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Fructose 1,6-Bisphosphate Aldolase from Rabbit Muscle

THE ISOMERIZATION OF THE ENZYME–DIHYDROXYACETONE PHOSPHATE COMPLEX

By ENRICO GRAZI and MAURIZIO BLANZIERI
Istituto di Chimica Biologica, Università degli Studi di Ferrara, Via Luigi Borsari 46, 44100 *Ferrara, Italy*

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The formation and dissociation of the aldolase–dihydroxyacetone phosphate complex were studied by following changes in A_{240} [Topper, Mehler & Bloom (1957) *Science* **126**, 1287–1289]. It was shown that the enzyme–substrate complex (ES) slowly isomerizes according to the following reaction:

$$E+S \text{ (keto form)} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} ES^*$$

the two first-order rate constants for the isomerization step being $k_{+2} = 1.3\,s^{-1}$ and $k_{-2} = 0.7\,s^{-1}$ at 20°C and pH 7.5. The dissociation of the ES complex was provoked by the addition of the competitive inhibitor hexitol 1,6-bisphosphate. At 20°C and pH 7.5, k_{+1} was $4.7\times10^6\,M^{-1}\cdot s^{-1}$ and k_{-1} was $30\,s^{-1}$. Both the ES and the ES* complexes react rapidly with 1.7 mM-glyceraldehyde 3-phosphate, the reaction being practically complete in 40 ms. This shows that the ES* complex is not a dead-end complex. Evidence was also provided that aldolase binds and utilizes only the keto form of dihydroxyacetone phosphate.

The formation of the aldolase–dihydroxyacetone phosphate complex is accompanied by an increase in A_{240} (Topper *et al.*, 1957; Mehler & Bloom, 1963). This signal was exploited to follow the formation as well as the dissociation of the enzyme–substrate complex. It was found that aldolase and dihydroxyacetone phosphate react to form an initial enzyme–dihydroxyacetone phosphate complex that slowly isomerizes to a second complex. The latter is not a dead-end complex, since it, like the first complex, reacts rapidly with glyceraldehyde 3-phosphate to form fructose 1,6-bisphosphate.

We also reconsider here the substrate specificity of the aldolase reaction and conclude that the enzyme binds and utilizes only the keto form of dihydroxyacetone phosphate.

Experimental

Materials

Fructose bisphosphate aldolase (EC 4.1.2.13) was prepared from rabbit muscle by the procedure of Taylor *et al.* (1948) and was recrystallized five times. The specific activity of the preparation used in these experiments was 16 units/mg of protein, where 1 unit is defined as the amount of enzyme that catalyses the cleavage of 1 μmol of fructose 1,6-bisphosphate/min under standard assay conditions. Dihydroxyacetone phosphate was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glycerol phosphate dehydrogenase was purchased from Boehringer, Mannheim, W. Germany. Hexitol 1,6-bisphosphate was prepared from fructose bisphosphate by the procedure of Ginsburg & Mehler (1966).

Dihydroxy[U-^{14}C]acetone phosphate was prepared and purified from commercial [U-^{14}C]fructose 1,6-bisphosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) as described by Ginsburg & Mehler (1966). The calcium salt of D-glyceraldehyde 3-phosphate was prepared by the procedure of Szewczuk *et al.* (1961) and converted into the sodium salt by treatment with Dowex 50 (H^+ form) followed by neutralization with NaOH.

Methods

Fructose bisphosphate aldolase activity was measured in the system described by Racker (1947).

Protein concentration was measured from the A_{280} by assuming that the absorbance of 1 mg of pure enzyme/ml (light-path 1 cm) is 0.91 (Baranowski & Niederland, 1949).

Stopped-flow measurements were performed with a Durrum D-110 rapid-mixing spectrophotometer. The dead time of the instrument was found to be 3 ms. The light-path of the instrument was 2 cm.

Traces were usually recorded at 0.1–0.2 s/division to measure the amplitude of the rapid phase and final rate, and at 20 ms/division to measure initial rate.

Reduction of $Fe(CN)_6^{3-}$ was followed at 420 nm;

the molar absorption coefficient was 1000 litre·mol^{-1}·cm^{-1}. The condensation of glyceraldehyde 3-phosphate with the aldolase–dihydroxyacetone phosphate complex was studied in the Durrum multi-mixer apparatus by mixing a solution containing 45.2μM-aldolase subunit and 32.5μM-dihydroxy[U-^{14}C]-acetone phosphate (specific radioactivity 770c.p.m./nmol) in 20mM-Tris/HCl buffer with an equal volume of a solution containing 3.4mM-glyceraldehyde 3-phosphate in 20mM-Tris/HCl buffer, pH7.5; the temperature was 20°C. At various time intervals the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 0.1M.

To 0.1ml of the quenched reaction mixture 0.25 μmol each of carrier dihydroxyacetone phosphate and fructose 1,6-bisphosphate were added followed by 0.02ml of 1.5M-NaOH. After 40min at 20°C, 0.12ml of 0.15M-HCl was added, followed by 0.3ml of 17mM-$ZnSO_4$ and 0.3ml of 17mM-$Ba(OH)_2$. The precipitate, containing newly formed radioactive fructose bisphosphate, was removed by centrifugation for 3min at 5000g; the supernatant solution, containing the product of the alkaline degradation of radioactive dihydroxyacetone phosphate, was assayed for radioactivity in a Packard Tri-Carb liquid-scintillation counter with 10ml of Bray's (1960) solution.

Results and Discussion

Evidence that aldolase binds and utilizes only the keto form of dihydroxyacetone phosphate

For aldolase, a conclusive demonstration that the keto form of dihydroxyacetone phosphate is the only active form is still lacking. The experiments of Reynolds *et al.* (1971) proved only that aldolase could bind the keto form of dihydroxyacetone phosphate, and the evidence from the experiments of Schray *et al.* (1975) was very indirect. Our previous proposal (Grazi, 1974*b*) that aldolase utilizes both the hydrated and the keto forms of dihydroxyacetone phosphate has also to be retracted. It was based essentially on the report that, at 4°C, only 17% of the dihydroxyacetone phosphate is in the keto form (Reynolds *et al.*, 1971), a report that we subsequently have not been able to confirm (E. Grazi, unpublished work). In fact, even at 4°C, the relative concentration of the keto form of dihydroxyacetone phosphate is never lower than 45%. The active site of aldolase can be titrated in the presence of excess dihydroxyacetone phosphate and ferricyanide (Scheme 1). In this system, (*c*) is the rate-limiting step, and the amplitude of the rapid phase (*b*) is proportional to the concentration of the active sites of the enzyme occupied by the substrate (Grazi, 1974*a*). This same technique can be used to investigate the nature of the active form of dihydroxyacetone phosphate, since the rate of step (*b*), in the presence of 0.6mM-ferricyanide, is much higher than the rate of the spontaneous conversion of the hydrated into the keto form of the substrate.

Increasing quantities of dihydroxyacetone phosphate were added to the enzyme (Fig. 1), at 20°C, where the concentrations of the keto and of the hydrated forms of dihydroxyacetone phosphate are approximately equal and the aldolase should be fully

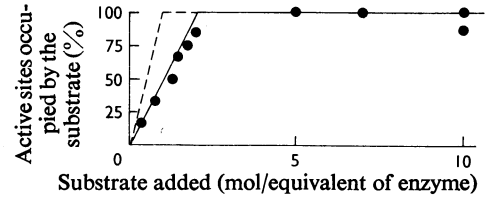
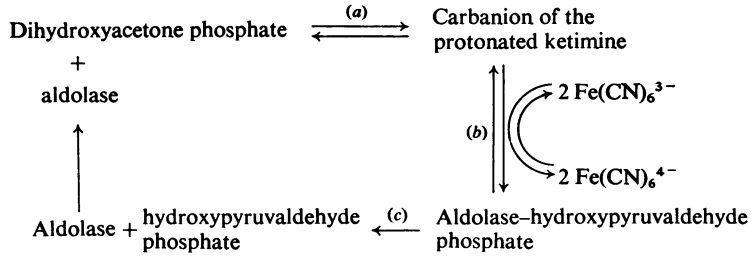


Fig. 1. *Titration of aldolase with dihydroxyacetone phosphate*

Aldolase subunit (41.2μM) dissolved in 20mM-Tris/HCl buffer, pH7.5, was mixed with an equal volume of the same buffer containing 1mM-ferricyanide and dihydroxyacetone phosphate as indicated in the Figure. Reduction of ferricyanide was followed at 420nm. Temperature was 20°C. The number of the active sites occupied by the substrate was calculated from the amplitude of the rapid phase of the reduction of ferricyanide, on the basis of a molar absorption coefficient of 1000 litre·mol^{-1}·cm^{-1} and of the uptake of 2mol of ferricyanide per active site.



Scheme 1. *Oxidation by ferricyanide of the aldolase–dihydroxyacetone phosphate complex*

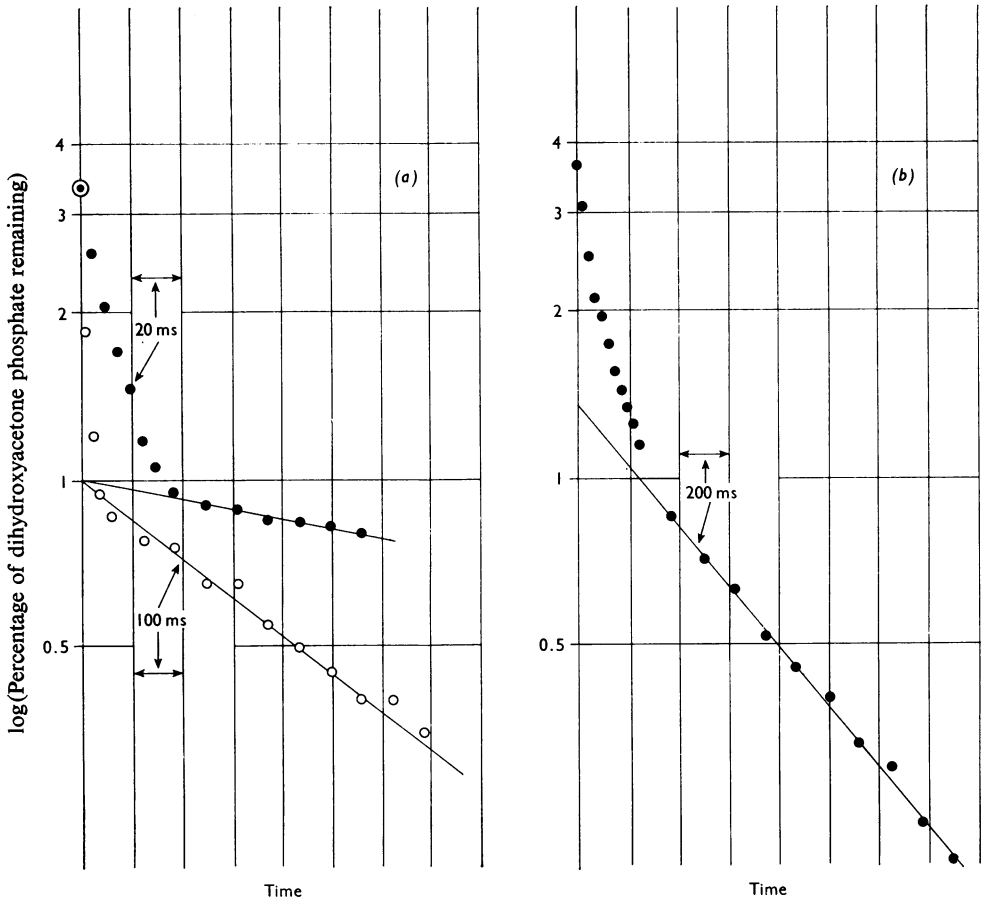


Fig. 2. *Keto form of dihydroxyacetone phosphate as a substrate for aldolase*

(*a*) Reduction of dihydroxyacetone phosphate by NADH and glycerol phosphate dehydrogenase. Dihydroxyacetone phosphate (27.5 μM) dissolved in 50 mM-triethanolamine/HCl buffer, pH 7.5, was mixed with an equal volume of the same buffer containing 0.2 mM-NADH and glycerol phosphate dehydrogenase (1 mg/ml). NADH oxidation was followed at 340 nm. Temperature was 37°C. (*b*) Oxidation by ferricyanide of the aldolase–dihydroxyacetone phosphate complex. Aldolase subunit (149.6 μM) dissolved in 50 mM-Tris/HCl buffer, pH 7.5, was mixed with an equal volume of the same buffer containing 34.6 μM-dihydroxyacetone phosphate and 1.32 mM-ferricyanide. Reduction of ferricyanide was followed at 420 nm. Temperature was 37°C.

titrated by the addition of: either (1) 1 molecule of substrate per active site of the enzyme, if both the forms are substrates for aldolase (Fig. 1, broken line); or (2) 2 molecules of substrate per active site of enzyme, if only one form of the sugar is a substrate for aldolase (Fig. 1, continuous line). Full titration of the enzyme was obtained by the addition of 2 molecules of substrate per active site (Fig. 1), as expected for case (2). Thus only one of the two forms of the substrate is bound and utilized. The proof that this form is the keto form was obtained by showing that at 37°C, in the ferricyanide system, 63% of the added substrate was oxidized rapidly (Fig. 2*b*), the relative concentration of the keto form being 70%, as determined by following the reduction of dihydroxyacetone phosphate by NADH in the presence of glycerol phosphate dehydrogenase (Fig. 2*a*). Thus aldolase binds and utilizes only the keto form of dihydroxyacetone phosphate.

Change in A_{240} after formation of the aldolase–dihydroxyacetone phosphate complex

Addition, at 20°C, of 0.6 mM-dihydroxyacetone phosphate to 37.6 μM-aldolase subunit, dissolved in 20 mM-Tris/HCl buffer, pH 7.5, increased A_{240} (light-

Table 1. *Slow change in the A_{240} on mixing of aldolase and dihydroxyacetone phosphate*

Aldolase subunit (26.8 μM), dissolved in 20mM-Tris/HCl buffer, pH7.5, was mixed with an equal volume of the same buffer containing dihydroxyacetone phosphate (30, 60, 150 and 170 μM). Final concentration of the enzyme subunit was 13.4 μM and of the substrate as in the Table. Temperature was 20°C. The increase in A_{240} was followed (light-path 2cm). In control experiments no change in the A_{240} was obtained by mixing aldolase with the buffer in the absence of dihydroxyacetone phosphate.

Expt.	Dihydroxyacetone phosphate (μM)	Rate constant (s^{-1})	ΔA_{240} observed
1	15	1.00	0.009
2	30	1.10	0.012
3	75	1.56	0.011
4	75	2.14	0.009
5	350	1.90	0.010

path 1cm) by 0.045, corresponding to a $\Delta\varepsilon_{240}$ of 1200 litre·mol^{-1}·cm^{-1}. In the rapid-mixing spectrophotometer the reaction took place in two phases. The first phase, which accounted for about two-thirds of the total A_{240} change, occurred in the dead time of the instrument. The second phase [A_{240} (light-path 2cm) = 0.009–0.012] took place as a first-order reaction with an apparent rate constant that increased with substrate concentration from 1 to 2 s^{-1} (Table 1). Since only the carbonyl form of dihydroxyacetone phosphate reacts with the enzyme, the slow phase could have been due to the slow re-equilibration of the hydrated with the keto form of dihydroxyacetone phosphate (rate constant of the spontaneous dehydration reaction 0.4 s^{-1}; Reynolds *et al.*, 1971). This possibility was excluded by increasing the concentration of the substrate well above the dissociation constant of the aldolase–dihydroxyacetone phosphate complex ($K_{diss.}$ = 4 μM at pH7.5, 20°C, *I* 0.02; Grazi & Trombetta, 1974), when the slow phase of the reaction was still observed (Table 1). The slow phase thus appears to be related to either (*a*) a slow isomerization step between two forms of the enzyme–substrate complex:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ES^*$$

or (*b*) a slow isomerization step between two forms of the free enzyme, only one form being able to bind the substrate:

$$E \rightleftharpoons E^* + S$$
$$\downarrow\uparrow$$
$$E^*S$$

Table 2. *Hexitol bisphosphate-induced dissociation of the aldolase–dihydroxyacetone phosphate complex*

Aldolase subunit (26.4 μM) and dihydroxyacetone phosphate (20 μM), dissolved in 20mM-Tris/HCl buffer, pH7.5, were mixed with an equal volume of the same buffer containing hexitol bisphosphate (0.35, 0.70 and 3.5mM). Temperature was 20°C. The reaction was followed at 240nm.

Expt.	Hexitol bisphosphate (mM)	Rate constants (s^{-1}) Slow phase	Rate constants (s^{-1}) Rapid phase	Amplitude (ΔA_{240}) Slow phase	Amplitude (ΔA_{240}) Rapid phase
1	0.175	0.4	30	0.0080	0.0130
2	0.175	0.4	30	0.0080	0.0130
3	0.35	0.46	23	0.0093	0.0093
4	0.35	0.46	23	0.0093	0.0093
5	1.75	0.48	29	0.0078	0.0115

Displacement of dihydroxyacetone phosphate from the aldolase–substrate complex by the addition of hexitol bisphosphate

The choice between the above two models was made by studying the dissociation of the aldolase–dihydroxyacetone phosphate complex, promoted by the addition of hexitol 1,6-bisphosphate, a competitive inhibitor of the enzyme ($K_{diss.}$ = 1.2 μM; Ginsburg & Mehler, 1966). The addition of this compound alone did not change the A_{240} of aldolase, provided that the experiment was performed at pH7.5 (at 240nm, light-path 1cm, the absorbance of 18.0 μM-aldolase subunit dissolved in 20mM-Tris/HCl buffer was increased by 0.008 at pH6.0 and decreased by 0.0076 at pH9.0 by the addition of 1.1 mM-hexitol bisphosphate).

When hexitol bisphosphate was mixed with the aldolase–dihydroxyacetone phosphate complex (Table 2), the decrease in A_{240} was independent of the concentration of hexitol bisphosphate and took place in two phases: a rapid one (rate constant 30 s^{-1}) and a slow one (rate constant 0.5 s^{-1}). The average amplitude of the two phases was 0.0112±0.0018 for the rapid phase and 0.0085±0.00075 for the slow one (means±S.E.M. for five experiments). This result is compatible with alternative (*a*) above, which predicts a rapid phase (the dissociation of ES to E and S) and a slow phase (the conversion of ES* into ES). It excludes alternative (*b*), which predicts, provided that the concentration of hexitol bisphosphate is sufficiently large (Expt. 5 of Table 2): (1) only a slow phase if hexitol bisphosphate binds only to E; (2) only a rapid phase if hexitol bisphosphate binds only to E* or to both E and E*.

We conclude that aldolase and dihydroxyacetone phosphate react rapidly to form the ES complex, which slowly isomerizes to the ES* complex.

The signal corresponding to the disappearance of ES (rapid phase, Table 2) can be interpreted in terms of the rate constant k_{-1} ($30\,s^{-1}$), since $k_{-1} \ll k_{+3}$ [X] and, very likely, $k_{+3}[X] > k_{+1}[S]$, where k_{+3} is the rate constant for the reaction of E with hexitol bisphosphate.

Equilibrium of the isomerization step

The release of dihydroxyacetone phosphate from the complex with the enzyme was also studied by following NADH oxidation in the presence of glycerol phosphate dehydrogenase. Reduction of dihydroxyacetone phosphate took place in two phases; the rate constant for the rapid phase was $15\,s^{-1}$ when aldolase was absent (Expt. 1, Table 3), and $5\,s^{-1}$ when excess aldolase was present (Expts. 2–5, Table 3). The ratio between the rapid and the slow phase changed in the two conditions, as expected, because of the formation of the aldolase–dihydroxyacetone phosphate complex. Thus, under the conditions of the experiments of Table 3, the equilibrium constant:

$$K_{diss.} = \frac{[\text{dihydroxyacetone phosphate (hydrated+keto)}]\,[\text{aldolase}]}{[\text{aldolase–dihydroxyacetone phosphate}]}$$

has a value of $4\,\mu M$ (Grazi & Trombetta, 1974).

Before mixing, the concentration of aldolase subunit was $41.2\,\mu M$ and dihydroxyacetone phosphate $27.5\,\mu M$. The substrate bound to the enzyme was thus $22.6\,\mu M$ (73%) and free substrate was $4.9\,\mu M$ [$2.7\,\mu M$ (10%) in the keto form and $2.2\,\mu M$ (7%) in the hydrated form]. Since the keto form of the substrate is rapidly reduced by the dehydrogenase whereas the hydrated form is reduced only after dehydration (Reynolds *et al.*, 1971), the amount of the keto form (10%) was subtracted from the rapid phase (39−10% = 29%) and the amount of the hydrated form (7%) was subtracted from the slow phase (61−7 = 54%). Thus 29% of the substrate was bound to the enzyme in a rapidly dissociable form and 54% was bound in a slowly dissociable form. The ratio of the two forms yields the ratio of the constants. Thus:

$$\frac{k_{+2}}{k_{-2}} = \frac{ES^*}{ES} = 1.86$$

From this ratio and from the value of $k_{+2}+k_{-2} = 2\,s^{-1}$ (Expts. 4 and 5, Table 1), the two first-order rate constants for the interconversion ES $\rightleftharpoons$ ES* were calculated to be: $k_{+2} = 1.3\,s^{-1}$ and $k_{-2} = 0.7\,s^{-1}$. The rate constant k_{+1} for the reaction of the keto form of dihydroxyacetone phosphate with aldolase was calculated from k_{-1} and from $K_{diss.}$ to be $4.7+10^6\,M^{-1}\cdot s^{-1}$ at 20°C and pH 7.5.

The rate constant of the slow phase ($0.5\,s^{-1}$) when dihydroxyacetone phosphate was displaced by the addition of hexitol bisphosphate (Table 2) agreed well with the value of k_{-2} calculated from reduction with the dehydrogenase (Table 3) as expected if the same phenomenon was studied in the two experiments.

The rate constant of the rapid phase was $5\,s^{-1}$ and $8.8\,s^{-1}$ respectively in the presence of 0.5 and 1 mg of glycerol phosphate dehydrogenase/ml, compared with $30\,s^{-1}$ for the displacement by hexitol bisphosphate, and reflected the high K_m of glycerol phosphate dehydrogenase and the low concentration of free dihydroxyacetone phosphate.

Condensation of glyceraldehyde 3-phosphate with the aldolase–dihydroxyacetone phosphate complex

When glyceraldehyde 3-phosphate was allowed to react with aldolase and dihydroxyacetone phosphate, the reaction of formation of fructose 1,6-bisphosphate took place in two phases (Fig. 3). In the first, rapid, phase (30–40ms), 76% of the dihydroxyacetone phosphate was taken up, a quantity that represented almost the whole of the dihydroxyacetone phosphate bound to the enzyme (i.e. 81% of the total dihydroxyacetone phosphate present, as calculated from the dissociation constant of $4\,\mu M$ for the aldolase–dihydroxyacetone phosphate complex). From the result of this experiment and from the consideration that the rate of production of fructose bisphosphate, in

Table 3. *Reduction of dihydroxyacetone phosphate released from the aldolase–dihydroxyacetone phosphate complex*

Aldolase subunit ($41.2\,\mu M$) and dihydroxyacetone phosphate ($27.5\,\mu M$), dissolved in 20mM-Tris/HCl buffer, pH 7.5, were mixed with an equal volume of the same buffer containing NADH (0.2mM) and glycerol phosphate dehydrogenase (1 mg/ml, Expts. 1–5; 2 mg/ml, Expts. 6 and 7). No aldolase was added in Expt. 1. Temperature was 20°C. NADH oxidation was followed at 340nm.

	Rate constants (s^{-1})		Amplitude (%)	
Expt.	Slow phase	Rapid phase	Slow phase	Rapid phase
1	0.5	15.3	47	53
2	0.5	5.1	64	36
3	0.5	4.5	61	39
4	0.6	5.0	58	42
5	0.5	5.2	62	38
6	0.48	8.8	55	45
7	0.48	8.7	55	45

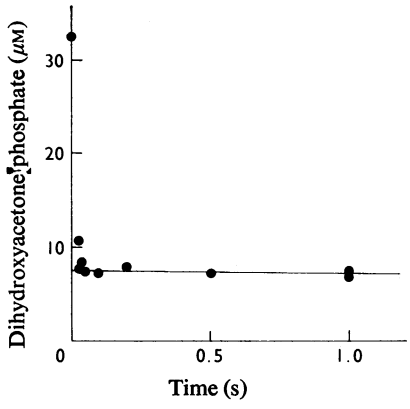


Fig. 3. *Condensation of glyceraldehyde 3-phosphate with the aldolase–dihydroxyacetone phosphate complex*

Glyceraldehyde 3-phosphate (3.4mM) dissolved in 20mM-Tris/HCl buffer, pH7.5, was mixed with an equal volume of a solution containing 45.2μM-aldolase subunit, 32.5μM-dihydroxy[U-^{14}C]acetone phosphate (specific radioactivity 770c.p.m./nmol) and 20mM-Tris/HCl, pH7.5. Temperature was 20°C. At various time intervals the reaction was stopped with trichloroacetic acid (final concentration 0.1M). Dihydroxyacetone phosphate was determined after alkaline hydrolysis, as described under 'Methods'.

the rapid phase, was much higher than the rate of the conversion of ES* into ES, it was concluded that both ES* and ES react rapidly with the aldehyde, and therefore that ES* is not a dead-end intermediate.

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