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## Chemistry of Proton Abstraction by Glycolytic Enzymes (Aldolase, Isomerases and Pyruvate Kinase)

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## Chemistry of proton abstraction by glycolytic enzymes (aldolase, isomerases and pyruvate kinase)

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Intermediates have been synthesized that are rapidly utilized by triose phosphate isomerase, yeast aldolase and pyruvate kinase. In each case the compounds have the properties of an enol expected for a stepwise proton transfer mechanism. Apparently the apparatus required for doing this chemistry is sufficiently unique for a large measure of structural homology to have been imposed upon the enzymes of this class during evolution.

### INTRODUCTION

A result of extraordinary importance has been the observation that triose phosphate isomerase, pyruvate kinase and KDPG aldolase show very great conservation in backbone architecture (Banner *et al.* 1975; Levine *et al.* 1978; Mavridis & Tulinsky 1976; Richardson 1977). These enzymes, with greatly different chemical roles in metabolism, would generally not be considered to be close relatives. They seem to have nothing more in common than that they each activate a C–H bond that is next to a carbonyl function. The aldolase is a Schiff's base aldolase. Pyruvate kinase surely makes use of K<sup>+</sup> and Mg<sup>2+</sup> in the enolization of pyruvate, and triose phosphate isomerase has no apparent requirements.

For this seeming dilemma to provide insights on the relation of structure to mechanism and to how enzymes evolve, the question must again be asked: how sure are we that we know the mechanisms of these reactions? How sure are we that there is so little else in common among the mechanisms than the juxtaposing of a basic residue of the enzyme and the substrate proton?

The first critical experiments on these three enzyme types (isomerases, aldolases and the enolpyruvate class of enzymes) were demonstrations that the abstracted proton was activated to undergo rapid exchange with protons in the medium (Topper 1957; Rieder & Rose 1959; Rose & Reider 1958; Meloche 1970; Rose 1960). For pyruvate kinase and all of the aldolases, the exchange was independent of the use that the enzymes were to make of the stabilized carbanion that must be developed. Thus ATP was not required for exchange by pyruvate kinase or glyceraldehyde-3-phosphate for exchange on a molecule of dihydroxyacetone phosphate (DHAP) by yeast or muscle FDP aldolase or on pyruvate by KDPG aldolase of bacterial origin. For muscle FDP aldolase the proton abstraction step, step 2, in the scheme could be

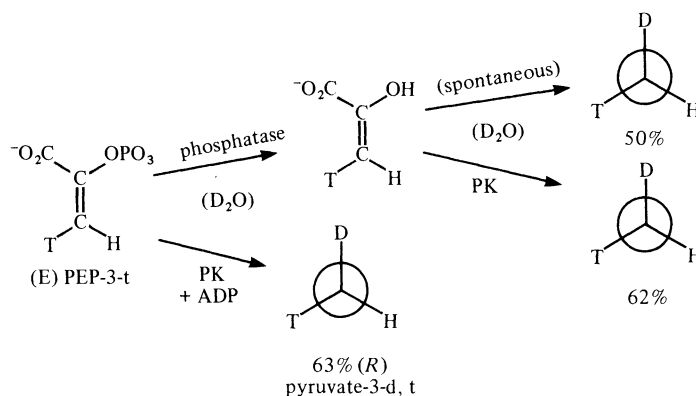


virtually inactivated by treatment of the enzyme with carboxypeptidase and yet not influence the ability of activated DHAP to condense with aldehydes as shown by the invariant exchange

rate for G3P into FDP (Rose *et al.* 1965). Pyruvate kinase has shown a remarkable plasticity in function that suggests separation of the kinase and enolization function: fluoride and hydroxylamine are good acceptors for phosphoryl transfer from ATP (Tietz & Ochoa 1958; Kupiecki & Coon 1960) and the decarboxylation of oxaloacetate catalysed by muscle pyruvate kinase is not influenced by the presence of ATP (Creighton & Rose 1976). Such pieces of evidence, the independence for exchange and the plastic properties, are undoubtedly chemical manifestations of a modular construction of these enzymes. An important structural question must then be: how do the modules or domains interact dynamically to make efficient use of the enol or enamine intermediates that are intrinsically unstable molecules? This suggests that there is sufficient cooperative interaction at the interface of the domains of these enzymes that counteracts the plasticity of the system that is manifest when some of the substrates are absent. This coincidence of plasticity and extreme organization is certainly at the heart of the ability of enzymes to bind very specific substrates both rapidly, which indicates accessibility, and tightly, which indicates multiple points of interaction as shown clearly by yeast hexokinase, where an apparent on-rate of *ca.*  $10^7 \text{ M}^{-1} \text{ s}^{-1}$  for glucose includes the structural changes that result in its almost complete envelopment through weak interactions (Bennett & Steitz 1978).

#### PYRUVATE KINASE

Direct evidence for the role of enolpyruvate as an intermediate in pyruvate kinase (PK) catalysis was recently obtained by the generation of the suspected intermediate by the action of a phosphatase on PEP and showing that the ketonization could be made stereospecific in the presence of kinase with the same specificity that the kinase has when PEP is used to phosphorylate ADP and produce pyruvate (Kuo & Rose 1978; Kuo *et al.* 1979) (scheme 1).



SCHEME 1

The enzymic ketonization requires both  $\text{K}^+$  and  $\text{Mg}^{2+}$ . Kinetic experiments could be done by studying the ability of enzyme to overcome the lag shown in the system PEP and phosphatase coupled to NADH and lactate dehydrogenase (figure 1). When the incubation was quenched with acid, an amount of pyruvate was found that agreed with prediction based on the length of lag in NADH oxidation and the steady-state rate (figure 2). This was the enolpyruvate, not available to the LDH, that had accumulated to its steady-state concentration

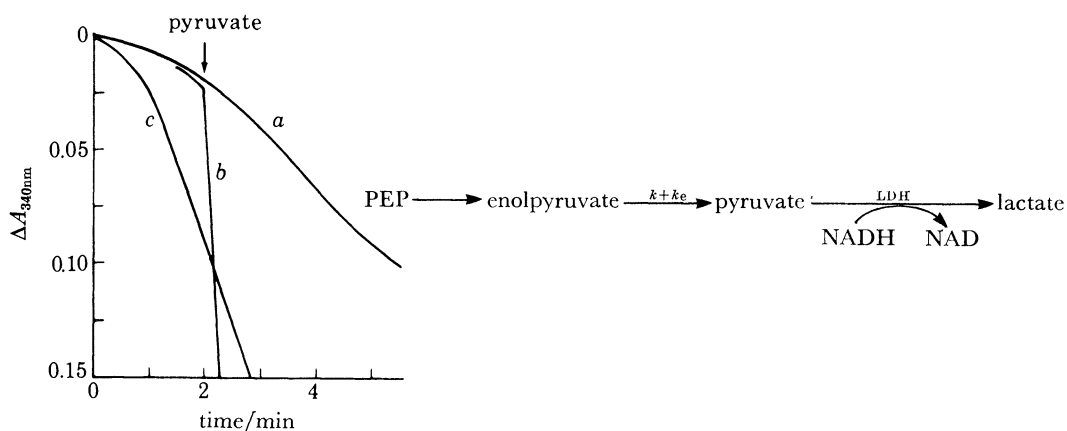


FIGURE 1. Effect of pyruvate kinase (*c*) on rate of pyruvate formation from PEP and phosphatase. The rate of NADH oxidation was followed by measuring  $A_{340 \text{ nm}}$  in a solution containing, in  $D_2O$  at  $15^\circ C$ : sodium maleate (40  $\mu M$ , pD 6.0), EDTA (0.1 mM),  $MgCl_2$  (1 mM), KCl (50 mM), NADH (0.12 mM),  $NAD^+$  (50  $\mu M$ ), acid phosphatase (Sigma IV, 150  $\mu g$ ), L-LDH (25  $\mu g$ ), and PEP (1 mM) added last. Pyruvate (0.1 mM) was added as noted by the arrow to a duplicate of (*a*) to show that LDH was fully active. Pyruvate kinase (25  $\mu g$ ) was present in *c* only.

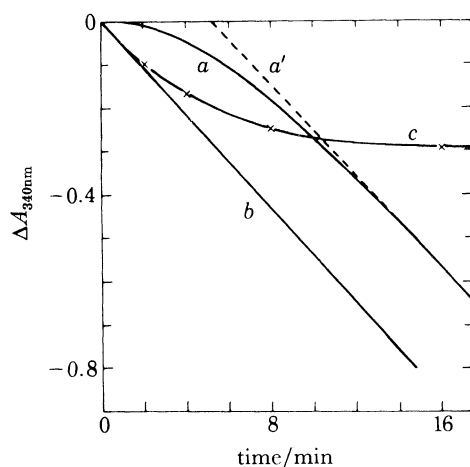


FIGURE 2. Kinetic evidence for an enolpyruvate intermediate. The incubation contained 10  $\mu mol$  of PEP, 1.25  $\mu mol$  of NADH, 44 units of LDH, 0.44 unit of acid phosphatase (Sigma IV), and 20  $\mu mol$  of maleate at pD 6.40,  $20^\circ C$ , in 5 ml of  $D_2O$ . The reaction was initiated with PEP and followed by measuring  $A_{340}$  decrease (curve *a*). Aliquots of 1 ml were quenched with 0.1 ml of 12 M  $HClO_4$ , at 2, 4, 8 and 16 min. After 10 min at room temperature, 0.6 ml of 2 M  $KHCO_3$  was added to neutralize the samples. Pyruvate was determined after centrifugation. Curve *b* is the steady-state rate. Curve *c* is derived from the absorbance of curve *b* minus curve *a* at that time. The four points on curve *c* represent the  $A_{340}$  of pyruvate found after quenching.

and collapsed to pyruvate upon acid quenching.† When pyruvate kinase was added, the amount of enolpyruvate was decreased and the lag was shortened. The greater the amounts of phosphatase or PEP used, the greater the concentration of enolpyruvate found for a given

† A solution of pyruvate, assayed with LDH under the same conditions, pD = 6.4,  $20^\circ C$ , will show biphasic kinetics. The slow reacting material, representing ca. 37%, is not enolpyruvate but the gem diol that has a half life of ca. 5 min. Although this is similar to that for enolpyruvate, ca. 3 min, it is not decreased when the pH is increased and such a high concentration is not consistent with the reported keto/enol equilibrium value of  $4 \times 10^{-6}$  (Burgner & Ray 1974).

level of pyruvate kinase (table 1). From such data, and by noting that PEP is a competitive inhibitor, the data gave an excellent linear plot when  $[\text{PEP}]/[\text{enolpyruvate}]$  was plotted against the reciprocal of the rate that could be attributed to the kinase (figure 3).

This representation is justified by the expectation that the enzymatic rate,

$$k_e = k_{\text{cat}} / \left( 1 + \frac{K_m}{[\text{enolpyruvate}]} \left( 1 + \frac{[\text{PEP}]}{K_i} \right) \right) \approx k_{\text{cat}} / \left( 1 + \frac{K_m}{K_i} \frac{[\text{PEP}]}{[\text{enolpyruvate}]} \right).$$

TABLE 1. KINETICS OF ENOLPYRUVATE KETONIZATION BY PYRUVATE KINASE

[phosphatase] μg	[PEP] μM	[enolpyruvate] μM	steady-state LDH rate nmol min <sup>-1</sup> ml <sup>-1</sup>	observed ( $k+k_e$ ) <sup>†</sup> min <sup>-1</sup>
150	964	27	6.7	0.25 (= $k$ )
150	948	12.2	13.3	1.09
150	460	8.4	10.3	1.55
300	175	7.5	16.0	2.13
300	472	10.6	19.3	1.82
600	443	18	37.8	2.10
1200	391	29	74.0	2.55

1 ml contained PEP and acid phosphatase, L-LDH (25 μg), KCl, MgCl, and pyruvate kinase (PK, 25 μg) in D<sub>2</sub>O, pD 6.0.  $A_{340 \text{ nm}}$  was followed into the steady-state when acid was added and [enolpyruvate] determined as pyruvate.

<sup>†</sup> ( $k+k_e$ ) = steady-state  $\Delta A_{340 \text{ nm}}/[\text{enolpyruvate}]$ .

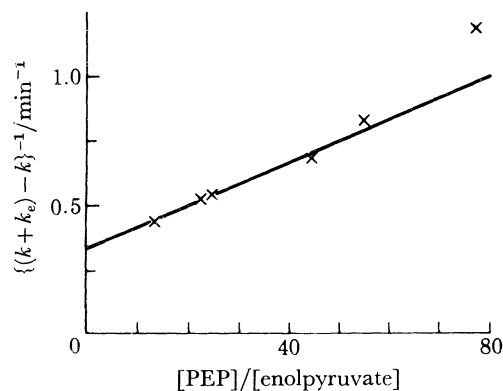


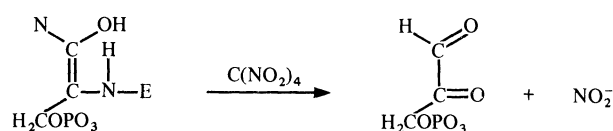
FIGURE 3. Determination of  $V_{\text{max}}$  and  $K_m$  of enolpyruvate for pyruvate kinase. The observed ketonization rate due to pyruvate kinase is plotted as its reciprocal against  $[\text{PEP}]/[\text{enolpyruvate}]$ ; data from table 1. At saturation,  $k_e = 3 \text{ min}^{-1}$  and  $K_m = \frac{1}{40} K_{i, \text{PEP}}$ .

The rate of enzymatic ketonization obtained from these data is only *ca.* 11% of the  $V_{\text{max}}$  for the production of ATP + pyruvate measured under the same conditions. To explain this low value one needs to remember that the ionization of the enolpyruvate species is a necessary step before ketonization. The  $K_m$  calculated for utilization of the intermediate is quite low, *ca.*  $10^{-8} \text{ M}$ , and must be due to the enol form of pyruvate. The enolate in solution would be present in only *ca.*  $10^{-6}$  of  $10^{-8} \text{ M}$  at the half-maximum condition at this pH (Burgner & Ray 1974), so it cannot contribute at all to the enzymatic rate. Whether the enol must undergo a chemical change before joining the reaction path is uncertain, although that would be the

straightforward explanation of its low  $k_{\text{cat}}$  for enzymatic ketonization. The enolate *per se* is thought of as an unpromising intermediate. In aqueous solution the enolate/keto equilibrium is *ca.*  $10^{-5}$  of the enol/keto equilibrium (Burgner & Ray 1974), so there must be a device for stabilizing the enolate. Unless some cationic group is used to neutralize the negative charge of the vinyl oxide anion, any enzyme residue that offers a hydrogen bond will give the enol. Sodium borohydride reduces pyruvate when it is bound to pyruvate kinase about  $10^4$  times faster than it does free pyruvate (Creighton & Rose 1976), which implies a strong polarization of the carbonyl, probably more than can be expected from simple hydrogen bond formation in the ground state. Enolpyruvate on the enzyme is ketonized about 200 times faster than the rate in solution at pD 6.0, where the rate is determined by the position of equilibrium to enolate and the rate-limiting protonation at C-3 (a sixfold slower rate is seen in  $\text{D}_2\text{O}$ ). It is not clear at this point whether the 200-fold stimulation by the enzyme is effected by enriching the concentration of enolate or speeding the ketonization of the enzyme-bound enolate.

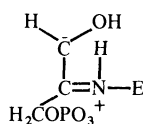
## ALDOLASE

Evidence for the enamine intermediate bound to muscle aldolase has accumulated in recent years from the use of the technique of catalytic oxidation of the intermediate by tetranitromethane or other oxidants (Christen & Riorden 1972; Healy & Christen 1972; Grazi



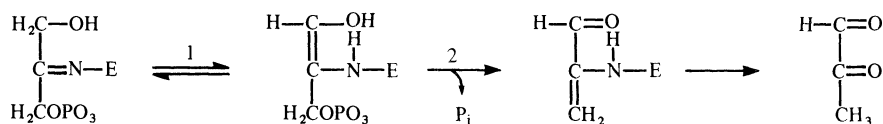
SCHEME 2

1975) (scheme 2). The form of the intermediate that is the reductant may be the carbanion



which is presumably the transient nucleophile for aldol condensation.

Recent evidence for the enamine intermediate for muscle aldolase has also come from the interesting observation that  $\text{P}_i$  and methylglyoxal are formed as side products of reaction with dihydroxyacetone phosphate (Grazi & Trombetta 1978, 1980) (scheme 3). Grazi & Trombetta (1978) made the important observation that when the mixture of DHAP and enzyme was quenched in acid, as much as 60% of the bound DHAP appeared as  $\text{P}_i$ , suggesting that a large amount of an acid-labile species is present among the enzyme-bound forms. This form must be reversibly connected to the main path of catalysis because it dis-



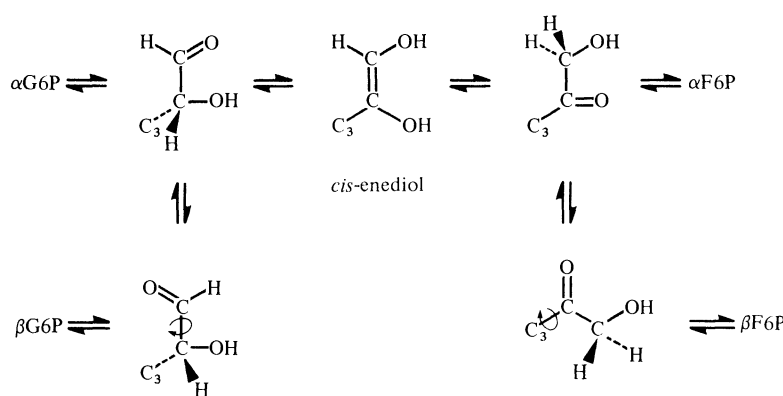
SCHEME 3

appeared if the DHAP was pulled or displaced from the enzyme before the addition of acid, or if glyceraldehyde-3-phosphate was added. That the bridge oxygen between C-1 and phosphorus is not broken in the course of the slow catalytic side reaction and before addition of acid can be shown by the absence of scrambling of the bridge oxygen to non-bridge positions in DHAP incubated with aldolase given sufficient time to exchange the C-3 pro *S* hydrogen *ca.*  $10^7$  times; i.e. step 2 is irreversible (Iyengar & Rose 1980*a*). It therefore seems that about 60% of bound DHAP is in the form of enamine. This displacement of an unfavourable equilibrium and the almost complete protection of the intermediate are cause for special note as they are both highly desirable characteristics of enzymes. More on these two points will follow.

## ISOMERASES

Turning to the aldose–ketose class of enzymes, we have different kinds of evidence to support a stepwise mechanism involving formation and utilization of an enol intermediate, more specifically a *cis*-enediol species. Table 2 summarizes our present view of the route from substrate to product, and it can be generalized to the eight isomerases that we have studied so far. There are several features that should be recognized (Rose 1975): (1) there is transfer of hydrogen between the neighbouring carbons of the same substrate molecule (not, for example, as in some coenzyme B<sub>12</sub>-dependent reactions (Frey *et al.* 1967)); (2) exchange also occurs, indicating that the hydrogen goes through an acid form that ionizes as a proton; (3) in many isomerases the transfer/exchange ratio exceed unity—indicating that the conjugate base is monoprotic; (4) the stereochemistry of the hydrogen transfer is always consistent with the interpretation that if an enediol is the intermediate it must be a *cis*-enediol; and (5) substrate specificity for one of the two possible anomeric ring forms for enzymes that act on pentoses and hexoses is predictable from the steric restriction imposed upon making C-2 tetrahedral, that ring closing can only occur from the face of the enediol that is opposite that from which the proton is added.

Implicit in the two stereochemical conclusions is the assumption of minimal motion of the enzyme and the intermediates that are proposed: the enediol and the two open-chain sugar forms that are tightly bound to the enzyme. The one exception to this assumption, the anomerase activity of phosphoglucose isomerase, must result from torsional freedom at the carbonyl carbon of the acyclic substrates that is completely restrained in the enediol intermediate (Schray *et al.* 1973) (scheme 4).



The mechanism proposed rests largely on the interpretation that the coincident occurrence of hydrogen transfer and exchange results from base catalysis. How secure is the stepwise enediol mechanism? Let us consider the negatives first: attempts to show carbanion character by use of oxidants have not succeeded with the simplest isomerase, triose phosphate isomerase plus DHAP (De La Mar *et al.* 1972). One could argue that the intermediate is highly protected

TABLE 2. STEREOCHEMICAL COURSE OF 2*R* AND 2*S* ISOMERASES 2*R*-ALDOSE ENZYMES

2 <i>R</i> -aldose enzymes		<sup>3</sup> H-exchange/transfer ratio
D-glyceraldehyde-3-phosphate	→ dihydroxyacetone phosphate	50
D-xylose	→ D-xylulose	10 <sup>-4</sup>
L-arabinose	→ L-ribulose	low
D-ribose-5-phosphate	→ D-ribulose-5-phosphate	high
D-glucose-6-phosphate	→ D-fructose-6-phosphate	1
D-glucosamine-6-phosphate	→ D-fructose-6-phosphate + NH <sub>3</sub>	200

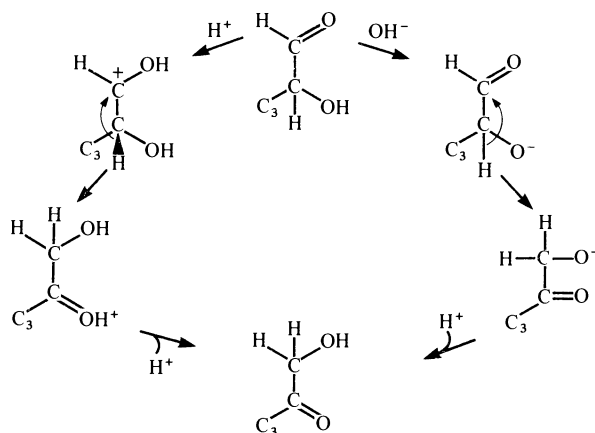
  
  

2 <i>S</i> -aldose enzymes		
D-arabinose	→ D-ribulose	low
D-mannose-6-phosphate	→ D-fructose-6-phosphate	1

in this enzyme. Perhaps a more troubling fact is that studies of acid- and base-catalysed isomerization of sugars in solution do not support the long accepted enolization mechanism (Topper & Stetten 1951). Direct H transfer observed in aqueous solution favours internal hydride mechanisms both in acid (Harris & Feather 1975; Ralapati & Feather 1978) and alkali (Gleason & Barker 1971) as shown in scheme 5. Of course, loss of some hydrogen to the medium is observed under alkaline conditions, but this may be related to destruction of the enol rather than to tautomerization to product.

Two arguments that I believe to be persuasive can be raised to support the interpretation that I have given for the enzymatic mechanism as opposed to an alternative of hydride transfer to explain the catalysis and a coincident but useless enolization to explain the exchange (Hines & Wolfe 1963). The first is kinetic: it has always been found that the partition of <sup>3</sup>H from tritiated water (an unimpeachable proton source) into aldose and ketose is the *same* in each direction that the reaction is studied (Rose & O'Connell 1961; Midelfort & Rose 1978). This implies a single intermediate species from which the exchange occurs. Agreement would not be expected if non-functional exchanges occurred at the E·aldose or E·ketose states at rates that should be unrelated.



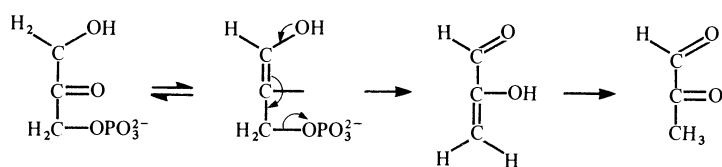


SCHEME 5

The second argument is chemical and is based on our recent synthesis of an unstable intermediate that is converted to triose phosphates by triose phosphate isomerase (Iyengar & Rose 1980*b*). It should be noted that neither the acid- nor base-catalysed hydride mechanism outlined above includes the synthesis of an intermediate that is not equivalent to substrate by simple ionization. The work with the synthetic intermediate leads to the important conclusion that the intermediate is an enediol, not an enediolate. Our evidence for the structure of the intermediate is that it is chemically very unstable, is not triose phosphate and is also used very well by yeast aldolase ( $\text{Zn}^{2+}$ -dependent) which, except for an enediol intermediate, one would not expect similarity in the mechanisms.

Our synthesis of the *cis*-enediol phosphate intermediate might more appropriately be termed biosynthesis. It was found that an incubation of [ $^{32}\text{P}$ ]DHAP and excess isomerase gave rise to the following three species after addition of acid: DHAP (42%), G3P (52%) and  $^{32}\text{P}_i$  (5%). The first two values establish the ratio of E·substrate forms to be close to unity instead of the value of 300:1 calculated for free DHAP:G3P when expressed only in their carbonyl form (Reynolds *et al.* 1971).

The observation of 5%  $\text{P}_i$  suggested that this amount of intermediate is present at equilibrium on the enzyme and that it decomposes as expected for an enediol-P by  $\beta$ -elimination to  $\text{P}_i$  and methylglyoxal (Brown *et al.* 1957) (scheme 6). The elimination of  $\text{P}_i$  by C-O cleavage



SCHEME 6

occurs if the enediol phosphate is produced in alkali (Midelfort & Rose 1976; Iyengar & Rose 1980*a*) or on the surface of methylglyoxal synthase (Summers & Rose 1977) and because both triose phosphates are perfectly stable in acid, we conclude that the labile character of the 5% intermediate derives from its loss of a proton at C-3. Although methylglyoxal has not been identified because of its low concentration in these experiments, we observe with others that there is a slow 'phosphatase' action (Webb *et al.* 1977) of isomerases, which gives rise to

methylglyoxal (Campbell *et al.* 1979) in equivalence to  $P_i$  (Iyengar & Rose 1980a). The rate of this side-reaction is *ca.*  $0.1 \text{ min}^{-1}$  at the half-optimal pH 6.0. When calculated for only 5% of  $E_t$  this is *ca.*  $0.03 \text{ s}^{-1}$ . The pH dependence curve for the triose phosphate isomerase formation of  $P_i$  and methylglyoxal is almost identical to that reported by Grazi & Trombetta (1978) for muscle aldolase (figure 4).

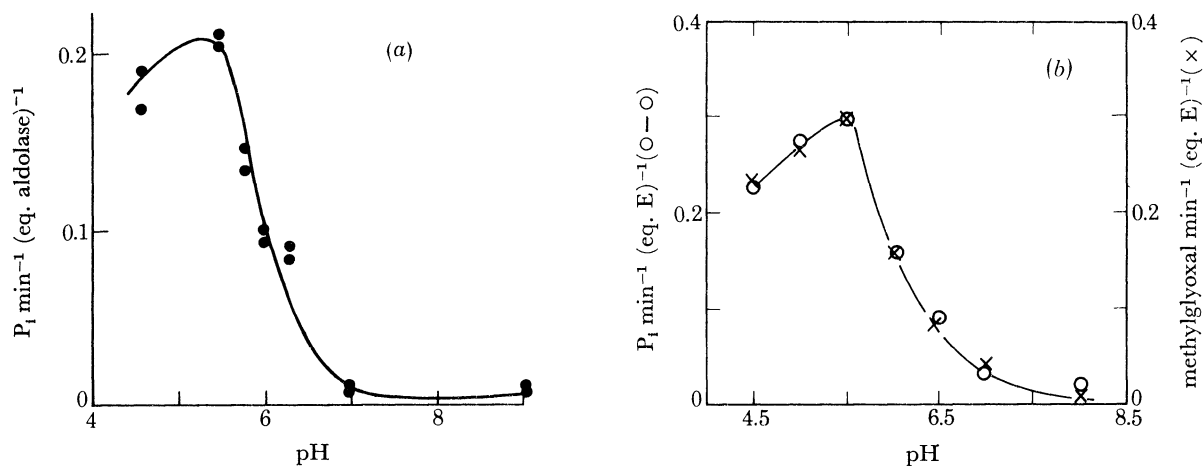
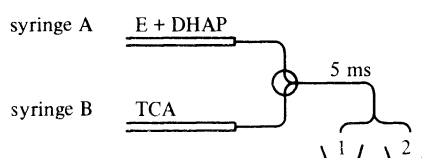


FIGURE 4. pH dependence of decomposition of DHAP by muscle FDP aldolase (a) (Grazi & Trombetta 1978) and triose phosphate isomerase (b) (Iyengar & Rose 1980a).

If an intermediate is liberated by acidification of the isomerase reaction, then we could study its properties if only we could stabilize it. This approach proved hopeless. Instead, we were forced to the strategy that to divert the presumed intermediate to another compound would give us a clue to its chemistry. The technique that proved successful was to use triose phosphate isomerase itself to capture the intermediate. The experiment allows the intermediate only 5 ms in TCA before it flows into a beaker that contains neutralizing buffer and varying amounts of isomerase (scheme 7). If beaker 1 contains only buffer, all 5% of the



SCHEME 7

initial [ $^{32}\text{P}$ ]DHAP is found as  $^{32}\text{P}_i$ . All but about 1% could be diverted to acid-stable and alkaline-labile form by sufficient amounts of isomerase in beaker 2. By varying the period spent in acid before reaching the enzyme in the beaker, the half-life of the intermediate was found to be *ca.* 15 ms from the slope of the decay curve (figure 5). From the intercept it is seen that the material that gives  $P_i$  fully retains its reactivity with enzyme after liberation from the enzyme in the acid. Therefore it cannot be a mixture of *cis*- and *trans*-enediols.

Similar data were obtained showing the lability of the intermediate in the range pH 4.5–8.5 by interposing a second mixer that introduced a selected buffer. Almost no effect was seen on the rate, which was *ca.*  $100 \text{ s}^{-1}$  throughout.

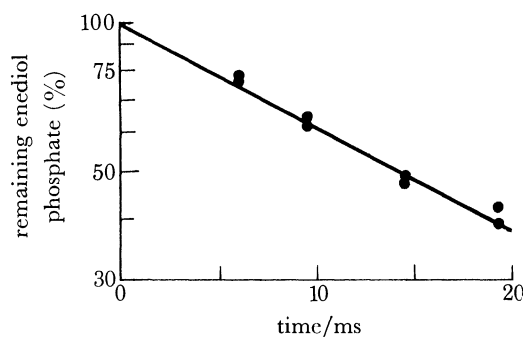


FIGURE 5. Rate of elimination of  $P_i$  from the enediol phosphate intermediate in TCA. Excess isomerase (10 nmol) was present in the trapping beaker. The time from the mixer to the beaker was varied.

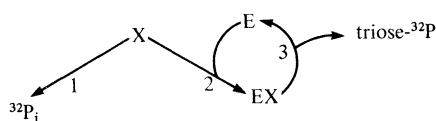
TABLE 3. EFFECT OF ISOMERASE AND INTERMEDIATE CONCENTRATIONS ON TRAPPING EFFICIENCY

[enediol phosphate]† nmol/ml	[isomerase]† nmol/ml	trapped	
		pH 5	pH 8
0.17	0	0	0
	0.033	0.05	0.06
	0.066	0.10	0.09
	0.1	0.13	0.14
	0.13	0.17	0.17
0.83	0	—	0
	0.06	—	0.40

Syringe A, 50  $\mu$ l ( $[^{32}P]$ DHAP (5 or 25 nmol) and isomerase (25 or 125 nmol) in 20 mM Na cacodylate, pH 6.5). Syringe B, 1 ml TCA (0.2 M). Beaker, 0.5 ml (neutralizing buffer (cacodylate or TEA-Cl) and fresh isomerase).

† Concentrations refer to the generated intermediate and the new enzyme in the beaker. Output was not split.

Knowing this and determining the amount of isomerase required to trap *ca.* 50% of the intermediate X at low concentration of X indicated the very large apparent second-order rate constant,  $k_2$ , of *ca.*  $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ . The same value was obtained at pH 5 and 8 (scheme 8;



SCHEME 8

table 3). With X greater than total enzyme, many times more than an equivalent of X can be diverted. By using a sufficiently large amount of X relative to  $E_T$  ( $EX > E$ ), it will be possible to determine the catalytic  $V_{max}$  and compare it with the  $V_{max}$ 's of the isomerase reaction. These experiments are in progress.

Therefore, there is a chemical species on triose phosphate isomerase, distinguishable from the substrates by being acid labile, that appears to be on the reaction path. Its ease of elimi-

nation of  $P_1$  implies the postulated enediol phosphate. Indeed, the high, pH-independent rate with which the enzyme reacts with X surely indicates that the enediol not the diolate reacts efficiently with the enzyme. The rate  $10^9 \text{ M}^{-1} \text{ s}^{-1}$  is so close to the diffusion limit that *all* of the enzyme and *all* of X must be involved in the interaction.

The enediol-P liberated from isomerase can be entirely diverted to FDP by yeast aldolase with the G3P also present. Therefore the aldolase reaction uses the *cis*-isomer of the enediol phosphate.

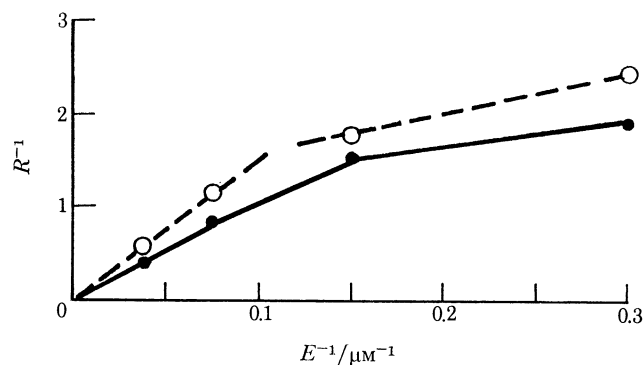


FIGURE 6. Dependence of yeast aldolase concentration on trapping of the isomerase intermediate. The first experiment of table 3 was reproduced with varying concentration of yeast aldolase ( $E$ ) in the beaker at pH 5.0 (●) or pH 8.0 (○). 0.01 M K acetate was included in the buffer. The ordinate gives the ratio of intermediate as  $P_1$  intermediate trapped.

A final comment concerns the much lower rate of decomposition of the intermediate on isomerase,  $0.03 \text{ s}^{-1}$  at pH 6.0 compared with *ca.*  $100 \text{ s}^{-1}$  in solution. The decomposition of the enediol phosphate in solution acts like a unimolecular process in that its rate is pH independent over the range pH 1–8.5 and is only weakly inhibited in  $\text{D}_2\text{O}$ . However, there must be interactions with the enzyme that are capable of stabilizing the enediol phosphate by *ca.* 3000-fold at pH 6 and much more at pH 8. We propose that these interactions hold the phosphate ester bridge oxygen in the plane of the molecule. The elimination reaction requires the C–O bond cleavage to occur out-of-the-plane just as nucleophilic addition to the double bond is favoured by  $\pi$ -orbital overlap. It may be that the very similar pH dependence for the catalytic formation of  $P_1$  and methylglyoxal by isomerase and aldolase reflects a relaxation from coplanarity that can occur when the phosphate group becomes monoanionic.

Returning to the initial puzzle of what aspects isomerase, pyruvate kinase and aldolase function could be imposed to maintain backbone homology throughout the course of evolution, I remain convinced that the mechanisms are fundamentally the same: the abstraction of a proton next to a carbonyl, immobilization of the intermediate in a highly planar form, and perhaps a commality in the nature and reinforcement of the functioning base. How much structure this requires and how unique the reinforcement of that structure must be are questions that need to be answered.

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