Role of Mono- and Divalent Metal Cations in the Catalysis by Yeast Aldolase†

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ABSTRACT: The rate of deuterium exchange between [1-(*S*)-^{2}H]dihydroxyacetone 3-phosphate and the solvent catalyzed by native and metal-substituted yeast aldolases has been measured. In the presence of 0.1 M potassium acetate at 15 °C, pH 7.3, the deuterium exchange reaction catalyzed by native yeast aldolase has a k_{cat} of 95 s^{-1}. In contrast to the 7-fold activity enhancement by 0.1 M potassium ion (relative to 0.1 M sodium ion) of the cleavage of D-fructose 1,6-bisphosphate catalyzed by native yeast aldolase, a negligible (1.1-fold) activation by 0.1 M potassium ion is observed in the rate of dedeuteration of [1(*S*)-^{2}H]dihydroxyacetone 3-phosphate. The order of reactivity of the yeast metalloaldolases in the deuterium exchange roughly parallels that seen in the fructose bisphosphate cleavage reaction. These findings suggest that the carbonyl groups of enzyme-bound D-fructose 1,6-bisphosphate and dihydroxyacetone phosphate are both polarized by the active site divalent metal cation. A mechanistic formulation consistent with the results of this and the previous paper is presented.

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) catalyzes the aldol condensation between dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. There are two families of aldolases: the enzymes from bacteria and yeast (class II aldolases) contain a tightly bound divalent metal cation, whereas the enzymes from higher eukaryotes (class I aldolases) have no metal ion requirement, the reaction proceeding via ketimine intermediates formed between the substrates fructose bisphosphate and dihydroxyacetone phosphate and an essential lysine residue (Grazi et al., 1962; Horecker et al., 1963; Model et al., 1968).

Yeast aldolase is prototypical of the class II aldolases. The enzyme has a molecular weight of 80 000 and is composed of two subunits of equal size, each of which is believed to bind one zinc ion that is essential for catalytic activity (Harris et al., 1969). The Zn(II) ions in the native enzyme can be removed and the resulting apoaldolase reconstituted with various divalent metal cations to give a series of metalloaldolases (Kobes et al., 1969). The fructose bisphosphate cleavage activity lost after removal of the Zn(II) ions is regained in increasing amounts when Ni(II), Mn(II), Fe(II), Co(II), and Zn(II) ions are used in the reconstitution. The rate of the cleavage reaction catalyzed by yeast aldolase is also affected by the presence of potassium ion, being increased about 7-fold by potassium ion at 0.1 M (Richards & Rutter, 1961a; Kobes et al., 1969).

It was originally proposed (Rutter, 1964) that the function of the zinc ion in yeast aldolase is to polarize the carbonyl groups of fructose bisphosphate and dihydroxyacetone phosphate by direct coordination to the carbonyl oxygen (Figure 1A). More recently, however, it has been proposed, based on nuclear relaxation rate measurements on the Mn(II) holoenzyme, that the distance between the metal ion and the carbonyl carbon of enzyme-bound dihydroxyacetone phosphate is 7.6 Å, which is too far for inner-sphere metal coordination of the carbonyl oxygen. Nevertheless, it appeared that the oxygen of the carbonyl group of the analogue acetol phosphate is oriented toward the metal ion. Based upon the estimated distances from the Mn(II) ion to the carbonyl oxygens of dihydroxyacetone phosphate and of acetol phosphate, it was suggested that the electrophilicity of the divalent metal cation is transmitted to the substrates by an intervening imidazole (Smith et al., 1980) (Figure 1B). In support of this hypothesis, NMR and chemical inactivation studies have indicated that 3 ± 1 imidazole groups are liganded to the metal ion in the enzyme (Smith & Mildvan, 1981). On this basis, and the fact that both classes of aldolases have been shown to proceed by an ordered reaction sequence in which dihydroxyacetone phosphate binds before glyceraldehyde phosphate (Rose et al., 1965; Hill et al., 1976), Smith et al. (1980) proposed that yeast aldolase follows the mechanism shown in Figure 1B.

There is new evidence, however, that requires the mechanistic model of Figure 1B to be reassessed. If the carbonyl groups of enzyme-bound fructose bisphosphate and dihydroxyacetone phosphate were strongly polarized on binding to the enzyme, a significant decrease in the values of their infrared stretching frequencies relative to those of free substrates in aqueous solution would be expected. For example, it has been shown by Fourier transform infrared spectroscopy that, on binding to triosephosphate isomerase, the carbonyl absorption of dihydroxyacetone phosphate is shifted by 19 cm^{-1} to lower frequency relative to that of the free substrate in aqueous solution, which absorbs at 1732 cm^{-1} (Belasco & Knowles, 1980). This result suggested that the isomerase polarizes the carbonyl group of dihydroxyacetone phosphate, facilitating the required enolization by providing an electrophilic component to the catalysis. In contrast, an analogous infrared spectroscopic investigation of yeast aldolase (Belasco & Knowles, 1983) has revealed a negligible effect (of ≤ 3 cm^{-1}) on the carbonyl stretching frequency of the ketonic substrates when these are bound at the active site. Yet the carbonyl stretching frequency of aldolase-bound glyceraldehyde phosphate is 24 cm^{-1} lower than that for the free substrate in aqueous solution (1730 cm^{-1}). These results demonstrate that the carbonyl group of glyceraldehyde phosphate is polarized by yeast aldolase much more strongly than those of fructose bisphosphate and dihydroxyacetone phosphate. From the infrared data alone, we must conclude that the putative imidazole residue that intervenes between the metal cation and the carbonyl oxygens of the ketonic substrates does not transmit a significant polarizing effect. It must be said, however, that a rather small change in carbonyl stretching frequency may be accompanied by a substantial change in chemical reactivity (Belasco & Knowles, 1983), and it is im-

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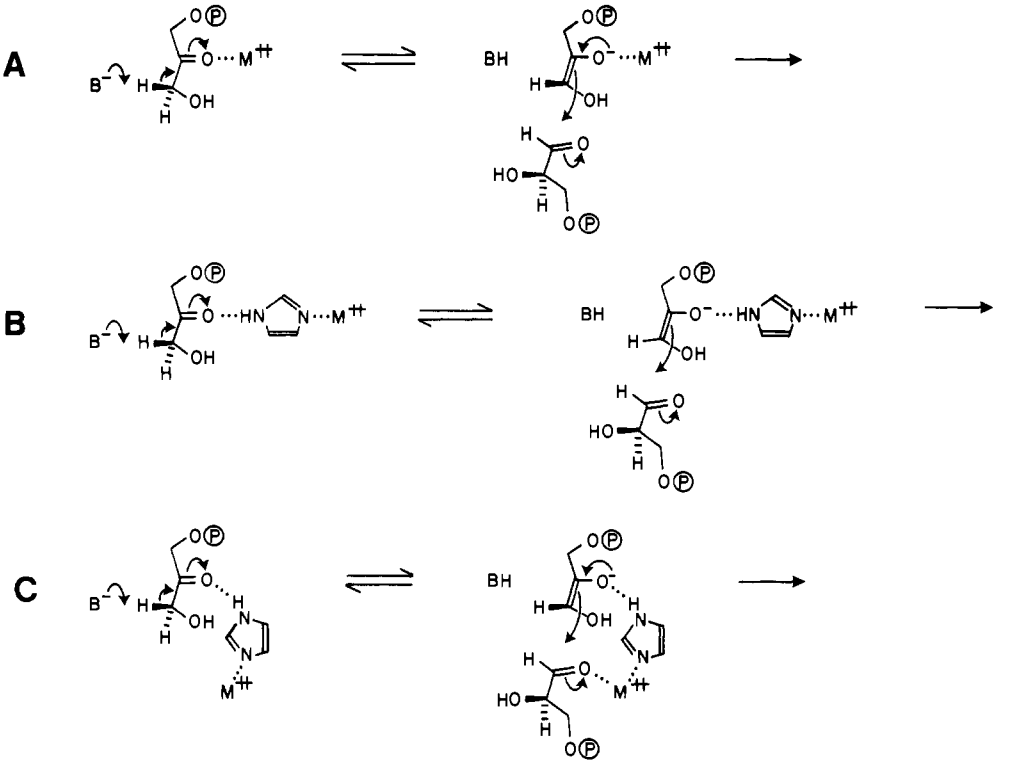


FIGURE 1: Some modes of substrate–metal interaction for yeast aldolase.

portant to relate infrared spectroscopic shifts to catalytic effects. In the present study, we attempt to make this correlation and to clarify the role of both monovalent and divalent cations in the catalysis mediated by yeast aldolase. The kinetic consequence of polarizing the carbonyl group of dihydroxyacetone phosphate has been investigated by studying the dependence of the rate of enolization of this substrate on the nature of the divalent metal cation. Simultaneously, we have followed the dependence of the rate of the fructose bisphosphate cleavage reaction on the nature of the dication, in order to probe the catalytic importance of the significant polarization of the carbonyl group of glyceraldehyde phosphate that is observed spectroscopically.

Materials and Methods

Aldolase (from bakers' yeast) was prepared as described in Belasco & Knowles (1983), following the procedure devised by Jack (1973). The enzyme was >95% homogeneous by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate and had a specific catalytic activity of 150 units/mg. An enzyme unit is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of fructose bisphosphate per min, under the assay conditions of Belasco & Knowles (1983). The concentration of solutions of yeast aldolase was determined by assuming $A^{0.1\%}_{280nm} = 1.02$ (Harris et al., 1969). Triosephosphate isomerase from chicken muscle was prepared by Dr. J. G. Belasco, according to Putman et al. (1972) and McVittie et al. (1972). Dihydroxyacetone phosphate as the bis(cyclohexylammonium) salt of the dimethyl ketal was either purchased from Sigma Chemical Co. (St. Louis, MO) or synthesized by R. T. Raines following the method of Ballou (1960). [1(*S*)-^{2}H]Dihydroxyacetone phosphate was prepared by using rabbit muscle aldolase (from which traces of triosephosphate isomerase activity had been removed by treatment with bromohydroxyacetone phosphate; de la Mare et al., 1972) in ^2H_2O by the method of Belasco & Knowles (1980) and was stored as the monoanion (pH 4.5). Aldolase (from rabbit muscle, as a crystalline suspension in ammonium sulfate), α-glycerophosphate dehydrogenase (from rabbit muscle, as a crystalline suspension in ammonium sulfate), D-fructose 1,6-bisphosphate (as the trisodium salt), reduced nicotinamide adenine dinucleotide (NADH)[1] (as the disodium salt), Sephadex G-25 (20–80-μm bead size), Sephadex G-150 (40–120-μm bead size), and Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked) were obtained from Sigma. Chelex-100 (Na^+ form, 200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA). DEAE-cellulose (DE-52) was obtained from Whatman, Inc. (Clifton, NJ). Deuterium oxide (99.7%) was the product of Merck Sharp & Dohme (Rahway, NJ). Yeast apoaldolase was reconstituted with solutions of Puratronic grade (Johnson Matthey Chemicals Ltd.; Royston, Herts., England) metal sulfate salts acquired from Alfa Products (Danvers, MA). The 2-mercaptoethanol used in the metal-free experiments was the highest purity available (less than 0.00001% Zn) and was purchased from Tridom Chemical Inc. (Hauppauge, NY).

Ultraviolet measurements were made on a Perkin-Elmer 554 spectrophotometer. Mass spectra were obtained on either a Kratos MS-50 or an AEI MS-9 instrument. Samples were introduced by direct probe insertion, and spectra were taken at a source temperature of 100 °C, an ionizing energy of 60 eV, and a pressure of 10^{-6} torr.

Dihydroxyacetone phosphate was assayed by reduction to *sn*-glycerol 3-phosphate by using α-glycerophosphate dehydrogenase/NADH. The reaction was initiated by the addition of dihydroxyacetone phosphate to a solution of 100 mM Hepes (NH_4^+ salt) buffer, pH 7.3, containing potassium acetate (100 μmol), α-glycerophosphate dehydrogenase (75 μg), and NADH (0.3 μmol) in a total volume of 1.035 mL. The change in the absorbance at 340 nm was recorded. Fructose bisphosphate was assayed by its conversion to 2 equiv

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl.

Table I: Fructose Bisphosphate Cleavage Rates Catalyzed by Various Metalloaldolases[a]

enzyme	k_{cat}[b,c] (s^{-1})	rel act.[c] (%)	metal ion equiv[d]	Zn(II) substitution rate[e]	k_{cat}[b,f] (s^{-1})	rel act.[f] (%)
native	34	91			12	95
apoenzyme	<0.18	<0.5		fast	0.04	0.3
Zn(II)	38	100	200		13	100
Co(II)	19	51	90	slow	9.7	75
Fe(II)	14	38	200	medium	8.1	63
Mn(II)	5.4[g]	14	>1800	fast	2.1	16
Ni(II)	<0.18	<0.5	(10-1000)	slow	1.2	9
Cu(II)	<0.18	<0.5	(10-530)	medium	<0.04[h]	<2[h]
Mg(II)	<0.18	<0.5	(10-1000)	fast	<0.04[h]	<2[h]

[a] Assays were performed as described under Materials and Methods. [b] Assuming a subunit molecular weight of 40 000 and an $A^{0.1\%}_{280nm}$ of 1.02. [c] This work. The estimated precision of the rate constants is ±2%. [d] The number of metal ion equivalents required per aldolase subunit for maximal activity. The numbers in parentheses show the range of metal ion equivalents added to inactive holoenzymes. [e] Zn(II) (200 equiv) was added to the metalloaldolase, and the rate of activity regain was measured: fast, 100% activity within 1 min; medium, >25% activity within 5 min; slow, <10% activity after 5 min. [f] Data of Kobes et al. (1969). [g] Extrapolated value; see Results. [h] In the absence of potassium ion.

of triose phosphate catalyzed by aldolase and triosephosphate isomerase, and the subsequent reduction of the dihydroxyacetone phosphate to *sn*-glycerol 3-phosphate by α-glycerophosphate dehydrogenase/NADH. The reaction was performed under conditions identical with those of the dihydroxyacetone phosphate assay except that rabbit muscle aldolase (100 μg) and triosephosphate isomerase (3 μg) were also present.

In all operations involving the preparation and handling of apo- and metalloaldolases, metal-free equipment and solutions were used. Metal-free water was prepared from distilled, deionized, and glass-redistilled water by passage through a column (1.6 cm × 18 cm) of Chelex-100 (NH_4^+ form). Glassware was soaked for a minimum of 24 h in concentrated sulfuric acid/concentrated nitric acid (1:1 v/v) and then rinsed thoroughly with metal-free water. Plasticware was soaked in Na_4EDTA (10 mM) for at least 24 h and then thoroughly rinsed with metal-free water. All aqueous solutions (buffers, substrates, and coupling enzymes) were passed through a column (1.6 cm × 12 cm or 0.5 cm × 3.5 cm) of Chelex-100 (NH_4^+ form) before use. The pH of metal-free buffer solutions was adjusted with metal-free aqueous ammonia, which was prepared by precipitating metallic impurities in liquid ammonia with sodium metal and then bubbling the ammonia gas evolved into metal-free water.

Yeast apoaldolase was prepared by a modification of the method of Kobes et al. (1969). The Zn(II) was removed with EDTA, and the apoprotein was separated from the chelating agent with a layered Chelex-100/Sephadex G-25/Chelex-100 column. All operations were performed at 4 °C.

A sandwich column (1.0 cm × 50 cm) was prepared with Chelex-100 (NH_4^+ form; 1.0 cm × 2.0 cm) in the bottom layer, Sephadex G-25 (1.0 cm × 41 cm) in the middle layer, and Chelex-100 (NH_4^+ form; 1.0 cm × 7.0 cm) in the top layer. The column was washed successively with 10 mM Na_4EDTA (300 mL), metal-free water (2 L), and then 25 mM Hepes (NH_4^+ salt) buffer, pH 7.3 (500 mL). Native yeast aldolase (approximately 5 mg) was concentrated in an Amicon Centriflo centrifugation membrane cone to about 0.25 mL and was then added to 100 mM EDTA at pH 7.5 (0.5 mL). This solution was stirred gently for 30 min. The mixture was then applied to the sandwich column described above, and the column was eluted with 25 mM Hepes (NH_4^+ salt) buffer, pH 7.3. Protein concentrations were determined by the absorbance at 280 nm. The fractions with the highest concentrations of apoaldolase were pooled, diluted with 0.5 volume of 25 mM Hepes (NH_4^+ salt) buffer, pH 7.3, containing glycerol (60% v/v), and then stored at −70 °C. The apoaldolase was stable for several months under these conditions. No regain of activity due to metal ion contamination could be detected, and the apoprotein could be completely reconstituted by the addition of Zn(II).

Measurement of the Fructose Bisphosphate Cleavage Rate of Yeast Metalloaldolases. The effect of metal substitution in yeast aldolase upon the catalysis of the cleavage reaction was studied under conditions similar to those used by Kobes et al. (1969). The absorbance of a solution (1.070 mL) of apoaldolase (typically 0.44 μg) in 25 mM Hepes (NH_4^+ salt) buffer, pH 7.3, containing potassium acetate (100 μmol), triosephosphate isomerase (3.75 μg), α-glycerophosphate dehydrogenase (62.5 μg), fructose bisphosphate (2.1 μmol), and NADH (0.3 μmol) at 15 °C was monitored at 340 nm for 6 min. If there was no detectable decrease (corresponding to a k_{cat} of less than 0.2 s^{-1}), it was concluded that divalent metal ion contamination was negligible. A portion (10 μL) of a Puratronic metal salt solution was then added, and the rate of decrease of the absorbance at 340 nm was recorded. Toward the end of the assay, zinc sulfate (10 μL, containing 2.2 × 10^{-9} mol) was added, to see if Zn(II) ion could displace the enzyme-bound metal cation. The results are presented in Table I.

Preparation of Tetrakis(trimethylsilyl)-sn-glycerol 3-Phosphate from Dihydroxyacetone Phosphate. Dihydroxyacetone phosphate (4.0 μmol) was added to a solution (0.4 mL) of 20 mM triethanolammonium acetate buffer, pH 7.5, containing NADH (10 μmol) and α-glycerophosphate dehydrogenase (50 units). The mixture was allowed to stand for 10 min and was then acidified with Dowex 50 (H^+) (1 mL). The slurry was filtered, and the filtrate was passed through charcoal to remove nucleotides. The filtrate was reduced to dryness on a rotary evaporator and then subjected to high vacuum (0.1 torr) for 10 min. To the resulting colorless film was added triethylamine (50 μL, 360 μmol; distilled from CaH_2) containing 4-(*N*,*N*-dimethylamino)pyridine (100 μg, 1 μmol) as a catalyst, and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (50 μL, 190 μmol) containing 1% trimethylchlorosilane. The silylation reaction was instantaneous at room temperature. The resulting tetrakis(trimethylsilyl)-*sn*-glycerol 3-phosphate was immediately analyzed on a mass spectrometer. Relative peak intensity values were obtained by averaging the data from 5 to 10 slow scans of the mass spectrum.

Dedeuteration Rate Assays. (A) Native Yeast Aldolase. To a solution (1.065 mL final volume) at 15 °C of 100 mM Hepes (NH_4^+ salt) buffer, pH 7.3, containing potassium acetate (100 μmol), zinc sulfate (0.21 nmol), and [1(*S*)-^{2}H]dihydroxyacetone phosphate (4.0 μmol) was added yeast

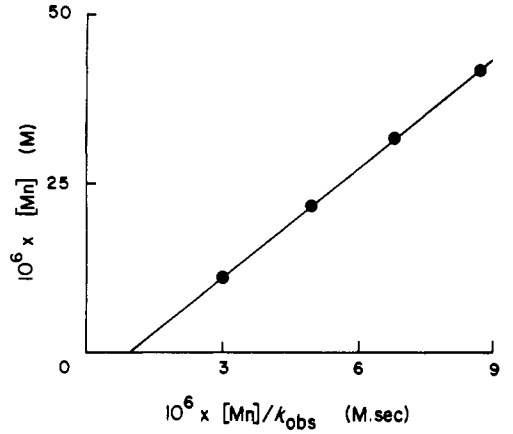


FIGURE 2: Plot of $[Mn]/k_{obsd}$ vs. [Mn] for the activity of yeast apoaldolase in the fructose bisphosphate cleavage reaction in the presence of varying concentrations of Mn(II) ion. k_{obsd} is the observed first-order rate constant, and [Mn] is the concentration of Mn(II).

aldolase (about 4 μg). After the solution was mixed, reaction samples were quenched at time intervals (0, 5, or 10 min) into a solution (0.4 mL) of 20 mM triethanolammonium acetate buffer, pH 7.5, containing NADH (10 μmol) and α-glycerophosphate dehydrogenase (100 units). The mixture was allowed to stand at room temperature for 10 min, and the resulting *sn*-glycerol 3-phosphate was isolated, silylated, and analyzed on a mass spectrometer as described above.

(*B*) *Metalloaldolases.* To a solution (1.065 mL final volume) of 100 mM Hepes (NH_4^+ salt) buffer, pH 7.3, containing potassium acetate (100 μmol), a Puratronic metal salt (typically 2.1–4.2 nmol), and yeast apoaldolase (about 3.1 μg) was added [1(*S*)-^{2}H]dihydroxyacetone phosphate (4.0 μmol). After the solution was mixed, reaction samples were quenched at time intervals (0, 5, or 10 min) into a solution (0.4 mL) of 20 mM triethanolammonium acetate buffer, pH 7.5, containing NADH (10 μmol) and α-glycerophosphate dehydrogenase (150 units). The mixture was allowed to stand at room temperature for 10 min, and the resulting *sn*-glycerol 3-phosphate was isolated, silylated, and analyzed on a mass spectrometer as described above.

Results

The effect of metal substitution upon the catalysis by yeast aldolase of the fructose bisphosphate cleavage reaction was studied under conditions identical with those used by Kobes et al. (1969), except that the rate measurements in the present work were made at pH 7.3, the pH optimum (Richards & Rutter, 1961b), rather than at pH 7.0. The results are presented in Table I. The relatively loose binding of Mn(II) to the apoenzyme precludes direct measurement of the specific activity of the Mn(II) holoaldolase, which was therefore determined from the dependence of the observed rate upon the Mn(II) concentration. Assuming that each active site independently binds one divalent metal cation, then if the concentration of Mn(II) is much greater than that of enzyme subunits, we have

$$[Mn] = k([Mn]/k_{obsd}) - K \quad (1)$$

where k is the k_{cat} (in s^{-1}) for the cleavage reaction catalyzed by the Mn(II) holoaldolase, [Mn] is the molar concentration of Mn(II), k_{obsd} is the observed k_{cat} (in s^{-1}) at a given [Mn], and K is the dissociation constant (in molar units) of Mn(II) from the catalytic site of an aldolase subunit. Figure 2 shows a graph of [Mn] vs. $[Mn]/k_{obsd}$ ($r^2 = 0.9998$), from which the specific activity, $k = 5.4\ s^{-1}$, and the dissociation constant, $K = 5.3 \times 10^{-6}$ M, can be derived.

The Co(II) holoaldolase was inert to Zn(II) substitution in the absence of any exogenous metal chelating species. To eliminate the remote possibility that addition of Co(II) results in the irreversible partial inactivation of the enzyme (e.g., by the oxidation of neighboring active site sulfhydryl groups), we removed the Co(II) in the holoenzyme with EDTA and replaced it with Zn(II). Full catalytic activity [of the Zn(II) holoenzyme] was regained. Under the conditions of the cleavage assay, there was no significant inhibition of either of the coupling enzymes, triosephosphate isomerase and α-glycerophosphate dehydrogenase, by the metal ions used. In accord with the findings of Richards & Rutter (1961a), a 7-fold enhancement of the cleavage activity of native yeast aldolase was observed in assays conducted in the presence of 0.1 M potassium acetate relative to those performed in 0.1 M sodium acetate.

The deuterium content of [1(*S*)-^{2}H]dihydroxyacetone phosphate was measured by mass spectrometry of tetrakis-(trimethylsilyl)-*sn*-[1(*S*)-^{2}H]glycerol 3-phosphate (Duncan et al., 1971; Harvey et al., 1972), which was prepared by silylation of the product from the reduction of [1(*S*)-^{2}H]dihydroxyacetone phosphate with NADH and α-glycerophosphate dehydrogenase. [An important feature of our analysis of deuterated dihydroxyacetone phosphate is the silylation reaction of *sn*-glycerol-3-phosphate. The conditions previously used have involved reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide in pyridine at room temperature for 1 h. We have found, however, that the reaction, in the presence of triethylamine instead of pyridine, is catalyzed by 4-(*N,N*-dimethylamino)pyridine. At room temperature, the silylation reaction is instantaneous, and the resulting tetrakis(trimethylsilyl) derivative is stable for about 6 h.] Since deuteration of dihydroxyacetone phosphate by the rabbit muscle aldolase results in deuterium incorporation at the *pro-S* position of C-3 as well as the *pro-S* position of C-1 (Lowe & Pratt, 1976; Galdes & Hill, 1978), it was important to determine the extent of deuterium substitution at both C-1 and C-3. This was done by measuring the relative peak intensities at $(m/z\ 446)/(m/z\ 445)$ ($M^+ - \cdot CH_3$) and $(m/z\ 358)/(m/z\ 357)$ ($M^+ - \cdot CH_2OSiMe_3$). The cleavage between C-1 and C-2 was shown to occur without hydrogen migration [see also Curstedt (1974)].

The rate of deuterium exchange between [1(*S*)-^{2}H]dihydroxyacetone phosphate and the solvent catalyzed by yeast aldolase was determined by monitoring the rate of loss of deuterium from the substrate upon incubation with the enzyme. The time course of the dedeuteration reaction followed the equation

$$\ln (x_0/x_t) = (k_x[E]/[S])t \quad (2)$$

where x_0 is the mole fraction of deuterium above natural isotopic abundance at the *pro-S* position of C-1 of [1(*S*)-^{2}H]dihydroxyacetone phosphate at time 0, x_t is the mole fraction of deuterium above natural isotopic abundance at the *pro-S* position of C-1 of [1(*S*)-^{2}H]dihydroxyacetone phosphate at time t, k_x is the first-order rate constant for the deuterium exchange reaction (in s^{-1}), [E] is the millinormal concentration of enzyme subunits, [S] is the total dihydroxyacetone phosphate species present in units of millimolar, and t is the time in seconds. In a typical assay, x_0 and x_t were measured by mass spectroscopy of tetrakis(trimethylsilyl)-*sn*-[1(*S*)-^{2}H]-glycerol 3-phosphate samples prepared from dedeuteration reaction mixtures, and the exchange activity, k_x, was determined by least-squares analysis of plots of $\ln (x_0/x_t)$ vs. t. A typical plot from a dedeuteration rate assay is shown in Figure 3. Under the conditions of the assay, spontaneous (nonen-

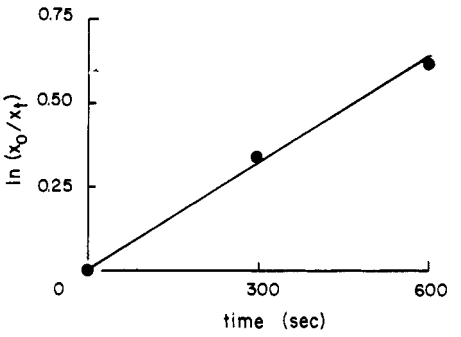


FIGURE 3: Dedeuteration of [1(*S*)-^{2}H]dihydroxyacetone 3-phosphate catalyzed by native yeast aldolase. x is the mole fraction of deuterium above natural abundance at C-1 of [1(*S*)-^{2}H]dihydroxyacetone phosphate at time zero (x_0) or time t (x_t).

Table II: Deuterium Exchange Rates of Various Metalloaldolases[a]

enzyme	k_{cat}[b] (s^{-1})	rel act. (%)	metal ion equiv[c]
native	95	94	
apoenzyme	<1	<1	
Zn(II)	101	100	30
Co(II)	20.5	20	30
Fe(II)	19.1	19	75
Mn(II)	26.5[d]	26	>500
Ni(II)	<1	<1	(15-150)
Cu(II)	<1	<1	(15-150)
Mg(II)	<1	<1	(15-150)

[a] Assays were performed as described under Materials and Methods. [b] Assuming a subunit molecular weight of 40 000 and an $A^{0.1\%}_{280nm}$ of 1.02. The estimated precision of the rate constants is ±5%. [c] The number of metal ion equivalents required per aldolase subunit for maximal activity. The values in parentheses indicate the range of metal ion equivalents added to inactive holoaldolases. [d] Extrapolated value; see Results.

zymic) washout of deuterium from [1(*S*)-^{2}H]dihydroxyacetone phosphate was not detectable.

Native yeast aldolase in the presence of 0.1 M potassium acetate at 15 °C, pH 7.3, has a first-order rate constant for deuterium exchange of 95 s^{-1}. Substitution of an equivalent amount of sodium acetate for potassium acetate under otherwise identical conditions results in an exchange rate constant of 87 s^{-1}. The activation of the deuterium exchange reaction by potassium ion at 0.1 M, relative to sodium ion at 0.1 M, is therefore 1.1-fold.

The deuterium exchange activities of several yeast metalloaldolases were measured, and the results are presented in Table II. For the Mn(II) holoenzyme, the exchange rate was determined from the dependence of the observed exchange activity upon the Mn(II) concentration. Least-squares analysis ($r^2 = 0.998$) of a plot of [Mn] vs. [Mn]/k_{obsd} (see Figure 4) gave a Mn(II) dissociation constant of 7.2×10^{-6} M and a value for k_x of 26 s^{-1}. K_m values of [1(*S*)-^{2}H]dihydroxyacetone phosphate in the dedeuteration reaction were found for the Zn(II), Co(II), Fe(II), and Mn(II) holoaldolases to lie between 0.5 and 1.0 mM.

Discussion

Rate-Limiting Transition State. The overall cleavage reaction catalyzed by yeast aldolase involves (1) the binding of fructose bisphosphate to the free enzyme, (2) the retroaldol cleavage to a complex containing bound glyceraldehyde phosphate and the anion of dihydroxyacetone phosphate, (3) the loss of glyceraldehyde phosphate, (4) the protonation of dihydroxyacetone phosphate, and (5) the loss of dihydroxyacetone phosphate and the regeneration of free enzyme

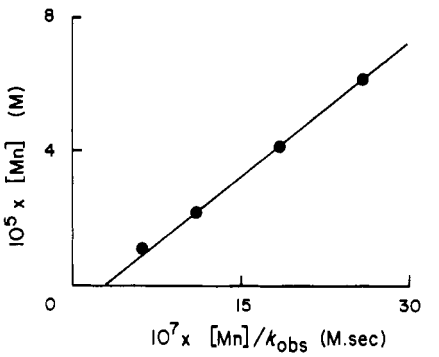
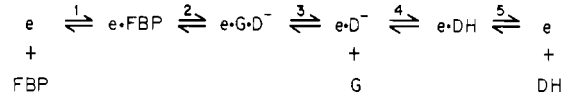


FIGURE 4: Plot of [Mn]/k_{obsd} vs. [Mn] for the activity of yeast apoaldolase in the dedeuteration of [1(*S*)-^{2}H]dihydroxyacetone phosphate in the presence of varying concentrations of Mn(II) ion. k_{obsd} is the observed first-order rate constant, and [Mn] is the concentration of Mn(II).

Scheme I: Reaction Path for Aldolase[a]



[a] FBP, fructose 1,6-bisphosphate; G, D-glyceraldehyde 3-phosphate; D^-, the anion of dihydroxyacetone phosphate; DH, dihydroxyacetone phosphate; e, enzyme.

(Scheme I). Under the normal conditions for the measurement of the catalytic activity of native [Zn(II)] yeast aldolase (pH 7.3, 0.1 M in potassium ion, 15 °C), the enzyme has a k_{cat} for the cleavage of fructose bisphosphate of 34 s^{-1}. Since the cleavage assay measures glyceraldehyde phosphate as it is liberated, the rate of the reaction at saturating levels of fructose bisphosphate must be limited either by steps 2 and 3 or by steps 4 and 5. Now, under similar conditions,[2] the enzyme catalyzes the dedeuteration of [1(*S*)-^{2}H]dihydroxyacetone phosphate with a k_{cat} of 95 s^{-1}. Since steps 4 and 5 must be *at least* as fast as the rate of dedeuteration of [1-(*S*)-^{2}H]dihydroxyacetone phosphate, it follows that the rate-limiting transition state in the cleavage reaction catalyzed by yeast aldolase must be that of step 2 or step 3. This conclusion is in accord with the observation of a secondary tritium kinetic isotope effect in the cleavage reaction of [3-^{3}H]fructose bisphosphate catalyzed by yeast aldolase (Biellmann et al., 1969). It must be emphasized, however, that the rate constants we have determined for the cleavage and exchange processes are not very different and that, as is increasingly being found for enzyme-catalyzed reactions, no single transition state is cleanly rate limiting in the overall reaction under near-physiological conditions of pH, temperature, and ionic strength.

Effect of Divalent Cations on the Cleavage Reaction. The cleavage rates for a variety of yeast metalloaldolases are compared with those reported by Kobes et al. (1969) in Table I. Despite differences in the absolute rates, the order of reactivity observed for the different metalloenzymes, i.e., Zn(II) > Co(II) > Fe(II) > Mn(II) > Ni(II), is identical. [The differences in the absolute values presumably derive from the different pH values used [pH 7.0 vs. pH 7.3: native yeast aldolase has a sharp pH optimum (Richards & Rutter, 1961b)] and the purity of the enzyme used.] The activities of the Co(II), Fe(II), and Mn(II) holoaldolases [relative to the native Zn(II) enzyme] observed in this study were all lower

[2] The cleavage reaction of fructose bisphosphate was performed in 25 mM Hepes buffer instead of 100 mM Hepes. We have shown, however, that this difference in the concentration of Hepes does not affect the rate of the cleavage reaction catalyzed by yeast aldolase.

than the values reported by Kobes and co-workers. In addition, the Ni(II) holoenzyme, which Kobes et al. found to possess 9.4% of the activity of the native Zn(II) enzyme, has been shown in the present work to be inactive. The simplest explanation for these discrepancies is a very small degree of Zn(II) contamination in the earlier experiments.

Effect of Divalent Cations on the Deuterium Exchange Reaction. It was reported by Rose & Rieder (1958) that the catalysis of the detritiation of [1(*S*)-^{3}H]dihydroxyacetone phosphate by yeast aldolase is inhibited by EDTA at 15 mM. We have confirmed this observation and have further shown that yeast *apo*aldolase, in the absence of EDTA, does not catalyze the deuterium exchange reaction. Reconstitution of the inactive apoenzyme with various divalent metal cations gives a series of metalloaldolases that display a range of specific deuterium exchange activities. The participation of the enzyme-bound divalent metal ion in the catalysis of the dedeuteration reaction by yeast aldolase is therefore clear. The nearly parallel order of reactivity of the metalloenzymes in the deuterium exchange and the cleavage reactions is consistent with the view that *the carbonyl groups both of bound fructose bisphosphate and of bound dihydroxyacetone phosphate interact (directly or indirectly) with the active site divalent metal ion.*

The dissociation constant of Mn(II), which evidently binds to yeast apoaldolase much less tightly than the other divalent cations investigated, has been determined from the dependence of the observed enzyme activity upon the concentration of Mn(II). For the cleavage and the dedeuteration reactions, the Mn(II) dissociation constants obtained were 5.3×10^{-6} and 7.2×10^{-6} M, respectively. These values are similar to that of 2.4×10^{-6} M determined by electron paramagnetic resonance (Mildvan et al., 1971). Although the "titration" of apoaldolase with a *stoichiometric* amount of Mn(II) to full catalytic activity of the holoenzyme has been described by Mildvan et al. (1971), saturation of aldolase with 1 equiv of Mn(II) is impossible at the concentrations of enzyme (0.36 μN) used in that work, and it seems likely that the reported titration is in error.

Effect of Potassium Ion. It has been well established that the rate of the cleavage reaction catalyzed by yeast aldolase is enhanced about 7-fold by 0.1 M potassium ion (relative to 0.1 M sodium ion). In contrast, we show here that the effect of potassium ion on the deuterium exchange reaction is negligible (1.1-fold). This indicates that the effect of potassium ion on the catalytic activity of yeast aldolase is directed mainly toward the transition state for step 2 and/or step 3 (Scheme I).

If we assume that the rate constant, k_1, for the formation of the enzyme–dihydroxyacetone phosphate complex from free enzyme and free substrate is about 5×10^8 M^{-1} s^{-1} [this appears to be the rate of a diffusion-limited process involving a protein and a relatively small substrate molecule (Hammes & Schimmel, 1970)], and that the dissociation constant of dihydroxyacetone phosphate from aldolase, K_s, is equal to the K_m of the labeled substrate in the deuterium exchange reaction (this is approximately 5×10^{-4} M), then the rate constant, k_{-1}, for the release of dihydroxyacetone phosphate from the enzyme–substrate complex ($k_{-1} = K_s k_1$) will be about 2.5×10^5 s^{-1}. Comparison of this estimate of k_{-1} with the k_{cat} for the dedeuteration reaction of 95 s^{-1} reveals that k_{-1} is much greater than the k_{cat} for isotope exchange. We may therefore conclude that there is essentially no potassium ion activation of the dedeuteration reaction (step 4, Scheme I) catalyzed by yeast aldolase and that the release of dihydroxyacetone phosphate from the enzyme–substrate complex (step 5) is not the slowest step in the isotope exchange reaction.

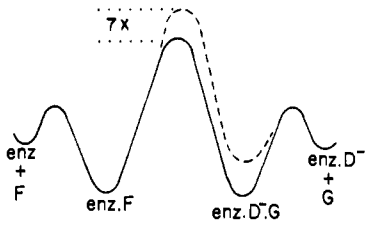


FIGURE 5: Free-energy profile illustrating the effect of potassium ion on the reaction catalyzed by yeast aldolase. enz is aldolase, F is fructose bisphosphate, D^- is the anion of dihydroxyacetone phosphate, and G is glyceraldehyde phosphate. Solid line, with potassium ion; dashed line, sodium ion replaces potassium ion.

In contrast to the results discussed above, a 2.5–6-fold activation by 0.1 M potassium acetate of the *detritiation* reaction of [1(*S*)-^{3}H]dihydroxyacetone phosphate catalyzed by yeast aldolase has been reported by Richards & Rutter (1961b). Since potassium ion is not expected to affect the isotope exchange reactions of [1(*S*)-^{2}H]- and [1(*S*)-^{3}H]dihydroxyacetone phosphate differentially, an alternative explanation was sought to resolve the discrepancy between our data and the earlier results. It was considered that the "activation" observed earlier may have derived from differences in ionic strength (sodium ion was not used as a reference in the previous study), and from ion effects on K_m, since Richards and Rutter used a lower substrate concentration in their experiments (1.3 mM vs. 3.7 mM). Both possibilities were investigated. The dedeuteration reaction at 3.7 mM [1(*S*)-^{2}H]dihydroxyacetone phosphate was found to proceed at a rate 1.4 times faster in assays containing 0.1 M potassium acetate than in those to which neither potassium acetate nor sodium acetate was added. Moreover, at 1.4 mM substrate, a 1.4-fold enhancement of the dedeuteration rate was observed in the presence of potassium acetate at 0.1 M relative to sodium acetate at 0.1 M. Presumably these effects are multiplicative, and the combined activation becomes $1.1 \times 1.4 \times 1.4 = 2.2$, which is near to the value of 2.5 found by Richards & Rutter (1961b) at pH 7.3.

Function of the Metal Ions. The 7-fold activation by potassium ion relative to sodium ion in the catalysis of the cleavage reaction by yeast aldolase is not observed in the "partial" reaction, the dedeuteration of [1(*S*)-^{2}H]dihydroxyacetone phosphate. The presence of potassium ion evidently accelerates step 2 and/or step 3 (Scheme I), that is, either the C–C bond cleavage or the release of glyceraldehyde phosphate from the enzyme. This phenomenon can be related to the effects of potassium noted in the preceding paper (Belasco & Knowles, 1983). From the infrared spectrum of fructose bisphosphate plus aldolase in the presence of potassium ion, we observe carbonyl absorption bands due to both the ketonic and the aldehyde substrates. With the assumption that the extinction coefficients of the bound species are not very different, this means that the free energies of the complexes of enzyme with fructose bisphosphate and with the two triose phosphates are about the same. In the absence of potassium, however, only the enzyme complex with fructose bisphosphate is seen, the internal equilibrium constant disfavoring the bound triose phosphates under these conditions (Belasco & Knowles, 1983). We have also shown that replacement of potassium ion by sodium ion slows the cleavage reaction by 7-fold, and these results can all be accommodated by the change in the energetics of the cleavage reaction illustrated in Figure 5. The primary effect of potassium ion appears to be to stabilize the complex of enzyme with the triose phosphates relative to the complex with fructose bisphosphate, with the concomitant

lowering of the free-energy barrier for the interconversion of these central complexes (step 2). [An analogous argument can be made if the "off" step for glyceraldehyde phosphate (step 3) is rate limiting for the cleavage reaction.] Additionally, in the presence of potassium (but not in its absence), a new infrared absorption band at 1748 cm^{-1} appears in the spectra of enzyme-bound fructose bisphosphate, dihydroxyacetone phosphate, or phosphoglycolohydroxamate, and this band has been tentatively ascribed to an enzymic carboxyl group in a nonpolar environment. How this change relates to the observed catalytic effect of potassium ion must await further experiments.

In contrast to the above, both the cleavage *and* the deuterium exchange activities of the metalloaldolases exhibit a strong dependence upon the enzyme-bound *di*valent metal cation. The effect of potassium ion on the catalysis of yeast aldolase is therefore independent of the action of the divalent metal cation. [In accord with this conclusion, Kobes et al. (1969) have shown that the cleavage rates of the Zn(II), Co(II), Fe(II), and Mn(II) metalloenzymes are all stimulated 6–7-fold by the presence of potassium ion.] Since the essential divalent cation affects both the cleavage and the isotope exchange reactions (indeed, the apoenzyme is inactive in both assays), the carbonyl group oxygens of both the ketonic substrates evidently sense (and are presumably somewhat polarized by) the cation. However, any polarization that does occur is not seen in the infrared spectra of these substrates, the carbonyl stretching frequencies of which are shifted negligibly on binding to the enzyme (Belasco & Knowles, 1983). It has been earlier noted, however, that a small change in carbonyl stretching frequency can correspond to a significant difference in chemical reactivity, and the arrangement suggested by Smith et al. (1980), where the electrophilicity of the divalent metal cation is transmitted to the carbonyl group of the ketonic substrates by an intervening imidazole ligand, could accommodate the observed results. Whether or not the divalent cation affects the carbonyl group of enzyme-bound glyceraldehyde phosphate cannot be determined from the kinetic results, though polarization of the carbonyl oxygen of this substrate by the metal (Figure 1C) is certainly consistent with the dramatic decrease (of 24 cm^{-1}) in the stretching frequency of glyceraldehyde phosphate upon binding to the enzyme (Belasco & Knowles, 1983). [This model would, however, require some reassessment of the absolute values of the metal–substrate distances reported by Smith et al. (1980).] Whatever the mechanism, strong polarization of glyceraldehyde phosphate has the attractive feature that the step in the overall reaction that we expect would be the slowest, that in which the carbon–carbon bond is made or broken, receives the maximum catalytic benefit from the enzyme.

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Registry No. Zn, 7440-66-6; Co, 7440-48-4; Fe, 7439-89-6; Mn, 7439-96-5; Ni, 7440-02-0; Cu, 7440-50-8; Mg, 7439-95-4; K, 7440-09-7; D-fructose 1,6-bisphosphate, 488-69-7; aldolase, 9024-52-6; [1(*S*)-^{2}H]dihydroxyacetone 3-phosphate, 83605-98-5; tetrakis(trimethylsilyl)-*sn*-glycerol 3-phosphate, 29881-28-5.

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