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Concentration of Activated Intermediates of the Fructose-1,6-bisphosphate Aldolase and Triosephosphate Isomerase Reactions†

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ABSTRACT: As discovered by Grazi & Trombetta [Grazi, E., & Trombetta, G. (1978) *Biochem. J. 175*, 361], fructose-1,6-bisphosphate aldolase of rabbit muscle causes the slow formation of inorganic phosphate (P_i) and methylglyoxal when incubated with dihydroxyacetone phosphate (DHAP). In addition, these authors found an acid-labile intermediate in equilibrium with the aldolase–dihydroxyacetone phosphate complexes representing ~60% of the enzyme-bound DHAP species. Experiments are reported here which argue that this acid-labile species is the enzyme-bound enamine phosphate or its equivalent that decomposes by β elimination in acid. A similar mechanism involving an enediol phosphate is proposed to explain a phosphatase action of triosephosphate isomerase that produces methylglyoxal and P_i at the rate of ~0.1 s^{-1} at pH 5.5. When DHAP with excess isomerase is quenched in strong acid, the formation of P_i indicates that ~5% of bound reactant is in the form of enediol phosphate. The remainder of the substrate is about equally distributed between bound forms of DHAP and D-glyceraldehyde 3-phosphate. This equilibrium differs by 300-fold from the appropriate equilibrium in solution. Yeast aldolase, contrary to expectation, does not catalyze formation of inorganic phosphate and methylglyoxal when incubated with DHAP and gives no evidence for an enediol phosphate intermediate when quenched in acid.

Grazi & Trombetta (1978) observed the slow production of inorganic phosphate and methylglyoxal when rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) and dihydroxyacetone phosphate (DHAP)[1] were incubated. This reaction occurred optimally in the pH range 5–6 with a rate ~10^{-4} that of the exchange of the C-3 hydrogen of DHAP with water. When the incubation was quenched with trichloroacetic acid (Cl_3CCOOH), an additional 0.6 equiv of inorganic phosphate (P_i) per equiv of enzyme was observed.

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[1] Abbreviations used: DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; FDP, fructose 1,6-bisphosphate; Cl_3CCOOH, trichloroacetic acid; TEA, triethanolamine; CP, carboxypeptidase; P_i, inorganic phosphate.

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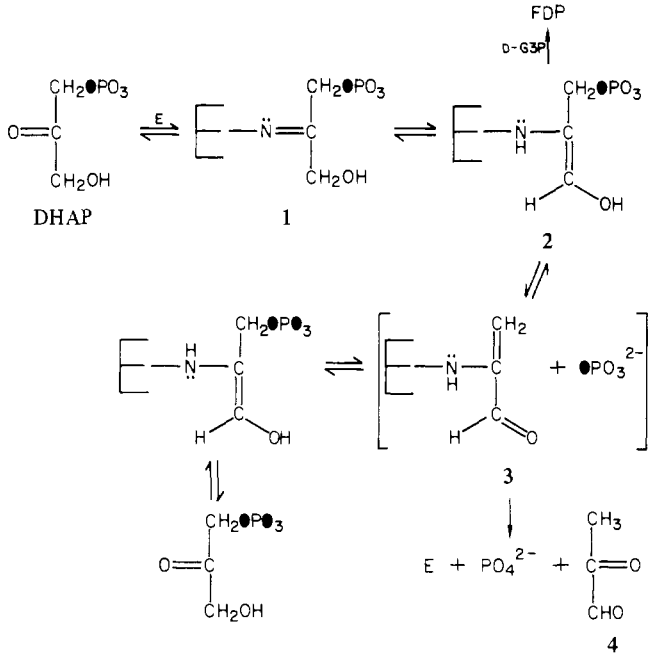
Scheme I: Positional Exchange of Bridge-Labeled [^{18}O]DHAP by the Methylglyoxal-Muscle Aldolase-Orthophosphate Complex



As shown in Scheme I, a new species, enzyme complex **3**, was proposed, which would either dissociate from the enzyme slowly, giving methylglyoxal **4** and P_i, or return rapidly to the enamine phosphate **2**. The need for such a complex was based on the knowledge that the triose phosphates are stable in acid and the apparent expectation that this would be true for the enamine phosphate intermediate **2**. In addition, indirect kinetic evidence was adduced for a second proton-abstracted species presumed to be **3**.

The question of whether C–O cleavage during P_i formation is reversible has been answered in this paper by looking for positional isotope exchange in recovered DHAP. With the assumption that the oxygens of bound P_i are torsionally symmetrical, the proposed reversible cleavage would lead to an interchange of the bridge and nonbridge oxygens of the DHAP. Further experiments comparing the amount of deprotonated forms of bound DHAP and Cl_3CCOOH-labilized phosphate support the conclusion that the eneamine phosphate **2** is acid labile.

Stoichiometry of P_i production and its kinetics with yeast aldolase and triosephosphate isomerase lead to conclusions about the amount and stability of enediol phosphate intermediates for these enzymes.

Materials and Methods

Materials. Rabbit muscle aldolase was obtained as a crystalline suspension in ammonium sulfate from Sigma and freed of traces of triosephosphate isomerase by treatment with glycidol phosphate (Rose & O'Connell, 1969b). Enzymatic activity was ~12 μmol of fructose 1,6-bisphosphate (FDP) cleaved min^{-1} mg^{-1} as determined spectrophotometrically by coupling FDP to α-glycerophosphate dehydrogenase and isomerase, measuring NADH oxidation at 340 nm. Protein concentration was measured from A_{280}, assuming the absorbance of 1 mg of pure aldolase/mL to be 0.91 cm^{-1} (Baranowski & Niederland, 1949) and 40000 daltons as the weight of the catalytic unit. Carboxypeptidase-treated aldolase was prepared by the method of Rose & O'Connell (1969b), resulting in a 96% decrease in the maximum rate of FDP cleavage as measured under standard conditions. No attempt was made to reisolate the modified aldolase. Zn-containing yeast aldolase (~85 units/mg) was a gift of Drs. G. M. Smith and A. S. Mildvan. Chromatography on a DEAE-cellulose column (Richards & Rutter, 1961) followed by treatment with glycidol phosphate (Rose & O'Connell, 1969b) was used to remove the contaminating triosephosphate isomerase. Protein concentration was determined by the absorbance at 280 nm, ϵ_{280} = 1.02 mL/mg (Harris et al., 1969). Triosephosphate isomerase (EC 5.3.1.1) of rabbit muscle was obtained from Boehringer and used without further purification. In addition, a portion of the enzyme was treated with glycidol phosphate followed by exhaustive dialysis to obtain ~99% inactivation of net isomerase activity (Rose & O'Connell, 1969b).

Other enzymes were obtained as follows: phosphofructokinase (rabbit muscle), phosphoglucose isomerase (yeast), alkaline phosphatase (*E. coli*), glycerol kinase (*E. coli*), glyoxalase I (yeast), and carboxypeptidase A-DFP were from Sigma; α-glycerophosphate dehydrogenase (rabbit muscle) and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were from Boehringer.

DHAP ketal from Sigma was hydrolyzed according to Ballou & Fischer (1956). The barium salt of DL-glyceraldehyde 3-phosphate diethyl acetal (Calbiochem) was hydrolyzed to the aldehyde. These compounds were assayed spectrophotometrically as the disappearance of NADH with α-glycerophosphate dehydrogenase alone or with added triosephosphate isomerase. There was no detectable contamination of glyceraldehyde 3-phosphate (G3P) with DHAP and vice versa. Glucose 6-phosphate (Na salt) was purchased from Sigma, Dowex-1 (Cl^- form, 200–400 mesh, 8% cross-linked) from Bio-Rad, [γ-^{32}P]ATP from New England Nuclear, and $H_2{}^{18}O$ from Miles-Yeda.

[1-^{18}O]Glucose 6-phosphate was prepared by exchange with $H_2{}^{18}O$ at acidic pH (Rittenberg et al., 1961). This was converted to [1-^{18}O]FDP by the combined action of phosphoglucose isomerase, phosphofructokinase, and ATP (with [γ-^{32}P]ATP added as tracer) in H_2O and then further reacted with aldolase and glyceraldehyde-3-phosphate dehydrogenase in the presence of 5 mM sodium arsenate. The [1-^{18}O]DHAP was isolated on a Dowex-1, Cl^- column and eluted with 0.03 N HCl (Bartlett, 1959).

[^{32}P]DHAP (8248 cpm/nmol at the time of preparation) was synthesized by phosphorylation of dihydroxyacetone by glycerol kinase with [γ-^{32}P]ATP and purified on a Dowex-1, Cl^- column (Rose & O'Connell, 1969a). It was free of G3P but contained ~5% of the total radioactivity as $^{32}P_i$.

Methods. The distribution of ^{18}O in DHAP was determined after conversion to P_i either by treatment with NaOH (0.5 N final concentration) for 15 min at 37 °C, giving C–O cleavage, or by O–P cleavage with alkaline phosphatase (2 units) in Tris-HCl, pH 7.8, containing $MgCl_2$ (0.01 M) for 3 h at 37 °C. Alkaline phosphatase does not catalyze the exchange of oxygens between water and inorganic phosphate under these incubation conditions (Eargle et al., 1977). The inorganic phosphate was prepared for mass spectral analysis by chromatography on Dowex-1, Cl^- and elution with 0.01 N HCl, followed by reaction with diazomethane, and analyzed as its trimethyl ester (Midelfort & Rose, 1976).

[1-^{18}O]DHAP (5 μmol) was incubated with rabbit muscle aldolase (250 nmol) in 1 mL of 0.02 M acetate buffer, pH 6.0. After 3 h at room temperature, DHAP was converted to phosphoglycolate by treating the reaction mixture with a 5-fold excess of periodic acid at pH 4.5 for 15 min at 10 °C. The excess periodic acid was destroyed with 50 μL of ethylene glycol. The phosphoglycolate, purified on Dowex-1, Cl^- by elution with 0.02 N HCl, was incubated with alkaline phos-

Table I: Test for Positional Exchange of Phosphate Oxygens by Muscle Aldolase

DHAP treated	mass spectral analysis	
	$P^{16}O_4$	$P^{16}O_3{}^{18}O_1$
starting (bridge [^{18}O]DHAP)		
alkali	25.7	74.3
phosphatase	99.2	0.79
incubated with aldolase[a]		
phosphatase	99.0	0.99
predicted for complete exchange	44.3	55.7

[a] Bridge [^{18}O]DHAP (5 μmol) was incubated with aldolase (250 nmol) in 1 mL of 0.02 M acetate buffer, pH 6.0, for 3 h at room temperature. Alkali treatment of DHAP produces P_i by C–O cleavage (Scheme II), and phosphatase treatment gives O–P cleavage by water.

phatase (conditions given above) and the inorganic phosphate analyzed as trimethyl phosphate by mass spectrometry as described previously.

Isotopic trapping experiments with muscle aldolase were done in which 20 μL of the premixed pulse containing approximately 250 μM aldolase subunits (equilibrated with buffer) and 211 μM [1-^{32}P]DHAP at 4 °C was added by forced delivery to 3 mL of vigorously stirred buffered solutions containing 1 mM DHAP and 1 mM G3P at 25 °C. HCl (0.1 N final concentration) was added from another Eppendorf pipet at about 2.5 s. An aliquot was analyzed for $^{32}P_i$ by 2-butanol extraction of the inorganic phosphate–molybdate complex (Berenblum & Chain, 1938). The labeled DHAP trapped as FDP was determined after a 15-min treatment with 0.5 N NaOH at 37 °C as that ^{32}P not extracted as a molybdate complex with 2-butanol. Another sample of the aldolase–DHAP pulse was added to 3 mL of Cl_3CCOOH (2 N final concentration) to liberate $^{32}P_i$ from an activated portion of the complex according to Grazi & Trombetta (1978). The $^{32}P_i$ was determined as the portion extracted as the molybdate complex with 2-butanol. Suitable controls were run without enzyme or with inactivated enzyme.

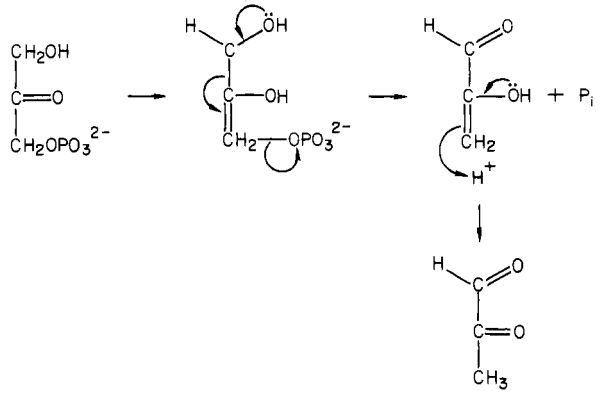
Experiments of a similar nature are reported with yeast FDP aldolase and triosephosphate isomerase of rabbit muscle.

Results

On kinetic grounds, Grazi & Trombetta (1978) proposed that a reversible interconversion of enamine phosphate **2** and enamine aldehyde···PO_4^{2-} **3** must occur (Scheme I). If this is true, one should expect the exchange of ^{18}O from the C–O–P to the $-PO_3^{2-}$ position in DHAP (Scheme I), assuming that the oxygens of the bound P_i are torsionally equivalent (Midlefort & Rose, 1976; Rose, 1979). For investigation of the equilibration of the proposed acid-labile intermediate **3** and the enamine **2**, DHAP labeled with ^{18}O in the C–O–P bridge position was incubated with muscle aldolase and recovered after what would have been approximately 10^4 turnovers of the entire DHAP pool, based on ^{3}H exchange data (Rose et al., 1965). As shown in Table I, mass spectral analysis of jthe trimethyl phosphate derived from the $-PO_3^{2-}$ group of the recovered DHAP showed negligible ^{18}O enrichment. The value obtained corresponded to $\sim 5 \times 10^{-3}$ turnovers of the DHAP pool. Therefore, oxygen randomization occurs at no more than $\sim 5 \times 10^{-7}$ times the tritium exchange rate. This absence of ^{18}O scrambling implies that the formation of inorganic phosphate and methylglyoxal occurs by an *irreversible* cleavage of a phosphorylated species, presumably one that has lost a proton at C-3.

The well-known alkaline lability of DHAP has been explained by an initial enolization followed by a 1,4-elimination

Scheme II: Mechanism for the Alkaline Lability of DHAP and Formation of Methylglyoxal and Inorganic Phosphate



(Scheme II). This mechanism explains the requirement for hydroxyl at C-3 as indicated from the stability of the 3-deoxy analogue of DHAP in alkali (Rose & O&Connell, 1969a). It seems reasonable to assume that a deprotonated species such as the enamine phosphate formed by reaction of DHAP and muscle aldolase might undergo an acid-catalyzed elimination of $O-PO_3H_2^-$. For determination of what components subsequent to C–H bond cleavage are the source of the P_i, a comparison was made of the size of "the enamine" pool and the amount of P_i that was generated in strong acid. For investigation of the former, use was made of the observation (Rose et al., 1965) that carboxypeptidase (CP) treatment of aldolase, which has no effect on the C–C bond-cleavage step, decreases C–H bond cleavage greatly, as shown by a 20-fold tritium discrimination in the enzymatic condensation of (3*S*)-[3-^{3}H]DHAP with glyceraldehyde. Because of this slow interconversion at the protonation step **1** ⇄ **2** (Scheme I), it seemed possible to estimate by isotope trapping with G3P the total size of the pool(s) at equilibrium, that is, subsequent to the C–H bond-cleavage step. In the isotopic trapping technique, labeled substrate and enzyme are premixed and then transferred to a rapidly stirred chase solution containing both unlabeled triose phosphate substrates. This dilutes the isotope of any unbound substrate and starts the formation of FDP (Rose, 1980). In this case, only nonprotonated species will be trapped since for **1** the rate of C–H bond cleavage is slower than the dissociation to free DHAP during the chase, as required by the isotope discrimination observed against (3*S*)-[3-^{3}H]DHAP in condensation reactions (Rose et al., 1965). Under similar conditions with CP–aldolase, Grazi & Trombetta (1980) observed biphasic ferricyanide reduction by a mixture of aldolase and DHAP, consistent with an "enamine" pool of ~0.6 equivalent capable of rapid oxidation and ~0.4 equivalent giving further reduction at a rate corresponding to the steady-state rate of reduction.

In isotope trapping experiments, a mixture of *native* aldolase and [^{32}P]DHAP at 4 °C was added to a magnetically stirred solution of unlabeled DHAP and G3P at 25 °C and followed by addition of acid in 2.5 s. Dilution of [^{32}P]DHAP by carrier in the chase solution limited its conversion to FDP to <1% of the total radioactivity present. However, all of the radioactivity was found to be stable to alkali. As expected from the absence of dicrimination in C–H bond cleavage with native aldolase, conversion to FDP was much more rapid than dissociation of the bound DHAP forms from the enzyme (Table II). Therefore, all of the enzyme-bound DHAP (33 500/34 000 cpm) can be trapped as FDP rather than dissociating to free DHAP. When this experiment was repeated with the *CP-treated aldolase–DHAP* complex, only 60% of the [^{32}P]DHAP

Table II: Native or CP–Aldolase–[^{32}P]DHAP Conjugates Converted to $^{32}P_i$ in Acid or Trapped as [^{32}P]FDP

aldolase/[^{32}P]DHAP	trapped by G3P[a] G3P (mM)	^{32}P trapped (cpm)	converted to $^{32}P_i$ by Cl_3CCOOH[b] (cpm)
native	0.2	33 500	20 150
CP treated	0.2	21 500	20 600
1.2:1	1.0	21 300	20 500
6:1	1.0	21 000	20 900

[a] The 20-μL pulse solution contained 5.08 nmol of native or carboxypeptide-modified aldolase subunits and 4.23 nmol of [^{32}P]DHAP (34 000 cpm) in 0.1 M TEA–HCl, pH 7.5, at 4 °C mixed into 3 mL of chase solution of DHAP (1 mM) and G3P (1 mM) at 25 °C. HCl (0.1 N final concentration) was added at 2.5 s. The counts not converted to $^{32}P_i$ after 15 min in alkali represent counts trapped as FDP. [b] For the acid stop, the pulse was added directly into 3 mL of Cl_3CCOOH (2 N final concentration). $^{32}P_i$ and [^{32}P]DHAP trapped were determined as described under Methods.

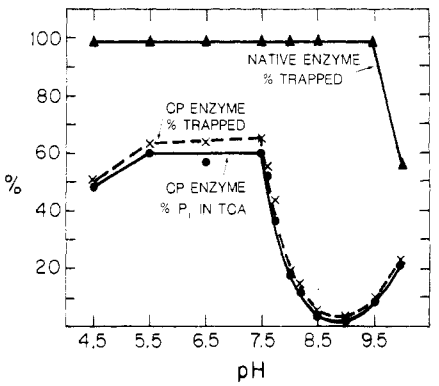


FIGURE 1: Effect of pH on muscle aldolase–DHAP, giving $^{32}P_i$ in acid or trapping as [^{32}P]FDP with G3P. The experiment described in Table II was reproduced at different pH values (pH 4.5, 0.02 M sodium acetate; 5.5–6.5, 0.02 M sodium cacodylate; 7.5–8.5, 0.1 M TEA–HCl; 9.0–10.0, 0.1 M glycine). ^{32}P trapped as FDP with native enzyme (▲) or CP–enzyme (×) or percent that goes to P_i in Cl_3CCOOH (TCA in figure) (●) with CP–enzyme.

was trapped. It was not possible to increase the amount of radioactivity trapped either by using 5 times as much enzyme, which indicates that all of the [^{32}P]DHAP present in the pulse was bound, or by increasing G3P concentration in the chase, which indicates saturation by the second substrate at the lowest concentration used. If the pulse solutions were added directly to acid, the amounts of inorganic phosphate formed from the complexes were not significantly different for the native and CP–aldolase complexes, approximately 0.6 equivalent; therefore, in the case of the CP-treated aldolase, the [^{32}P]DHAP that was chased to FDP with G3P prior to dissociation from the enzyme corresponds precisely to the inorganic phosphate produced in the acid chase.

Similar experiments, conducted over the pH range 4.5–10 (Figure 1), showed that $^{32}P_i$ formed and [^{32}P]DHAP trapped were always equal when the CP–enzyme was used. The native form, except at pH 10, gave 100% trapping, and the elimination of $^{32}P_i$ in Cl_3CCOOH agreed with that found with CP–enzyme. The agreement in the size of the acid-labile "carbanion" pool for the native and CP–enzyme complexes shows that the carboxypeptidase treatment, while uncoupling the C–C and C–H cleavages as if they were on different domains of the enzyme, did not alter the apparent pK_a of the proton abstraction step. The sharp fall in DHAP trapped with CP–aldolase from pH 7.8 to 8.2 was examined in detail (Figure 2) and shown to depend on a change of approximately two protons with similar pK values.

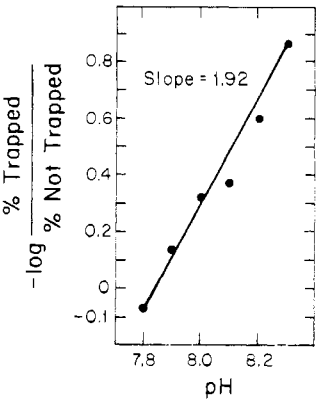


FIGURE 2: Plot of –log K vs. pH for the trapping of the CP–aldolase–DHAP complex by G3P.

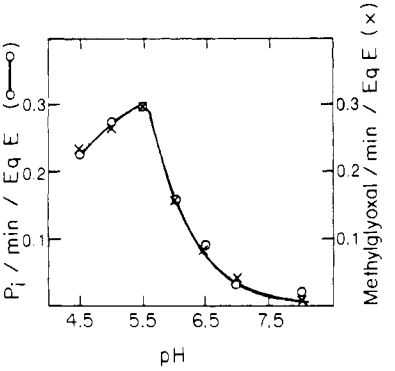


FIGURE 3: Effect of pH on the rate of elimination of P_i (×) and methylglyoxal (○) from the triosephosphate isomerase–DHAP complex. The incubation mixture (0.5 mL) contained DHAP (0.94 mM, 5×10^4 cpm) and triosephosphate isomerase subunits (20 μM) in buffer (pH 4.5, 0.02 M sodium acetate; 5.5–6.5, 0.02 M sodium cacodylate; 7.5–8.5, 0.1 M TEA–HCl). After 1 h at 37 °C, the $^{32}P_i$ was determined. Methylglyoxal was assayed enzymatically (Racker, 1957).

Triosephosphate Isomerase. Mention has been made in the literature by Webb et al. (1977) that triosephosphate isomerase has a "phosphatase" action that is sensitive to an active site directed inactivator. This phenomenon had been reported earlier by Browne et al. (1976) with the additional observation of methylglyoxal and P_i but not dihydroxyacetone as the reaction products. Subsequent experiments and speculation as to the cause of the degradation were made by Campbell et al. (1979). The observations of Grazi & Trombetta (1978, 1980) and the above experiments with muscle aldolase suggested that β-OPO_3 elimination from enzyme-bound enediol phosphate as proposed for the reaction in alkali (Scheme II) might be occurring with the enzyme acting as the base. That this is an elimination reaction and not a simple hydrolysis is confirmed by the fact that methylglyoxal and P_i are formed at the same rate (Figure 3). The pH dependence for the enzymatic elimination is shown to be very similar to that observed by Grazi & Trombetta (1978) with muscle aldolase. The rate of elimination is ~0.3 min^{-1}, with isomerase assuming all of the substrate in the enediol phosphate form. For evaluation of the amount in this form, a pulse of enzyme and [^{32}P]DHAP was incubated briefly and then acidified to determine how much $^{32}P_i$ was formed in excess of that expected from the turnover (Table III). A significant difference was seen when active and inactivated enzymes were used, indicating that ~5% of the total triose phosphate on the enzyme at pH 6.5 was acid labile and therefore presumably in the enediol phosphate form. No difference was observed between no enzyme and inactivated enzyme. The 4–5% $^{32}P_i$ present in these incubations was

Table III: Elimination of O-PO_3H^- from the Triosephosphate Isomerase-DHAP Complex in Cl_3CCOOH[a]

pH	triosephosphate isomerase subunits/ [^{32}P]DHAP	% of total ^{32}P present: A, $^{32}P_i$ formed with native enzyme	% of total ^{32}P present: B, ^{32}P formed with glycidol phosphate treated enzyme	$^{32}P_i$ due to enzyme (A − B)
4.5	5:1	7.5	4.5	3
5.5	5:1	9.8	5.0	4.8
6.5	5:1	9.0	4.0	5.0
6.5	10:1	9.5	4.5	5.0
7.5	5:1	9.2	4.8	4.4
8.5	5:1	7.9	5.0	2.9

[a] The pulse solution (20 μL at 25 °C), containing [^{32}P]DHAP (5 nmol, 45 000 cpm), triosephosphatase isomerase (native or inactivated as indicated), and buffer (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), was added directly to Cl_3CCOOH (3 mL, 2 N). $^{32}P_i$ was assayed after extraction of the molybdate complex.

Table IV: Relative Concentrations of Isomerase-Bound Species[a]

treatment of quenched pulse before addition of alkali	% of total ^{32}P present: inactive isomerase pulse quenched directly	native isomerase: pulse quenched directly	native isomerase: pulse diluted 150-fold before quench
glycerolphosphate dehydrogenase	5.0	59.4	5.2
glycerolphosphate dehydrogenase and isomerase	4.8	9.9	5.0

[a] The 20-μL pulse solution at 4 °C (25 nmol of native or inactivated isomerase and 5 nmol of [^{32}P]DHAP, 5560 cpm/nmol in 0.02 M sodium cacodylate, pH 6.5) was quenched after 2.5 s either directly with Cl_3CCOOH (3 mL, 2 N) or after 150-fold dilution in cacodylate buffer. After neutralization the indicated enzymes were added with NADH followed by NaOH (0.5 N final concentration). The resulting $^{32}P_i$ was determined. E·G3P/E·DHAP = 49.5/40.6 = 1.2; E·enediol = 5%.

carried through from the starting [^{32}P]DHAP.

If the pulse of isomerase and [^{32}P]DHAP was briefly diluted 20-fold before addition of Cl_3CCOOH, no $^{32}P_i$ above the control was observed. This is consistent with the idea that the species that gave rise to $^{32}P_i$ in acid was capable of reversing to stable triose phosphates, when dissociation of the enzyme–substrate complexes was favored by dilution of the enzyme.

When 5% of bound radioactivity was present as enediol phosphate, the quantities of bound D-G3P and DHAP were found from the experiment in Table IV to be 49.4% and 40.6% (100% − 59.4%), respectively. It seems most likely that these bound species represent the carbonyl forms only because the *gem*-diol forms have been shown not to be substrate for isomerase (Trentham et al., 1969). Therefore, the observed equilibrium constant, E·G3P/E·DHAP ≃ 1.2, is very much shifted from the value in solution, ~0.003, calculated by Reynolds et al. (1971).

Yeast Aldolase. Similar experiments have been done with Zn-containing yeast aldolase, which is known to enolize DHAP at a rate sufficient to account for the aldol condensation (Rose & Reider, 1958). Unlike muscle aldolase, prolonged incubation of [^{32}P]DHAP with yeast aldolase in the pH range 4.5–7.5 did not result in significant formation of $^{32}P_i$ (Table V). By assuming that the largest difference relative to the nonenzymatic control is a significant difference, one estimates a turnover of DHAP to be $\leq 2.5 \times 10^{-6}$ of the aldolase exchange process. Furthermore, on the assumption that the enzyme-bound enediol may not be labile in the pH range 4.5–7.5, a concentrated mixture of aldolase and [^{32}P]DHAP was acidified to determine the size of the bond enediol phosphate pool (Table VI). The amount of $^{32}P_i$ formed was not significant. Under similar conditions of the pulse, by the isotope trapping technique, using a chase solution containing unlabeled triose phosphates, the trapping of 20% of the total [^{32}P]DHAP indicated that this was the minimal amount of DHAP that would have been bound. Hence, the enediol phosphate species represents less than 1% of the occupied enzyme.

Table V: Cleavage of [^{32}P]DHAP by Yeast Aldolase[a]

pH	subunits of enzyme (nmol)	% of total ^{32}P: $^{32}P_i$ after 3 h	% of total ^{32}P: $^{32}P_i$ due to enzyme
4.5	0	4.9	
	10	5.1	0.2
5.5	0	5.1	
	10	5.3	0.2
6.5	0	5.8	
	10	6.1	0.3
7.5	0	5.9	

[a] The incubation mixture (50 μL) contained [^{32}P]DHAP (20 nmol, 1956 cpm/nmol), potassium acetate (0.1 M), mercaptoethanol (0.001 M), and yeast aldolase subunits as indicated in buffer (pH 4.5, 0.02 M potassium acetate; pH 5.5 and 6.5, 0.02 M potassium cacodylate; pH 7.5, 0.1 M TEA-HCl). $^{32}P_i$ was determined after 3 h at 37 °C.

Table VI: Acid-Catalyzed Elimination of O-PO_3H^- from the Yeast Aldolase-DHAP Complex[a]

yeast aldolase subunits (mM)	[^{32}P]DHAP (mM)	^{32}P formed > B in 2 N Cl_3CCOOH (mM)
0	1.2	B = 4.8%
1.2	0.83	0.013
1.5	1.6	0.004

[a] The pulse solution of 15 μL at 4 °C contained yeast aldolase and [^{32}P]DHAP (4523 cpm/nmol), as indicated, in β-mercaptoethanol (0.001 M), and potassium cacodylate (0.02 M, pH 6.5) was added directly to Cl_3CCOOH (1 mL, 2 N). $^{32}P_i$ was determined.

Discussion

The rate of phosphate ^{18}O positional isotope exchange observed with native muscle aldolase and DHAP was only $\sim 10^{-7}$ the rate of exchange that would be observed between DHAP and ^3H_2O under these conditions (Rose et al., 1965). This would seem to rule out the proposal by Grazi & Trombetta (1978, 1980) of an equilibration of the enamine phosphate form of DHAP and enamine aldehyde···PO_4 **3** on the enzyme which was based on the following argument.

Grazi & Trombetta (1980) observed similar rates for the stereospecific tritium exchange from (3*S*)-[3-^{3}H]DHAP and for the formation of the supposed acid-labile intermediate. By comparison of the rate of detritiation and oxidation of the native–DHAP complex by ferricyanide, a 2.5-fold isotope effect was detected. On the basis of this tritium discrimination, it was concluded that part of the deprotonated species, presumably the enamine present, was unable to hydrolyze to P_i upon strong acidification but that an additional species was required. There is good reason to believe that the tritium

discrimination reported may be an artifact of the technique used. The observation in Table II that all of the [^{32}P]DHAP bound to native aldolase can be trapped by G3P rules out the possibility of demonstrating such a discrimination, which requires that the step in which DHAP bind to the enzyme be readily reversible relative to condensation. If formation of the enamine is not rate limiting for the condensation reaction, it would also not be limiting for the exchange reaction because the reaction subsequent to proton abstraction is the isotopically irreversible exchange with the medium. A direct test for discrimination against [3-^{3}H]DHAP in condensation with G3P was made by Rose & Reider (1958), and none was found. Inefficient trapping of the unprotonated species by ferricyanide could have resulted in some isotope discrimination.

The irreversible β-elimination step, that leads to formation of E–enamine aldehyde + P_i (Scheme I), must be the rate-limiting step for the side path that also must include the Schiff's base form of methylglyoxal. If this were not the case, there would be a significant accumulation of enzyme-bound unphosphorylated species in the steady state. The excellent agreement with CP–aldolase between the amount that can be trapped with G3P and the P_i formed upon acidification over the pH range 4.5–9.5 (Figure 1) excludes this. It is therefore most reasonable to conclude that the acid-labile species is at the same oxidation state of/or is the enamine phosphate intermediate itself.

The observed apparent equilibrium constant between imine and enamine of $\sim$1.5 over the pH range 5.5–7.5 reflects the relative pK_a of the carbon acid and the conjugate acid of the protein base as well as that of the imine and amine forms of the complexed ϵ-NH_2 group of lysine used in forming the Schiff's base. A similarity in the pK_a of groups involved in proton transfer has been considered to be necessary for the high isotope effect for proton abstraction seen with CP–aldolase (Rose et al., 1965; Klinman, 1978). The pK_a of the basic group involved in C–H cleavage appears not to titrate in this pH range or up to pH 9 inasmuch as this would cause displacement toward enamine rather than the reverse, as seen. A pK_a lower than 5.5 would be consistent with a carboxylate group as the enzymatic base. Although active-site labeling of muscle FDP aldolase has not yet indicated a functional carboxylate group, it is noteworthy that a glutamate has been identified by Meloche (1970) in the active site of the Schiff base aldolase 2-keto-3-deoxygluconate-6-phosphate aldolase by reaction with an affinity-site inactivator.

Earlier studies of muscle triosephosphate isomerase have failed to detect the high amount of G3P bound at equilibrium. Thus, Veech et al. (1969) were unable to explain the abnormal ratio of the triose phosphates in terms of enzyme-bound forms found in tissue extracts and in studies with purified enzyme. Belasco & Knowles (1980) have interpreted the infrared carbonyl spectrum of DHAP with isomerase, assuming the presence of only a small amount of E·G3P, and binding constants for DHAP have assumed it to be the main species on the enzyme (Albery & Knowles, 1976). The ratio of bound G3P to DHAP is found to be 1.2–1.4 over the pH range 5.5–7.5 with the rabbit enzyme and approximately the same (at pH 6.5 and 7.5) with the enzyme from chicken muscle, kindly provided by Dr. J. R. Knowles.

It may be asked if the values obtained for DHAP, G3P, and enediol phosphate after sudden acidification of enzyme at equilibrium may result, in part, from changes that occur during denaturation. The values for E·DHAP and E·G3P seem correct because they are similar if the E·S mixture is diluted in a solution of the competitive inhibitor phosphoglycolate and quenched with acid. The exact concentration of the enzyme-bound intermediate is important for an evaluation of the rate constants for the reactions in which it participates although not for the ratio of these rates. The value of $\sim$5% for the enediol phosphate at equilibrium at pH 6.5 is not changed if higher amounts of acid are used for denaturation or if the acid is introduced in bulk or with a rapid mixing cell. Furthermore, pH 6.5 provided the maximum amount of "intermediate" in the pH range 4.5–8.5, indicative of a regular pH-dependent equilibrium. This would not be observed if changes that occur during acid denaturation made a significant contribution to the result. Whether the intermediate is in the form of diol or diolate requires additional studies.

The rate of decomposition of E·DHAP to P_i and methylglyoxal increases below its half-maximal pH of 6.0. This is also true for muscle aldolase. In both cases, the slow rates assure that none of the steps of the normal catalysis is rate limiting, and, therefore, the elimination step is being titrated, all other steps being at equilibrium. In the aldolase case, the P_i elimination must occur from the enzyme-bound enamine phosphate form, but the rate for isomerase could reflect the release of the enediol phosphate intermediate from the enzyme rather than from reaction on the enzyme. However, this interpretation seems inconsistent with the result that although the pH of the half-maximum rate of G3P formation from DHAP is also pH 6 (Wolfenden, 1970; Plaut & Knowles, 1972) and that the release of G3P from the enzyme is believed to be rate determining (Maister et al., 1976) the sensitivity to pH change is in the opposite sense to that of the elimination reaction.

The pH dependence of P_i formation by triosephosphate isomerase seems best interpreted to reflect a favored reaction for the singly charged phosphate ester; i.e., $-OPO_3H^-$ is a much better leaving group than $-OPO_3^{2-}$. This result would therefore establish the pK_a of the phosphate ester on the enzyme. The observation that V_{max} for isomerase action follows the precise inverse pH dependence favors the interpretation that only the dianion forms of the substrates and intermediate enediol phosphates are functional at saturation. Presumably, the second negative charge is necessary for the proper conformation of the active site or orientation of the phosphate group.

No evidence for an enediol phosphate structure was obtained in the yeast aldolase–DHAP system either by catalytic production of P_i and methylglyoxal or by their generation upon acidification. If it is assumed that the enediol phosphate on yeast aldolase and triosephosphate isomerase decompose at pH 6 at similar rates, only 10^{-5} of the DHAP bound to yeast aldolase would be present as enediol phosphate. However, the ability to react catalytically with tetranitromethane (Riorden & Christen, 1969) and reduce ferricyanide (Christen et al., 1976) with formation of pyruvaldehyde 3-phosphate certainly indicates carbanionic character in the aldolase intermediate. On the basis of stereochemical comparison the enediols of the isomerases are expected to have the cis (or *Z*) structure (Rose, 1975). One might suppose that the greater stability toward β elimination and greater ease of oxidation are characteristics of a trans (or E) enediol phosphate on the aldolase. This possibility is considered in the following paper.

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Liberation of the Triosephosphate Isomerase Reaction Intermediate and Its Trapping by Isomerase, Yeast Aldolase, and Methylglyoxal Synthase†

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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_i) 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 10^9 M^{-1} s^{-1}. 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 wiith triose-phosphate 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_i)[1] and methylglyoxal equally at a measurable

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[1] Abbreviations used: DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; Cl_3CCOOH, trichloroacetic acid; TEA, triethanolamine; P_i, inorganic phosphate; enediol phosphate, 1,2-dihydroxy-1-propene 3-phosphate; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide adenine nucleotide.

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