Metal-Replacement Studies in *Bacillus stearothermophilus* Aldolase and a Comparison of the Mechanisms of Class I and Class II Aldolases

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A comparison of the product-inhibition patterns during cleavage of D-fructose 1,6-diphosphate by aldolases from yeast, rabbit muscle and *Bacillus stearothermophilus* shows an ordered reaction sequence for all three enzymes, with dihydroxyacetone phosphate the last-leaving product. Addition of Zn^{2+} , Co^{2+} , Fe^{2+} , Mn^{2+} or Cd^{2+} ions to the inactive apo-(*Bacillus stearothermophilus* aldolase) restores activity to different extents, whereas Ni^{2+} , Mg^{2+} or Cu^{2+} ions have no effect. The cleavage activity of this aldolase is not enhanced by added K^{+} ion. The effects of metal replacement on thermal stability, K_m and $V_{\max}$ are given and the possible role of the metal is discussed in the light of these results.

D-Fructose 1,6-diphosphate aldolases (EC 4.1.2.13) have been subdivided into two main classes (Rutter, 1964). The class I enzymes, typified by rabbit muscle aldolase, have been well studied and extensively reviewed (Morse & Horecker, 1968; Lai & Horecker, 1972). The mechanism is presumed to involve formation of a Schiff base between dihydroxyacetone phosphate and an active-site lysine (Horecker *et al.*, 1963; Model *et al.*, 1968). The class II enzymes, typified by yeast aldolase, are metalloenzymes inhibited by chelating agents (Rutter, 1964). They are much less well studied, but it has been suggested that the metal has a function analogous to that of the active-site lysine of class I aldolases, serving as a Lewis acid, thereby aiding bond polarization and subsequent cleavage of substrate (Rutter, 1964; Mildvan *et al.*, 1971).

As part of a programme designed to investigate the detailed mechanistic role of the metal ion in these and related enzymes, we describe in this paper results obtained not only from yeast and rabbit muscle aldolases, but also from a study of aldolase from a thermophilic bacterium, *Bacillus stearothermophilus*. It is known to be a metalloenzyme containing two atoms of zinc per molecule (Sugimoto & Nosoh, 1971), and consisting of two identical subunits of molecular weight 30 500 (Jack, 1973). Its molecular weight is thus significantly lower than the value for yeast aldolase, which is 80 000 (Harris *et al.*, 1969) and there is evidence that little sequence homology exists between the two (Jack, 1973), or with rabbit muscle aldolase (Jack & Harris, 1971), the three apparently being the result of convergent evolution.

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Experimental

Yeast aldolase was prepared by a modification (Jack, 1973) of the procedure of Rutter (Rutter *et al.*, 1966), in the Enzyme Preparation Laboratory of the Oxford Enzyme Group. *B. stearothermophilus* aldolase was prepared by previously established procedures (Jack, 1973). Both enzymes were homogeneous by electrophoretic criteria.

Rabbit muscle aldolase, glyceraldehyde 3-phosphate dehydrogenase, α -glycerophosphate dehydrogenase, triose phosphate isomerase, NAD^{+} , NADH , fructose 6-phosphate, and triethanolamine hydrochloride were from Boehringer (Mannheim) Ltd., London W5 2TZ, U.K. Fructose 1,6-diphosphate and fructose 1-phosphate were from Wessex Biochemicals, Bournemouth, Hants., U.K. 8-Hydroxyquinoline-5-sulphonic acid, Trizma base, dihydroxyacetone phosphate as the dimethylketal dicyclohexylammonium salt, and DL-glyceraldehyde 3-phosphate as the diethylacetal monobarium salt, were from Sigma Chemical Co., London S.W.6, U.K. Dihydroxyacetone phosphate and DL-glyceraldehyde 3-phosphate were prepared from their salts as described by the suppliers, and their concentrations were determined before use enzymically with α -glycerophosphate dehydrogenase and triose phosphate isomerase. KCl, sodium arsenate, sodium EDTA, dithizone and Hepes were of the highest grade available from BDH, Poole, Dorset, U.K. $\text{Cd}(\text{NO}_3)_2$, CoSO_4 , ZnSO_4 and MnSO_4 were spectroscopically pure samples from Johnson, Matthey and Co., Wembley, Middx., U.K. Bromohydroxyacetone phosphate was a gift from Dr. J. R. Knowles, Harvard University.

Assays were carried out by adding a suitable portion of aldolase to a cuvette containing 0.1 M-KCl/0.1 M-triethanolamine hydrochloride (pH 7.3 unless otherwise stated) and the solution was made up to 3 ml by the addition of: 50 μ l of 0.1 M-fructose 1,6-diphosphate, 50 μ l of NADH (10 mg/ml), and 20 μ l of α -glycerophosphate dehydrogenase/triose phosphate isomerase mixture (10 mg/ml) (assay A); 50 μ l of 0.1 M-fructose 1,6-diphosphate, 50 μ l of NADH (10 mg/ml), and 20 μ l of α -glycerophosphate dehydrogenase (10 mg/ml) (assay B); or 50 μ l of 0.1 M-fructose 1,6-diphosphate, 50 μ l of NAD⁺ (10 mg/ml), 50 μ l of 60 mM-sodium arsenate, and 20 μ l of glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) (assay C). In each case the change in absorbance at 340 nm was recorded immediately, by using a Unicam SP.800 instrument, and aldolase activity expressed as μ mol of fructose 1,6-diphosphate cleaved/min per mg of enzyme at 25°C.

In all experiments with metalloaldolases, trace metals were removed from all buffers and assay constituents (except coupling enzymes) by extraction with dithizone in carbon tetrachloride (Thiers, 1957), residual traces of the organic solvent being removed by the passage of White-Spot nitrogen through the solution.

Apo-(*B. stearothermophilus* aldolase) was prepared in a similar way to that described for yeast aldolase (Kobes *et al.*, 1969). Native enzyme, freeze-dried or as the (NH₄)₂SO₄ precipitate, was dissolved in 0.1 M-EDTA, pH 8.0, and incubated at 25°C for 1 h. Apoenzyme was separated from EDTA and metal ion either by exhaustive dialysis against metal-free 50 mM-Hepes* or triethanolamine hydrochloride pH 7.5, or by gel filtration through a Sephadex G-25 column which had been freed of metal ions by washing with 8-hydroxyquinoline-5-sulphonic acid at pH 8.0, and then equilibrated with 50 mM-triethanolamine hydrochloride or -Tris, pH 8.0. The apoenzyme thus obtained was found to exhibit some 2–6% of native activity by assay, or 2–6% of the metal content of the native enzyme by atomic absorption.

Apoenzyme in metal-free buffer was reconstituted by addition of small volumes of spectroscopically pure Zn²⁺, Mn²⁺, Co²⁺ or Cd²⁺ ions in metal-free water, giving holoenzyme solutions with a 5–10-fold excess of metal ion.

Any triose phosphate isomerase contaminant was removed from all enzymes except yeast aldolase by addition of bromohydroxyacetone phosphate, a potent triose phosphate isomerase inhibitor (De la Mere *et al.*, 1972), and excess of reagent removed by gel filtration through Sephadex G-25, or by dialysis against 20 mM-Tris, pH 7.5. Sulphate was removed from coupling enzymes by extended dialysis against 20 mM-Tris, pH 7.5.

* Abbreviation: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

Equilibrium-dialysis experiments were carried out in acid-washed plastic dialysis cells of 1 ml capacity. Membranes were boiled in EDTA to remove trace metals, and exhaustively washed with metal-free water. The ammonium salt of D-[U-¹⁴C]fructose 1,6-diphosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) of specific radioactivity 250 mCi/mmol, was diluted into unlabelled fructose 1,6-diphosphate of known concentration. A calibration of count rate against substrate concentrations was determined by standard liquid-scintillation in a Hewlett-Packard Tri-Carb instrument, portions of standard substrate solutions being diluted into the scintillator described by Bray (1960). In all experiments allowance was made for background by subtraction of the count of a control under comparable conditions, the value of this count being at least 10-fold lower than that of the most dilute substrate solution measured. Holo- or apo-aldolase (0.5 ml) in metal-free 0.1 M-KCl/0.1 M-triethanolamine hydrochloride, pH 7.3, was dialysed against metal-free ¹⁴C-labelled substrate (0.5 ml) in the same buffer for 48 h at 18°C. After this time, samples were removed from both sides of the cell and the substrate concentration was determined from the count rate and the calibration curve. Enzyme concentration was determined by assuming an *E*₂₈₀ of 1.02 for a 1 mg/ml aldolase solution (Jack, 1973). From the data obtained, binding constants were determined from standard double-reciprocal plots of mol of bound substrate per mol of enzyme against free substrate concentration at equilibrium (Klotz, 1974).

The effect of temperature on *V*_{max} for the zinc and cobalt forms of *B. stearothermophilus* aldolase was determined in 50 mM-Hepes, pH 7.5, by using assay C. The buffer pH was adjusted such that a pH of 7.5 $\pm$ 0.05 was obtained at the desired temperature of assay. The cuvette and buffer were incubated for several minutes in the spectrometer at the desired temperature. When equilibrated, the other assay constituents, and a portion of enzyme were added quickly in small volumes. The rate rapidly became linear, and all points were double-checked with excess of coupling enzyme to ensure that its own turnover was not rate-limiting.

Atomic-absorption measurements on cobalt *B. stearothermophilus* aldolase, exhaustively dialysed against metal-free 50 mM-Hepes, pH 7.5, to remove residual metal ions, were carried out in a Unicam SP.90 Atomic Absorption Spectrometer.

Results

Metal content and its relation to activity

Although *B. stearothermophilus* aldolase is a zinc metalloenzyme (Sugimoto & Nosoh, 1971), it is prepared here in the more thermally stable cobalt

form (see below). Analysis of metal content by atomic absorption gives a value of 0.9 cobalt atom per enzyme subunit, based on a subunit molecular weight of 30 500 (Jack, 1973). Addition of spectroscopically pure CoSO₄ in metal-free water to the inactive apoenzyme results in a linear increase in activity until two atoms of cobalt per molecule of aldolase have been added. Further addition of cobalt results in no further increase in activity (Fig. 1).

Basic kinetic data for *B. stearotherophilus* aldolase

Assay A is essentially a form of the coupled assay used to determine aldolase activities for many years (Racker, 1947; Beisenherz *et al.*, 1953), triose phosphate isomerase being included to double sensitivity. By using this assay, the values of *K_m* for fructose 1,6-diphosphate, and *K_i* for the competitive inhibitors fructose 1-phosphate and fructose 6-

phosphate, were obtained with a suitable sample of the cobalt form of *B. stearotherophilus* aldolase. In line with other class II enzymes (Rutter, 1964) this aldolase is specific for fructose 1,6-diphosphate, its *K_m* for fructose 1-phosphate being too high to measure accurately. The data are given in Table 1, with comparable data for rabbit muscle and yeast aldolases.

Product inhibition data

Assay B is a modification of assay A, in which all traces of triose phosphate isomerase are eliminated by treatment of coupling enzyme (α -glycerophosphate dehydrogenase) and the aldolases to be assayed with bromohydroxyacetone phosphate as described in the Experimental section. Under these conditions no interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate occurs and it is possible to hold the glyceraldehyde 3-phosphate concentration constant during the assay.

Assay C is a modification of the assay of Rutter (Richards & Rutter, 1961) in which glyceraldehyde 3-phosphate, rather than dihydroxyacetone phosphate, is coupled to the NAD⁺/NADH redox system, by using glyceraldehyde 3-phosphate dehydrogenase. If triose phosphate isomerase is again excluded from this system then the dihydroxyacetone phosphate concentration may be held constant during the assay.

There is evidence that both yeast and rabbit muscle aldolase (E) follow an ordered reaction sequence in which dihydroxyacetone phosphate is the last-leaving product (Rose *et al.*, 1965):

Fig. 1. Metal content and aldolase activity

The Figure shows the correlation between specific activity expressed as μ mol of fructose 1,6-diphosphate cleaved/min per mg of enzyme and metal content on addition of Co²⁺ ion to apo-(*B. stearotherophilus* aldolase) in 50mM-Hepes, pH7.3.

Table 1. Aldolase kinetic constants

Michaelis and inhibition constants for the cobalt form of *B. stearotherophilus* aldolase with substrate, substrate analogues and products were determined as described in the text. Comparable constants for yeast and rabbit muscle aldolases are quoted from the following sources; (a) Richards & Rutter (1961); (b) Rutter (1964); (c) Spolter *et al.* (1965).

Parameter	Class II source		Class I source
	<i>B. stearotherophilus</i>	Yeast	Rabbit muscle
<i>K_m</i> (fructose 1,6-diphosphate)	(M) 8.0×10^{-5}	(M) 3.7×10^{-4} (b)	(M) 1.0×10^{-5} (b)
<i>K_m</i> (fructose 1-phosphate)	—	—	1.0×10^{-2} (b)
<i>K_i</i> (fructose 1-phosphate)	4.5×10^{-3}	1.0×10^{-3} (a)	—
<i>K_i</i> (fructose 6-phosphate)	8.0×10^{-3}	3.8×10^{-3} (a)	1.0×10^{-3} (c)
<i>K_i</i> (dihydroxyacetone phosphate)	1.2×10^{-4}	1.9×10^{-4}	4.8×10^{-5}
<i>K_i</i> (glyceraldehyde 3-phosphate)	3.8×10^{-5}	4.0×10^{-5}	1.5×10^{-5}

where Fru-1,6- P_2 is fructose diphosphate, Grn-P is dihydroxyacetone phosphate and Gra-3-P is glyceraldehyde 3-phosphate. The rate equation for such a mechanism has been previously formulated (Spolter *et al.*, 1965), or may be derived by standard techniques (King & Altman, 1956) and is:

$$v = (V_{\max} \cdot S) / \left(K_m + S + \frac{K_m}{K_p} \cdot P + \frac{K_m}{K_q} \cdot Q + \frac{K_1}{K_p} \cdot PS + K_2 \cdot P \cdot Q \right)$$

where S, P, and Q are the concentrations of substrate (fructose 1,6-diphosphate), first-leaving product (glyceraldehyde 3-phosphate) and last-leaving product (dihydroxyacetone phosphate) respectively, and the kinetic constants $V_{\max}$, K_m , K_p , K_q , K_1 and K_2 are functions of one or more of the individual rate constants $k_{+1} \dots k_{-4}$.

Under the conditions of assay A, the standard aldolase assay, the concentration of both products is

negligible, and thus both P and Q approximate to zero. The rate equation is then:

$$v = (V_{\max} \cdot S) / (K_m + S)$$

corresponding to standard Michaelis-Menten kinetics.

Under the conditions of assay C, only the glyceraldehyde 3-phosphate concentration (P) is negligible, and the rate equation becomes

$$v = (V_{\max} \cdot S) / \left[K_m \left(1 + \frac{Q}{K_q} \right) + S \right]$$

which is the form for competitive inhibition (Webb, 1963).

Under the conditions of assay B, only the dihydroxyacetone phosphate concentration (Q) is negligible, and the rate equation becomes

$$v = (V_{\max} \cdot S) / \left[K_m \left(1 + \frac{P}{K_p} \right) + S \left(1 + \frac{K_1}{K_p} \cdot P \right) \right]$$

Fig. 2. Aldolase product-inhibition patterns

Reciprocal reaction velocity, in arbitrary units, plotted against reciprocal substrate concentration, for the cleavage of fructose 1,6-diphosphate by (a) Co(II) *B. stearothermophilus* aldolase in the presence of 0, 50, 100 or 150 μ M-dihydroxyacetone phosphate (●), or 0, 30, 60 or 90 μ M-glyceraldehyde 3-phosphate (■); (b) rabbit muscle aldolase in the presence of 0, 40, 80 or 120 μ M-dihydroxyacetone phosphate (●), or 0, 30, 60, 90 or 120 μ M-glyceraldehyde 3-phosphate (■); (c) Zn(II) yeast aldolase in the presence of 0, 100 or 200 μ M-dihydroxyacetone phosphate (●), or 0, 120 or 240 μ M-glyceraldehyde 3-phosphate (■).

which corresponds to non-competitive or mixed inhibition, in which both the slope and the intercept of a double-reciprocal plot of $1/v$ against $1/[S]$ vary with inhibitor concentration.

The nature of the inhibition of the two cleavage products was determined by adding known concentrations of dihydroxyacetone phosphate or glyceraldehyde 3-phosphate to the cuvette and assaying the cobalt form of *B. stearothermophilus* aldolase, zinc yeast aldolase or rabbit muscle aldolase. By using assay C dihydroxyacetone phosphate was found to be a competitive inhibitor, and by using assay B glyceraldehyde 3-phosphate a mixed inhibitor, in line with the above reaction sequence, in all three cases. The K_i for the inhibition of rabbit muscle aldolase by dihydroxyacetone phosphate is in agreement with values obtained by other methods, such as fluorescence quenching (Rose & O'Connell, 1969), u.v. difference spectroscopy (Mehler & Bloom, 1963), or de-tritiation studies (Rose & Reider, 1958). The data are summarized in Fig. 2; kinetic constants are given in Table 1.

Fig. 3. Equilibrium-dialysis data

The Figure is a double-reciprocal plot of mol of substrate bound per mol of enzyme (R) against free substrate concentration at equilibrium, for Co(II) holo- (○) and apo- (●) (*B. stearothermophilus* aldolase).

Equilibrium-dialysis experiment

To investigate the effect of the metal ion on substrate binding, the binding constant of $[U-^{14}C]$ fructose 1,6-diphosphate to holo- and apo-(*B. stearothermophilus* aldolase) was determined by equilibrium dialysis, as described in the Experimental section. The dissociation of substrate to holoenzyme was $1.1 \times 10^{-4} M$, in agreement with the experimentally determined K_m (Table 1). Binding to the apoenzyme, shown to contain some 5% native cobalt by atomic absorption, was significantly decreased, with a measured constant of 4 mM. The data are summarized in Fig. 3, both plots being consistent with two independent binding sites.

Metal replacement studies

Addition of Co^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} or Cd^{2+} ion to inactive apo-(*B. stearothermophilus* aldolase) in 50 mM-Tris pH 8.0, resulted in restoration of cleavage activity in the order $Cd^{2+} > Co^{2+} > Fe^{2+} > Zn^{2+} > Mn^{2+}$. Addition of Cu^{2+} , Ni^{2+} or Mg^{2+} ion had no effect, and addition of Hg^{2+} ion abolished the trace of residual activity remaining in the apoenzyme. In view of the inhibitory effects of Tris buffer (see below), subsequent work was confined to apoprotein reconstituted with spectroscopically pure Mn^{2+} , Co^{2+} , Zn^{2+} or Cd^{2+} , in 50 mM-Hepes, brought to pH 7.5 with carbonate-free NaOH.

Effect of added K^+ ion on activity. We find that, in 50 mM-Hepes or triethanolamine hydrochloride buffers, at pH values between 6.5 and 8.5, the presence of 100 mM- K^+ ion, as either the chloride or the acetate, has no stimulatory effect whatever on any of the four

metalloaldolase forms of *B. stearothermophilus*. At the lower pH values, in fact, this concentration of K^+ ion is somewhat inhibitory to the zinc, manganese or cadmium enzymes.

Effect of Tris buffer on activity. In 50 mM-Tris, pH 8.5, the activity of all four metal forms is inhibited. Added K^+ ion (100 mM) then stimulates cleavage activity to values comparable with those obtained in 50 mM-Hepes pH 8.5, in the absence of K^+ ion (Table 2). At pH 7.5 no inhibition was detected in this buffer.

Effect of acetaldehyde on activity. Addition to the assay cuvette of acetaldehyde to a final concentration of 87 mM enhances the rate of fructose 1,6-diphosphate cleavage, as measured by assay C, for the zinc, cobalt and manganese enzymes at both pH 7.5 and 8.5 in 50 mM-Hepes. At lower pH values no stimulation is observed, whereas the cadmium enzyme is unaffected by added acetaldehyde at any pH (Table 2).

Effect of temperature on activity. The effect of temperature on the cleavage activity of the zinc and cobalt enzymes, as measured by assay C, is summarized as plots of $\log(1/V_{max})$ against the reciprocal of the absolute temperature (Fig. 4). V_{max} is expressed in arbitrary units, and the V_{max} values of both metal forms at 25°C have been set equal for comparative purposes. Both plots are very similar and are independent of added K^+ ion at any temperature. In agreement with previous observations on this system (Sugimoto & Nosoh, 1971), a discontinuity in the plots occurs in the region of 50°C, possibly owing to a conformational change.

Thermal stability. The four metal forms of *B.*

Table 2. *Specific activities of B. stearothermophilus aldolase metal forms*

The variation of specific activity with bivalent metal ion, buffer, pH, added aldehyde and added K^+ ion for the active metal forms of the class II aldolase from *B. stearothermophilus* was determined as described in the text and is expressed as μmol of fructose 1,6-diphosphate cleaved/min per mg of enzyme.

System	Specific activity (μmol of fructose 1,6-diphosphate per min/mg) for:			
	Mn enzyme	Co enzyme	Zn enzyme	Cd enzyme
50mM-Hepes pH7.5	12.0	20.5	11.0	13.4
As above, with added acetaldehyde (fold stimulation)	1.8	1.7	1.2	—
50mM-Hepes, pH8.5	7.8	11.0	6.2	27.8
As above, with added acetaldehyde (fold stimulation)	1.7	3.8	1.6	—
50mM-Tris, pH8.5	5.2	8.2	3.0	20.8
50mM-Tris, 100mM- K^+ , pH8.5	7.6	11.0	6.6	28.5

Fig. 4. *Temperature variation of $V_{\max}$.*

The log of the reciprocal maximum velocity, expressed in arbitrary units, plotted against reciprocal absolute temperature, for the zinc ($\circ$) and cobalt ($\bullet$) forms of *B. stearothermophilus* aldolase.

stearothermophilus aldolase were incubated in 50mM-Hepes, pH7.5, at 64°C. Samples were withdrawn at suitable intervals and their cleavage activity was measured at 25°C by using assay C. After a few minutes the cadmium form begins to precipitate, and the zinc enzyme forms an opaque solution, whereas the cobalt and manganese aldolases remain in clear solution. Plots of log residual activity against time are given in Fig. 5. A sample of yeast aldolase under similar conditions precipitates and is totally inactive before it can be assayed.

Variation of K_m and $V_{\max}$ with pH. The effect of pH on $V_{\max}$ for all four metal enzymes was

Fig. 5. *Thermal stabilities of metalloaldolases*

The log of the residual activity plotted against time on incubation of the cobalt ($\blacksquare$), manganese ($\square$), zinc ($\bullet$) and cadmium ($\circ$) forms of *B. stearothermophilus* aldolase at 64°C in 50mM-Hepes pH7.5.

measured in 50mM-Hepes buffer. The effect of pH on K_m for all four forms was measured in 100mM-KCl/100mM-triethanolamine hydrochloride buffer. The quoted pH is that of the cuvette solution after assay.

The data are summarized in Figs. 6 and 7. The K_m values of the manganese, zinc and cobalt forms are so similar, as are the shapes of their pH profiles and the position of their pH optima, so only the data for the cobalt form is presented. The only differences lie in the absolute values of $V_{\max}$, which differ by less than a factor of two over almost the whole pH range studied.

However, the cadmium form of the enzyme differs in kind. Its K_m profile is still very similar, though somewhat higher values are obtained, but the pH

Fig. 6. pH variation of kinetic constants for Co(II) *B. stearothermophilus* aldolase

The Figure shows the variation with pH of Michaelis constant (○) and maximum velocity (●) for fructose 1,6-diphosphate cleavage by Co(II) *B. stearothermophilus* aldolase at 25°C.

Fig. 7. pH variation of kinetic constants for Cd(II) *B. stearothermophilus* aldolase

The Figure shows the variation with pH of Michaelis constant (○) and maximum velocity (●) for fructose 1,6-diphosphate cleavage by Cd(II) *B. stearothermophilus* aldolase at 25°C.

profile is different, and the pH optimum is shifted by almost 1 pH unit. This profile is essentially the same in either Hepes or triethanolamine hydrochloride buffers.

Discussion

In this paper we demonstrate an absolute requirement for bivalent metal ion in the dimeric class II aldolase from *B. stearothermophilus*. Atomic absorption measurements on exhaustively dialysed protein show that two mol of metal ion per mol of enzyme are tightly bound. Removal of the metal gives a totally inactive apoenzyme, and re-addition results in a linear increase in activity until two mol of metal ion per mol of enzyme have been added. Addition of Zn^{2+} , Co^{2+} , Mn^{2+} or Cd^{2+} to the apoenzyme gives active aldolase. Equilibrium dialysis studies of holo- or apo-enzyme against fructose 1,6-diphosphate show that the metal is required for tight binding of

substrate. In addition, the nature of the metal is critical to thermal stability, the cobalt and manganese forms of the enzyme being much more resistant to thermal inactivation than those of the zinc or cadmium.

Evidence from isotope-exchange studies (Rose *et al.*, 1965) suggests that both the yeast and rabbit muscle aldolase reactions proceed almost exclusively by the ordered reaction sequence given earlier. Yet arguments against such a sequence of product release, and for a random order of reaction, may be cited from kinetic evidence in the literature (Spolter *et al.*, 1965; Mehler & Cusic, 1967). In a further paper (Rose & O'Connell, 1969) designed to explain such anomalies, the authors indicate that the enzymic reactions of muscle and liver aldolases, in both the native and carboxypeptidase-treated forms, do indeed proceed via the ordered sequence, although inhibition studies by any of the known acceptors of dihydroxyacetone phosphate (Spolter *et al.*, 1965) apparently imply a random order. Yet it was later suggested (Mildvan *et al.*, 1971) that the condensation reaction catalysed by the manganese form of yeast aldolase obeys a rapid-equilibrium random kinetic scheme, based on relaxation studies of substrate and products with this class II enzyme by n.m.r. By including data from *B. stearothermophilus* aldolase we show that the product-inhibition patterns of three analogous aldolases are identical, and are entirely consistent with an ordered sequence in which dihydroxyacetone phosphate is the last-leaving product of fructose 1,6-diphosphate cleavage.

In addition to the demonstration of a general requirement for a bivalent metal ion in class II aldolases (Rutter, 1964; Kobes *et al.*, 1969), the function of this metal ion in the active site may be localized to the initial stages of the above reaction sequence, that is in the formation of the dihydroxyacetone phosphate carbanion. Stimulation of the rate of fructose 1,6-diphosphate cleavage by added K^+ ion, at an optimum concentration in the region of 100 mM, is considered a characteristic of class II aldolases, and 2–35-fold activation has been observed for more than ten enzymes from a wide variety of sources (Rutter, 1964). In yeast aldolase, added K^+ ion is found to enhance the cleavage rate to a similar extent for five different active metal forms, even though the absolute V_{max} values for these forms differ by up to a factor of 10 (Kobes *et al.*, 1969). These data imply that both K^+ ion and the bivalent metal ion present must independently affect the same rate-limiting step. K^+ ion in yeast aldolase has been shown to enhance the cleavage rate by increasing the rate of carbanion formation (Riordan & Christen, 1969). The rate-limiting step in yeast aldolase, and indeed in most class II aldolases, at least at pH values of optimum activity in the region of 7.0 to 7.5, is therefore carbanion formation, a proposal consistent

with other data on this enzyme (Biellmann *et al.*, 1969). That the nature of the bivalent metal ion affects the rate of this step is consistent with its proposed role in these enzymes as an electrophile polarizing the carbon-carbon bond through the carbonyl group of the dihydroxyacetone phosphate moiety of the substrate (Rutter, 1964; Mildvan *et al.*, 1971).

At pH values outside this range, however, steps in the reaction sequence after carbanion formation, which are likely to be independent of the bivalent metal ion, can become rate-limiting. These are protonation of the dihydroxyacetone phosphate carbanion or release of product from the enzyme. A possible test for such a rate-limiting step is stimulation of the cleavage rate by added aldehyde, such as acetaldehyde. The presence of another aldehyde in the system (in addition to glyceraldehyde 3-phosphate) may result in a thermodynamically favoured condensation reaction between the dihydroxyacetone phosphate carbanion and aldehyde at a rate higher than the rate-limiting step of substrate cleavage. In such a case, a rate enhancement will be observed, provided that an assay is used which does not measure dihydroxyacetone phosphate production alone (e.g. assays A or C, but not assay B). This effect has been seen with carboxypeptidase-treated rabbit muscle aldolase, where protonation of the dihydroxyacetone phosphate carbanion is rate-limiting (Rose *et al.*, 1965) and also with the class II aldolase from *Fusarium oxysporum* (Ingram, 1967; Ingram & Hochster, 1967), where an 8-fold stimulation of rate is observed at pH 7.5 with added K^+ ion, whereas added acetaldehyde is without effect. At pH 8.5, however, acetaldehyde stimulation is observed, indicating a change of rate-limiting step with pH.

As stimulation by K^+ ion is considered a characteristic of class II aldolases, and as it has been reported that the zinc form of *B. stearothermophilus* aldolase is also stimulated by K^+ ion at pH 8.5 (Sugimoto & Nosoh, 1971), many experiments, some reported in the present paper, were carried out as a routine in the presence of 100 mM- K^+ ion. However, some experiments with the cobalt form of this enzyme suggested that its effects were small or negligible, and so a full investigation was carried out in this system. We find, in fact, that added K^+ ion has no effect at any pH between 6.5 and 8.5 on the cleavage rate of any of the active metal forms of *B. stearothermophilus* aldolase, and this aldolase is, to our knowledge, the first class II enzyme for which this phenomenon has not been observed. Further, we show that the variation of V_{max} with pH is very similar for the zinc, cobalt and manganese forms of this enzyme, the absolute values of V_{max} differing by less than a factor of 2 over the pH range studied. These data, combined with the lack of K^+ ion stimulation for any of the four metal forms, imply that bivalent metal-ion-dependent carbon-carbon bond cleavage is never the rate-limiting step

in this thermophilic aldolase. This view is further substantiated by the variation of V_{max} with temperature at pH 7.5, in which the increasing rates are independent of added K^+ ion, and are closely parallel for both zinc and cobalt forms, implying a metal-independent rate-limiting step over the whole temperature range studied. In addition we find a rate enhancement on addition of acetaldehyde for the zinc, cobalt and manganese forms at both pH 7.5 and 8.5, suggesting that even at pH 7.5 carbanion protonation or loss of dihydroxyacetone phosphate is rate-limiting. It should be pointed out that only a positive stimulation with added aldehyde is a conclusive result. Lack of stimulation may merely imply that the condensation reaction, if it occurs at all, proceeds at a lower rate than the rate-limiting step of substrate cleavage. Bearing in mind that the observed rate enhancements are small (Table 2), the lack of stimulation of the cadmium enzyme, or any of the four metal forms at lower pH values, is inconclusive.

Interestingly, when assayed in 50 mM-Tris buffer at pH 8.5 under conditions similar to those where the zinc form of *B. stearothermophilus* aldolase is reported to be stimulated by added K^+ ion (Sugimoto & Nosoh, 1971) we find that the activity of all four metal forms is inhibited (Table 2). Further, when K^+ ion is now included in the assay system, the activity is raised to a value comparable with that in a neutral buffer in the absence of K^+ ion at the same pH (Table 2). Tris is a strong base and is known to interact with the active-site metal of metalloenzymes (Vallee & Wacker, 1970), and should be used with care in such systems. It is likely that this buffer interacts with the metal at the active centre, causing metal-dependent carbon-carbon bond cleavage to become rate-limiting. Under such conditions the expected K^+ ion stimulation occurs, restoring the original rate-limiting step. In line with these observations, the reported V_{max} and K_m for zinc *B. stearothermophilus* aldolase in Tris buffer (Sugimoto & Nosoh, 1971) are some 10-fold lower and 40-fold higher respectively than we observe under optimum conditions for the zinc form at the same pH.

Rabbit muscle aldolase has a substrate K_m of about 1–10 μ M, and a turnover number of about 10–15 μ mol of fructose 1,6-diphosphate cleaved/min per mg of enzyme (Spolter *et al.*, 1965; Lai & Horecker, 1972). In general, class II aldolases are less efficient than their class I counterparts by virtue of their higher K_m values (typically 10^{-4} M or higher; see Table 1) and low turnover numbers in the absence of K^+ ion, when the true catalytic ability of the active-site metal ion is usually seen [some 3–7 μ mol of fructose 1,6-diphosphate/min per mg for aldolases from yeast (Richards & Rutter, 1961) and *Fusarium oxysporum* (Ingram & Hochster, 1967)].

In the present paper we show that *B. stearothermophilus* aldolase is an atypical class II aldolase. It has

no thiol requirement (Jack, 1973), unlike other class II enzymes (Rutter, 1965), shows no K^+ ion stimulation, and its active metal forms are efficient at carbon-carbon bond cleavage, with metal-independent turnover numbers approaching $30 \mu\text{mol}$ of fructose 1,6-diphosphate cleaved/min per mg for the cobalt or cadmium forms, whereas K_m values under optimum conditions, in the absence of K^+ ion, may be as low as $8 \mu\text{M}$, and thus directly comparable with values for class I enzyme. In those zinc metalloenzymes where metal-replacement studies have been carried out, it is found that substitution of cadmium for zinc generally results in total loss of activity. Even in carboxypeptidase A, where the metal ion is thought to polarize the carbonyl oxygen of the peptide (Quiocho & Lipscomb, 1971) and which, along with class II aldolases, shows the most flexibility in such metal substitutions, peptidase activity is completely abolished. Further, Cd(II) yeast aldolase is reported to be inactive (Kobes *et al.*, 1969). Such results are generally rationalized on the basis of the differences in size and preferred co-ordination of zinc and cadmium, and substitution of the latter is assumed to occur with significant configurational distortion of the active-site region (Makinen, 1975). Here we show not only that Cd(II) *B. stearrowthermophilus* aldolase may achieve catalysis, but that at its pH optimum it is in fact the most active form of this enzyme, again demonstrating the uncharacteristic behaviour of this class II enzyme. Nevertheless, the cadmium aldolase differs in kind from the first-row transition-metal forms. Its pH profile differs in shape, and the pH optimum is shifted by about 1 pH unit. Essentially the same results are obtained in either triethanolamine hydrochloride or Hepes buffers, demonstrating that the data are not generated by an artifact of the buffer, such as Cl^- ion.

There is growing evidence, both indirect (Riordan & Christen, 1969; Heron & Caprioli, 1974) and direct (Mildvan *et al.*, 1971) to support the original suggestion (Rutter, 1964) that the bivalent metal ion in class II aldolases causes bond polarization through the carbonyl group of the dihydroxyacetone phosphate moiety of the substrate, thus having a function analogous to the active-site lysine in class I enzymes. Although the essential catalytic or functional role of the metal is beyond doubt, its overall role in these systems is relatively ill-defined. Its influence on substrate-binding or on enzyme structure, for example, is not clear. Here we show that the metal ion in *B. stearrowthermophilus* aldolase is required for tight binding of substrate, but whether this is a direct or indirect effect is not shown by our results. Replacement of zinc by other first-row transition metal ions in yeast aldolase (Kobes *et al.*, 1969) affects V_{max} , but has little effect on K_m (although the data were all obtained at one pH value, and no K_m values were quoted). It has therefore been suggested that, although

the metal is essential for activity, substrate binding is unaffected by the metal species present. Relaxation studies by n.m.r. of aldolase substrates indicate that for the manganese form of yeast aldolase, the phosphorus of dihydroxyacetone phosphate, as well as its carbonyl oxygen, is close to the metal (Mildvan *et al.*, 1971). From these data it was suggested that the metal is involved in the orientation and binding of substrates as well as in catalysis. Our results agree with and enlarge on those for the yeast enzyme, in that the variation of K_m with pH is essentially identical for the zinc, cobalt and manganese forms of *B. stearrowthermophilus* aldolase, and very similar for the cadmium form, although the K_m values of the latter are slightly higher. These data indicate that, although the metal ion is required for tight substrate binding, the nature of the metal exerts little influence on that binding. That is, either the substrate binds in a similar manner to each of the metal ions, or it does not bind significantly to the metal. We favour the former explanation, and consider that the functional role of the metal lies as much in the correct orientation of the substrate as in its ability to act as an electrophile. However, the overall role of the metal is clearly not apparent from these studies, but it is obviously multi-faceted, and its direct study awaits thorough spectroscopic investigation.

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