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# Chemical Trapping of Complexes of Dihydroxyacetone Phosphate with Muscle Fructose-1,6-bisphosphate Aldolase†

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ABSTRACT: Dihydroxyacetone phosphate (DHAP) in equilibrium with FDP aldolase of muscle is present in the form of two major covalent complexes. One, representing ~60% of total bound substrate, decomposes to $P_i$ and methylglyoxal upon acid denaturation of the enzyme as first reported by Grazi and Trombetta [Grazi, E., & Trombetta, G. (1979) *Biochem. J. 175*, 361–365]. This is now shown to be the enzyme–eneamine phosphate reaction intermediate since $P_i$ formation is prevented if the acid denaturation is done in the presence of potassium ferricyanide, an oxidant of the eneamine. The enzyme–eneamine aldehyde·$P_i$ **6**, presumed to be an intermediate of the slow methylglyoxal synthetase reaction of aldolase, must not be a significant source of the $P_i$ produced upon denaturation and is probably not a significant component of the equilibrium. The oxidation product, the enzyme–imine of phosphopyruvaldehyde, is sufficiently stable in 1 N HCl, $t_{1/2}$ = 76 min at 0 °C, to be isolated with the trichloroacetic acid precipitated protein. A second covalent complex, ~20–24% of bound dihydroxyacetone [$^{32}$P]phosphate, remains with the protein during acid denaturation and centrifugation. This acid-stable complex is formed rapidly and is chased rapidly by unlabeled substrate. Its stability in 1 N HCl is similar to that of the ferricyanide-oxidized derivative mentioned above. From this and its reactivity with cyanoborohydride in acid, this complex is thought to be the imine adduct of DHAP with aldolase **4** and/or the carbinolamine complex **3** present in the initial equilibrium. D-Glyceraldehyde 3-phosphate in the carbonyl form also forms an acid-precipitable complex with aldolase. However, its dissociation from the enzyme under conditions usual for studying the aldolase reaction is slow, $t_{1/2} > 20$ s at 25 °C, so that the complex must not be functional. The ability to trap and isolate covalent complexes in the Schiff-base class of enzymes offers a new and versatile approach to the study of these reaction intermediates in the steady state and at equilibrium and to explaining more precisely the consequences to the catalytic process of protein modification, solution pH, and alteration in substrate structure.

The sequence for interaction of aldolase and dehydroxyacetone phosphate (DHAP)[1] shown in Scheme I for steps prior to reaction with aldehyde is strongly indicated by several arguments: (1) proton exchange with water is catalyzed in the absence of aldehyde (Rieder & Rose, 1955), (2) reductive inactivation by $NaBH_4$ in the presence of excess DHAP is stoichiometric giving the secondary amine as expected for an imine with a unique $N^{\epsilon}$-lysine (Grazi et al., 1963), (3) exchange of the C2 oxygen of substrate with $H_2O$ is at least as rapid as aldol cleavage (Model et al., 1968), and (4) oxidants such as $K_3Fe(CN)_6$ are cosubstrates for the catalytic oxidation of DHAP by way of the eneamine **5** to pyruvaldehyde 3-phosphate (Healy & Christen, 1973).

We wished to examine the possibility that the imine and carbinolamine intermediates **4** may be sufficiently stable in acid to survive as covalent complexes with acid-denatured enzyme that can be brought down by centrifugation. This possibility is based on the expectation that (1) for DHAP to be released from the imine complex **4** it must pass through the zwitterion state, (2) acid conditions would favor the protonated carbinolamine, $pK_a$ = 8–9, slowing the release, and (3) dehydration of the carbinolamine to the imine is acid catalyzed and favored in aqueous solution (Jencks, 1969; Hine

† This work was supported by U.S. Public Health Service Grants GM-20940 (I.A.R.) and CA-06927 and RR-05539 (Institute for Cancer Research) and was also supported by an appropriation from the Commonwealth of Pennsylvania.

[1] Abbreviations: $BH_3$, sodium cyanoborohydride; DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; MG, methylglyoxal; TCA, trichloroacetic acid.

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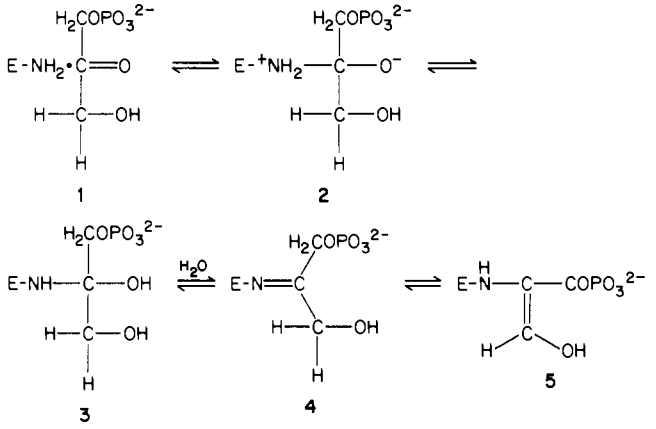
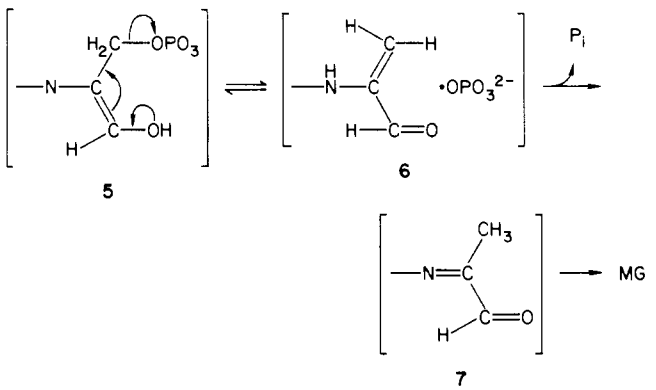
Scheme I



et al., 1970; Sayer et al., 1974; Pollack et al., 1977). As an example (Hine et al., 1970), hydrolysis of the imine formed between methylamine and isobutyraldehyde at 35 °C changed from an observed rate of $\sim 1\ s^{-1}$ at pH 4 to $\sim 10^{-3}\ s^{-1}$ at pH 1. Conjugated imines should be even more stable.

An additional covalent complex derived by $\beta$-elimination and thought to contain tightly bound $P_i$, **6**, has been proposed



as a reversible intermediate in the slow conversion of DHAP to $P_i$ + methylglyoxal (MG) through ketimine **7** (Grazi & Trombetta, 1978). This eneamine aldehyde·P complex was presumed to be the source of $P_i$ and MG that forms when an equilibrium of enzyme with DHAP is quenched in acid (Grazi & Trombetta, 1980). On the basis of the agreement between the size of the burst in DHAP oxidized by $Fe(CN)_6^{3-}$ with enzyme and the $P_i$ formed upon acidification, ~60% of the bound DHAP, Grazi & Trombetta (1980) conclude that **5** and **6** are in rapid equilibrium and that the amount of **6** must exceed that of **5** in the equilibrium. On the contrary, **6** will be shown to make a negligible contribution to the enzyme–DHAP equilibrium. All of the $P_i$ results from decomposition of the eneamine–phosphate intermediate since its formation is almost completely suppressed by $K_3Fe(CN)_6$ oxidation of **5** under strongly denaturing conditions.

## Materials and Methods

Rabbit muscle aldolase was obtained as a crystalline suspension in ammonium sulfate from Calbiochem or isolated by reported procedures (Penhoet et al., 1969). Aldolase was freed of triosephosphate isomerase activity (TIM) by treatment with glycidol phosphate (Rose & O'Connell, 1969b). Aldolase specific activity was ~10 $\mu$mol of fructose 1,6-bisphosphate (FDP) cleaved per minute per milligram at 23 °C as determined spectrophotometrically by coupling FDP to $\alpha$-glycerophosphate dehydrogenase and isomerase, measuring NADH oxidation at 340 nm. This corresponds to $k_{cat} \sim 6.7\ s^{-1}$. Protein concentration was determined from $A_{280}$, assuming the absorbance of 1 mg of pure aldolase/mL to be 0.91 $cm^{-1}$ (Baranowski & Niederland, 1949) and a subunit of $M_r$ of 40000.

DHAP ketal from Sigma was hydrolyzed according to the procedures recommended by the manufacturer. [$^{32}$P]DHAP (9000 cpm/nmol at the time of preparation) was prepared by phosphorylation of dihydroxacetone by glycerol kinase with [$\gamma$-$^{32}$P]ATP and purified on a Dowex 1-Cl column (Iyengar & Rose, 1981). It was free of G3P but contained 1% of the total radioactivity as [$^{32}$P]$P_i$. [$^{32}$P]-D-G3P was prepared by reaction of [6-$^{32}$P]FDP, prepared enzymatically, with aldolase and $\alpha$-glycerolphosphate dehydrogenase followed by isolation on Dowex 1-$Cl^{-1}$. $^{32}$P in both triosephosphates was determined by conversion to $P_i$ in 0.5 N NaOH, 37 °C and 15 min. Prior treatment with $\alpha$-glycerolphosphate dehydrogenase and NADH removed the DHAP, leaving the G3P as the source of alkali-labile phosphate. With triosephosphate isomerase present, the D-G3P was also stabilized. These reactions were followed to completion at 340 nm with added DHAP and G3P. Preparation of [6-$^{32}$P]FDP is described by Rose & Warms (1985).

Incubations of aldolase and [$^{32}$P]DHAP at ice temperature contained 50 mM triethanolamineacetate at pH 7.5 and lasted 90 s in order to give time for dehydration of the more abundant gem-diol species of DHAP. Cold HCl (3–5 volumes of 1 N) was added followed by a like volume of 1 N trichloroacetic acid. Centrifugation in a cold room was limited to 1 min to avoid heating of the samples in the Eppendorph Model 5412 centrifuge. Longer centrifugation decreased the recovery of radioactivity in the pellet (Rose & Warms, 1985). After removal of supernatant the tubes were rinsed with 150 $\mu$L of cold 1 N trichloroacetic acid without disturbing the pellet. $^{32}$P was determined by Cerenkov counting. To count acid-insoluble protein, the washed pellet was dissolved in 0.2 ml of 0.1 N NaOH and then mixed with 5 ml of $H_2O$ and 1 ml of $Me_2SO$. The molybdate–2-methyl-2-propanol extraction method for $P_i$ isolation (Berenblum & Chain, 1938) was used to determine [$^{32}$P]$P_i$ in the supernatant: the 2-methyl-2-propanol fraction (~0.9 mL) was mixed with 5 mL of 2-methyl-2-propanol and 1 mL of $Me_2SO$, and the aqueous layer (~1.1 mL) containing free [$^{32}$P]DHAP was mixed with 5 ml of $H_2O$ and 1 ml of $Me_2SO$. Each solution was counted before and after addition of an internal standard to correct for differences in counting efficiency.

## Results

*Detection of an Acid-Precipitable Complex of Aldolase with DHAP.* When [$^{32}$P]DHAP, 2.5–400 $\mu$M, and aldolase, ~500 $\mu$M, were incubated at 0 °C for 90 s followed by addition of acid and by rapid centrifugation of the protein, about 25% of the label was found with the protein precipitate. Only ~15% of total $^{32}$P was found as DHAP in the acid supernatant (alkali labile unless reduced with $\alpha$-glycerolphosphate dehydrogenase), and the remainder was [$^{32}$P]$P_i$. The appropriate data are shown in Table I on the basis of the experiment of Figure 1. The same values were obtained for incubations from 45 s to 5 min. Therefore, 90 s was long enough for the inactive gem-diol form to have been fully converted to the reactive keto form. Although the gem diol exceeds the keto form by ~6-fold at 4 °C (Reynolds et al., 1971), its dissociation constant for aldolase will probably be in the range of 5 mM on the basis of values reported for monophosphate analogues of DHAP (Hartman & Barker, 1965) or about $10^3$ less favorable than that of DHAP. Therefore, at equilibrium in enzyme excess the diol form of DHAP should be present at <1% including

Table I: Intermediates of Aldolase-Bound DHAP with Excess Enzyme

| conditions | percent of total [$^{32}$P]DHAP | | |
|---|---|---|---|
| | on pellet | $P_i$ (sup) | DHAP (sup) |
| standard[a] | 20.8 ± 1.1 | 64.2 ± 1.2 | 15 ± 2.0 |
| 5 mM DHAP before acid[b] | 0.7 | 1.5 | 97.8 |
| 5 mM DHAP before enzyme[b] | 0.4 | 1.5 | 98.1 |

[a] Data from Figure 1 including the seven samples with ≤0.6 mM DHAP and 0.5 mM enzyme. [b] Aldolase (0.5 mM) plus [$^{32}$P]DHAP (0.1 mM) in 2 μL; 50 μL of DHAP (5 mM) added 1 s before acid or before enzyme.

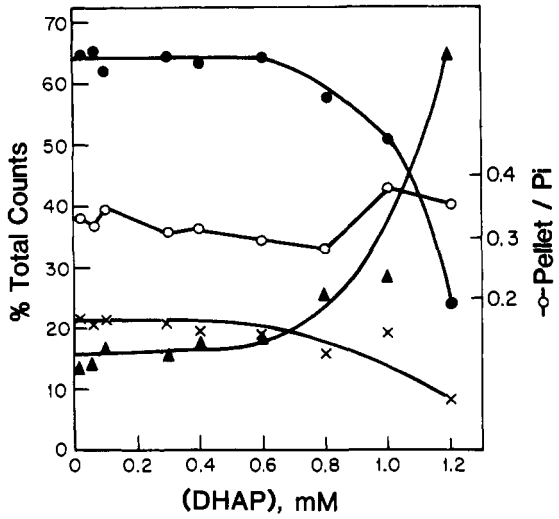



FIGURE 1: Aldolase complexes as a function of DHAP concentration. Aldolase (0.5 mM), the noted DHAP (6000 cpm total), and triethanolamineacetate (50 mM, pH 7.5) in 10 μL were quenched after 90 s at 0 °C with 50 μL of cold 1 N HCl followed within 5 s by 50 μL of cold 1 N TCA. The insoluble and soluble fractions were separated by centrifugation. Counts in pellet (×) and $P_i$ (●) and DHAP (▲) in the supernatant were determined. Also shown is the ratio of $^{32}$P counts in pellet to $P_i$ (○).

bound and free forms, and the values observed in Table I should represent an equilibrium distribution of catalytically active species. In keeping with this, the species responsible for the $P_i$ of the supernatant and the DHAP of the pellet fraction were both chased within 1 s by unlabeled DHAP, Table I. The amount of $P_i$ generated from enzyme-bound DHAP upon acidification is in agreement with the observations of Grazi & Trombetta (1978, 1980). The acid-insoluble radioactivity was identified as DHAP. When extracted from the pellet by neutral buffer, it was assayed as $P_i$ after treatment with alkali unless first exposed to α-glycerolphosphate dehydrogenase and NADH under conditions that gave complete reduction of added DHAP. Therefore, the pellet counts represent a form of DHAP, probably the enzyme-bound carbinolamine and imine forms of DHAP, present at the time of acidification. The pools represented by the pellet and $P_i$ counts are in constant proportion (Figure 1) over a wide concentration range including excess substrate. This would be expected if the two pools represent two enzyme-bound species in equilibrium. The DHAP found in the acid supernatant under conditions of enzyme excess could represent the enzyme–DHAP Michaelis complex present in the equilibrium or it could be derived via the carbinolamine complex during the acidification.

*Comparison of Complexes of DHAP and* D-*G3P.* Both triosephosphates form covalent complexes with aldolase that are TCA insoluble, Figure 2. The reaction between G3P and excess enzyme is slow, $t_{1/2}$ = 10 s (curve A), probably limited

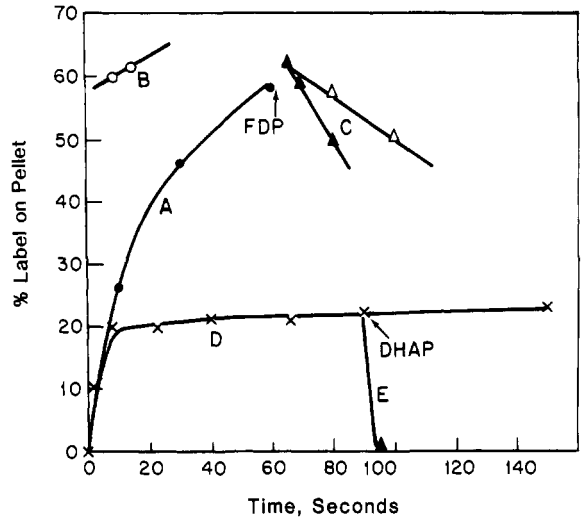
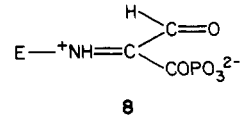



FIGURE 2: Rate of formation and dissociation of acid-stable DHAP and D-G3P protein complexes. (A) [$^{32}$P]-D-G3P (3 μM, 5000 cpm) + aldolase (100 μM), 25 °C. (B) [5-$^3$H]FDP (8 μM, 5000 cpm) + aldolase (100 μM), 25 °C. Quench at 8 and 16 s. Counts made volatile by alkali in the pellet and supernatant are compared to measure the percent of total [2-$^3$H]G3P that is bound. (C) Like (B) except allow 60 s before chasing with FDP (30 mM) (△) or with ~100 units of triosephosphate isomerase (▲); $t_{1/2}$ = 80 and 40 s, respectively. (D) [$^{32}$P]DHAP (5 μM, 2000 cpm) + aldolase (100 μM), at 0 °C. (E) Like (D), at 90 s add unlabeled DHAP (4 mM).

by the slow dehydration of the dominant, 97% (Trenthan et al., 1969), gem-diol form, $t_{1/2}$ = 8 s at 20 °C. When [5-$^3$H]FDP is incubated with excess enzyme in the absence of DHAP, the *free* aldehyde of G3P is produced. It interacts rapidly with enzyme to form the TCA-precipitable species (curve B). [2-$^3$H]G3P was identified in the pellet and supernatant by conversion of $^3$H to volatile form after brief incubation with alkali. With both sources of G3P, about 60% of the total alkali-labile counts were acid precipitable, indicating that ~40% might be in a noncovalent binary complex in equilibrium with the aldimine, E–G3P/E·G3P ≃ 1.5.

The enzyme–DHAP and enzyme–D-G3P complexes differ importantly in their rate of dissociation as shown by chase experiments, Figure 2. With [2-$^3$H]G3P, the acid-stable complex disappears from the equilibrium very slowly, $t_{1/2}$ > 20 s at 25 °C, under three sets of chase conditions: (1) displacement by FDP or (2) detritiation by triosephosphate isomerase (curve C) and (3) reaction with DHAP in the presence of FDP (not shown). On the other hand, the covalent enzyme–DHAP complex, also formed rapidly (curve D), is chased completely in 5 s at 0 °C (curve E).

*Origin of the $P_i$ When E·DHAP Is Acidified.* Two proposals have been advanced to explain the $P_i$ and methylglyoxal found after acid quenching: (1) decomposition by β-elimination of the E–eneaminephosphate **5** (Iyengar & Rose, 1981) and 2) *dissociation* of $P_i$ and ketonization of the eneamine aldehyde, from complex **6** presumed to be an intermediate in the methylglyoxal synthetase activity of muscle aldolase (Grazi & Trombetta, 1978, 1980, 1984). To distinguish between these proposals, we tested the possibility that $K_3Fe(CN)_6$, known to oxidize the eneamine phosphate complex of the native enzyme (Healy & Christen, 1973), might also oxidize acid-denatured **5**. The product of this reaction would be the imine aldehyde **8**. Ferricyanide would not be expected to oxidize



**8**

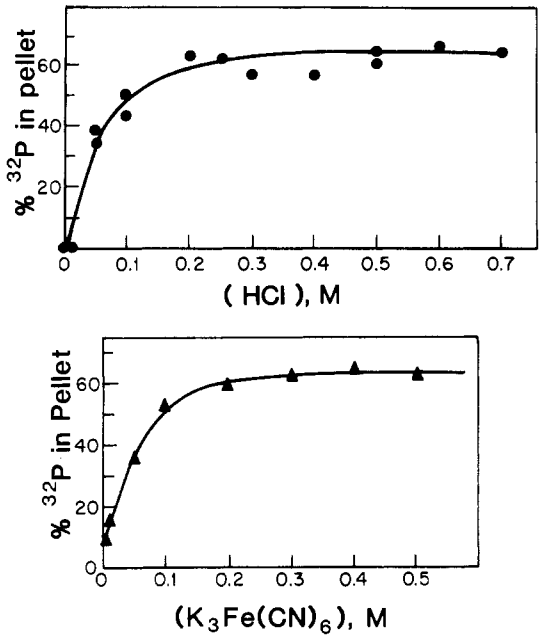



FIGURE 3: Denaturation/oxidation by ferricyanide. (A) An incubation as in Figure 1 with 2 $\mu$M [$^{32}$P]DHAP was quenched after 90 s at 0 °C with 5 volumes of cold 0.5 M $K_3Fe(CN)_6$ in the noted concentrations of HCl followed after 5 s by 5 volumes of cold 1 M TCA. (B) $K_3Fe(CN)_6$ varied as indicated was used in cold 0.6 N HCl to quench. In (A) and (B) controls were run containing a parallel amount of potassium ferrocyanide to determine the amount of DHAP recovered in the pellet without oxidation. These values were subtracted from the plus ferricyanide results and expressed as percent of total counts in each incubation that were made insoluble due to ferricyanide.

Table II: Effect of Ferricyanide in Trapping the Acid-Denatured Eneamine Phosphate Complex

| conditions[a] | distribution of $^{32}$P (% of total) pellet | supernatant $P_i$ | supernatant $P_{org}$ |
|---|---|---|---|
| (A) DHAP + aldolase + $K_3Fe(CN)_6$ | 75 | 3 | 22 |
| (B) DHAP + aldolase | 28 | 54 | 18 |

[a] The incubation (6 $\mu$L), containing 1.5 × $10^3$ cpm [$^{32}$P]DHAP and 0.5 mM aldolase, at pH 7.5 and 0 °C was mixed after 90 s with 30 $\mu$L of 0.3 M $K_3Fe(CN)_6$ in 0.5 N HCl (A) or with 30 $\mu$L of HCl alone (B), followed by 50 $\mu$L of 1 M TCA and separation of supernatant and pellet fractions as usual. The counts observed represent the percent of total corrected for contaminating [$^{32}$P]$P_i$, 150 cpm.

complex **6**, which would therefore decay to $P_i$ and methylglyoxal. Only if the conversion **6** to **5** occurred rapidly on the enzyme would one expect the oxidant to prevent $P_i$ formation from **6**. With 0.5 M $K_3Fe(CN)_6$ in HCl over the range 0.2–0.7 N, shown in Figure 3A, the $^{32}$P otherwise found as $P_i$ was found to coprecipitate with protein after acidification, thus providing another example of the acid stability of imine adducts to proteins. Raising the acid concentration itself does not decrease the $P_i$ formed, suggesting that the $\beta$-elimination is much more rapid than protonation of the "carbanion" **5** to give DHAP. The fact that increasing concentration of acid does not cause an increase in $P_i$ indicates that a conversion of **6** to **5** is not the explanation for the curtailed $P_i$ formation with ferricyanide. Furthermore, the oxidation seems to be occurring with the denatured form of **5**. Half-maximal trapping at 50 mM HCl may result from a shift with pH in the eneamine concentration to a new equilibrium that occurs before denaturation is complete. Ferricyanide concentration dependence during denaturation in 0.6 N HCl is shown in Figure 3B. As shown in Table II, all but ~6% of the $P_i$ generated by acid denaturation could be trapped as organic phosphate. This represents the maximum amount of complex **6** that could be present at equilibrium.

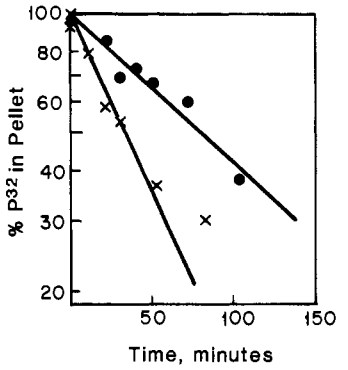



FIGURE 4: Stability of acid-trapped and acid/$K_3Fe(CN)_6$-trapped complexes. A standard 10-$\mu$L incubation as in Figure 1 was quenched with either 1 N HCl (×) or 0.5 M $K_3Fe(CN)_6$ in HCl at 2 °C (●). Cold TCA (1 M) was added at the indicated times. At zero time, 20 and 55% of the radioactivity was found in the acid pellet for the HCl and HCl + $Fe(CN)_6^{3-}$ quenching, respectively.

Table III: Borohydride Reaction with Acid-Stable Complexes[a]

quench $\xrightarrow{TCA}$ pellet $\xrightarrow[10\ min]{pH\ 7.6}$ TCA solubilized

| quench | pellet (% of total) | TCA soluble (% of pellet) |
|---|---|---|
| HCl | 24 | 97 |
| HCl + $BH_3$ | 20 | 20 |
| HCl/$Fe(CN)_6^{3-}$ | 58[b] | 100 |
| HCl/$Fe(CN)_6^{3-}$ + $BH_3$ | 65[b] | 8 |

[a] An enzyme + [$^{32}$P]DHAP equilibrium, 15 $\mu$L, was quenched with 50 $\mu$L of 1 N HCl or 50 $\mu$L of 1 N HCl containing 0.5 M $K_3Fe(CN)_6$. $NaCNBH_3$ (50 $\mu$L, 1 M) when used was added after 1 min. TCA-insoluble protein was separated by centrifugation, and each pellet was extracted with 0.1 mL of 1 M Tris-HCl, pH 7.6, at 37 °C for 10 min followed by TCA, centrifugation, and determination of solubilized counts. [b] The lower than expected value probably results from loss of the enzyme–imine phosphate complex from the pellet when 0.5 M $Fe(CN)_6^{3-}$ is present in the HCl and can be reproduced if $K_2Fe(CN)_4$ replaces $K_3Fe(CN)_6$.

*Comparison of the Two Acid-Stable Enzyme–Schiff Bases.* The two enzyme-associated acid-insoluble complexes show similar stabilities in 1 N HCl at 0 °C, Figure 4. The greater stability of the ferricyanide oxidation product may arise from the conjugative effect of the carbonyl **8**. Additional evidence that the acid-stable complexes are imines or are able to form imines without dissociating is their almost quantitative reaction with $NaCNBH_3$ after denaturation or denaturation/oxidation, Table III. Unlike the minus borohydride controls, the reduced complexes remain associated with the protein after exposure to neutral buffer. On the other hand, $NaCNBH_3$ does not trap the eneamine phosphate under conditions of acid denaturation, there being no increase in $^{32}$P in the pellet when $CNBH_3$ was included with the acid denaturant. Evidently the $\beta$-elimination reaction is much more rapid than formation of the imine on the denatured enzyme in contrast to native aldolase, which possibly maintains the bond to be broken in an unfavorable stereochemistry.

## Discussion

Methods of enzyme denaturation that either stabilize or simultaneously derivatize reaction intermediates without altering their concentrations are essential for intermediate identification and quantitation by chemical means to succeed. The stability of imines and carbinolamines in acid seems to

have been overlooked as an approach to characterizing these reaction intermediates. Evidence that the method can work quantitatively comes from the experiment using acid/ferricyanide to denature. In this case virtually all of the intermediate that normally produces $P_i$ is found with the denatured protein pellet. The oxidation product has the properties expected for the imine **8** on the basis of reactivity with $CNBH_3^-$, Table III. It cannot be stated with similar confidence that acid denaturation also traps the enzyme–DHAP imine species quantitatively. The value reported in Table I indicates that within a small experimental error about 20% of the bound DHAP is present in this form after correction for nonspecific binding, but this could represent a reproducible fraction of the pool of **3** plus **4**. In more accurate work it would be desirable to correct the observed value for loss by hydrolysis, which occurs with $t_{1/2}$ = 40 min in the quench medium, Figure 4. A systematic study evaluating the effect of temperature, mixing efficiency, and acid concentration at the time of denaturation would add confidence that neither an enzyme-catalyzed or noncatalyzed partition occurs during denaturation to influence this value of 20%. This question is considered further in the following paper (Rose & Warms, 1985).

It is important to be certain that a complex suspected of being on the reaction path be shown to be kinetically competent in conversions to normal reaction products. This can often be done by chase methods. The covalent complex formed between aldolase and D-G3P, while it is formed rapidly, dissociates much too slowly to be on the reaction pathway. A number of very slowly dissociating inhibitory covalent complexes are known to be formed between muscle aldolase and a variety of aldehydes (Horecker et al., 1972). These are generally assumed, from protection experiments, to arise by direct interaction with the substrate binding region: erythrose 4-phosphate gives rise to a metastable inhibitory complex with the loss of one –SH group/subunit (Wagner et al., 1972), a cysteine present in the active lysine peptide. Formation of gem diamines as found with pyridoxal phosphate–protein complexes may be involved in the aldolase–pyridoxal phosphate adduct. Even an allosteric site has been postulated to explain the effect of L-G3P, a slowly reversed inhibitor with no effect on FDP binding as shown by the unaltered transaldolase activity of the complex (Rose & O'Connell, 1969a). Suggestion that the acid-precipitable adduct of DHAP with aldolase represents a functional intermediate comes from its rapid dissociation and the fact that DHAP in excess of the enzyme does not lead to complex formation greater than predicted from the equilibrium with enzyme in excess.

The complex that is degraded to $P_i$ upon acid denaturation and trapped by denaturation/oxidation can be deduced to represent the kinetically competent eneamine **5** that is on the path of enzyme-catalyzed oxidation of DHAP by ferricyanide giving the free keto aldehyde **8** (Healy & Christen, 1973; Grazi, 1975). By use of carboxypeptidase-treated aldolase, which is specifically defective in carrying out the interconversion of DHAP imine and eneamine (Rose et al., 1965), the amount of ferricyanide that was rapidly reduced by an enzyme–DHAP equilibrium mixture was shown to agree closely with the amount of $P_i$ produced upon acid denaturation of that mixture (Grazi & Trombetta, 1980). These authors have chosen to ascribe this equality to a rapid interconversion of species **5** and **6**, concluding that **6** was the major source of $P_i$. Kinetic resolution of these species has been a subject of much additional work by these authors (Grazi & Trombetta, 1980; Grazi et al., 1983) primarily based on a comparison of the catalytic rate of detritiation of labeled DHAP with the rate of formation of the $P_i$-forming complex over a range of temperatures and pH. However, the present study, Table II and Figure 3, shows that oxidation and fragmentation are reactions of the same intermediate in as much as rapid mixing with cold 1 N HCl would prevent conversion of **6** to **5**. This assumes, of course, that this enzymatic conversion and the oxidation of eneamine phosphate by 0.2 M ferricyanide is not more rapid than the change in pH that would be expected to prevent it. This caveat is a general one in all attempts to measure intermediates by quenching methods. That simple mixing of two solutions can be expected to adequately quench reactions with turnovers of the range 100–1000 $s^{-1}$ can be judged from the observation of central equilibria close to unity in many cases (Ray & Long, 1976; Barman et al., 1978; Wilkinson & Rose, 1979). Were the inactivations not instantaneous relative to $k_{cat}$ in these cases, values closer to the solution equilibria differing by $\sim 10^3$ would be expected. It seems unlikely that aldolase, which in the cold has a rate of $\sim 1$ $s^{-1}$ and like many enzymes seems to have a balanced kinetic pattern, would be one of the enzymes for which this problem would be expected.

$^{13}C$ NMR can be useful to study intermediates at equilibrium that are too labile to detect off the enzyme (MacKenzie et al., 1984) though the long data collection times rule out studies of the normal steady state. A pertinent example of this approach with muscle aldolase is the investigation of Ray et al. (1983) with [1-$^{13}C$]glycoaldehyde phosphate, which produced a sharp resonance in the region expected for a carbinolamine. The quantitative application of this approach and the acid precipitation assay would allow an estimate to be made of the imine present, but apparently not visualized by the magnetic resonance approach.

**Registry No.** Fructose-1,6-diphosphate aldolase, 9024-52-6.

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# Complexes of Muscle Aldolase in Equilibrium with Fructose 1,6-Bisphosphate†

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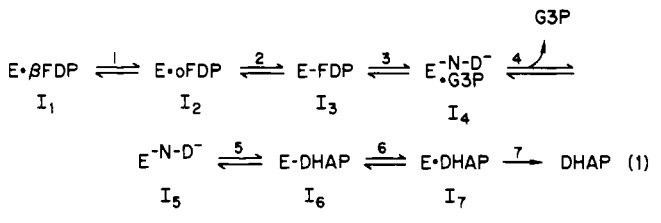
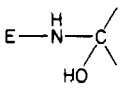
ABSTRACT: Minimum values for the content of covalent intermediates in the equilibria of muscle aldolase with its cleavable substrates have been determined by acid denaturation/precipitation. Ribulose 1,5-bisphosphate, a nonsubstrate that binds well to aldolase in the native state, does not form a covalent complex that is acid precipitable. The insoluble protein complexes with substrates fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate, representing ~50% and ~60% of total bound substrate, are much more stable in acid and alkali than that with substrate 5-deoxyfructose 1,6-bisphosphate, suggesting that they have the form of protein-bound N-glycosides. Whether such complexes exist on the enzyme in the native state in addition to being formed subsequent to denaturation is unresolved. Both the acid-precipitable and nonprecipitable forms of fructose 1,6-bisphosphate are converted to triose phosphate products at the same rate, providing no kinetic evidence for a pool that is not on the main reaction path. Total fructose 1,6-bisphosphate liganded to enzyme returns to the free solution about 9 times for each net cleavage reaction. It is still not clear whether this is limited by the cleavage step or by release of glyceraldehyde phosphate.

The currently understood reaction pathway for Schiff base dependent fructose-1,6-bisphosphate aldolase of rabbit muscle consists of several steps of chemical modification as shown in abbreviated form in eq 1 (Horecker et al., 1972). Steps 1–7




are as follows: (1) ring opening of the major solution species, βFDP;[1] (2) imine formation, E–FDP, from the open form of FDP, $I_2$, includes addition to $N^{\epsilon}$ lysine-221 giving a carbinolamine



followed by dehydration; (3) –C–C– bond cleavage of this open-chain imine, $I_3$, to the "ternary" complex, $I_4$, containing the eneamine form of dihydroxyacetone phosphate, DHAP, $E^{-N-D-}$, and D-glyceraldehyde 3-phosphate, G3P; (4) dissociation of G3P from the ternary complex; (5) proton addition to carbon of the eneamine, $I_5$, giving the imine of DHAP, $I_6$; (6) hydrolysis of the imine, which includes the carbinolamine of DHAP; (7) dissociation of DHAP from the binary Michaelis complex, $I_7$. Each of the seven intermediate states is demonstrably accessible to solvent as shown by proton and carbonyl–$H_2O$ exchange and by oxidation of the eneamine by a variety of chemical oxidants.

Our objective is to interpret the overall enzyme reaction kinetics in terms of the component intermediates, a process that has made significant progress for the analogous pyridoxal phosphate class of enzymes making use of differential UV spectroscopy. Without this asset, pending further applications of NMR to tightly held complexes with large proteins (Mackenzie et al., 1984), a chemical approach to quantitating intermediates in the sugar aldolase reaction is necessary. The preceding paper (Kuo & Rose, 1985) describes a simple assay for the imine plus carbinolamine complexes of aldolase with

† This work was supported by U.S. Public Health Service Grants GM-20940 (I.A.R.) and CA-06927 and RR-05539 (Institute for Cancer Research) and was also supported by an appropriation from the Commonwealth of Pennsylvania.

[1] Abbreviations: c and o, cyclic and open forms; FDP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; RuBP, ribulose 1,5-bisphosphate; Sed, sedoheptulose; Sor, Sorbose; TCA, trichloroacetic acid.

0006-2960/85/0424-3952$01.50/0 