction with its dehydrogenase (pH 8.0 with 0.1 mM EDTA, 3 mM $HAsO_4^{2-}$, 3 mM NAD^+). Unlabeled G3P was added after a few minutes and the change in absorbance was followed to completion. T-DHAP was isolated on DE-52, eluted with a salt gradient at pH 7.6, and kept at −80 °C at pH 4.5. It was free of T-G3P and T-PGA.

T-water (5 Ci/mL) was obtained from Amersham and used directly.

Results

Tritium Present on TIM Exchanges into Triose Phosphates. In pulse/chase experiments with triose-P isomerases from yeast, rabbit, or human using T-water in the pulse and DHAP in the chase, the counts trapped are always greater than those for control incubations. As will be shown, many fewer counts were trapped with G3P, a more rapidly used substrate. The T fixed in the control indicates the turnover rate of the enzyme in the chase solution and a correction to be made for incorporation from the medium rather than from E·T. An example is given in Table I with the yeast enzyme where, with 5 mM

Table I: T-TIM (Yeast) + DHAP → T-Triose-Ps[a]

	fixed in triose-Ps			
	cpm	cpm − control cpm		equiv trapped
control 1	68			
control 2	206	138	53 nmol/3 s[b]	
P/C	1507	1301	0.76 nmol[c]	2.18

[a] The pulse solution contained in 3 μL triethanolamine (TEA-Cl⁻, pH 7.53, 100 mM), TOH (1700 cpm/nanoatom), and yeast TIM (0.35 nmol). The magnetically stirred chase solution of 2 mL at ~0 °C contained TEA-Cl⁻ (pH 7.5, 50 mM) with DHAP (5 mM) in the pulse/chase (P/C). In control 1, TCA was added to the chase solution before the pulse solution was added. In control 2, the DHAP was added 1 min after the pulse was diluted. TCA was added at 3 s. The difference between the controls measures the rate of enzyme turnover by T incorporation. [b] Using 2.55 cpm/nanoatom as the specific activity of the TOH in the chase, the T incorporated corresponds to a turnover rate of 53 nmol fixed by 0.35 nmol of enzyme in 3 s, or 53 s^{-1}. [c] Using 1700 cpm/nanoatom as the specific activity of the TOH in the pulse.

Table II: Trapping with G3P or DHAP[a]

substrate (mM)	cpm/nmol of E		equiv trapped[b]
	control	chase	
DHAP (10)	942	5650	1.18
G3P (10)	593	1050	0.11
DHAP (10) + G3P (10)	1166	5770	1.15

[a] The procedures were as in Table I. The columned samples were incubated ± TIM, and the counts labilized include T in both DHAP and G3P. [b] (Chase − control)/SA of pulse (4000 cpm/nanoatom).

DHAP in the chase, ~2.2 equiv of T was fixed after correction for the control. Of the 5 × 10^8 cpm in each incubation only 68 cpm was found in control 1 in which TCA was added to the chase solution prior to addition of the pulse, showing that the enzyme was free of substrate. When the substrate was added *after* the pulse solution had been diluted in substrate-free buffer, control 2, an additional 138 cpm was fixed, corresponding to 150 cycles of enzyme reaction with DHAP at the specific activity of the water in the chase. These are cycles of T incorporation from water, not corrected for any isotope effect. Therefore, in the pulse/chase incubation the enzyme should have undergone enough turnovers with DHAP to have partitioned all the enzyme-bound T relevant to catalysis. The 53 nmol of exchange labeling found in the control indicates the amount of exchange out of fixed counts that might occur during the chase. This is clearly negligible compared with the 10 μmol of DHAP present.

Much less is trapped when G3P is used in the chase instead of DHAP, Table II. Under conditions of this chase ~270 nmol of DHAP was formed at 0 °C in 3 s in 2 mL of 5 mM G3P with 1 nmol of yeast TIM. This value (0.135 mM) may represent all of the aldehyde form of the G3P present, $K_{eq} \simeq 3 \times 10^{-3}$ (Reynolds et al., 1971). To examine the possibility that the trapping seen with G3P was due to the DHAP formed, the DHAP concentration dependence for trapping was determined. These data could be used to determine the rate of exchange and the minimum size of the pool of active hydrogens.

Kinetic Parameters of Trapping with DHAP. The pulse/chase results as a function of DHAP concentration with enzyme from yeast, rabbit, and human are shown in double-reciprocal form in Figure 1. Simple saturation kinetics are seen. The maximum number of enzyme equivalents trapped is determined by extrapolation to be ~1.0 with the rabbit and human enzyme and 2 and 2.8 with two samples of yeast TIM. The concentrations of substrate at which half-maximum trapping is found, $K_{1/2}$, for rabbit and human TIM were 1.0

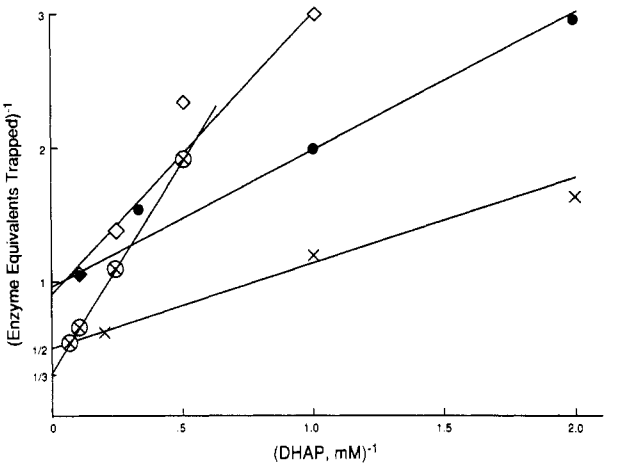


FIGURE 1: T-TIM trapped by DHAP. Conditions given in Table I, with rabbit (●), human (□), and yeast (× and ⊗) enzyme. The second sample of yeast example (⊗) came from the first, stored at −80 °C.

Table III: Effect of Competitive Inhibitors on Trapping and Rate[a]

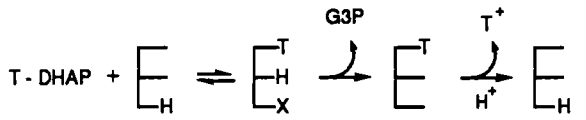
conditions (mM)	control rate (nmol/3 s)	P/C (equiv trapped)	ratio of effects[b]
DHAP (5)	196	1.04	
+D-glycerol-P (10)	47	0.29	0.95
+P-glycolate (0.4)	47	0.90	0.17
DHAP (10)	92	1.14	
+P-glycolate (2)	12	0.92	0.22
DHAP (5)	210	0.72	
+D-glycerol-P (20)	31	0.17	0.90
+P_i (50)	86	0.60	0.28

[a] Three independent experiments were done, as in Table I, with yeast TIM at 0 °C. DHAP, either alone or with inhibitor, was used in the control and pulse/chase (P/C) experiments. [b] Ratio of effects = (extent of inhibition of trapping)/(extent of inhibition of the control rate).

and 3.2 mM, respectively. For the yeast sample with which ~3 equiv was trapped, $K_{1/2}$ was 9.1 mM. The maximum rate of T incorporation in the controls was ~50 s^{-1}. Since most of the T that was trapped was found in DHAP, it was necessary to know the kinetic constants pertinent to exchange of T between DHAP and medium. The K_m for T-DHAP was determined from its exchange kinetics over the range 0.2–8 mM at pH 7.5 and 0 °C. A linear double-reciprocal plot was obtained from which the values K_m = 0.24 mM and k_{cat} ~ 43 s^{-1} for exchange were determined. Dissociation into the medium of the three T of the free enzyme is calculated from $k_{off}^T = K_{1/2}k_{cat}/K_m$ (Rose et al., 1974) and is ~1600 s^{-1}. Exchange of T is too rapid, the concentration of DHAP required too great, to attribute the counts trapped when G3P is used for trapping to the 0.135 mM DHAP formed, i.e., $\leq 0.135/9 \times 1.5 = 0.023$ equiv as the upper limit compared with 0.11 equiv found.

Effects of Competitive Inhibitors. What are the characteristics of DHAP that decrease the exchange rates of the active site T's from 1600 to ~0 s^{-1} so that 3 equiv can be trapped? Three competitive inhibitors, inorganic phosphate (K_i = 5 × 10^{-3} M), D-α-glycerol-P (K_i = 10^{-4} mM), and 2-phosphoglycolate (K_i = 6.5 × 10^{-6} M) [values for chicken TIM, pH 7.7, reported by Johnson & Wolfenden (1970)], were tested for their ability to affect trapping by DHAP, Table III. If an inhibitor completely blocks the exchange of bound T, it will *not* influence the amount of T that will be trapped by a given amount of DHAP as long as enough time is allowed before the addition of TCA for all of the expected T to be processed. On the other hand if T exchange occurs in the complex formed with the inhibitor, the amount trapped would

Scheme III



be decreased to the extent that the inhibitor rather than substrate occupies the active site as shown by the extent of inhibition of incorporation in the control. A comparison of these two values shows that bound P_i and phosphoglycolate allow little exchange to occur whereas α-glycerol-P, a somewhat better inhibitor than P_i, has less of an effect on inhibiting the exchange. A more extended study would be required to determine the T exchange rate of the inhibited enzyme compared with that of the free enzyme.

Discussion

A proton transfer mechanism was proposed for phosphoglucose isomerase (Rose & O'Connell, 1961; Rose, 1962) on the basis of the occurrence of transfer of hydrogen between C_1 and C_2 of the same molecule and exchange with medium of that which was not transferred. The exchange, for which no role was given in the mechanism, was taken as evidence for a proton transfer mechanism. It was assumed to occur by proton dissociation at the intermediate state as an alternative to transfer to C-1 or C-2 of the tightly bound enediol intermediate. It was clear that such a mechanism for exchange would lower the concentration of the functional donor in the intermediate state depending on its pK_a and the pH of the medium. Transfer is a major factor with this enzyme; ~45% of the T of substrate was transferred to product at 37 °C, so that catalytic efficiency could only be increased 2-fold by preventing the exchange. The model of proton dissociation at the intermediate state poses a significant disadvantage for TIM, if, as suggested by the low extent of transfer, the intermediate state is close to dissociative equilibrium. The value 3–6% is most usually given for transfer (Herlihy et al., 1976; Nickbarg & Knowles, 1988). The "degradation" of the intermediate state by proton dissociation would be a disadvantage that evolution would need to overcome by use of a base with a high pK_a in the enzyme–substrate complex or by shielding the active site from the medium at the intermediate stage of the reaction so that loss of the donor H could not occur. Glu_{165} has a low pK_a of 3.9 in the free enzyme form (Hartman et al., 1975). To reconstitute the protonated intermediate, as would be necessary to maintain the maximum rates that are seen at pH 10, would require a 5 or 6 order increase in basicity of this group, a property that is contraindicated by the rapid loss of the T from the intermediate in the first place. A mechanism that uses two bases to explain exchange (Scheme III), possibly with a small amount of transfer between them, would very well explain the high "apparent" exchange in the conversion of T-DHAP to G3P, but it would not explain the rapid back-exchange of the medium hydrogen into starting substrate under steady-state conditions. This can only occur by hydrogen exchange from the noncommitted intermediate state and would be independent of whether or not the enzyme uses a second hydrogen donor in the formation of product.

The conclusion that TIM uses internal H exchange for the back-labeling of substrate assumes that the pulse/chase results reflect steps that occur in the steady state. If the same process occurs at a sufficient rate during the steady state, both back-labeling in T-water and exchange from T-DHAP would be explained by the combination of internal and external exchange as in Scheme II. By use of yeast TIM, the rate of the exchange of enzyme-bound T with the medium was calculated from k_{cat}/K_m for the detritiation of T-DHAP under the same reaction conditions and from the concentration of DHAP required for half-maximal trapping to be ~1600 s^{-1}. The external exchange rate of the three T of the active site in free enzyme is therefore much faster than required to explain the exchange of T of T-DHAP with the medium, ~43 s^{-1} under the same conditions.

Very significant structural changes occur upon addition of substrate to TIM as shown by crystallography (Banner et al., 1975; Alber et al., 1981) and from changes in the backbone T exchange pattern (Browne & Waley, 1974). In the latter study DHAP inhibited the exchange with water of more than 100 amide hydrogens. The linearity of the trapping data of Figure 1 implies that only a small number of sites, one to three, are available for back-exchange with the substrate proton, and the high rate of their exchange with the medium indicates that they are not amide in origin. Although the trapped T theoretically represents a minimum of the number of enzyme protons that participate in the exchange, the linearity of the reciprocal plots, Figure 1, suggests that additional positions would need to exchange much more rapidly than those trapped. Protons of the active site that exchange much more rapidly than 10^3 s^{-1} would not retain T from the pulse long enough to be trapped by 20 mM DHAP.

Trapping of more than 1 enzyme equiv of T requires the active site to be stable through several reaction cycles. That this is not seen with the mammalian enzymes may indicate some characteristic of the active site that is different in the yeast TIM. For example, the active site of yeast enzyme may be found to have a lower thermal mobility factor when examined in the crystalline state than the other TIMs. Greater thermal mobility of the active site regions of the rabbit and human enzymes may make it difficult to trap more than 1 of 3 equiv available for exchange with the donor proton during extra catalytic cycles of these enzymes. It may be relevant to this that the pulse/chase properties of the yeast enzyme preparation have been observed to change with time of storage. With the same procedures, the $K_{1/2}$ for the same sample of yeast enzyme, kept at −80 °C, has increased from ~1 to 9 mM with time. The higher $K_{1/2}$ indicates a faster exchange rate with the medium. The limit value also tended to increase from 2 to 3.

With none of the three enzymes were the counts trapped with G3P greater than ~10% of those trapped with DHAP although back-labeling in T-water is as firmly established for G3P as for DHAP. Raines and Knowles (1987) using the isotope counterflow method of Britton (1973) showed that DHAP and G3P use different forms of chicken TIM. The poor trapping with G3P could reflect an unfavorable distribution of isoforms in the pulse. Possibly the T of the DHAP-specific form undergoes extensive exchange during conversion to the G3P-reactive form.

G3P would be less effective in trapping if its dominant *gem*-diol form, ~97% of the total (Trentham et al., 1969), caused the discharge of T during the chase in competition with the active aldehyde form of the substrate. An approach to this question was made by determining whether trapping by the two substrates was additive. As seen in Table II any effect of G3P or its *gem*-diol in facilitating the detritiation of the enzyme does not apply to the T that is trapped by DHAP, or is much less than would be required to explain so much less trapping with G3P.

Relevance to Earlier Reports. X-ray crystallographic studies of chicken and yeast TIM (Banner et al., 1975; Alber

et al., 1981) have identified several residues within 5 Å of Glu_{165} that contain exchangeable hydrogens. Two of these, His_{95} and Lys_{13}, have been proposed as H donors to polarize the substrate carbonyl. A third residue, Ser_{96}, was identified as forming an H-bond with the carboxyl of Glu_{165}. All of these should become labeled in the pulse incubation. It may be that Glu_{165} or some nearby group of low pK_a plays a role in external exchange of some or all of these in the free enzyme. Imposing substrate between these groups could explain the lack of exchange in E·T·DHAP. Since even the simplest of competitive inhibitors, P_i, causes significant stabilization of E·T, it appears that isolation of the active site from the medium is an early consequence of substrate binding. Which residue(s) participates in the internal exchange with Glu_{165} and what are the routes of internal transfer will require further study. Since internal exchange occurs at the state of the tightly bound enediol-P intermediate and must be extremely rapid, identification of the responsible active site protons should help in understanding the structure of the intermediate complex. Candidate residues and the active site base should be located at the *re* face of the sp^2 carbons of the substrates (Rose et al., 1969). It is tempting to ascribe the difference of two T trapped between the yeast and mammalian enzyme to an active site water molecule. Rapid exchange of this water during the chase could occur with the latter.

The exchange of hydrogen abstracted from substrate with water is a side reaction of no known significance to the TIM reaction. However, given the correct model for describing the mechanism and the position in the scheme at which the exchange occurs, it is possible to obtain insight into the kinetic pattern of the enzyme from exchange studies. An example of this approach and its uses may be seen with TIM using the central exchange model, Scheme I (Albery & Knowles, 1976). Since exchange, in fact, occurs at the level of free enzyme, some changes may be needed in the interpretations presented by these authors. In the central exchange mechanism, discrimination observed in the formation of each product in TOH will be independent of discrimination that occurs in forming the other. In the closed model with a single base mechanism, T from water into alternate products at high substrate concentration will be partitioned to reflect relative isotope effects in the two limbs of the reaction at early times [p 219 of Rose (1977)]. When the reaction is taken to completion, no discrimination will be seen. It should be noted that in examining models to explain their observation that the percent transfers from 1L-DHAP to 2L-G3P (where L is either T or ^2H) are the same at complete reaction with chicken TIM, Fisher et al. (1976) discuss one model in which rapid equilibration occurs between the donor hydrogen and a pool of enzyme-bound H_2O molecules. The fractionation factor between $-CO_2L$ and H_2O is expected to be ~ 1. Release of the bound water, not involving –O–L bond cleavage, in each cycle would not show an isotope effect. Therefore, this internal/external exchange model was deemed acceptable to explain exchange. The effects of specific amino acid replacement on T trapping should be useful in revealing changes in dynamics not visible by steady-state studies.

Raines et al. (1986) reported that when the active site base Glu_{165} of chicken TIM was replaced by an Asp residue, decreasing the forward rate 240-fold, T transfer from T-DHAP to G3P still could be observed, 2% as opposed to ~4% under the same conditions as with the wild-type yeast enzyme. On the basis of the central exchange model, this observation seems contrary to the assignment of Glu_{165} as the base involved in breaking and making –CH bonds, since it requires that the rates of *both* exchange and transfer are decreased to about the same extent by the change of Glu to Asp. However, in the new model, Scheme II, the extent of transfer need not decrease when transfer to the enediol-P is made slower, since both exchange and transfer require passage through the same inhibited step.

Yeast TIM $His_{95} \rightarrow Gln$ was shown by Nickbarg et al. (1988) to have a 140-fold decreased forward k_{cat}. In examining the origin of this effect, it was found that incorporation of T from TOH into product was still observed but that back-exchange into substrate had been lost. In particular, this observation was made for the reaction studied in either direction. These are characteristics of a "two-base mechanism". The central exchange model does not provide a simple explanation for the preferential inhibition of back-labeling. The present observation that labeling of substrates occurs by exchange of the donor proton with hydrogens of the enzyme makes it likely that His_{95} is a necessary component of the internal exchange process. For the rabbit and human isomerase, His_{95} may be the only labeled residue in the internal exchange "pool" since only 1 enzyme equiv could be trapped (Figure 1). Complete loss of back-exchange in the $His \rightarrow Gln$ yeast mutant where three hydrogens of the wild-type enzyme participate as shown by pulse/chase suggests that access to the donor hydrogen of any of the three depends on the presence of His_{95}. Lacking interaction with His_{95}, one of the remaining 2 equiv might be the source of hydrogen used to form the product, giving the appearance of a two-base mechanism.

Caveats and Conclusions. It is important to point out that the isotope trapping experiment does not necessarily reflect the behavior of the enzyme in the steady state in T-water. For example, an excess of time is given for labeling the enzyme in the pulse of these experiments whereas labeling of enzyme forms from the medium in the steady state must occur in fractions of a second. In addition, in the continuous presence of T-water the processing of T that is incorporated into the enzyme is not sensitive to its exchange rate in the same way that it is in a pulse/chase experiment in which the medium has been changed to allow exchange to be observed. Nevertheless, the finding that the donor hydrogen once labeled, however circuitously, can be exchanged with the abstracted proton of the substrate without exchange with the medium simply by use of high substrate concentration suggests that the mechanism of back-labeling shown in Scheme I is not correct. The additional observation that binary complexes with such distant substrate analogues as P_i strongly block the exchange of T of the active site with water leads to the same conclusion.

Generalizations. It is proposed that upon binding of substrate the active sites of enzymes must become isolated from the medium in order to prevent loss of hydrogens that are required for processing the reaction intermediates. Minimizing the binding energy required to insulate the active sites from proton exchange with the medium at committed states may be an important factor in the evolution of enzyme structure and may be one of the achievements of the $(\alpha\beta)_8$ barrel configuration frequently seen in enzymes that abstract and transfer protons. Three of the 14 enzymes known to have this structure [see Lebioda and Stec (1988) for a list] have been shown so far to lack direct proton exchange with the medium during catalysis: TIM, xylose isomerase (Rose et al., 1969), and pyruvate kinase in which the proton needed for the ketonization of enolpyruvate is bound to the enzyme before the full complex with substrates is formed and is fully retained during the preceding phosphoryl transfer step (Rose & Kuo, 1990).

Xylose isomerase is an interesting example in that no exchange with the medium could be found during prolonged incubation (Rose et al., 1969). Crystallographic data from two laboratories disagree as to the placement and role of an active site histidine (Carrell et al., 1989; Collyer et al., 1990) but agree in finding the active site very hydrophobic and free of other prototropic amino acids with which internal exchange might occur. Other enzymes not known to have the β barrel motif that show substrate-blocked proton exchange are aconitase (Kuo & Rose, 1987), fumarase (Kuo and Rose, unpublished results), and phosphoglucose isomerase (Seeholzer and Rose, unpublished results). In addition, many single-substrate enzymes have broad V_{max} versus pH profiles despite significant mechanistic complexity in their reactions. These may also be examples of the inaccessibility of the active site to medium imposed by substrate binding, in which case values interpreted as pK_a's may be more complex.

Unlike hydrogen exchange with the medium, *exchange* between groups on a protein will not lead to a change in proton potential, and therefore, its occurrence per se may not be viewed as an important factor in the evolution of protein structure. Such proton exchange networks in special cases make possible the mobility of protons for proton relays between the active site and the medium and for rapid charge rearrangement even at a distance from a signal. Given the frequency of hydrogen bonding, the presence of unshared electron pairs on amino acid residues containing N, O, and S, and the presence of bound H_2O molecules, internal proton exchange must be a constant, though silent, phenomenon of proteins. "Silent" except when it provides access to a proton pool with which hydrogen from a stable position of the substrate exchanges.

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References

Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. London, B 293*, 159–171.

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627–5631.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., & Waley, S. G. (1975) *Nature 255*, 609–614.

Britton, H. G. (1973) *Biochem. J. 133*, 255–261.

Browne, C. A., & Waley, S. G. (1974) *Biochem. J. 141*, 753–760.

Browne, C. A., Campbell, I. D., Kiener, P. A., Phillips, D. C., Waley, S. G., & Wilson, I. A. (1976) *J. Mol. Biol. 100*, 319–343.

Burton, P. M., & Waley, S. G. (1968) *Biochim. Biophys. Acta 151*, 714.

Carrell, H. L., Glusker, J. P., Burger, B., Manfre, F., Tritsch, D., & Biellmann, J.-F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4440–4444.

Collyer, C. A., Hendrick, K., & Blow, D. M. (1990) *J. Mol. Biol.* (in press).

Corran, P. H., & Waley, S. G. (1973) *FEBS Lett. 30*, 97–99.

Coulson, A. F. W., Knowles, J. R., & Offord, R. E. (1970) *Chem. Commun.*, 7–8.

Fisher, L. M., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5621–5626.

Hartman, F. C., La Muraglia, G. M., Tomozawa, Y., & Wolfenden, R. (1975) *Biochemistry 14*, 5274–5279.

Herlihy, J. M., Maister, S. G., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 3601–3606.

Johnson, L. H., & Wolfenden, R. (1970) *J. Mol. Biol. 47*, 93–100.

Krietsch, W. K. G., Pentchev, P. G., Klingenbürg, H., Hofstätter, T., & Bücher, T. (1970) *Eur. J. Biochem. 14*, 289–300.

Kuo, D. J., & Rose, I. A. (1987) *Biochemistry 26*, 7589–7596.

Lebioda, L., & Stec, B. (1988) *Nature 333*, 683–686.

Lu, H. S., Yuan, P. M., & Gracy, R. W. (1984) *J. Biol. Chem. 259*, 11958–11968.

Maister, S. G., Pett, C. P., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5612.

Miller, J. C., & Waley, S. G. (1971) *Biochem. J. 122*, 209–218.

Nickbarg, E. B., & Knowles, J. R. (1988) *Biochemistry 27*, 5939–5947.

Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5948–5960.

Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 310–311.

Raines, R. T., & Knowles, J. R. (1987) *Biochemistry 26*, 7014–7020.

Raines, R. T., Sutton, E. L., Straus, D. R., Gilbert, W., & Knowles, J. R. (1986) *Biochemistry 25*, 7142–7154.

Reider, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007–1010.

Reynolds, S. J., Yates, D. W., & Pogson, C. I. (1971) *Biochem. J. 122*, 285–297.

Richard, J. (1984) *J. Am. Chem. Soc. 106*, 4926–4936.

Rose, I. A. (1962) *Brookhaven Symp. Biol. 15*, 293–309.

Rose, I. A. (1977) *Isotope Effects on Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 209–233, University Park Press, Baltimore, MD.

Rose, I. A. (1980) *Methods Enzymol. 64*, 47–59.

Rose, I. A. (1984) *Biochemistry 23*, 5893–5894.

Rose, I. A., & O'Connell, E. L. (1961) *J. Biol. Chem. 236*, 3086–3092.

Rose, I. A., & Kuo, D. J. (1989) *Biochemistry 28*, 9579–9585.

Rose, I. A., O'Connell, E. L., & Mortlock, R. P. (1969) *Biochim. Biophys. Acta 178*, 376–379.

Rose, I. A., O'Connell, E. L., Litwin, S., & Bar Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168.

Simon, H., Medina, R., & Müllhofer, G. (1968) *Z. Naturforsch. 23*, 59–64.

Trentham, D. R., McMurray, C. H., & Pogson, C. T. (1969) *Biochem. J. 114*, 19–24.

Waley, S. G., Miller, J. C., Rose, I. A., & O'Connell, E. L. (1970) *Nature 227*, 181.