Fructose 1,6-Diphosphate Aldolase from Rabbit Muscle

EFFECT OF pH ON THE RATE OF FORMATION AND ON THE EQUILIBRIUM CONCENTRATION OF THE CARBANION INTERMEDIATE

By ENRICO GRAZI

Istituto di Chimica Biologica, Università di Ferrara, Ferrara, Italy

(Received 8 April 1975)

The rate of oxidation by ferricyanide of the aldolase–dihydroxyacetone phosphate complex was measured under different conditions. The following conclusions are drawn. 1. In the cleavage of fructose diphosphate, catalysed by native aldolase, the steady-state concentration of the enzyme–dihydroxyacetone phosphate carbanion intermediate represents less than 6% of the total enzyme–substrate intermediates. 2. Fructose diphosphate and dihydroxyacetone phosphate compete for the four catalytic sites on aldolase, the binding of fructose diphosphate being about twice as tight. 3. The equilibrium concentration of the carbanion intermediate formed by reaction of carboxypeptidase-treated aldolase with dihydroxyacetone phosphate is independent of pH between 5.0 and 9.0. The rates of formation of the carbanion intermediate and of the reverse reaction are, however, concomitantly increased by increasing pH between 5.0 and 6.5.

The study of the transient kinetics of an enzymic reaction is largely facilitated by the availability of a specific signal for the different enzyme–substrate intermediates. Healy & Christen (1973) have shown that $\text{Fe}(\text{CN})_6^{3-}$ is reduced when added to a solution containing fructose diphosphate aldolase and dihydroxyacetone phosphate. In this reaction the oxidized species is the enzyme–dihydroxyacetone phosphate carbanion intermediate (Healy & Christen, 1973; Christen & Riordan, 1968), which yields 1 mol of hydroxypyruvaldehyde phosphate with the consumption of 2 mol of $\text{Fe}(\text{CN})_6^{3-}$, and the enzyme retains its catalytic activity.

The study of the transient kinetics of this reaction has proved very useful. It has allowed the determination of the number of catalytic sites on native aldolase, the determination of the concentration, at equilibrium, of the carbanion intermediate in the reaction catalysed by carboxypeptidase-treated aldolase (Grazi, 1974a) and the study of the nature of the active forms of the substrates of aldolase (Grazi, 1974b).

The present paper reports the results of studies on the effect of H^+ concentration on the steady state and on the equilibrium concentrations of the enzyme–dihydroxyacetone phosphate carbanion intermediate formed in the reactions catalysed by native and carboxypeptidase-treated aldolases.

Experimental

Materials

Fructose diphosphate aldolase 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 a unit is defined as the amount of enzyme that catalyses the cleavage of 1 μmol of fructose 1,6-diphosphate per min under standard assay conditions.

Dihydroxyacetone phosphate and carboxypeptidase A (treated with di-isopropyl phosphorofluoridate) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Dihydroxy[^{14}C]acetone phosphate was prepared from commercial [$\text{U}-^{14}\text{C}$]fructose 1,6-diphosphate by the procedure of Horecker *et al.* (1963) as modified by Ginsburg & Mehler (1966). $\text{K}_3\text{Fe}(\text{CN})_6$ was obtained from Merck, Darmstadt, W. Germany.

Methods

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

Sephadex G-50 filtration was performed at 22°C on columns (1.2 cm $\times$ 37 cm) equilibrated with dihydroxy[^{14}C]acetone phosphate and 50 mM-Tris-HCl buffer, pH 6.0. Ionic strength was adjusted to 0.05 by the addition of NaCl. The flow rate was 4 ml/min and fractions (1 ml) were collected.

Radioactivity determinations were made in a Packard Tri-Carb liquid-scintillation counter in 10 ml of Bray's (1960) solution. Corrections for quenching were introduced after the effect of protein on counting efficiency was tested directly by the addition of known scalar amounts of aldolase to a sample of known radioactivity. Protein concentration was measured from the E_{280} by assuming that the absorbance of 1 mg of pure enzyme/ml (light-path 1 cm) is 0.91 (Baranowski & Niederland, 1949). The number of molecules of substrate bound to the enzyme was estimated from the specific radioactivity of the radio-

active substrate by assuming a molecular weight for the enzyme of 159000 (Kawahara & Tanford, 1966).

Digestion of aldolase with carboxypeptidase A was performed as described by Rutter *et al.* (1961) except that the aldolase/carboxypeptidase ratio was 70. After digestion most of the carboxypeptidase was removed as described by Spolter *et al.* (1965). The specific activity of carboxypeptidase-treated aldolase was 0.27 unit/mg of protein.

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. Measurements were made at 420 nm, the absorption maximum of $\text{Fe}(\text{CN})_6^{3-}$. The molar extinction coefficient was 1000 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. The light-path of the instrument was 2 cm, which was taken into account in the calculations. All the concentrations of the reagents refer to concentrations after mixing in the stopped-flow mixing chamber. The amplitude of the rapid phase was estimated as the difference between the starting point of the reaction and the point obtained, on the ordinate axis, by linear extrapolation of the slow phase of the reaction to zero time.

The initial concentration of the carbanion intermediate was calculated from the formula $A[\gamma/(\gamma-1)]$ where A is the amplitude of the rapid phase and γ is the ratio of the initial to the final rate. Traces were usually recorded at 0.1 s/box to measure the amplitude of the rapid phase and final rate, and at 20 ms/box to measure initial rate. Initial slopes were fitted by eye.

Results

Transient kinetics of $\text{Fe}(\text{CN})_6^{3-}$ reduction by the native aldolase-dihydroxyacetone phosphate complex

$\text{Fe}(\text{CN})_6^{3-}$ is rapidly reduced when added to a solution containing aldolase and dihydroxyacetone phosphate (Fig. 1). Reduction is practically undetectable in the presence of aldolase alone and does not take place at all with dihydroxyacetone phosphate

alone. In the complete system, between pH 5.0 and 9.0, the reaction occurs through a rapid and a slow phase independently of the order of mixing of the reagents. The kinetics of the rapid phase was studied as a function of $\text{Fe}(\text{CN})_6^{3-}$ concentration and, for simplicity, at quasi saturation of the enzyme with substrate (Grazi & Trombetta, 1974), so that the concentration of the enzyme-substrate complex could be equated with good approximation to the concentration of the total enzyme. Under these conditions, between pH 5.5 and 9.0, the rapid phase follows the kinetics of a second-order reaction, being pseudo-first-order with respect to both the enzyme-substrate complex and to $\text{Fe}(\text{CN})_6^{3-}$ (Table 1). At pH 5.0 the reaction was independent of $\text{Fe}(\text{CN})_6^{3-}$ concentration. This behaviour, which probably depends on a

Fig. 1. Transient kinetics of $\text{Fe}(\text{CN})_6^{3-}$ reduction by the aldolase-dihydroxyacetone phosphate complex

Enzyme and substrate were pre-mixed at pH 6.0 in Tris-HCl buffer 0.05 M; I 0.05. Aldolase ($2.3 \mu\text{M}$), dihydroxyacetone phosphate (0.28 mM), $\text{Fe}(\text{CN})_6^{3-}$ (0.51 mM). Temperature 24°C.

Table 1. Rate constants of the rapid phase and rates of the slow phase of the oxidation by $\text{Fe}(\text{CN})_6^{3-}$ of the enzyme-dihydroxyacetone phosphate complex

Enzyme and substrate were pre-mixed. Native aldolase (specific activity 16 units/mg of protein) was $4.7 \mu\text{M}$, and dihydroxyacetone phosphate 0.5 mM. The system was buffered either with 0.05 M-Tris-0.05 M-sodium acetate at pH 5.5 or 0.05 M-sodium acetate at pH 5.0 or 0.05 M-Tris-HCl at pH 7.0 and 9.0. Ionic strength was 0.05 mol/litre. Temperature was 26°C. The rate of reduction of $\text{Fe}(\text{CN})_6^{3-}$ is referred to per mol of aldolase.

			Pseudo-first-order rate constants of rapid phase (s $^{-1}$)				Second-order rate constants of rapid phase (mM $^{-1}$ $\cdot$ s $^{-1}$)				Rate of $\text{Fe}(\text{CN})_6^{3-}$ reduction in the slow phase (M $\cdot$ s $^{-1}$)			
$[\text{Fe}(\text{CN})_6^{3-}]$ (mM)	pH	...	5.0	5.5	7.0	9.0	5.0	5.5	7.0	9.0	5.0	5.5	7.0	9.0
0.45			13.2	—	9.1	4.3	—	—	20.3	9.5	3.9	—	3.3	3.7
0.26			13.2	10.4	5.5	2.5	—	40.0	21.0	9.6	3.7	3.0	2.7	3.4
0.14			11.3	6.3	3.2	1.5	—	45.0	23.0	10.7	3.5	3.3	1.7	2.1
0.08			—	4.0	—	—	—	50.0	—	—	—	3.0	—	—

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

Table 2. Rate constants of the rapid phase and rates of the slow phase of the oxidation by Fe(CN)_6^{3-} of the carboxypeptidase-aldolase-dihydroxyacetone phosphate complex

Enzyme and substrate were pre-mixed. Carboxypeptidase-treated aldolase (specific activity 1.0 unit/mg of protein; $6.0 \mu\text{M}$) and dihydroxyacetone phosphate (0.5 mM). The system was buffered with 0.05 M -sodium acetate buffer at pH 5.0, and 0.05 M -Tris-HCl buffer at pH 7.0 and pH 9.0; 0.05 . Temperature was 26°C . The rate of reduction of Fe(CN)_6^{3-} is referred to per mol of aldolase.

$[\text{Fe(CN)}_6^{3-}]$ (mM)	pH	...	Pseudo-first-order rate constants of rapid phase (s^{-1})			Second-order rate constants of rapid phase ($\text{mM}^{-1} \cdot \text{s}^{-1}$)			Rates of Fe(CN)_6^{3-} reduction in the slow phase ($\text{M} \cdot \text{s}^{-1}$)		
			5.0	7.0	9.0	5.0	7.0	9.0	5.0	7.0	9.0
0.4			39.5	13.5	10.3	98.7	33.7	25.7	0.8	4.2	3.5
0.24			—	7.0	5.7	—	29.2	23.8	—	4.1	2.8
0.2			26.4	—	—	132.0	—	—	0.7	—	—
0.13			—	3.9	3.0	—	30.0	23.0	—	3.9	2.8
0.10			13.8	—	—	138.0	—	—	0.9	—	—

change in the rate-limiting step of the rapid phase, needs more precise kinetic analysis to be clarified.

The effect of the ferricyanide concentration is also evident on the rate of the slow phase at pH 7.0 and 9.0. This is probably due to the increase in the stationary concentration of the enzyme-hydroxypyruvaldehyde phosphate complex.

The time-course of the reaction is not affected by the pre-mixing conditions [enzyme and substrate pre-mixed or substrate and Fe(CN)_6^{3-} pre-mixed]. This indicates that between pH 5.5 and 9.0, the rate of the rapid phase is limited by the rate of the oxidation of the carbanion intermediate (step *c* of Scheme 1) and not by the rate of its formation (steps *a* and *b* of Scheme 1). Consequently the rate-limiting step of the

slow phase must be the release of the oxidized substrate from the enzyme (step *d*).

Transient kinetics of the reduction of Fe(CN)_6^{3-} by the carboxypeptidase-aldolase-dihydroxyacetone phosphate complex

Fe(CN)_6^{3-} also oxidizes the complex formed by reaction of dihydroxyacetone phosphate with carboxypeptidase-aldolase, and the reaction follows second-order kinetics between pH 5.0 and 9.0, being pseudo-first-order with respect to both the enzyme-substrate complex and to Fe(CN)_6^{3-} (Table 2). In this case, however, and over the whole range of pH tested, the rapid phase is detected only when enzyme and substrate are pre-mixed. This means that the

rate-limiting step is before step (c) (Scheme 1). Since it is known that the slowest step of the reaction catalysed by carboxypeptidase-aldolase is the formation of the carbanion intermediate (Scheme 1 step b) (Rose *et al.*, 1965), this must be the rate-limiting step of the overall sequence.

The rate of the rapid phase decreases twofold between pH 5.0 and 9.0 (Fig. 2a) and it is generally larger for carboxypeptidase-aldolase than for native aldolase. Direct comparison of the native and carboxypeptidase-treated enzyme, under exactly the same conditions, showed that, at pH 6.0 (50 mM-Tris-HCl buffer, *I* 0.05) and 27°C, the second-order rate constant for the rapid phase is $18\,500\text{ M}^{-1}\cdot\text{s}^{-1}$ for native aldolase and $38\,000\text{ M}^{-1}\cdot\text{s}^{-1}$ for carboxypeptidase-aldolase.

The rate of the slow phase increases rapidly from pH 5.0 to 6.5, reaches a maximum between pH 6.5 and 8.0, and decreases again at higher pH values (Fig. 2b). The rate of reduction is not influenced by changes

Fig. 2. Effect of pH on the rate of oxidation by $\text{Fe}(\text{CN})_6^{3-}$ (rapid and slow phases) of the carboxypeptidase-aldolase-dihydroxyacetone phosphate complex

Enzyme and substrate were pre-mixed. Carboxypeptidase-aldolase (specific activity 0.27 unit/mg of protein; $6.5\text{ }\mu\text{M}$), dihydroxyacetone phosphate (0.4 mM) and $\text{Fe}(\text{CN})_6^{3-}$ (0.67 mM). The system was buffered with 0.05 M-sodium acetate at pH 5.0 and 5.5 and with 0.05 M-Tris-HCl buffer from pH 6.0 to 9.0; *I* 0.05. Temperature was 29°C. (a) Pseudo-first-order rate constants for the rapid phase; (b) rates of the slow phase were expressed as mol of $\text{Fe}(\text{CN})_6^{3-}$ reduced/s per mol of enzyme.

Fig. 3. Effect of pH on (a) the amplitude of the rapid phase of the oxidation by $\text{Fe}(\text{CN})_6^{3-}$ of the carboxypeptidase-aldolase-dihydroxyacetone phosphate complex and on (b) the equilibrium concentration of the carbanion intermediate

Data are taken from Fig. 2 and calculations were done as described in the Experimental section.

in the concentration of $\text{Fe}(\text{CN})_6^{3-}$ (Table 2). The rate-determining step of this phase must therefore be before step (c) (Scheme 1), and since the slowest step before step (c) is the formation of the carbanion, this must be the rate-limiting step. It turns out therefore that the rate of formation of the carbanion intermediate is strongly influenced by pH. The effect of pH on the slow phase is more pronounced in the experiment in Fig. 2 than in that in Table 2. This is probably a result of the different specific activities of the carboxypeptidase-aldolase used in the two experiments.

The amplitude of the rapid phase decreases slowly from pH 5.0 (E_{420} 0.028) to pH 9.0 (E_{420} 0.022) (Fig. 3a).

Substrate-binding sites on carboxypeptidase-aldolase

The number of substrate-binding sites on carboxypeptidase-aldolase was directly determined by filtration of the enzyme through a Sephadex G-50 column equilibrated with radioactive dihydroxyacetone phosphate. It was shown that carboxypeptidase-aldolase, like the native enzyme, possesses four substrate-binding sites (Fig. 4).

Competition between dihydroxyacetone phosphate and fructose 1,6-diphosphate for native aldolase

The initial rate of the rapid phase of the reduction of $\text{Fe}(\text{CN})_6^{3-}$ decreases when fructose 1,6-diphosphate is added together with dihydroxyacetone phosphate. The phenomenon follows the

Fig. 4. Effect of dihydroxyacetone phosphate concentration on the number of mol of substrate bound per mol of native and carboxypeptidase-treated aldolase

The mixture (0.5ml) contained either 2mg of aldolase (specific activity 16units/mg of protein) or 2mg of carboxypeptidase-treated aldolase (specific activity 0.27 unit/mg of protein) dissolved in 0.05M-Tris-HCl buffer, pH 6.0. The mixture was filtered through Sephadex G-50 columns equilibrated with the same buffer and with dihydroxy- ^{14}C acetone phosphate at the concentrations indicated. Temperature was 22°C. The reciprocal of the number of mol of substrate bound/mol of enzyme was plotted against the reciprocal of substrate concentration. ○, Native aldolase; ●, carboxypeptidase-treated aldolase.

Fig. 5. Effect of the addition of fructose diphosphate on the initial rate of $\text{Fe}(\text{CN})_6^{3-}$ reduction

Enzyme and substrates were not pre-mixed. Native aldolase (specific activity 16 units/mg of protein; $2.3\mu\text{M}$), $\text{Fe}(\text{CN})_6^{3-}$ (0.64mm), dihydroxyacetone phosphate (0.28mm) and fructose diphosphate (as indicated in the Figure). The system was buffered with 0.05M-Tris-HCl buffer, pH 7.5. Temperature was 25°C.

pattern expected for competitive inhibition. For these experiments aldolase was added to one syringe and dihydroxyacetone phosphate, fructose diphosphate and $\text{Fe}(\text{CN})_6^{3-}$ were added to the other. A plot of the reciprocal of the initial velocity of the rapid phase against the concentration of the inhibitor yields a

straight line (Fig. 5). From this the dissociation constant of the aldolase-fructose 1,6-diphosphate complex ($3.3\mu\text{M}$) was calculated by the equation of Dixon & Webb (1964):

$$\frac{1}{v_1} = \frac{K_s}{[S] \cdot v} + \frac{1}{v} + \left(\frac{K_s}{[S] \cdot v} \right) \frac{[I]}{K_i}$$

where v_1 and v are respectively the initial velocity in the presence and in the absence of the inhibitor; K_s is the dissociation constant of the aldolase-dihydroxyacetone phosphate complex [$6.7\mu\text{M}$ at pH 7.5 and $1/0.05$ (Grazi & Trombetta, 1974)]; $[S]$ and $[I]$ are the concentrations of the substrate and inhibitor respectively and K_i is the dissociation constant of the enzyme-inhibitor complex.

Reduction of $\text{Fe}(\text{CN})_6^{3-}$ also takes place in the presence of native aldolase and with fructose diphosphate as the only substrate. In this case, however, a steady state is established from the very beginning of the reaction and the rapid phase is not detectable. At pH 7.5 and 22°C, in the presence of $3.1\mu\text{M}$ -aldolase, 0.3mm-fructose diphosphate and 0.64mm- $\text{Fe}(\text{CN})_6^{3-}$, the rate of reduction of $\text{Fe}(\text{CN})_6^{3-}$ is 3.2mol/s per mol of enzyme. The rate of cleavage of fructose diphosphate cannot be rate-limiting since it is much higher (42mol/s per mol of enzyme at pH 7.5 and 22°C). The slow rate of oxidation in the presence of fructose diphosphate can thus only be related to a low steady-state concentration of the carbanion intermediate. This cannot be larger than 6% of the total concentration of all the enzyme-substrate intermediates since, under the same conditions and with dihydroxyacetone phosphate as the only substrate, the initial rate of the rapid phase is 54mol/s per mol of enzyme (Fig. 5).

Discussion

The carbanion intermediate, formed by reaction of aldolase with dihydroxyacetone phosphate, is oxidized by $\text{Fe}(\text{CN})_6^{3-}$ (Healy & Christen, 1973).

With native aldolase the transient kinetics of the reaction display a rapid and a slow phase, independently of the pre-mixing conditions of the reagents. This reveals that step (c), and not steps (a) and (b) (Scheme 1), is rate-limiting for the rapid phase. By elimination, the rate-limiting step of the slow phase must be the release of the oxidized substrate from the enzyme (step d).

Under these conditions the rapid phase represents a single turnover of the enzyme and its amplitude measures the number of the catalytic sites of aldolase. These are four (Grazi, 1974a) in agreement with the number of the equivalent and independent binding sites (Grazi *et al.*, 1973; Grazi & Trombetta, 1974).

The rate of the slow phase is practically independent of pH. The pseudo-first-order rate constant of the

rapid phase decreases about twofold from pH 5.5 to 9.0. Since the reduction potential of $\text{Fe}(\text{CN})_6^{3-}$ is independent of pH and the formation of the carbanion, between pH 5.5 and 9.0, never becomes rate-limiting, this decrease could depend on a change in the equilibrium concentration of the carbanion. Alternatively, a decrease in positive charges at the active site could hinder the approach of $\text{Fe}(\text{CN})_6^{3-}$ to the carbanion and decrease the rate constant of the rapid phase.

Starting from fructose diphosphate as the only substrate the rate of reduction of $\text{Fe}(\text{CN})_6^{3-}$ reaches a steady state from the very beginning of the reaction, the rate being 17 times lower than the initial rate of the rapid phase that is obtained when dihydroxyacetone phosphate is used as substrate. This means that during the cleavage of fructose 1,6-diphosphate the concentration of the carbanion intermediate cannot be higher than 6% of the total concentration of the enzyme-substrate intermediates.

By studying the competition between dihydroxyacetone phosphate and fructose 1,6-diphosphate the dissociation constant of the aldolase-fructose 1,6-diphosphate complex was evaluated. Fructose 1,6-diphosphate appears to bind to the enzyme about twice as tightly as dihydroxyacetone phosphate.

With carboxypeptidase-treated aldolase the rapid phase is detected only when enzyme and substrate are pre-mixed. This indicates that the rate-limiting step must be before step (c). It is, in fact, step (b), which is the formation of the carbanion intermediate (Rose *et al.*, 1965). The rapid phase thus measures the difference between the original (equilibrium) and steady-state concentration of the carbanion intermediate. Taking this into account the original equilibrium concentration of the carbanion intermediate was calculated and shown to be practically constant between pH 5.0 and 9.0 (Fig. 3b).

The rate of the slow phase, i.e. the rate of formation of the carbanion intermediate, increases dramatically from pH 5.0 to 6.5. This implies that the rate of the reverse reaction, the protonation of the carbanion,

increases accordingly, since the equilibrium concentration of the carbanion is not affected by pH.

The rate constant of the rapid phase also changes with pH. In this case, however, only one of the two explanations offered for the native enzyme is tenable, namely that a change of charge at the active site influences the reactivity of $\text{Fe}(\text{CN})_6^{3-}$.

This work was supported by grant no. 74.00206.04 from the Italian Consiglio Nazionale delle Ricerche and by NATO grant 751.

References

- Baranowski, T. & Niederland, T. R. (1949) *J. Biol. Chem.* **180**, 543–548
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Christen, P. & Riordan, J. F. (1968) *Biochemistry* **7**, 1531–1538
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, p. 328, Longmans, London
- Ginsburg, A. & Mehler, A. H. (1966) *Biochemistry* **5**, 2623–2634
- Grazi, E. (1974a) *Biochem. Biophys. Res. Commun.* **56**, 106–111
- Grazi, E. (1974b) *Biochem. Biophys. Res. Commun.* **59**, 450–454
- Grazi, E. & Trombetta, G. (1974) *Biochim. Biophys. Acta* **364**, 120–127
- Grazi, E., Sivieri-Pecorari, O., Gagliano, R. & Trombetta, G. (1973) *Biochemistry* **14**, 2583–2590
- Healy, M. J. & Christen, P. (1973) *Biochemistry* **12**, 35–41
- Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T. & Tchola, T. (1963) *Biochem. Z.* **338**, 36–51
- Kawahara, K. & Tanford, C. (1966) *Biochemistry* **5**, 1578–1584
- Racker, E. (1947) *J. Biol. Chem.* **167**, 843–852
- Rose, I. A., O'Connell, E. L. & Mehler, A. M. (1965) *J. Biol. Chem.* **240**, 1758–1765
- Rutter, W. J., Richards, O. C. & Woodfin, B. M. (1961) *J. Biol. Chem.* **236**, 3193–3197
- Spolter, P. D., Adelman, R. C. & Weinhouse, S. (1965) *J. Biol. Chem.* **240**, 1327–1337
- Taylor, J. F., Green, A. A. & Cori, G. T. (1948) *J. Biol. Chem.* **173**, 591–598