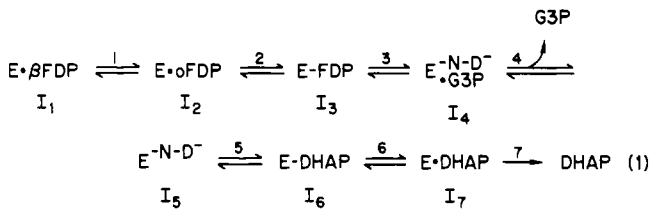
Pollack, R. M., Kayser, R. H., & Damewood, J. R., Jr. (1977) *J. Am. Chem. Soc. 99*, 8232–8237.
Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 3993.
Ray, B. D., Harper, E. T., & Fife, W. K. (1983) *J. Am. Chem. Soc. 105*, 3731–3732.
Reynolds, S. J., Yates, D. W., & Pogson, C. I. (1971) *Biochem. J. 122*, 285–297.
Rose, I. A., & Rieder, S. V. (1955) *J. Am. Chem. Soc. 77*, 5764–5769.
Rose, I. A., & O'Connell, E. L. (1969a) *J. Biol. Chem. 244*, 126–134.
Rose, I. A., & O'Connell, E. L. (1969b) *J. Biol. Chem. 244*, 6548–6550.
Rose, I. A., & Warms, J. V. B. (1985) *Biochemistry* (following paper in this issue).
Rose, I. A., O'Connell, E. L., & Mehler, A. H. (1965) *J. Biol. Chem. 240*, 1758–1763.
Sayer, J. M., Pinsky, B., Schonbrunn, A., & Washtien, W. (1974) *J. Am. Chem. Soc. 96*, 7998–8009.
Trenthan, D. R., McMurray, C. H., & Pogson, C. I. (1969) *Biochem. J. 114*, 19–24.
Wagner, J., Lai, C. Y., & Horecker, B. L. (1972) *Arch. Biochem. Biophys. 152*, 398.
Wilkinson, K. D., & Rose, I. A. (1979) *J. Biol. Chem. 254*, 12567.

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

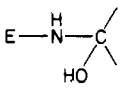
Irwin A. Rose* and Jessie V. B. Warms
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111
Received September 18, 1984

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^{ϵ} 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

DHAP showing them to be stable enough in acid to be isolated with the acid-denatured protein precipitate. In this paper the same approach is used to analyze the reaction equilibria of aldolase with FDP and other ketose substrates. The question arises as to whether, when allowed by a C-5 hydroxyl, the ketimine intermediate may normally be present in part in the cyclic form, in analogy with the substrate, step 1. Such enzyme N^{ϵ} Lys N-glycosides would not be on the direct reaction pathway and may provide a solution for the lack of correlation noted here between the amount of acid-precipitable substrate and the maximum rates of aldol cleavage reported for different substrates.

Materials and Methods

Aldolase was prepared from rabbit muscle (Pelfreeze) according to Penhoet et al. (1969) and treated with glycidol phosphate to lower the triose phosphate isomerase activity to ~0.01% of that of the aldolase, which had a specific activity of ~12 units/mg. DL-G3P and D-erythrose 4-phosphate were obtained from Calbiochem. L-G3P was a solution of DL-G3P from which the D-G3P had been removed with α-glycerol-phosphate dehydrogenase. Propionaldehyde 3-phosphate was prepared in an earlier work (Midelfort et al., 1976). [1-^{32}P]- and [6-^{32}P]FDP were prepared by reaction of [γ-^{32}P]ATP with phosphofructokinase (Boehringer) or yeast hexokinase (Sigma), and the steps of FDP synthesis were as reported earlier (Biellmann et al., 1965). [1-^{32}P]RuBP was prepared according to Jaworowski et al. (1984). [5-^{3}H]FDP was prepared from [5-^{3}H]glucose (Amersham). At least 97% of the tritium was at C-5 as shown with aldolase and α-glycerol-phosphate dehydrogenase followed by either triose/phosphate isomerase (Boehringer) or alkali and analysis for volatile counts. [^{32}P]DHAP was prepared as reported (Kuo & Rose, 1985).

All experiments were conducted at pH 7.5 in 20 mM triethanolamineacetate buffer at ~25 °C by manual mixing for times of 1 s or longer or with the Update Instrument Inc. System 1000 for rapid mixing and quenching. In studying the complexes of labeled FDP with aldolase present in great excess, it was important not to allow the label to appear in complexes containing only one of the triose phosphates. Extensive cleavage of FDP can occur when enzyme is in excess due to the covalent interaction of the G3P formed and enzyme in an irrelevant side reaction (Kuo & Rose, 1985). Unlabeled G3P was therefore used to prevent formation of labeled DHAP complexes from [1-^{32}P]FDP. Likewise, with [6-^{32}P]FDP it was necessary to include DHAP so that the distribution of isotope would reflect the combined equilibrium of steps 1–3. Only a short interval of ~3 s was allowed to establish the equilibrium. This was adequate time to allow the nonsubstrate αFDP, ~18% of the initial solution equilibrium (Midelfort et al., 1976), to be converted to the reactive open-chain and β forms but not long enough for the added G3P to take over the aldolase. The adequacy of this methodology is shown by observing the same distribution of isotope with either [1-^{32}P]- or [6-^{32}P]FDP. Care was taken to avoid additional anions such as Cl^- that have the effect of raising the K_m of FDP as the second power of their concentration (Rose & O'Connell, 1969). Enzyme–eneamine phosphate, I_5, was measured as P_i produced by acid quenching, by using 2-methyl-2-propanol extraction of the acid–phosphomolybdate complex (Berenblum & Chain, 1938). Acid precipitation of enzyme from a 15-μL incubation mixtue was initially done by addition of 100 μL of cold 0.5 N TCA, centrifuged in an Eppendorf Centrifuge 5412 for 8 min in the cold room, condition 1. The tubes were found to heat up during this prolonged centrifugation, and lower pellet-associated substrate was observed than if centrifugation was limited to 1.5 min and 200 μl of cold 1 M TCA used in the precipitation, condition 2. Supernatants were carefully removed and the tubes rinsed with cold TCA without disturbing the pellet. Pellets and supernatants were counted by Cherenkov in an Intertechnique SL30 scintillation counter. The aqueous and organic phases obtained in the extraction of P_i were counted with an ethanol–toluene–phosphor mixture. Labilization by alkali when used as a test for ^{32}P or ^{3}H in triose phosphate was done in 0.5 N NaOH for 15 min at 37 °C. [1-^{32}P]FDP was hydrolyzed to the extent of ~5% by this treatment. No ^{3}H was labilized from [5-^{3}H]FDP.

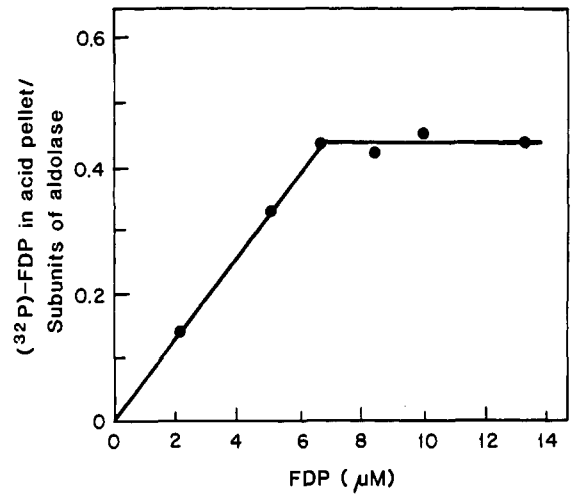


FIGURE 1: Acid-insoluble FDP used to titrate aldolase. The incubations contained in 15 μL aldolase (5.6 μM), DHAP (3.3 μM), and varied [6-^{32}P]FDP (~10^8 cpm/μmol) in 50 mM triethanolamine acetate (pH 7.5). After 3 s at 25 °C, TCA (100 μL of 9.2%) was added, and precipitable counts were determined.

Results and Discussion

Acid-Insoluble Complex of Enzyme–FDP. When [6-^{32}P]FDP and excess enzyme (~80 μM) were mixed and the addition of TCA made within a few seconds, the resulting insoluble protein obtained by centrifugation contained more than 40% of the FDP present in the incubation. That this product relates to a single site per subunit was shown by repeating the experiment with increasing amounts of [6-^{32}P]FDP, 1.5–10 μM, added to 4.25 μM aldolase subunits, Figure 1. In this experiment, DHAP (2.5 μM) was included to minimize formation of [^{32}P]G3P in the equilibrium.

The amount of acid-precipitated radioactivity reached a plateau of 43% of the total enzyme active sites in the presence of a slight excess of FDP. In the presence of excess enzyme (5.6 μM) and 2 μM FDP, about 39% of the FDP was in the acid pellet. The sharp end point observed at equimolar enzyme and FDP suggests a dissociation constant even lower than the K_m of ~2.8 μM (Hartman & Barker, 1965). Approximate agreement between the radioactivity fixed when calculated as percent of protein in excess [^{32}P]FDP or as percent of FDP in excess enzyme indicates that at least 40% of FDP bound to a single enzyme site was present on the enzyme at equilibrium in covalent linkage prior to acidification.

Identical extents of labeling of the acid precipitate were obtained with FDP labeled in the 1- and 6-positions when D-G3P and DHAP, respectively, were also present to suppress accumulation of labeled triose phosphate in the equilibrium. The acid pellet radioactivity that could be eluted into buffer had the properties expected for FDP; i.e., with [6-^{32}P]FDP, the precipitated label was stable to alkali but could be made labile if treated with aldolase and α-glycerolphosphate dehydrogenase before addition of the alkali.

Table I: Acid Precipitability and Substrate Specificity

ketose[a]	relative V_{max}[b]	% percent in pellet condition 1	% percent in pellet condition 2	% P_i[c]
DHAP		<8	23	60
FDP	1.0	42	52	4.5
SedBP	0.6	60	59	2
5-deoxy-FDP	2.0	1	23	3
L-SorbBP	0.06	1	9	~10
RuBP	0		0	0

[a] Aldol condensations were used to generate 1-^{32}P-labeled ketoses: aldolase (100 μM), [1-^{32}P]DHAP (5 μM), aldehyde (none or ~200 μM D-G3P, D-erythrose 4-phosphate, 2-deoxy-G3P, or L-G3P), pH 7.5, 25 °C. After at least 5 s, TCA was added and the protein precipitate obtained by condition 1 or 2 as given under Materials and Methods. The values given are the means of several determinations. In a similar experiment with [1-^{32}P]RuBP, 2.5 μM of RuBP was used instead of DHAP and aldehyde. [b] Taken from Richards & Rutter (1961) and Midelfort et al. (1976). [c] [^{32}P]P_i was determined in the supernatant to indicate E-DHAP (eneamine) remaining in the equilirbium and is corrected for [^{32}P]P_i present in the starting material.

Formation of the aldolase–FDP complex that is acid precipitable, presumably the imine complex I_3, was complete within 1 s of mixing at 5 °C. The radioactivity to be found in the pellet was decreased to <1% if 1 μmol of FDP, 10^3E_{total}, was added to the equilibrium about 1 s before the acid. Therefore, the identified enzyme–FDP complex was in rapid exchange with free FDP and/or triose phosphate products, consistent with the role of a reaction intermediate. The presence of unlabeled FDP in the quenching acid was without effect on the distribution of label between the protein pellet and the supernatant.

Although conditions used to obtain the TCA precipitate in the experiment shown in Figure 1 were found to be reproducible, they did not give the maximum yield. Changes in the way acid was added to terminate the incubation and in the length of centrifugation were shown to alter the counts pelleted with the protein. It was important to agitate the incubation as the acid (10 volumes) was being added. Once mixed it was important to centrifuge immediately at 4 °C and to keep the sample cold during the brief centrifugation. Loss of counts during the acid wash of the pellet surface and tube was not a problem when done in the cold. Conditions of mixing, acid concentration, and temperature were particularly important when the same experiment was performed with some of the other aldolase substrates, as will be shown. For the conditions given under Materials and Methods with excess enzyme, about 53% of total FDP present was usually found in the acid precipitate.

Do Other Ketose Substrates Yield Acid-Stable Complexes with Aldolase? The Schiff base FDP aldolase are highly specific for DHAP but can use a variety of aliphatic aldehydes from formaldehyde to erythrose and phosphoesters of aldehydes from glycolaldehyde phosphate to erythrose phosphate as condensation partners. To test the ability of other substrates to form acid-precipitable complexes with aldolase, [^{32}P]DHAP was incubated with an excess of a chosen aldehyde and amounts of aldolase that would assure good binding of the rapidly formed condensation product, Table I. Sufficient time was allowed for the nonenzymatic conversion of the unreactive DHAP *gem*-diol to DHAP, $k \simeq 1.5\ s^{-1}$ (Reynolds et al., 1971). Given the low affinity of *mono*phosphate ketoses relative to bisphosphates generally (Hartman & Barker, 1965), only aldehydes bearing a phosphate group were tested. Since bisphosphates have K_i values of the order 1–10 μM, the presence of 100 μM aldolase should be adequate to bind most of the product formed in these condensations. An internal test of the extent to which the [^{32}P]DHAP had been utilized for

Table II: Stability of the Acid-Stable Complex to Alkali[a]

(E + ketose) $\xrightarrow{TCA}$ pellet $\xrightarrow{OH^-}$ $\xrightarrow{TCA}$ pellet + Sup

ketose	acid pellet (% of total)	acidify and spin: pellet	acidify and spin: Sup P_i (% of first pellet)	acidify and spin: Sup P_{org}
DHAP	23	0	92	8
5-deoxy-FDP	24	1	78	21
FDP	50	39	15	46
SedBP	60	78	8	14

[a] As in Table I. The TCA-insoluble pellet obtained by condition 2 was dissolved in 0.5 N NaOH (50 μL). After 5 min at 25 °C, TCA (100 μL, cold, 1.75 M) was added. The pellet and supernatant were separated and counted. The supernatant was extracted to determine [^{32}P]P_i and other ^{32}P; P_{org} is probably the ketose derived by dissociation by way of the imine.

product formation is the decrease in the enzyme–eneamine phosphate form of DHAP, measured as [^{32}P]P_i formed upon acidification (Kuo & Rose, 1985). Under the conditions found to give the greatest yield of acid-insoluble FDP, only RuBP, a strong competitive inhibitor, failed to give pellet counts. By using the rate of dialysis method of Colowick & Womack (1969), RuBP was shown to be more than 95% complexed to the protein under the conditions of the experiment. Therefore the *S* configuration at C-3, which distinguishes the substrate xylulose 1,5-bisphosphate from the inhibitor RuBP, is important for Schiff-base formation to occur as well as for –C–C– cleavage. Aldolase catalyzes the detritiation of acetolphosphate, $CH_3COCH_2OPO_3^{2-}$ (Rose & O'Connell, 1969). From this it would seem that Schiff-base formation does not require a hydroxyl substituent at C-3 and that the failure of RuBP to do so arises from an inhibitory effect of its C-3 hydroxyl group.

From the preliminary experiments using less stringent condtiions for obtaining the acid precipitate (condition 1), it was believed that DHAP and 5-deoxy-FDP, each with high affinity as substrate, were either present as imines in only very low concentration in the enzymatic equilibrium or that these intermediates were particularly labile. As shown in Table I, the latter is the case. As is evident in Table I, 5-deoxy-FDP forms a much less stable complex than FDP; in 1 M TCA and 25 °C, it dissociated about 20 times faster than FDP. At 0 °C the half-life was still appreciable, ~25 min.

A simple explanation for the greater stability of the covalent enzyme–FDP and –sedoheptulose 1,7-bisphosphate complexes in acid is that these imines are able to cyclize upon acid denaturation, producing more stable furanosyl and pyranosyl derivatives. Such cyclization reactions are very rapid and probably readily reversible on the time scale of hydration and deamination of the imine, eq 2.

Stability of Acid-Precipitable Intermediates or Their Derivatives. In all cases studied, the release of label from the acid-precipitable state after acid denaturation could be represented by a single rate constant over at least two half-lives, Figure 2A, suggesting either that only a single species is present on the protein or that interconversion of two or more forms occurs rapidly in relation to the dissociation step. Stability was always greatest at low pH, Figure 2B. At very high pH, stability was correlated with the capacity of the imine to cyclize.

As shown in Table II, 0.5 N NaOH (5 min at 25 °C) destroyed the acid precipitability of the DHAP and 5-deoxy-FDP adducts whereas FDP and sedoheptulose 1,7-bisphosphate were significantly recovered in the subsequent TCA precipitate. When studying the effect of alkali on

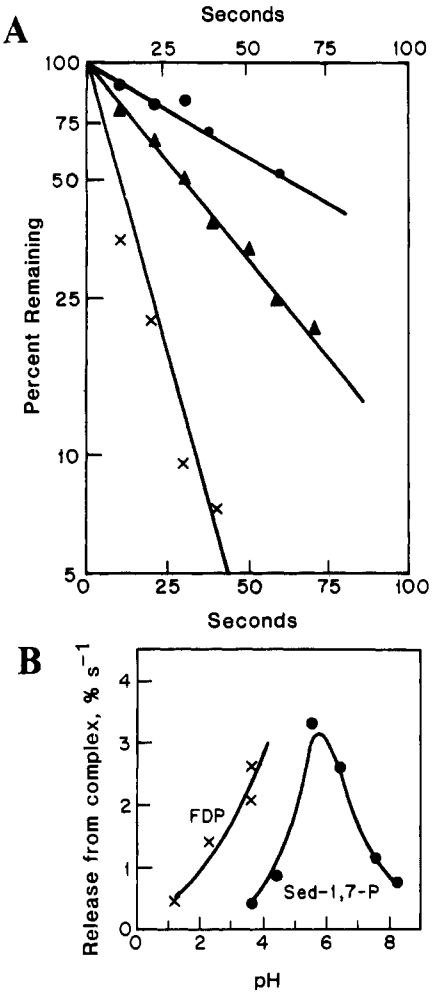
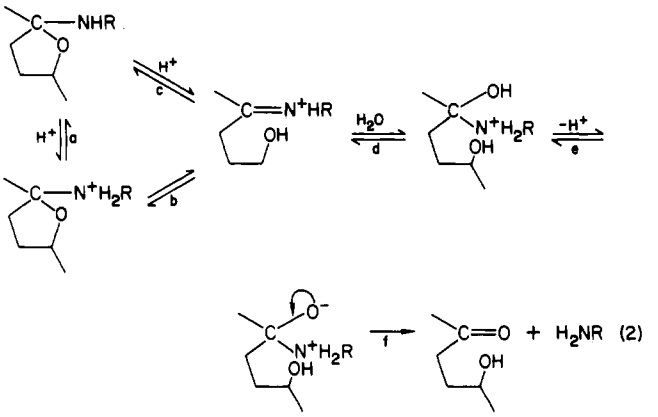


FIGURE 2: Stability of acid-precipitable complexes as a function of pH. SedBP or FDP were generated as in Table I in the presence of excess aldolase for 10 s and quenched in HCl–4 M urea. Stabilities of TCA-precipitable label were measured at 23 °C either directly (pH 1.2) or after 10-fold dilution into 0.1 M buffer with enough NaOH to give the indicated pH. Buffers used were as follows: glycine–NaOH, pH 2.3; acetate–NaOH, pH 3.6 and 4.4; sodium cacodylate, pH 5.5 and 6.5; triethanolamine, pH 7.5 and 8.3. TCA (1 M) was added after intervals, and counts in the washed pellet were determined. Rate constants were obtained over the whole pH range from data such as that in (A) with sedoheptulose 1,7-bisphosphate at pH 3.6 (▲) and 7.5 (●) and with FDP at pH 7.5 (×). Rates of dissociation are given as percent of the TCA precipitable radioactivity that is no longer precipitable in each successive second of time.

acid-precipitable counts, one must consider the β-elimination of $-OPO_3$ from the base-generated eneamine as well as the dissociation of the cyclic and acyclic complexes according to eq 2. From Table II, dissociation takes precedence over degradation in the case of enzyme-bound FDP, but clearly the extent of degradation is almost 10 times greater than would be observed with free FDP, which would be degraded to the extent of about 1.5% in 5 min under the same conditions. Therefore, the imine form of enzyme–FDP must partition approximately 3 to 1 between dissociation and the eneamine elimination pathway, which is most likely limited by proton abstraction. Since a similar ratio would be expected for the imine of 5-deoxy-FDP, it is likely, in that case, that most of the P_i was generated from 5-deoxy-FDP after dissociation. Exposure to strong alkali did not change the nature of the acid-precipitable species as shown with sedoheptulose 1,7-bisphosphate. The dissociation rate of this adduct was found to be 0.8% s^{-1} at pH 4.36 before and after 5 min in 0.5 N NaOH.

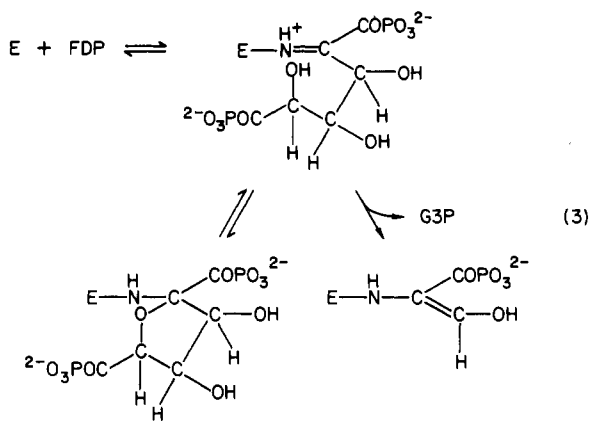
One may interpret the pH rate profile for hydrolysis of the N-glycoside of sedoheptulose 1,7-bisphosphate in solution (Figure 2B) on the basis of a consideration of eq 2. Ring



opening to the protonated imine will be at equilibrium since its reversal, cyclization, should be much more rapid than hydrolysis. For example, it has been determined that cyclization of FDP occurs at ~1400 s^{-1} at pH 7.2, 25 °C (Midelfort et al., 1976). Any implied homogeneity of the label in the pellet fraction based on the first-order decay of protein-boudn label, Figure 2A, may therefore be misleading. Since the hydration requires the protonated imine, the rate increase with acidity can be attributed to protonation of the imine. The decline in rate at lower pH results from the unfavorable effect of H^+ on the ionization of the carbinolamine, step e of eq 2 (Jencks, 1969; Hine et al., 1970).

Are There N-Glycosides on the Enzyme prior to Denaturation? It is established that aldolase catalyzes ring opening of β-FDP at the substrate level (Wurster & Hess, 1973; Grazi, 1974; Schray et al., 1975; Rose & O'Connell, 1977) and therefore cyclization as well so that one might consider the possibility that a part of the acid-stable adduct is already present as an N-glycoside before denaturation, i.e. that cyclization and ring opening are catalyzed with the enzyme–imine as well as the enzyme–FDP.

Such a species should not be on the main reaction path but would join with it through conversion to the imine by catalyzed ring opening, eq 3. In this case, the rate at which the N-



glycoside was formed and utilized in the aldolase reaction need have no relation to the rate of conversion of FDP to G3P. If any part of labeled pellet-forming material were shown to form products more slowly than label from the rest of the FDP, it could not be on the direct reaction path. To examine this possibility, [5-^{3}H]FDP and enzyme were first rapidly mixed and allowed to reach equilibrium for 6 s with added DHAP. This established the enzyme–FDP equilibrium under conditions

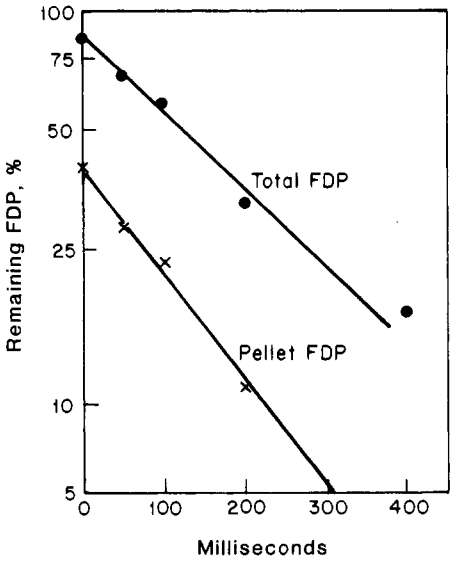


FIGURE 3: Conversion of acid-stable and total FDP complexes to triosephosphate. [5-^{3}H]FDP (300 pm), DHAP (4000 pm), and aldolase (1600 pm) in 30 μL were together for 6 s before being mixed with triosephosphate isomerase (960 units in 30 μL) and after intervals up to 400 ms quenched in a jet mixer with 2 volumes of 0.35 M TCA. As shown, with longer reaction times in manual experiments the appearance of ^{3}HOH proved to be biphasic. The data plotted have been corrected for a slower second species of ~9% of total. Release of label from the acid-precipitable pool and from all FDP forms by action of isomerase on [2-^{3}H]G3P is compared.

of minimal formation of [2-^{3}H]G3P. This was followed by rapid addition of triosephosphate isomerase so that as G3P was formed it would be readily detritiated instead of returning to bound [5-^{3}H]FDP by reaction with E–DHAP. This reaction mixture at 25 °C was precipitated with TCA with times up to 400 ms, and the loss of pellet counts and the overall aldol cleavage as measured by appearance of [^{3}H]H_2O were determined. The data in Figure 3 show approximate agreement between the two rates, the loss of pellet counts being first order from 40% to at least 6% of the total counts with $t_{1/2} \simeq 110$ ms. When corrected for a significantly slower second rate, representing ~9% of the FDP [possibly due to bound α-FDP (Wurster & Hess, 1973)] with a $t_{1/2}$ of 4 s, detritiation of the remaining total or 91% of bound FDP occurred with $t_{1/2} \simeq$ 160 ms; clearly the loss of acid-stable species is not slower than the overall catalytic rate when measured by tritium appearance in G3P. Therefore, the occurrence of an N-glycoside form of the substrate in a side path cannot be established kinetically.

It will be noted in Table I that the concentrations of acid-precipitable intermediates do not correlate with the relative maximum velocities of the overall reaction. This is inconsistent with the expectation that steps 3 and 4 are important in determining V_{max} unless these concentrations include additional species as shown in eq 3. Before this direction of argument can be pursued as suggestive evidence for a dead-end covalent complex, one would need to know the concentrations of intermediates with enzyme in the steady state of reaction with these substrates. However, the distributions between imine and Michaelis complexes, for example, I_3 vs. I_1 and I_2 in eq 1, are also unexpected on the basis of equilibrium consideration. For example, in the equilibrium with excess enzyme, the presence of the cyclic form of FDP, I_1 should decrease the proportion of substrate in normal acyclic ketimine form, I_3, relative to the value to be expected with 5-deoxy-FDP. The lower amount of covalent complexes present with 5-deoxy-FDP is unexpected and requires the hydroxyl at C-5 of FDP to stabilize I_3 more than its combined effect in stabilizing the

Table III: [E–FDP(imine) $\rightleftharpoons$ $E^{-DHAP(eneamine)}_{-G3P}$]$_{eq}$[a]

DHAP (μM)	pellet (%)[a]	^{3}HOH (%)[b]
26	54	4.15
53	54	3.68
106	55	2.75
210	54	2.05

[a] Each incubation in 30 μL contained aldolase (1600 pm), [5-^{3}H]-FDP (100 pm, 5900 cpm), and DHAP as noted. TCA added after 3 s at 25 °C contained FDP (1 μm) and DL-G3P (0.08 μmol). [^{3}H]G3P was determined as volatile counts after conversion of D-G3P to glycerol phosphate by triosephosphate isoermase and α-glycerolphosphate dehydrogenase. [b] Corrected for 3% of counts not in FDP and for 0.25% volatile in the absence of enzyme.

cyclic- and acyclic-bound substrate, I_1 and I_2. It may be appropriate to attempt to resolve this problem of the presence of N-glycosides by ^{13}C NMR examination of the reaction equilibrium.

*Concentration of Aldolase–Eneamine–*D*-G3P Complex at Equilibrium.* The equilibrium concentration of the I_4 complex that precedes D-G3P release in the aldol cleavage reaction can be determined from the amount of G3P liberated in an acid quench of an incubation containing FDP and a great excess of aldolase with enough DHAP to assure the absence of free G3P. By using [5-^{3}H]FDP, the TCA supernatant was examined for [2-^{3}H]G3P as counts made volatile, [^{3}H]H_2O, by reaction with triosephosphate isomerase, Table III. The isomerase reaction was followed by using added carrier G3P and coupling its isomerization to reduction by α-glycerolphosphate dehydrogenase and NADH. Similar experiments were performed using [1-^{32}P]FDP with excess aldolase and G3P (50–250 μM), making use of the conversion of the bound eneamine phosphate to [^{32}P]P_i when TCA is added to assay the complex. In both cases, the ternary complex at equilibrium was found to be no greater than 2% of total bound FDP. The ratio $I_4/I_3 = K_3 \leq 0.04$.

In previous steady-state experiments with β-FDP, the concentration of the complex containing DHAP–eneamine and G3P, I_4, was found to be very low, <2% of the enzyme occupied in catalysis (Rose & O'Connell, 1977). As shown here, this complex is also very low in equilibrium with bound FDP. If it had been found that I_4 had a higher level in the equilibrium than in the steady-state, the kinetic data would have been used to argue that the –C–C– cleavage step must be rate limiting, resolving the ambiguity inherent in the large isotope effects observed by Biellmann et al. (1969) with [3-^{3}H]- and [4-^{3}H]FDP. These secondary α effects may be interpreted as either kinetic or equilibrium effects, leaving uncertian whether cleavage or the G3P release step is rate limiting.

Partition of Aldolase–FDP Complex. If instead of quenching with TCA as in Table III a chase solution containing FDP and triosephosphate isomerase is added followed by TCA, the distribution of counts, volatile or not, will indicate the relative rates of formation of triose phosphate and FDP from all of the bound species, Table IV. The results were the same for a 1- and 5-s chase period as they should be if the isomerase reaction is rapid enough to detritiate all of the G3P formed within 1 s and the unlabeled FDP prevented any significant redistribution of [^{3}H]FDP 5 s after the partition. About 11% of the bound radioactivity, much more than the ~2% present in the G3P–product complex, appears as free G3P in the partition . This result tells the extent to which either step 3 or 4 is less than fully rate limiting. The fact that such a small amount of kinetic leakage occurs is consistent with the large values of the tritium α-secondary isotope effects reported for the cleavage reaction and the FDP–$H_2{}^{18}O$ exchange measurements of Model et al. (1968). A solution to

Table IV: The Partition[a] E-FDP → FDP + E; G3P + DHAP + E

chase (s)	pellet (%)	^{3}HOH (%)	E-FDP → FDP / G3P
none	50.5	2.5	
1	0	14	83/11.5[b]
5	0	13	84/10.5[b]

[a] Each incubation contained in 30 μL E (53 μM), [5-^{3}H]FDP (3.3 μM, 5000 cpm), and DHAP (200 μM). After 3 s, either TCA was added or 200 μL of the chase solution [FDP (1 μmol) + triosephosphate isomerase (150 units)] followed after 1 or 5 s by TCA. ^{3}HOH was determined after microdistillation as in Table III. [b] These values represent the partition of all the bound FDP (corrected for 97% purity and presence of 2.5% as the ternary complex).

the problem of which of these steps is limiting would be reached if the partition of the E–eneamine–G3P, I_4, could be determined. However, its low concentration at equilibrium seems to rule out this approach to the problem.

References

Berenblum, I., & Chain, E. (1938) *Biochem. J. 32*, 295.

Biellmann, J. F., O'Connell, E. L., & Rose, I. A. (1969) *J. Am. Chem. Soc. 91*, 6484–6488.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 2306.

Grazi, E. (1974) *Biochem. Biophys. Res. Commun. 59*, 450–454.

Hartman, F. C., & Barker, R. (1965) *Biochemistry 4*, 1068–1075.

Hine, J., Craig, J. C., Jr., Underwood, J. G., II, & Via, F. A. (1970) *J. Am. Chem. Soc. 92*, 5194–5197.

Horecker, B. L., Tsolas, O., & Lai, C. Y. (1972) *Enzymes (3rd Ed.) 7*, 213–258.

Jaworowski, A., Hartman, F. C., & Rose, I. A. (1984) *J. Biol. Chem. 259*, 6783–6789.

Kuo, D. J., & Rose, I. A. (1985) *Biochemistry* (preceding paper in this issue).

Mackenzie, N. E., Malthouse, J. P. G., & Scott, A. I. (1984) *Science (Washington, D.C.) 225*, 883–889.

Midelfort, C. F., Gupta, R. K., & Rose, I. A. (1976) *Biochemistry 15*, 2178–2185.

Model, P., Ponticorvo, L., & Rittenberg, D. (1968) *Biochemistry 7*, 1339–1347.

Penhoet, E. E., Kochman, M., & Rutter, W. J. (1969) *Biochemistry 8*, 4391–4395.

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

Richards, O. C., & Rutter, W. J. (1961) *J. Biol. Chem. 236*, 3185–3192.

Rose, I. A., & O'Connell, E. L. (1969) *J. Biol. Chem. 244*, 126–134.

Schray, K. J., Fishbein, R., Bullard, W. P., & Benkovic, S. J. (1975) *J. Biol. Chem. 250*, 4883–4887.

Wurster, B., & Hess, B. (1973) *Biochem. Biophys. Res. Commun. 55*, 985–992.

Active Site Histidine in Spinach Ribulosebisphosphate Carboxylase/Oxygenase Modified by Diethyl Pyrocarbonate†

Yasuo Igarashi, Bruce A. McFadden,* and Ted El-Gul

Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-4660

Received July 11, 1984

ABSTRACT: [^{3}H]Diethyl pyrocarbonate was synthesized [Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 251–258] from [^{3}H]ethanol prepared by the reduction of acetaldehyde by NaB^3H_4. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from spinach was inactivated with this reagent at pH 7.0 the presence of 20 mM Mg^{2+}, and tryptic peptides that contained modified histidine residues were isolated by reverse-phase high-performance liquid chromatography. Labeling of the enzyme was conducted in the presence and absence of the competitive inhibitor sedoheptulose 1,7-bisphosphate. The amount of one peptide that was heavily labeled in the absence of this compound was reduced 10-fold in its presence. The labeled residue was histidine-298. This result, in combination with our earlier experiments [Saluja, A. K., & McFadden, B. A. (1982) *Biochemistry 21*, 89–95], suggests that His-298 in spinach RuBisCO is located in the active site domain and is essential to enzyme activity. This region of the primary structure is strongly conserved in seven other ribulosebisphosphate carboxylases from divergent sources.

Various chemical modifications of D-ribulose-1.5-bisphosphate carboxylase/oxygenase (RuBisCO,[1] EC 4.1.1.39) have shown that lysyl, cysteinyl, histidyl, tyrosyl, arginyl, and methionyl residues may be located in the active site domain of this enzyme [for a review, see Miziorko & Lorimer (1983)]. Among those, the locations of only lysyl (Lys-175 and Lys-334) and cysteinyl (Cys-202 and Cys-459) residues have been determined in the large subunit of spinach RuBisCO to date. The requirement of Lys-175 for activity and its placement at the active site are especially well established (Schloss et al.,

† This research was supported in part by NIH Grant GM-19,972.

[1] Abbreviations: DEP, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NEM, *N*-ethylmaleimide; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SBP, D-sedoheptulose 1,7-bisphosphate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/85/0424-3957$01.50/0