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## Liberation of the Triosephosphate Isomerase Reaction Intermediate and Its Trapping by Isomerase, Yeast Aldolase, and Methylglyoxal Synthase<sup>†</sup>

Radha Iyengar and Irwin A. Rose\*

**ABSTRACT:** When a mixture of triosephosphate isomerase (rabbit muscle) and dihydroxyacetone phosphate (DHAP) is quenched with acid, a compound is liberated, presumed to be the *cis*-enediol 3-phosphate, that decomposes to inorganic phosphate (P<sub>i</sub>) and methylglyoxal [Iyengar, R., & Rose, I. A. (1981) *Biochemistry* (preceding paper in this issue)]. The decomposition can be prevented by rapid neutralization if a catalytic amount of fresh isomerase is present. Varying the time between acidification and rescue gave a half-life of the liberated compound of ~12–17 ms. Varying the concentration of enzyme used for rescue gave a minimum second-order rate constant for trapping of 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup>. These results add further evidence favoring a stepwise mechanism for the aldose–ketose isomerase reactions in which a chemically defined enzyme-bound intermediate is formed. The high rate of trapping over a wide pH range indicates that the enediol phosphate, not the

enediolate phosphate, is the intermediate. One property of the enzyme is to stabilize the intermediate with respect to its fragmentation in solution by >1000-fold. Yeast aldolase is also able to rescue all of the isomerase intermediate, though higher concentrations of enzyme are required. Although different enantiotopic protons of DHAP are abstracted by isomerase and aldolase, both enzymes use the same enediol phosphate intermediate. Methylglyoxal synthase at a 50-fold greater concentration was unable to compete with triosephosphate isomerase for *cis*-enediol phosphate. Either the synthetase has a low *V*/*K* for the *cis* isomer or it uses the *trans*-enediol phosphate form specifically. A new strategy for the chemical and enzymological characterization of enzyme reaction intermediates is provided here based on the liberation of the intermediate from the reaction equilibrium and its recovery by fresh enzyme or another enzyme species.

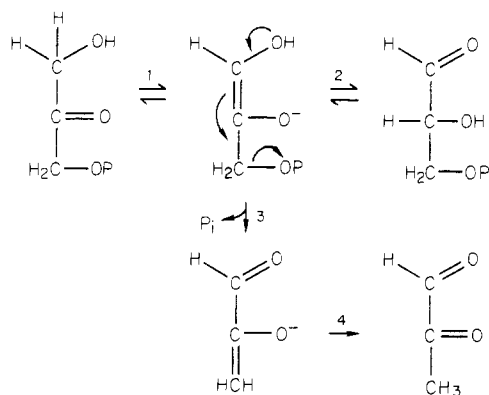
The observation of intramolecular hydrogen transfer in acid- and base-catalyzed aldose–ketose interconversion in solution has provided evidence that the long-favored enediol mechanism may be incorrect (Gleason & Barker, 1971; Harris & Feather, 1975; Lookhart & Feather, 1978). On the other hand, enzymatic isomerizations are most easily explained if a single base on the enzyme abstracts the α proton which is then

partitioned between the neighboring carbons of a *cis*-enediol with varying degrees of exchange with the medium (Rose & O'Connell, 1961; Rose, 1975). Chemical support favoring an enediol phosphate intermediate in the triosephosphate isomerase reaction was obtained recently (Iyengar & Rose, 1981) with the observation that isomerase produced inorganic phosphate (P<sub>i</sub>)<sup>1</sup> and methylglyoxal equally at a measurable

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<sup>1</sup> Abbreviations used: DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; Cl<sub>3</sub>CCOOH, trichloroacetic acid; TEA, triethanolamine; P<sub>i</sub>, inorganic phosphate; enediol phosphate, 1,2-dihydroxy-1-propene 3-phosphate; ATP, adenosine 5'-triphosphate; NADH, reduced nicotinamide adenine nucleotide.

Scheme I



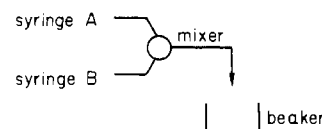
fraction of the normal enzyme catalytic rate. This was best explained as a side reaction of the enediol phosphate intermediate (step 3, Scheme I), followed by ketonization (step 4) (Brown et al., 1957). Further support for an enediol phosphate intermediate was the formation of  $^{32}P_i$  when a mixture of isomerase and [ $^{32}P$ ]DHAP was denatured in acid. On the basis of this, the presumed enediol phosphate would represent ~5% of the total of bound substrates. Generation of the enediol phosphate in this way has now been used to study its properties in solution and its ability to react with isomerase itself and with enzymes for which it might serve as a reaction intermediate. Full diversion from  $^{32}P_i$  formation proves that the intermediate was liberated from isomerase without chemical change and supports the argument for a stepwise mechanism in the isomerase reaction. Reaction with yeast aldolase demonstrates that both enzymes use the same intermediate species. Observations with methylglyoxal synthase place limits to the possibilities for its intermediate.

## Materials and Methods

Rabbit muscle triosephosphate isomerase (EC 5.3.1.1) was obtained from Boehringer, Mannheim, and used without further purification. Protein concentration was measured from the  $A_{280}$  by assuming the absorbance of 1 mg of pure isomerase/mL to be 1.21/cm and the weight of the catalytic subunit to be 26 000 daltons (McVittie et al., 1972). Triosephosphate isomerase was assayed with DL-glyceraldehyde 3-phosphate (G3P) as the substrate and  $\alpha$ -glycerophosphate dehydrogenase as the coupling enzyme (Beisenherz, 1955). Zn-containing yeast aldolase (~85 units/mg) was a gift from Drs. G. Smith and A. S. Mildvan. It was treated with glycidol phosphate (Rose & O'Connell, 1969b) to inactivate contaminating triosephosphate isomerase. Protein concentration was determined by absorbance at 280 nm,  $\epsilon_{280} = 1.02$  mL/mg, and an equivalent weight of 40 000 (Harris et al., 1969). Methylglyoxal synthase was isolated from trypticase soy broth grown *Proteus vulgaris* cells (ATCC 13315) by the procedure of Tsai & Gracy (1976) and had a specific activity of 8–10 units/mg, similar to that reported for the crystalline enzyme. Synthase activity was measured by the glyoxalase I coupled spectrophotometric method (Racker, 1957). The preparation contained no detectable triosephosphate isomerase or aldolase activity. The enzyme was stored in 1 mM  $P_i$  which was removed prior to use. Other enzymes were obtained as follows: glycerol kinase (*Escherichia coli*) and glyoxalase I were from Sigma Chemical Co.;  $\alpha$ -glycerophosphate dehydrogenase (rabbit muscle) was from Boehringer.

Dihydroxyacetone phosphate ([ $^{32}P$ ]DHAP) (7780 cpm/nmol at the time of preparation) was synthesized by phosphorylation of dihydroxyacetone by glycerol kinase with [ $\gamma$ -

Scheme II



$^{32}P$ ]ATP and was purified on a Dowex-1 ( $Cl^-$ ) column (Rose & O'Connell, 1969a). Most of the contaminating  $^{32}P_i$  was removed by precipitation as the barium salt. The preparation was free of G3P but contained ~1% of the total radioactivity as  $^{32}P_i$ . [ $\gamma$ - $^{32}P$ ]ATP was purchased from New England Nuclear and 2,4-dinitrophenyl acetate, from Eastman Kodak.

A rapid-flow apparatus diagramed in Scheme II was used in most of the experiments. The mixer cell was the design of Dr. D. Ballou (Ballou & Palmer, 1974). In the usual experiment a solution containing triosephosphate isomerase and  $^{32}P$ [DHAP] is injected from syringe A into the mixer where 0.2 N trichloroacetic acid ( $Cl_3CCOOH$ ) introduced from syringe B inactivates >99.8% of the enzyme. The acidic reaction mixture is delivered into a beaker containing a vigorously stirred, buffered solution. Variable reaction times were obtained by maintaining a constant flow rate but changing the length of tubing between mixer and beaker. After the addition to the beaker (5 s),  $Cl_3CCOOH$  (0.2 N final concentration) was added.  $^{32}P$  present as inorganic phosphate ( $^{32}P_i$ ) was analyzed by isobutyl alcohol extraction of the molybdate complex (Berenblum & Chain, 1938). Reaction times for the rapid quench experiments were standardized by the hydrolysis of 2,4-dinitrophenyl acetate in base (Froehlich et al., 1976).

## Results

It has been shown that the so-called phosphatase activity of triosephosphate isomerase acting on DHAP is due to the slow production of  $P_i$  and methylglyoxal (Iyengar & Rose, 1981). In addition, when a mixture of [ $^{32}P$ ]DHAP and excess isomerase was added to  $Cl_3CCOOH$ , ~5% of the  $^{32}P$  was found to be present as  $^{32}P_i$ . As both substrates of the isomerase reaction are stable under these conditions, on the basis of the mechanism for the alkaline lability of DHAP and earlier considerations of the active intermediate for the normal isomerase reaction (Reider & Rose, 1959; Rose, 1962), an enediol phosphate was proposed as the source of the  $^{32}P_i$ . If this is the case and if the intermediate has sufficient stability in the acid, it should be possible to prevent its decomposition to  $P_i$  by a fresh sample of enzyme at neutral pH. On the other hand, if the intermediate is altered in the acid, none or perhaps only part of it would be trapped by the intervention of isomerase before decomposition to  $P_i$ .

The addition of fresh isomerase to an acid-quenched mixture of isomerase and [ $^{32}P$ ]DHAP had no effect on the yield of  $^{32}P_i$  if the addition was made by hand 2 s after the acid. This indicated that either the intermediate was altered during denaturation or that it was very unstable once it was liberated from the enzyme. When the experiment was repeated in the rapid-mixing device so that the acidified mixture reached the new isomerase solution after ~5 ms (Scheme II), the results of Table I were obtained. When the quenched solution was mixed either into the stirred, pH 7.6 buffered solution or into  $Cl_3CCOOH$ , the expected ~5% of initially bound [ $^{32}P$ ]DHAP was found as  $^{32}P_i$ . However, a maximum of 80% of the acid-labile intermediate could be diverted to a form that was alkaline labile, i.e., triose phosphates, when isomerase was present in the buffer. No such effect was seen if the isomerase was pretreated with the active site directed inactivator, glycidol phosphate. Hence it was concluded that at least 80% of a very

Table I: Labile Compound Liberated by Acidifying Triosephosphate Isomerase and [ $^{32}\text{P}$ ]DHAP, an Isomerase Substrate

additions to beaker	[ $^{32}\text{P}$ ]-DHAP (cpm)	$^{32}\text{P}_i$ (cpm)	$^{32}\text{P}_i$ > con- by triose- trol <sup>a</sup> (%)	fraction trapped phosphate isomerase
none	26 032	1692	5.0	0
$\text{Cl}_3\text{CCOOH}$ (0.2 N final concn)	25 232	1715	5.3	0
isomerase (5 nmol)	28 712	748	1.1	0.79
isomerase (10 nmol)	28 416	710	1.0	0.81
inactivated isomerase (10 nmol)	23 205	1554	5.2	0

<sup>a</sup> 1.5%  $^{32}\text{P}_i$  when no enzyme is in syringe A. The pulse solution in syringe A, 50  $\mu\text{L}$ , containing [ $^{32}\text{P}$ ]DHAP (5 nmol, ~5000 cpm/nmol) and triosephosphate isomerase (25 nmol) in 0.02 M sodium cacodylate, pH 6.5, was mixed with  $\text{Cl}_3\text{CCOOH}$  (1 mL, 0.2 N) from syringe B. At 5.6 ms the reaction mixture was delivered to a beaker containing a rapidly stirred mixture of buffer (0.5 mL, 0.1 M TEA-HCl, pH 7.6 final concentration) and the enzymes indicated.  $\text{Cl}_3\text{CCOOH}$  (0.2 N final concentration) was added 5 s later, and  $^{32}\text{P}_i$  was assayed directly. In all cases, further treatment with 0.5 N NaOH for 15 min at 37 °C yielded >99% of the radioactivity as  $^{32}\text{P}_i$ .

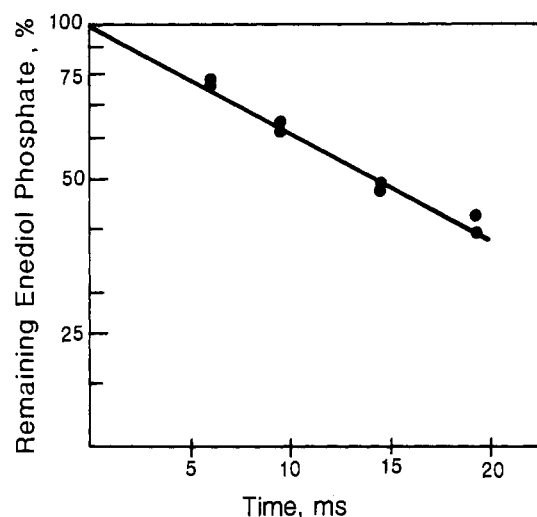


FIGURE 1: Rate of elimination of  $\text{P}_i$  from the enediol phosphate intermediate. With excess isomerase (10 nmol) in the trapping beaker (as in Table I) the times in 0.2 N  $\text{Cl}_3\text{CCOOH}$  from the mixer to the beaker were varied: remaining enediol phosphate  $\equiv [({}^{32}\text{P}_i)_\infty - ({}^{32}\text{P}_i)_t] / [({}^{32}\text{P}_i)_\infty - ({}^{32}\text{P}_i)_0]$ , where  $({}^{32}\text{P}_i)_0$  is the  $^{32}\text{P}_i$  contaminant of the substrate,  $({}^{32}\text{P}_i)_\infty$  is the  $^{32}\text{P}_i$  present if isomerase is omitted from the beaker, and  $({}^{32}\text{P}_i)_t$  is that present at elapsed time  $t$  between the generation and utilization of the intermediate.

labile intermediate could be recovered 5 ms after liberation from its bound form but none at 2000 ms.

To determine if the 20% of the  $^{32}\text{P}_i$  that could not be trapped by isomerase simply reflected the amount of the intermediate that was destroyed prior to reaching the beaker or if it represented an unstable compound that could not react with isomerase, it was necessary to calculate the time dependence for the formation of  $\text{P}_i$  in  $\text{Cl}_3\text{CCOOH}$ . Figure 1 shows that the earliest point obtained falls on the experimental line that extrapolates to 100% trapping by isomerase and therefore supports the thesis that only one compound with a  $t_{1/2}$  in 0.2 N  $\text{Cl}_3\text{CCOOH}$  of ~15 ms is released from the enzyme.

By extending the rapid mixing device to include a third syringe that carries buffer to neutralize the effluent of the quench mixer in a second mixer before it reaches the beaker, it was possible to determine the effect of pH on the rate of the elimination reaction. As shown in Figure 2 no more than

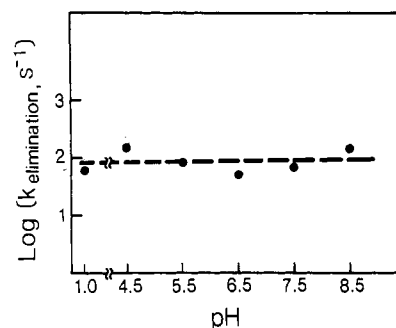
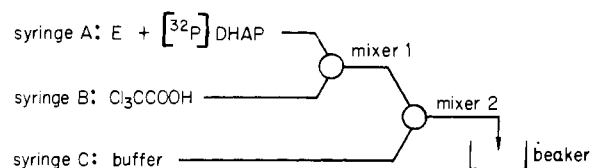
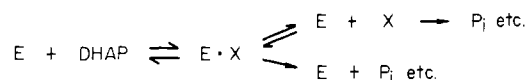


FIGURE 2: pH dependence of  $k_{\text{elim}}$ . In mixer 1 was added 50  $\mu\text{L}$  of pulse solution from syringe A containing [ $^{32}\text{P}$ ]DHAP (5 nmol, 7780 cpm/nmol) and triosephosphate isomerase (25 nmol) in 0.02 M sodium cacodylate (pH 6.5) which was mixed with  $\text{Cl}_3\text{CCOOH}$  (1 mL, 0.2 N) from syringe B. Buffer (1 mL; final pH 4.5, 0.02 M sodium acetate; pH 5.5 and 6.5, 0.02 M sodium cacodylate; pH 7.5 and 8.5, 0.1 M TEA-HCl) from syringe C was injected into mixer 2 where the pH of the deactivated pulse was instantaneously adjusted. The solution was allowed to react during the time it travels from mixer 2 to the trapping beaker containing excess isomerase (10 nmol) as in Table I. After the addition to the beaker (5 s),  $\text{Cl}_3\text{CCOOH}$  (0.2 N final concentration) was added and  $^{32}\text{P}_i$  was determined. The reaction time from mixer 1 to the beaker was 9.6 ms:



The difference between  $^{32}\text{P}_i$  formed when isomerase is omitted entirely from the trap and when it was included in syringe C gives the total amount of X available at the point of pH change. Because the distance between the two mixers was very small, this was 4–5 times the  $^{32}\text{P}_i$  present at the entry to mixer 2. With enzyme in the beaker the amount decomposed in the buffers during the time interval from mixer 2 to the beaker can be calculated.

Scheme III



a 3-fold change in rate was observed between pH 4.5 and pH 8.5, and the lowest rate at pH 6.5 of 60  $\text{s}^{-1}$  was approximately the same as that observed in 0.2 N  $\text{Cl}_3\text{CCOOH}$ , pH ~1. At all test pH values the amount of  $^{32}\text{P}_i$  resulting when enzyme was absent from the beaker was ~5% of the total  $^{32}\text{P}$ , indicating that any differences in rate with pH did not result from a partition of the free enediol phosphate to products other than  $\text{P}_i$ ; that is, tautomerization does not seem to be a competing process at any pH.

**Relationship between the Enediol Phosphate Intermediate and Triosephosphate Isomerase.** The instability of the enediol phosphate in solution can be contrasted to its highest apparent rate constant of decomposition of 0.1  $\text{s}^{-1}$  on the enzyme at pH 5.5 (Iyengar & Rose, 1981). It is not immediately obvious if this value represents the rate at which the intermediate dissociates from the enzyme or the rate of its decomposition in the special environment of the active site. Were the intermediate liberated prior to the elimination reaction, one would expect any uncomplexed isomerase to convert it to triose phosphates as was observed in the rapid quench experiment (Table I). These alternatives are shown in Scheme III, where X represents enediol phosphate. As seen in Figure 3 the rate of  $\text{P}_i$  formation increased until DHAP and isomerase were equivalent and remained constant in the presence of as much as 50  $\mu\text{M}$  free isomerase. Formation of free G3P itself could not explain the production of  $\text{P}_i$  because the lowest amount

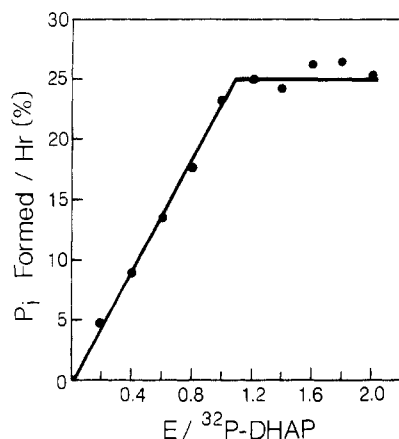


FIGURE 3: Effect of excess enzyme on  $\beta$  elimination by isomerase with  $[^{32}\text{P}]\text{DHAP}$ . The incubation mixture (0.1 mL, 37 °C) contained  $[^{32}\text{P}]\text{DHAP}$  (5 nmol, 7780 cpm/nmol), triosephosphate isomerase as indicated (1–10 nmol), and buffer (0.1 M TEA-HCl, pH 7.5). The  $^{32}\text{P}_i$  that was formed in 1 h was determined. A control of 10%  $\text{P}_i$  formed/h in the absence of enzyme was subtracted from all points although this would be incorrect as free substrate becomes negligible with increasing enzyme.

Table II: Effect of Isomerase Concentration on Trapping the Intermediate<sup>a</sup>

isomerase subunits ( $\mu\text{M}$ )	$^{32}\text{P}_i$ formed (%)		rel fraction trappable by isomerase	
	pH 5	pH 8	pH 5	pH 8
0	5.3	5.2	0	0
0.033	4.0	3.8	0.31	0.34
0.047	3.5	3.4	0.43	0.44
0.066	2.8	3.0	0.60	0.54
0.1	2.0	1.8	0.79	0.83
0.13	1.2	1.1	1.0	1.0
0.66	1.1	1.2	1.0	1.0

<sup>a</sup> The syringes were set up as in Table I. Isomerase concentration was varied in the stirred trapping solution at a final concentration of pH 5.0 (0.02 M sodium cacodylate) or pH 8.0 (0.1 M TEA-HCl). The initial concentration of the generated intermediate was  $\sim 0.17 \mu\text{M}$ .

of enzyme employed would be sufficient to establish equilibrium. Therefore, the decomposition occurs on the enzyme at  $\sim 10^{-3}$  the rate observed in solution at pH 5.5 and  $3 \times 10^{-5}$  as fast as pH 7.5.

Because the  $\text{pK}_a$  values of enols are likely to be  $>10$ , the free intermediate at pH 5–8 will be present mainly in the diol form. A requirement that the enzyme interact with only a diolate phosphate could be excluded by measuring the effect of pH on the enzyme concentration dependence for trapping of the liberated intermediate. If the rate constant for elimination in solution is known, the rate constant for the trapping reaction can be derived by determining the partition of X between triose phosphate and  $\text{P}_i$  as a function of  $[\text{E}]$  as shown in eq 1. This equation assumes that every time E and X

$$^{32}\text{P}_{\text{trapped}}/^{32}\text{P}_i = k_{\text{trapped}}[\text{E}][\text{X}]/(k_{\text{elimination}}[\text{X}]) \quad (1)$$

interact the X goes to stable product rather than return to free X and that  $[\text{E}\cdot\text{X}] < \text{E}$ .

At both pH 5 and pH 8,  $7 \times 10^{-8}$  M isomerase subunits caused half of the intermediate to be trapped (Table II). The rate constant for this interaction must be  $\sim 110 \text{ s}^{-1}/7 \times 10^{-8} \text{ M} = 1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , or greater if all of the enzyme molecules are not equally accessible to the intermediate. This high and pH-independent value shows that the trapping reaction is limited by the rate of encounter of enzyme with the entire

Table III: Does Isomerase Act Catalytically in the Trapping Reaction?<sup>a</sup>

[enediol phosphate] ( $\mu\text{M}$ )	[isomerase] ( $\mu\text{M}$ )	$[^{32}\text{P}]\text{DHAP}$ trapped ( $\mu\text{M}$ )
0.17	0	0
0.83	0	0
0.17	0.06	0.077
0.83	0.06	0.40
0.17	0.10	0.10
0.83	0.10	0.51

<sup>a</sup> The conditions of Table I were reproduced with isomerase and  $[^{32}\text{P}]\text{DHAP}$  present in syringe A at 5 and 25 nmol each, respectively. The enediol phosphate formed in the two cases after neutralization was 0.17 and 0.83  $\mu\text{M}$  and the isomerase was present as noted.

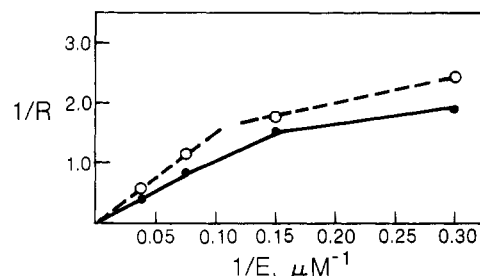


FIGURE 4: Dependence of yeast aldolase concentration on trapping of the isomerase intermediate. The experiment described in Table II was reproduced with varying concentration of yeast aldolase in the trapping solution at pH 5.0 (●) or pH 8.0 (○). Potassium acetate (0.01 M) was included in the buffer. The ordinate is the ratio of intermediate found as  $\text{P}_i$  to that which is trapped.

pool of X in solution. Because of its high  $\text{pK}_a$  this most certainly includes the enediol phosphate. This does not exclude the possibility that the enolate in solution is also trapped.

The high rate constant calculated implies that every encounter of isomerase and X is productive of a stabilized form of X. This form could be an  $\text{E}\cdot\text{X}$  that is not a normal isomerase intermediate, does not dissociate, and only slowly goes to the species on the catalytic path. To determine if the enzyme acts catalytically in the trapping reaction, it was necessary to measure the effect of having X in excess of enzyme on the partition of X between triose phosphate and  $\text{P}_i$  (Table III). The absence of an effect assures that free E is regenerated at a rate sufficient to maintain the same concentration in the two experiments. Therefore, the enzyme is acting catalytically during the time that it competes for the intermediate.

*Does Yeast Aldolase Act on the Enediol Phosphate Released by Acid from Triosephosphate Isomerase?* Yeast aldolase is known to catalyze stereospecific exchange of the C-1 proton of DHAP in the absence of G3P (Rose, 1958; Rose et al., 1965). An enediol or enediolate phosphate is the most reasonable intermediate, there being no evidence for a Schiff base (Horecker et al., 1963; Kobes et al., 1969). Although enantiotopic protons are abstracted by isomerase and aldolase (Reider & Rose, 1959), it is possible that both enzymes use the same enediol phosphate. Unlike the isomerase, no chemical evidence for an enediol phosphate could be obtained with the aldolase; i.e.,  $^{32}\text{P}_i$  and methylglyoxal formation occurred at  $<0.3\% \text{ h}^{-1}$  (equiv of  $\text{E}\cdot[^{32}\text{P}]\text{DHAP complex}^{-1}$ ) and the addition of  $\text{Cl}_3\text{CCOOH}$  to a mixture of aldolase and  $[^{32}\text{P}]\text{DHAP}$  failed to produce a significant amount of  $^{32}\text{P}_i$ . The possibility that yeast aldolase might use the enediol phosphate liberated from isomerase was tested by examining the ability of aldolase to prevent its decomposition to  $^{32}\text{P}_i$ . Figure 4 presents the result

Table IV: Is *cis*-Enediol Phosphate the Product of Methylglyoxal Synthase?<sup>a</sup>

time (min)	<sup>32</sup> P <sub>i</sub> formed (%)		
	none <sup>b</sup>	isomerase <sup>b</sup>	aldolase <sup>b</sup>
0	1.5	1.7	1.4
1	19.0	21.1	20.3
5	45.0	46.3	45.1
10	56.1	54.6	55.6
20	65.2	63.8	66.7

<sup>a</sup> The incubation mixture (1 mL, 0.05 M imidazole, pH 7.0, 37 °C) contained [<sup>32</sup>P]DHAP (1 mM) and methylglyoxal synthase (0.30 unit, 0.57 μM). The effect of added triosephosphate isomerase (5 μM subunits) or yeast aldolase (20 μM subunits) on <sup>32</sup>P<sub>i</sub> formation was determined. <sup>b</sup> Trapping enzyme added.

of such an experiment at pH 5 and pH 8 in which the observed ratio  $x/(1-x)$ ,  $R^{-1}$ , is plotted against  $[\text{aldolase}]^{-1}$ , where  $x$  represents the fraction of the available enediol phosphate that is found as P<sub>i</sub> in the presence of a particular concentration of aldolase in the beaker. Both pH values yield biphasic relations, perhaps reflecting a shift to dimers at higher concentrations (Harris et al., 1969). As seen from eq 1 the slope of such a reciprocal plot corresponds to the ratio  $k_{\text{elim}}/k_{\text{trapped}}$ . The interaction between the isomerase intermediate and aldolase at high aldolase concentration occurs with a rate constant of  $\sim 6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ . A significantly higher value seems to be obtained at lower aldolase concentration. The data could be extrapolated to  $\sim 100\%$  trapping as enzyme concentration is increased, indicating complete correspondence between the isomerase and aldolase in the ability to use the liberated enediol phosphate as a substrate. The aldolase product was shown to be alkaline labile unless  $\alpha$ -glycerophosphate dehydrogenase and NADH were also present in the beaker with the aldolase. The addition of G3P with the aldolase also resulted in an alkaline-stable product. These are the expected results if [<sup>32</sup>P]DHAP was produced in the trapping reaction and either glycerol phosphate or fructose 1,6-bisphosphate was produced thereafter.

**Methylglyoxal Synthase.** The conversion of DHAP to methylglyoxal and P<sub>i</sub> by the *E. coli* enzyme was found to involve specific abstraction of the pro-S hydrogen at C-1 of DHAP and nonstereospecific formation of the  $-\text{CH}_3$  group at the carbon from which  $-\text{OPO}_3$  is eliminated (Summers & Rose, 1977). The most likely explanation for the latter is that methylglyoxal is formed in solution rather than on the enzyme. This was indicated also by the failure of methylglyoxal to act as a product inhibitor. As considered above for isomerase, it is possible that the true product of the enzymatic reaction is the enediol phosphate. In that case, and assuming the product to be identical with the isomerase intermediate, the presence of aldolase or triosephosphate isomerase should prevent formation of P<sub>i</sub> and methylglyoxal by the synthase. That this was not observed (Table IV) shows that the true enzyme product is not the *cis* isomer. Nevertheless, the enzyme may have the *cis*-enediol phosphate as an *intermediate*, in which case, as is probable for isomerase, the product released by the synthase is the enol aldehyde (step 3, Scheme I).

For determination of *cis*-enediol phosphate as a synthase intermediate, an antitrapping experiment was done as follows: isomerase with [<sup>32</sup>P]DHAP was mixed with Cl<sub>3</sub>CCOOH to produce the intermediate shown in Scheme II. The beaker contained only enough isomerase to trap about half of the enediol phosphate. Further addition of synthase to the beaker could be expected to reverse the isomerase effect if both enzymes use the same intermediate. One complicating aspect of this experiment is that DHAP present from the original

 Table V: Does Methylglyoxal Synthase React with the *cis*-Enediol Phosphate?<sup>a</sup>

additions to beaker (μM final concn)	<sup>32</sup> P <sub>i</sub> > control <sup>b</sup> (%)	<sup>32</sup> P <sub>i</sub> after 15 min in NaOH <sup>c</sup>
none	5.2	56.6
synthase (5)	5.3	54.5
isomerase (0.1)	2.1	2.4
synthase (5) and isomerase (0.1)	2.2	2.3

<sup>a</sup> The protocol followed Table I with the presence, in addition, of  $\alpha$ -glycerophosphate dehydrogenase (40 nmol) and NADH (40 nmol) in the beaker. Methylglyoxal synthase and triosephosphate isomerase were also present as noted. <sup>b</sup> 1.5% <sup>32</sup>P<sub>i</sub> when no enzyme is in syringe A. <sup>c</sup> The alkaline-labile <sup>32</sup>P<sub>i</sub> represents the G3P present on the isomerase when quenched with Cl<sub>3</sub>CCOOH (Iyengar & Rose, 1981).

incubation must be removed very rapidly to prevent its reaction with the synthase added to the beaker. This could be done very well with NADH and  $\alpha$ -glycerophosphate dehydrogenase as shown in Table V. The results of this and similar experiments of this kind failed to show competition by synthase for the isomerase intermediate.

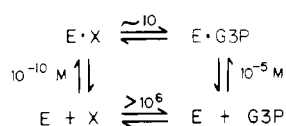
## Discussion

The compound that is liberated upon acid denaturation of isomerase when it is interconverting substrates has the properties of an enediol phosphate intermediate of the isomerase reaction. Its degradation to P<sub>i</sub> and methylglyoxal occurs *after* release from the denatured enzyme as shown by the fact that it can be converted to triose phosphate by fresh isomerase (Table I). On the other hand, the catalytic degradation of substrate that is a normal side reaction of the enzyme occurs on the enzyme as shown by its failure to be prevented by excess enzyme (Figure 3). The material found after acid denaturation does not represent a mixture of *cis*- and *trans*-enediols or one would not expect complete trapping by isomerase, believed to be specific for the *cis* isomer (Reider & Rose, 1959; Rose, 1962). Therefore, it seems that the bound intermediate of the isomerase reaction and the released species are identical, although precise comparison of its enzymatic rate of conversion to triose phosphate with the standard  $k_{\text{cat}}$  has not yet been made. As shown (Table III), the trapping process is catalytic and therefore represents conversion to a released product, found to be alkaline labile, well within the time,  $<10^{-2} \text{ s}$ , for spontaneous elimination of P<sub>i</sub>.

Demonstration of a minor species in the acid-quenched isomerase-substrate system with the properties of an intermediate should dispell arguments for a hydride-transfer mechanism based on either prior proton addition to the carbonyl or proton removal from the adjacent hydroxyl group. Such reaction intermediates preceding the hydride-transfer step would not be kinetically distinguishable from substrates upon their liberation from triosephosphate isomerase in acid.

The affinity of the enediol phosphate isomerase seems extraordinary and is appropriate for its role as a highly reactive enzyme-bound intermediate. Its dissociation from the enzyme is undetectable relative to its decomposition rate of  $<0.1 \text{ s}^{-1}$ , depending on pH (Iyengar & Rose, 1981). The association rate of  $\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , about the diffusion limit, suggests that free enediol phosphate is highly accessible to the enzyme-active site in the normal state of the enzyme. The resulting dissociation constant of  $<10^{-10} \text{ M}$  is therefore below that reported for the so-called transition state analogue, phosphoglycolate,  $\sim 10^{-6} \text{ M}$  (Wolfenden, 1969). It has been estimated that the interaction of isomerase with the aldehyde form of G3P is also

diffusion limited (Albery & Knowles, 1976). However, its rate of release as a product is  $\sim 1000 \text{ s}^{-1}$  based on  $k_{\text{cat}}$  for the forward reaction (Albery & Knowles, 1976) and the expectation that about half of the enzyme would be in the E-G3P form in the steady state. Therefore, there must be a unique mechanism to explain the tight binding of the enediol phosphate. A similar phenomenon was observed between enol-pyruvate and pyruvate kinase (Kuo & Rose, 1978; Kuo et al., 1979). Although in both cases the enolate species may be the active form on the enzyme, it is not reasonable to attribute more than a  $10^3$ -fold effect to ionic interaction alone. The remaining  $\sim 10$  kcal of binding energy must be derived from a significant change in the active-site conformation upon contact with the intermediate. This process also has the extraordinary effect of shifting the equilibrium between G3P and enediol phosphate on the enzyme to  $<0.1$  from  $\sim 10^{-6}$  in neutral solution. This is seen from the following cycle:



The form of the free intermediate that reacts so rapidly with isomerase is certainly the enediol form. The unfavorable position of the diolate/diol equilibrium in solution and the absence of a strong pH effect on trapping (Table II) require this. Furthermore, it seems from this observation that one negative charge on the phosphoryl group is sufficient for recognition of the intermediate. This agrees with the result of Jones & Waley (1979) that the binding of glycerol phosphate is not much affected between pH 5.5 and pH 8.5 although a more complex interpretation of the  $^{31}\text{P}$  NMR line broadening data suggested weaker binding of the monoanion (Campbell et al., 1979). If the enediol itself is not on the direct reaction path of isomerase action, there must be a mechanism for it to join the path by proton removal from one of the hydroxyls by an apparently gratuitous base. The simplest model, however, is to suppose that the enolizations of DHAP and G3P are general-acid, general-base catalyzed. Ground-state polarization of substrate carbonyl has been adduced from facilitated borohydride reduction of substrate in the presence of enzyme (Webb & Knowles, 1974) and by the appearance of a new band in the infrared carbonyl region (Belasco & Knowles, 1980), which could reflect the presence of a cationic acid in the environment and could proceed to the enediol during C-H bond cleavage. A histidine and lysine residue in the active site of chicken isomerase could serve this role (Phillips et al., 1977). On the other hand, the results favoring an enediol phosphate intermediate would rule out any polarization mechanism based on a structural dipole from the protein (Hol, 1978) as well as any scheme requiring the neighboring hydroxyl of the substrate to supply the proton for the carbonyl  $\rightarrow$  carbinol conversion.

The reaction of the free isomerase intermediate with yeast aldolase is similar to its reaction with isomerase except, of course, that ketonization must occur with protonation from the *si* face at C-1 with the aldolase. Recent studies by Smith et al. (1980) measuring the distance between  $\text{Mn}^{2+}$  and atoms of a DHAP analogue suggest that the metal, normally  $\text{Zn}^{2+}$ , is too distant to cause much polarization of the DHAP carbonyl group. Rather, a histidine residue, coordinated to the  $\text{Zn}^{2+}$ , could serve that purpose. However, metal coordination raises the pK of the remaining imidazole nitrogen to  $\sim 10$  so that it should be largely protonated and could serve in a hydrogen bond but would not lead to an enediol. This seems contrary to the fact that the enediol phosphate is active and

would require conversion to the enediolate on the enzyme. Failure to show a pH effect on the binding rate is not unexpected since the fraction present as enediolate at pH 8 must be very small. The mechanism of deprotonation of the enediol therefore remains obscure unless, in fact, the histidine proposed by Smith et al. (1980) acts as a general acid to activate enolization rather than as a structural element limited to hydrogen bonding.

The combination of experiments reported in Tables IV and V suggests that methylglyoxal synthase does not liberate *cis*-enediol phosphate in the course of its normal reaction with DHAP nor employs it as an intermediate as judged by failure of the synthase to alter the effectiveness of a low amount of isomerase in trapping experiments. The latter experiment (Table V) shows that if the synthase activates the fragmentation of free *cis*-enediol phosphate, it must be  $<10^{-3}$  as effective as the isomerase is in tautomerizing it. The minimum rate required of any step of the synthase reaction, where  $k_{\text{cat}}$  is only  $10 \text{ s}^{-1}$ , seems too small to rule out the *cis*-enediol phosphate as an intermediate using this approach, even when using greater amounts of enzyme. The same approach using *trans*-enediol phosphate awaits a method for generating that compound. The possibility that the enzyme only recognizes the enolate form emphasizes the difficulty in interpreting a negative result without appropriate additional information about the catalytic properties of the enzyme.

It is of interest to ask what properties of the interaction between an enzyme and the enediol phosphate are important in determining the rate of the fragmentation reaction. As already noted, fragmentation of the enediol phosphate in aqueous medium is virtually pH independent in the range pH 4.5–8.5. This implies that the charge on the phosphate group is not important and that the enol itself is the reacting species; i.e., the abstraction of the enol proton by water is concerted with loss of  $-\text{OPO}_3$ . Alternatively, it may be supposed that the fastest reaction path is the one with the enolate form with a monoanionic phosphate group. These alternatives have not been further examined except to note that the rate in  $0.2 \text{ N Cl}_3\text{CCOOH}$ , pH  $\sim 1$ , was in the same range, favoring the first interpretation. In the least, this would rule out a mechanism in which the phosphate acts as a base for removal of the C-1 enol hydroxyl. An understanding of the factors important in determining this apparent unimolecular fragmentation rate is important before the greater stability of the enediol phosphate on isomerase can be understood.

The interconversion of enol and enolate on triosephosphate isomerase must be much more rapid than the fragmentation of the enediol on the enzyme, since it is part of the much more rapid isomerization catalysis of the enzyme. Also, it would seem unlikely that enol/enolate equilibrium would be less favorable on the enzyme than in water since any basic residue of the enzyme would be a better base than water. Therefore, some other factor is required to explain the  $>1000$ -fold greater kinetic stability of the enediol phosphate on the enzyme. Of importance may be the orientation of the C-O bond to be split relative to the plane defined by the 3-carbon  $\pi$  system in the transition state. One expects a coplanar orientation to give greatest stability and an orthogonal arrangement to give the least, just as, in the reverse direction, the best route of approach of  $\text{O}_3\text{PO}^-$  to the enolaldehyde would be from out of the plane.

The "imperfection" in triosephosphate isomerase betrayed by the phosphate elimination reaction is progressively greater as the pH falls between pH 7.5 and pH 5.5 with an apparent pK of 6.5 (Iyengar & Rose, 1981). This was interpreted to reflect a preference for monoanionic phosphate as the leaving

group. In light of the above discussion, however, a more likely explanation could relate to the effect that charge of the phosphate group might have on fixing the linking oxygen relative to the plane of the enediol. The dianion form could be least free to relax from a geometry in which the bridge oxygen is coplanar with the allylic system. An enzyme such as methylglyoxal synthase, where conditions for the elimination might be optimized, may be expected to have a more out of plane geometry for the bridge oxygen.

**General.** Isotope-exchange methods have provided a most useful approach for implicating intermediates in multisubstrate reactions. The method is successful when the labeled test species readily dissociates from the enzyme complex under partial reaction conditions. In special cases the nature of a tightly bound complex can be implied if one of the products is torsionally symmetrical so that the mechanism can be characterized by intramolecular isotopic rearrangement (Midelfort & Rose, 1976). The approach that has been taken in the present work calls for identification of the unstable reaction intermediate by its generation from a terminated catalytic mixture and by its utilization as a supersubstrate with the same or other enzymes. An alternative in which the unstable intermediate is generated continuously in situ is illustrated by phosphatase action on phosphoenolpyruvate generating enolpyruvate for test with pyruvate kinase (Kuo & Rose, 1978; Kuo et al., 1979). The effort involved in the "de novo synthesis" of a source of the proposed intermediate in a form that can lead to continuous generation of the intermediate under mild conditions will limit the usefulness of the synthetic approach. The analytical approach used in this paper has the important characteristic that it leaves the sophisticated synthetic work to the enzyme. Obviously any approach that characterizes the liberated intermediate or its degradation products (destruction analysis) will provide evidence for the discussion of the chemical reaction mechanism. Structural information may be lost by allowing the liberated intermediate to decompose so that it will often be desirable to intercede by chemical or enzymatic derivatization. As was expected, the intermediate liberated from isomerase by acid could be prevented from decomposition if ferricyanide was present under neutral or mild alkaline conditions. This contrasts with the reported failure to oxidize the isomerase-bound form (de la Mar et al., 1972). The same intermediate, present in much lower concentration on yeast aldolase, is oxidizable at a significant rate by tetranitromethane (Riorden & Christen, 1969). Perhaps the difference reflects the fact that the aldolase complex must be accessible to the medium for approach of the second substrate, G3P, whereas the isomerase reaction requires no communication with solvent after substrate binding.

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