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Presteady-State Kinetic Evidence for a Ring-Opening Activity in Fructose-1,6-(bis)phosphate Aldolase

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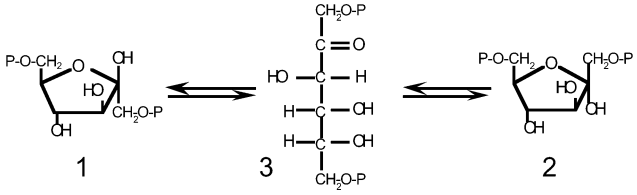
In this report, the use of transient-state kinetics in single-turnover experiments demonstrates that aldolase utilizes only one anomeric form of fructose-1,6-(bis)phosphate (Fru-1,6-P_2) and that the overall rate of Schiff-base formation from this substrate is faster than that of sugar ring-opening in solution, thus requiring catalysis of this step by the enzyme. This resolves a long-standing issue regarding the substrate for aldolase and provides insight into the nature of the active site.[1−3]

The central pathway for sugar metabolism in the cell, glycolysis, converts the six-carbon sugar, glucose, to two three-carbon pyruvate molecules. The carbon−carbon bond cleavage reaction is catalyzed by Fru-1,6-P_2 aldolase (EC 4.1.2.13). The substrate for this enzyme, Fru-1,6-P_2, exists in solution chiefly as a furanose ring (**1** and **2**) (Scheme 1).[4] The relative amounts of the β- and α-anomers of Fru-1,6-P_2 in solution are about 80:20 at 35 °C with 2% in the free keto (**3**) or the hydrate forms.[4] However, the mechanism of the aldolase reaction requires a ring-opened ketose (**3**), whose carbonyl can be polarized by a metal ion, as in the case of the class II enzymes,[5] or which can react to form a Schiff-base intermediate, as in the case of class I enzymes.[6] Whether class I aldolase is actively involved in catalysis of the conversion of the furanose ring to the open-chain ketose or whether it binds chiefly the open-chain form and pulls the ring-opening reaction(s) by mass action has been an unsolved mystery for many years.

Scheme 1. Anomeric Reactions Prior to the Aldolase Reaction



Part of the reason this question has remained unanswered is the equivocal evidence regarding the true form of the hexose substrate in the aldolase reaction. Using a variety of techniques for measuring binding to aldolase, one or more of several possible forms of hexose substrate Fru-1,6-P_2 (β-furanose (**1**), α-furanose (**2**), and keto (**3**)) have been proposed as possible substrates.[1−3] Furthermore, anomerase activity ($\beta \leftrightarrow \alpha$) has been measured for the class II yeast aldolase, but not detected for the class I rabbit muscle aldolase using the same assay.[2] This assay, however, would not have detected the half-reaction ($\beta \rightarrow$ keto or $\alpha \rightarrow$ keto), which is minimally required in the aldolase mechanism.

A transient kinetic approach has been useful for direct detection of Schiff-base formation and carbon−carbon bond cleavage.[7] Combined with the use of site-directed mutagenesis and X-ray crystallography, transient-state kinetics has helped discern the aldolase reaction mechanism.[8,9] The structural studies have revealed

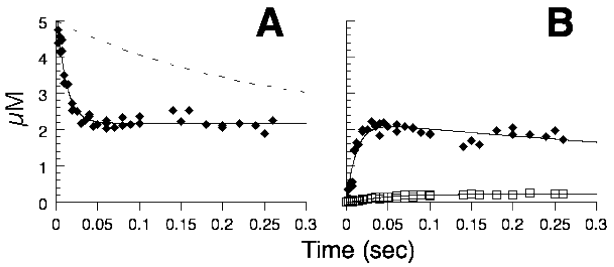


Figure 1. Single-turnover experiment at 4 °C for aldolase A with Fru-1,6-P_2. The reaction was started by mixing a solution of [U-^{14}C]Fru-1,6-P_2 with a solution of enzyme, yielding a final concentration of 5.0 and 75 μM, respectively. The concentrations of unreacted substrates, covalent intermediates, and both triosephosphate products were measured. Symbols represent remaining unreacted Fru-1,6-P_2 (◆) (panel A), covalently bound Fru-1,6-P_2 (◆) (panel B), and triose-phosphate products (□) (panel B). The curves were fit as described in Supporting Information (for panel A, r = 0.976; panel B, r = 0.936 and 0.967. The dashed line is calculated from the rate of ring-opening in solution at these concentrations.

Table 1. Rate Constants from Single-Turnover Experiments Determined at 4 °C

	Fru-1,6-P_2 Schiff-base intermediate formation		triosephosphate production	
enzyme	Fru-1,6-P_2 depletion k_1 (s^{-1})[a]	Schiff-base formation k_1 (s^{-1})[a]	Fru-1,6-P_2 cleavage k_2 (s^{-1})[a]	k_{cat} (s^{-1})[b]
aldolase A[c]	−	95 ± 5	6 ± 1	0.7
aldolase A	82 ± 6	83 ± 10	31 ± 3	1.0
aldolase B	60 ± 7	60 ± 8	20 ± 4	0.06

[a] Rate constants were calculated from data in Figure 1, A or B, using the kinetic model in Scheme 2 (see Supporting Information). [b] From Table S1. [c] From ref 7.

different binding modes for the substrates.[10] However, the initial binding reactions preformed by aldolase have remained unresolved.

Steady-state kinetics was performed at 4 °C on recombinant aldolases A and B[8,11] such that comparison to presteady-state kinetics was possible (see Supporting Information). The initial reactions with Fru-1,6-P_2 were examined under single-turnover conditions[7] by rapidly mixing enzyme and a limiting amount of substrate. The reaction was monitored from 3 to 300 ms as shown in Figure 1. Table 1 summarizes the derived rate constants. The data in Figure 1A show an exponential depletion of noncovalently bound Fru-1,6-P_2 as a function of time from 3 to 300 ms ($t_{1/2} \approx 10$ ms). The data in Figure 1B show that the amount of Fru-1,6-P_2 incorporated into covalent intermediates also increased exponentially as the Schiff-base intermediates were formed. The incorporated Fru-1,6-P_2 subsequently decreased as cleavage occurred and glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) were produced (□, Figure 1B). There are two observations that are immediately apparent from these data. First, that rate of reaction to form the Schiff-base intermediate is faster than ring-opening in solution (dotted line, Figure 1A).[12] Second, the Fru-

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1,6-P_2 remaining in solution was not fully depleted and the radioactivity in the acid precipitant reached a maximum of ~40% of the [U-^{14}C] Fru-1,6-P_2 present in the incubation during the time-frame of the experiment. This second observation indicated that aldolase likely prefers one form of the substrate (**1**−**3**), and this can be revealed by the equilibrium amounts of each anomer in solution.

The single-turnover kinetic values were determined by modeling the data according to the irreversible reaction depicted in Scheme 2.

Scheme 2. Presteady State Reactions

$$E + \text{Fru-1,6-P}_2 \xrightarrow{k_1} E = \text{Fru-1,6-P}_2 \xrightarrow{k_2} \text{G3P} + \text{DHAP} + E$$

In Scheme 2, E = Fru-1,6-P_2 is the Schiff-base intermediate whose rate of formation is k_1. The apparent rate constant, k_1, represents the combination of several rates leading to its formation. The rate of generation of the products, G3P and DHAP, is k_2. The rate constant, k_2, represents only the carbon−carbon bond-cleavage step since acid quench immediately releases both G3P and noncovalently bound DHAP product.

The incomplete reaction with Fru-1,6-P_2 is consistent with earlier investigations concerning the actual isomeric form of the sugar bound at the aldolase active site, which has been controversial.[1−3] Experiments performed for longer times (3 ms to 3 s) and in the presence of triosephosphate isomerase to pull the equilibrium and lower the concentration of Fru-1,6-P_2 revealed a slow phase of the reaction (see Supporting Information). The data indicate that aldolase A initially reacts in a fast reaction with 70−80% of the β-D-Fru-1,6-P_2 anomer present at equilibrium in solution[4] and the remaining 20−30% of the α-D-Fru-1,6-P_2 anomer reacts in a slow phase only after it converts to the β-anomer. This slow phase (ca. 1 s^{-1}) is slower than the $\alpha \rightarrow$ keto reaction (8 s^{-1})[3] and is consistent with earlier reports that form **2** binds unproductively.[2]

The dotted line in Figure 1A shows that rate of ring-opening in solution (**1** → **3**) calculated the same as that for Fru-1,6-P_2 depletion.[13] The rate of conversion to the reactive keto form by ring-opening in solution was clearly slower than the rate of reaction with the keto form to form the Schiff base. The most likely explanation for this is that aldolase catalyzes the ring-opening step as it binds its β-furanose substrate.

In conclusion, the rate of Schiff-base formation measured here includes all necessary steps from Fru-1,6-P_2 binding to formation of the covalent complex with the enzyme. Given that at equilibrium most of the substrate in solution is form **1** and appears to be that used by the enzyme, the spontaneous ring-opening of furanose Fru-1,6-P_2 at 4 °C (~4 s^{-1}) is not fast enough to be consistent with the rates of Schiff-base formation measured here (≥60 s^{-1}). This was also the case for aldolase B. Thus, both isozymes must catalyze this ring-opening step. By inference, this means that the catalysis of ring-opening is stereospecific with respect to the C-2 of the furanose ring of the β-anomer. It has been suggested in a crystal structure of Fru-1,6-P_2−enzyme complex that hexose binding to aldolase and the formation of the Schiff-base intermediate may occur in different sites.[10b] However, the location of the active-site residues responsible for this hemiketal cleavage, either as general acids/bases or as residues that may indirectly aid in a phosphate-catalyzed ring-opening,[14] have not been identified.

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Supporting Information Available: More detailed information for experimental rationale, design, interpretation, and controls (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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