Fructose-1,6-bisphosphate Aldolase from Rabbit Muscle. Kinetic Resolution of the Enamine Phosphate from the Enamine–Aldehyde Intermediate at Low Temperature†

Enrico Grazi,* Giorgio Trombetta, and Vincenzo Lanzara

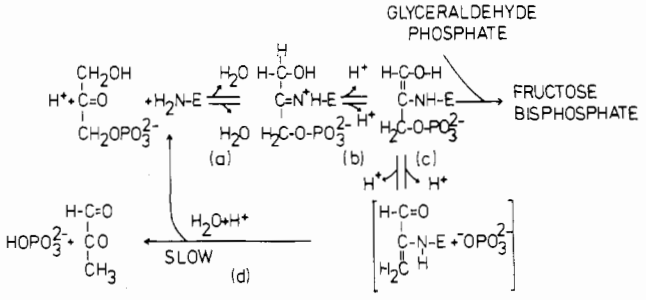
ABSTRACT: At or below −12 °C and in the presence of 40% ethylene glycol, only two out of the four dihydroxyacetone phosphate binding sites of aldolase are catalytically active. At these same temperatures and at pH* 8.3, the equilibrium between the pre-enamine and the enamine plus the post-enamine intermediates is largely shifted in favor of the latter. The enamine phosphate and the enamine–aldehyde phosphate intermediates have been resolved by studying the rate of their formation at −13 °C and pH* 5.28 and the trapping by DL-glyceraldehyde 3-phosphate at −24 °C and pH* 5.24.

We have previously observed (Grazi & Trombetta, 1978, 1980) a slow production of inorganic phosphate and methylglyoxal when rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) and dihydroxyacetone phosphate are incubated. This reaction occurs optimally in the pH range 5–6 with a rate $\sim 10^{-4}$ that of the exchange of the C-3 hydrogen of dihydroxyacetone phosphate with water. When the incubation mixture is quenched with trichloroacetic acid, an additional release of 0.6 equiv of inorganic phosphate/equiv of enzyme is observed with the muscle aldolase (Grazi & Trombetta, 1978) and 0.25 equiv with the rabbit liver aldolase (Grazi & Trombetta, 1979). To explain these phenomena, we have proposed a new enzyme–substrate intermediate: the enamine–aldehyde phosphate (acid labile) (the species in brackets in Scheme I), which is reversibly produced from the enamine phosphate intermediate (Scheme I).

By studying the formation of the aldolase–dihydroxyacetone phosphate complex at temperatures lower than −12 °C, we have found that, under these conditions, (a) only two out of the four dihydroxyacetone phosphate binding sites of aldolase are catalytically active, (b) the bound substrate does not exchange with that free in the medium even after 30 s of incubation, and (c) at pH* 8.3 the equilibrium between the pre-enamine and the enamine plus the post-enamine intermediates is largely shifted in favor of the latter.

Because of these peculiarities, it has been possible to obtain evidence that, at −13 °C and pH* 5.28, the rate of the formation of the enamine phosphate is larger than that of the acid-labile species (the enamine–aldehyde phosphate). We show also that at −24 °C and pH* 5.24, after addition of DL-glyceraldehyde 3-phosphate to the aldolase–dihydroxyacetone phosphate complex, more hexose bisphosphate is produced than acid-labile species is consumed, data evidently incompatible with the acid-labile species and the enamine phosphate being the same intermediate.

Scheme I



Materials and Methods

Fructose-bisphosphate aldolase from rabbit muscle was purchased from Boehringer, Mannheim, West Germany. The specific activity was 11 IU/mg of protein. (3*S*)-[3-^{3}H]Dihydroxyacetone phosphate was prepared according to Rose & Rieder (1955). Dihydroxy[^{14}C]acetone phosphate was prepared from commercial [^{14}C]fructose 1,6-bisphosphate (The Radiochemical Centre, Amersham, U.K.) by the procedure of Ginsburg & Mehler (1966). DL-Glyceraldehyde 3-phosphate–diethyl acetal dicyclohexylammonium salt was purchased from Boehringer, Mannheim, West Germany.

Fructose-bisphosphate aldolase activity was measured in the test system described by Racker (1947). The protein concentration was measured from the A_{280}, with the assumption that the absorbance of 1 mg of pure aldolase/mL (light path 1 cm) is 0.91 (Baranowski & Niederland, 1949). In the presence of ethylene glycol, the protein concentration was measured by the Coomassie Blue method (Bradford, 1976).

The subunit concentration was determined on the basis of a molecular weight of 159 000 for the tetrameric aldolase (Kawahara & Tanford, 1966). Orthophosphate was determined according to Tashima & Yoshimura (1975) on the deproteinized sample. Readings were taken at 650 nm after 20 min of color development. In the experiments where the reaction was quenched by the addition of 10 mM trichloroacetic acid, the quenching agent was subsequently increased to 0.1 M to allow protein precipitation.

To study the aldolase reaction at low temperature, the water–ethylene glycol mixture was selected. Ethylene glycol appears to have little effect on protein conformation and does not specifically interact with the chromophores of aldolase (Donovan, 1969). At room temperature it does affect neither the dissociation constant nor the stoichiometry of the ligand binding to aldolase (Crowden et al., 1973). At the concentrations employed in our experiments, it does not inhibit the aldolase reaction (data not shown).

pH* in the ethylene glycol solutions was measured by an Ingold LoT 401-TT electrode; standardization was performed as described by Larroque et al. (1976).

The rapid quenching experiments were performed with the Durrum multimixer apparatus. The effectiveness of the quenching of the reaction by either 10 mM or 0.1 M trichloroacetic acid was checked in control experiments where

† From the Istituto di Chimica Biologica, Università di Ferrara, Ferrara, Italy. *Received January 18, 1983.* This work was supported by grants from the Italian Consiglio Nazionale delle Ricerche and from the Ministero della Pubblica Instuzione.

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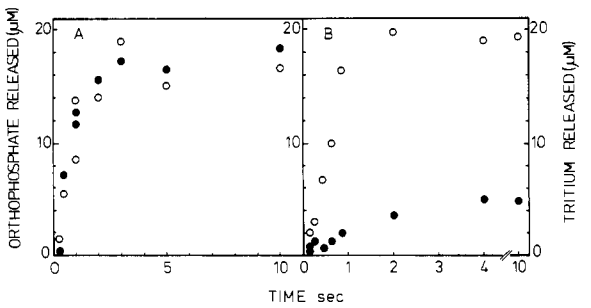

FIGURE 1: Comparison of rates of release of tritium from (3*S*)-[3-^{3}H]dihydroxyacetone phosphate and of formation of the acid-labile intermediate at pH* 8.33 and 5.28. (A) The incubation mixtures contained 30 μM aldolase subunit, 0.25 mM (3*S*)-[3-^{3}H]dihydroxyacetone phosphate (specific radioactivity 100 cpm/nmol), 30% (v/v) ethylene glycol, and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer. pH* was 8.33; temperature was −12 °C. (B) The incubation mixtures contained 50 μM aldolase subunit, 0.20 mM (3*S*)-[3-^{3}H]dihydroxyacetone phosphate (specific radioactivity 100 cpm/nmol), 30% (v/v) ethylene glycol, and 10 mM acetate buffer. pH* was 5.28; temperature was −12 °C. At various time intervals, the reaction was stopped by the addition of an equal volume of a solution containing 20 mM trichloroacetic acid and 30% (v/v) ethylene glycol. The release of tritium (○) and orthophosphate (●) was determined as was described under Materials and Methods.

the "enzyme syringe" contained buffered aldolase solution, the "substrate syringe" contained buffer alone, and the third syringe contained the quenching agent plus the substrate.

To measure the release of tritium from (3*S*)-[3-^{3}H]dihydroxyacetone phosphate, 0.1 mL of the incubation mixture was treated with 0.1 mL of 20 mM trichloroacetic acid solution containing either 30 or 40% (v/v) ethylene glycol, diluted with 0.2 mL of 50% ethanol poured on a column (0.5 g dry weight) of Dowex 1-X2 (acetate form, 200–400 mesh), and washed with 0.8 mL of water. Determination of tritiated water was made in the effluent and washings, total volume 1.2 mL.

To determine [^{14}C]hexose bisphosphate, formed by condensation of dihydroxy[^{14}C]acetone phosphate and DL-glyceraldehyde 3-phosphate, the deproteinized reaction mixtures (0.1 mL), containing 0.1 M trichloroacetic acid, after the addition as a carrier of 0.5 μmol of fructose bisphosphate, were treated with 0.1 mL of 2 M NaOH to degrade the triose phosphate into orthophosphate and lactic acid. After 20 min of incubation at 22 °C, the mixtures were neutralized with 0.1 mL of 1.9 M HCl and treated with 0.3 mL of 20 mM $ZnSO_4$, followed by 0.3 mM of 20 mM $Ba(OH)_2$. With the Zn^{2+}/Ba^{2+} treatment, fructose bisphosphate was precipitated, and the lactic acid produced by the degradation of triose phosphates remained in the supernatant solution. The precipitate containing the radioactive and the carrier fructose bisphosphate was suspended in 10 mL of Bray's (1960) solution and counted in a Packard liquid scintillation counter.

Results

Partition of Aldolase between the Pre-Enamine and the Enamine plus Post-Enamine Intermediates at −12 °C. The formation of the aldolase–dihydroxyacetone phosphate complex was studied at pH* 8.33 and −12 °C by mixing 30 μM aldolase subunit and 0.25 mM (3*S*)-[3-^{3}H]dihydroxyacetone phosphate (Figure 1A). After various times of incubation, the reaction mixtures were quenched with trichloroacetic acid and assayed for the presence of tritium ion and of inorganic orthophosphate. Both these species were found in quantities that were increasing at the same rate with the time of incubation (apparent first-order rate constant 0.92 s^{-1}). At equilibrium, 17.5–18 nanoatoms of tritium and 17.5–18 nmol of orthophosphate, corresponding to 58–60% of the total binding sites of the enzyme, were present in the quenched incubation mixtures.

Table I: Number of Binding Sites of Aldolase for Dihydroxyacetone Phosphate at pH* 8.3 and at Temperatures of either −13 or 20 °C[a]

temp (°C)	dihydroxy-[^{14}C]acetone phosphate (equilibrium concn) (μM)	dihydroxyacetone phosphate bound per aldolase tetramer
−13	0.6	2.3
−13	9.6	2.4
20	1.8	1.1
20	10.0	2.6
20	25.0	3.2

[a] The incubation mixtures (0.5 mL) contained 50 μM aldolase subunit, 80 μM dihydroxy[^{14}C]acetone phosphate (specific radioactivity 355 cpm/nmol), 40% (v/v) ethylene glycol, and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer. pH* was 8.3; temperature was either −13 or 20 °C. The mixtures were submitted to gel filtration on Sephadex G-50 columns (0.9 × 27 cm), operated by means of a Minipuls 2 Gilson peristaltic pump. The columns, equilibrated with the ethylene glycol-buffer solutions indicated above plus the radioactive substrate at the concentrations indicated in the table, were operated either at −13 or at 20 °C. After the injection of the sample in the column, the flow was stopped for 4 min to allow temperature equilibration. Fractions, collected at a flow rate of 0.65 mL/min, were analyzed for radioactivity and for protein content.

If one takes into account the mechanism of the aldolase reaction (eq 1), the limited release of tritium obtained by

$$E + AH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EAH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\begin{matrix}A^- \\ H^+\end{matrix} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EA^- + H^+ \quad (1)$$

reacting aldolase with the tritiated dihydroxyacetone phosphate can be explained by either one of the following mechanisms: (a) All four binding sites of aldolase are catalytically active, and $k_3 = 0$ and $k_2 = k_{-2}$. In this case, the tritium ion does not exchange with the medium, and since [EAH] = [H^+–E–A^-], the quenching of the reaction with trichloroacetic acid releases tritium only from half of the molecules of the substrate bound to the enzyme. (b) Only two out of the four binding sites of aldolase are catalytically active.

As we have previously shown (Grazi et al., 1983), mechanism b is the operating one. In the same work, it was also found that no exchange is detectable, even after 30 s of incubation at −12 °C, between the dihydroxyacetone phosphate bound to the enzyme and that free in the medium.

The binding of dihydroxyacetone phosphate to aldolase was also studied by filtration of the enzyme through Sephadex G-50 columns equilibrated with the radioactive substrate (Table I). It was found that at −13 °C and pH* 8.3 approximately 2 molecules of substrate was bound per aldolase tetramer both at the substrate concentration of 0.6 and 9.6 μM. In the control experiments performed at 20 °C, the number of molecules of the substrate bound to the aldolase tetramer increased from 1.12 to 3.2 when the substrate concentration was increased from 1.8 to 25 μM, becoming approximately 4 at infinite substrate concentration.

The results of the experiments illustrated in Figure 1A and in Table I support the conclusion that at −13 °C and pH* 8.3 the equilibrium of the reaction is largely displaced in favor of the enamine and post-enamine intermediates, the carbinolamine and the ketimine intermediates representing only a few percent of the total intermediates.

A quite different situation is prevailing at 22 °C, at least for the carboxypeptidase-treated aldolase, where, between pH 5.0 and 7.5, the enamine plus the post-enamine intermediates

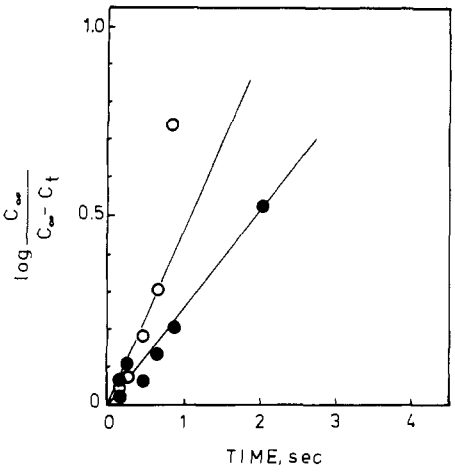

FIGURE 2: Semilogarithmic plot of tritium and orthophosphate release from the aldolase–dihydroxyacetone phosphate complex at pH* 5.28 and −12 °C. Data are taken from Figure 1B. Tritium release (○); orthophosphate release (●).

represent about 60% of the total intermediates (Grazi & Trombetta, 1980). The difference is even more pronounced at pH 8.3 where, according to Rose & Iyengar (1981), these latter intermediates represent only about 10% of the total intermediates.

Kinetic Resolution of the Enamine Phosphate from the Enamine–Aldehyde Phosphate Intermediate. While at pH* 8.33 and −12 °C the release of tritium and the release of orthophosphate proceed at the same rate, the two processes are resolved if the pH* is lowered to 5.28. Under these conditions, in the presence of 50 μM aldolase subunit and 0.2 mM (3*S*)-[3-^{3}H]dihydroxyacetone phosphate, it is found that the release of tritium from the enzyme–substrate complex occurs with an apparent first-order rate constant of 1.03 s^{-1} The release of the orthophosphate is slower and takes place with an apparent first-order rate constant of 0.57 s^{-1} (Figures 1B and 2). Furthermore, the quantities of tritium and of orthophosphate released are quite different, thus showing that the total enamine formed (20 nmol of tritium released in 10 s) and the orthophosphate-yielding species (5 nmol formed in 10 s) are two different intermediates.

The lower amount of orthophosphate released at pH* 5.28 than at pH* 8.3 cannot be due to the change in the partitioning of the intermediates back to substrate during the quenching because the quenching is done under the same conditions with trichloroacetic acid.

Trapping by DL-*Glyceraldehyde 3-Phosphate of the Enamine Phosphate Intermediate at pH* 5.24 and −24 °C.* The observations that below −12 °C the dihydroxyacetone phosphate bound to aldolase does not exchange with that free in the medium and that at low temperature and pH* 5.24 the enamine intermediate is resolved from the orthophosphate-yielding species prompted us to utilize the trapping by DL-glyceraldehyde 3-phosphate as a tool to further differentiate the enamine phosphate from the enamine–aldehyde intermediate.

It was found that by mixing at −24 °C and pH* 5.24 the aldolase–dihydroxyacetone phosphate complex with DL-glyceraldehyde 3-phosphate hexose bisphosphate was produced in larger amount than the acid-labile species was consumed (Figure 3). In control experiments it was shown that at −24 °C the release of tritium from the aldolase–(3*S*)-[3-^{3}H]dihydroxyacetone phosphate complex was complete in 3–4 min and no more release of tritium occurred in an additional 20 min. It was shown also that, in 20 min at −24 °C, no exchange

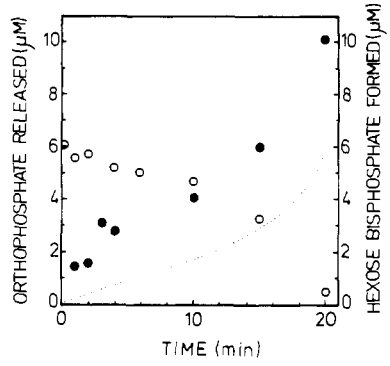

FIGURE 3: Stoichiometry of formation of hexose bisphosphate from the acid-labile intermediate and DL-glyceraldehyde 3-phosphate at pH* 5.24 and −24 °C. The incubation mixtures contained 70 μM aldolase subunit, 53 μM dihydroxy[^{14}C]acetone phosphate (specific radioactivity 355 cpm/nmol), 2 mM DL-glyceraldehyde 3-phosphate, 0.01 M Tris–0.01 M acetate buffer, and 40% (v/v) ethylene glycol. Temperature was −24 °C; pH* was 5.24. Aldolase and dihydroxy-[^{14}C]acetone phosphate were mixed first at 2 °C and immediately cooled to −24 °C. After 6 min of equilibration at −24 °C, to the 0.17 mL of the incubation mixtures was added 0.03 mL of a 13.3 mM DL-glyceraldehyde 3-phosphate–40% ethylene glycol solution. At various time intervals, the reaction was stopped by the addition of 0.2 mL of a 0.2 M trichloroacetic acid solution. The release of the inorganic phosphate (○) and the formation of the hexose bisphosphate (●) were determined as described under Materials and Methods. The dotted line represents the acid-labile species consumed.

of dihydroxy[^{14}C]acetone phosphate of the medium occurred with the unlabeled dihydroxyacetone phosphate bound to the enzyme. The exchange being studied as it was previously described (Grazi et al., 1983).

Discussion

In our previous papers (Grazi & Trombetta, 1978, 1979, 1980) we have described a new intermediate of the aldolase reaction: the enamine–aldehyde phosphate, which is reversibly formed from the enamine phosphate intermediate. This proposal has been questioned by Yiengar & Rose (1981). These authors have found that aldolase does not promote the exchange of ^{18}O from the C–O–P to the PO_3^{2-} position in dihydroxyacetone phosphate; they have therefore concluded that step c of Scheme I is irreversible and that the enamine phosphate is the acid-labile species. In our opinion, the lack of the exchange proves only the irreversibility of step d but not at all the irreversibility of step c of Scheme I.

The mechanism we have proposed does not necessarily require the "scrambling" of the phosphate group bound to the enzyme at the enamine–aldehyde intermediate stage. In starch phosphorylase, for example, positional exchange of the C–O–P bridge and nonbridge phosphoryl oxygens of glucose 1-phosphate is only seen in the presence of *malto*-triose as a primer but not during starch elongation (Kokesh & Kakuda, 1977). Our present data prove the following.

(1) Below −12 °C, only half of the sites of aldolase are catalytically active. Under these conditions and at pH* 8.3, the enamine plus the post-enamine intermediates prevail largely over the carbinolamine and the ketimine intermediates.

(2) At −13 °C and pH 5.28*, the acid-labile species (as measured from the yield on orthophosphate by quenching the reaction with trichloroacetic acid) is formed at a slower rate that the enamine phosphate intermediate (as measured from the release of tritium from the tritiated dihydroxyacetone phosphate). These data thus exclude that the acid-labile species and the enamine phosphate are the same intermediate. The total amount of tritium released in the course of the reaction represents all of the species present on the enzyme

that are losing their tritium by exchange through the formation of the enamine phosphate. Unfortunately, no data are at present available on the rate of this exchange and, thus, on the actual concentration of the enamine phosphate intermediate.

(3) At −24 °C and pH* 5.24, the amount of hexose bisphosphate formed by reaction of the aldolase–dihydroxyacetone phosphate complex with DL-glyceraldehyde 3-phosphate is larger than the amount of the acid-labile species consumed, thus showing that an additional species, besides the acid-labile one, is trapped by DL-glyceraldehyde 3-phosphate. This species cannot be represented by the molecules of the substrate free in the medium since no exchange was detected in the course of the experiment between dihydroxy[^{14}C]acetone phosphate of the medium and the unlabeled dihydroxyacetone phosphate bound to the enzyme. The additional trapping species are thus the other enzyme–substrate intermediates, including the enamine phosphate that is directly involved in the trapping.

Registry No. Fructose-1,6-bisphosphate aldolase, 9024-52-6; dihydroxyacetone phosphate, 57-04-5.

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Applicability of the Induced-Fit Model to Glyceraldehyde-3-phosphate Dehydrogenase from Sturgeon Muscle. Study of the Binding of Oxidized Nicotinamide Adenine Dinucleotide and Nicotinamide 8-Bromoadenine Dinucleotide†

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ABSTRACT: A new method of calculation, based on a direct fitting of the protein fluorescence intensity observed upon coenzyme binding (H.-P. Lutz, unpublished results), is used to study the negative cooperative behavior of glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle. The calculation procedure simultaneously elaborates data obtained for four different protein concentrations, and it is able to compare different models by computing the minimal and critical sum of squares. Using this approach, it is shown that the induced-fit model [Koshland, D. E., Jr., Nemethy, G., & Filmer, D. (1966) *Biochemistry 5*, 365] and the dimer of dimer model [Malhotra, O. P., & Bernhard, S. A. (1968) *J. Biol. Chem. 243*, 1243–1252] can both be applied for explaining the negative cooperativity observed upon coenzyme binding to sturgeon glyceraldehyde-3-phosphate dehydrogenase. In addition to the progressive modification of the binding affinity during ligand binding, different maximal fluorescence quenchings for the binding steps must be postulated; and furthermore, the binding capability decreases by decreasing the protein concentration. The fact that the induced-fit model can also be applied is rather in contradiction with the view generally accepted of a dimer of dimer structure of sturgeon glyceraldehyde-3-phosphate dehydrogenase. By use of the same approach, nicotinamide 8-bromoadenine dinucleotide is shown to bind to glyceraldehyde-3-phosphate dehydrogenase from sturgeon in a negative cooperative manner.

Monod et al. (1965) have proposed a model that explains the positive cooperativity of ligand binding to oligomeric proteins. On the other hand, Koshland et al. (1966) have introduced the "induced-fit" model, which explains both positive and negative cooperativity. Both models assume complete symmetry of the nonligated and fully ligated states but differ in so far as the intermediate states are not symmetric in the induced-fit model. Negative cooperativity in ligand binding has been also interpretated by Malhotra & Bernhard (1968) and MacQuarrie & Bernhard (1971) on the basis of preexisting asymmetry in the protein oligomer. In such a case, it is assumed that dimers or tetramers are never symmetric

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0006-2960/83/0422-4437$01.50/0