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A Functional Role of Metal Ions in a Class II Aldolase*

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ABSTRACT: A crucial role of the metal ion in the catalytic activity of yeast aldolase has been demonstrated. Zinc can be removed from the native enzyme resulting in an inactive apoenzyme. The activity can be reconstituted by addition of Zn^{2+} or by certain ions of the first transition period, namely Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+}. The Zn^{2+} protein has the highest specific activity; the other metal ions restore the enzymatic activity to varying degrees.

A number of other metal ions (Cu^{2+}, Hg^{2+}, Cd^{2+}, Mg^{2+}, and Fe^{3+}) fail to restore activity to any measurable extent. All active metalloaldolases exhibit similar K_m values for fructose diphosphate and are stimulated by K^+ ion.

Studies on fructose diphosphate aldolases from phylogenetically divergent organisms suggest that these enzymes can be grouped into two distinct classes (Rutter, 1964). The class I enzymes are found in animals, plants, protozoa, and algae. Rabbit muscle aldolase, the prototype of the class I enzymes, has a molecular weight of 160,000, a tetrameric structure (Kawahara and Tanford, 1966; Penhoet *et al.*, 1966, 1967), and a mechanism which involves Schiff-base formation between dihydroxyacetone phosphate and a lysyl residue on the protein molecule (Grazi *et al.*, 1962; Horecker *et al.*, 1963; Model *et al.*, 1968).

Class II aldolases are found in most bacteria, fungi, yeast, and blue-green algae (Rutter, 1964). Yeast aldolase, the prototype of the class II enzymes, has a molecular weight of approximately 80,000, a dimeric structure (C. E. Harris, R. D. Kobes, D. C. Teller, and W. J. Rutter, in preparation), and is activated by K^+ ion. Little is known about the catalytic mechanism of the yeast enzyme.

Warburg and Christian (1943) first observed that yeast aldolase was inhibited by chelating agents and proposed that it was activated by a metal ion (presumably Fe^{2+}). Later it was found that significant quantities of zinc, and only traces of iron were present in this enzyme prepared by various methods. Thus, zinc contents of 1800 μg/g of protein (Rutter and Ling, 1958), 1150 μg/g of protein (Richards and Rutter, 1961), and 1200 μg/g of protein (B. L. Vallee, unpublished observations) have been found, although much lower zinc contents (465–643 μg/g of protein) have been reported by other workers (Vanderheiden *et al.*, 1962). These results suggested that yeast aldolase might be a zinc metalloprotein, but evidence for participation of the metal atom in either the structure or the catalytic action of this enzyme has not been reported.

Previous attempts to study the molecular and catalytic properties of this enzyme were hampered by the instability of the purified preparations. We have recently been able to obtain yeast aldolase in a stable and homogeneous form, thus permitting us to undertake studies on the molecular architecture and mechanism of action of this enzyme.

In this context, we now have been able to directly dem-

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onstrate the essentiality of the divalent metal ion for the enzymatic activity. Removal of zinc from the native aldolase abolishes activity, while the readdition of zinc to the apoenzyme restores activity fully. In fact, the addition of other divalent metal ions, *e.g.*, Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+} also restores activity to the inactive apoenzyme. All these yeast metalloaldolases are activated by K^+ ion, similar to the native enzyme.

Materials and Methods

Yeast aldolase was prepared by a modification of the procedure of Rutter *et al.* (1966). In our preparations, the instability problem has been alleviated by including phenylmethylsulfonyl fluoride, a potent proteolytic inhibitor, in all steps of the purification procedure. Under these conditions, the crystalline aldolase stored in 80% saturated $(NH_4)_2SO_4$ is stable for 2 months. These preparations were essentially homogeneous on polyacrylamide disc gel electrophoresis at pH 8.3 and in the ultracentrifuge. The enzymatic activities of such preparations were 85–105 μmoles of fructose diphosphate cleaved per min per mg of protein assayed as described by Richards and Rutter (1961).

Apoaldolase was prepared by incubation of 1–100 mg of native aldolase in 0.5 ml of 0.05 M EDTA (pH 8.0) for 1 hr at 4°. This mixture was then applied to a Bio-Gel P-6 column (0.9 × 15 cm) which had been freed of metals by washing with 0.01 M 8-hydroxyquinoline-5-sulfonic acid (pH 8.0) and then equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The protein was eluted with this latter buffer, separated from the chelating agent and all metals. Apoenzyme prepared in this fashion contained less than 1% of the original zinc content.

The apoenzyme was reconstituted with solutions of the spectrographically pure metal sulfate salts (Johnson Matthew Co., Ltd.) in metal-free water. Trace metal contaminants were removed from all substrates and buffers either by extraction with dithizone in CCl_4, or by passage over a Chelex-100 (Bio-Rad Corp.) column (Himmelhoch *et al.*, 1966). Other precautions to prevent contamination with adventitious metal ions were taken as previously described (Thiers, 1957).

Aldolase activity was determined at 15°. A suitably defined aliquot of aldolase (usually 44 μg) was added to a mixture containing 75 μmoles of *N*-Z-hydroxyethylpiperazine-*N'*-Z-ethanesulfonic acid buffer (pH 7.0), 3 μmoles of fructose diphosphate, 0.9 μmole of DPNH, and 100 μg of a mixture of crystalline α-glycerol phosphate dehydrogenase and triose phosphate isomerase in a total volume of 3.0 ml. The decrease in absorbance at 340 mμ was recorded immediately.

Protein concentrations were determined at 280 mμ using 1.00 as the absorbance of the enzyme at a concentration of 1 mg/ml. This absorptivity was confirmed by determination of protein dry weight after trichloroacetic acid precipitation (Hoch and Vallee, 1953). A Zeiss PMQII spectrophotometer was employed for determination of absorbance at discrete wavelengths. A Unicam SP.800 spectrophotometer was used to measure the disappearance of DPNH in enzymatic assays.

Zinc was determined by atomic absorption spectrometry (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964). Metal content and molar enzyme concentrations are expressed on the basis of a molecular weight of yeast aldolase of 80,000 (C. E. Harris, R. D. Kobes, D. C. Teller, and W. J. Rutter, in preparation).

Results

Removal of zinc from yeast aldolase results in an essentially inactive apoenzyme, when assayed as defined under Materials and Methods (Table I).[1] When increments of zinc are added to the apoenzyme, or alternatively, to the assay mixture before the addition of the apoprotein, activity is restored to values equal to or greater than those observed with the native protein (Table I). This recovery of activity is optimal at a molar ratio of zinc to aldolase of 2:1. At lower molar excesses of metal ion, lower activities are observed, while much higher excesses of metal ions inhibit activity.

TABLE I: Activities of Metalloaldolases.[a]

Enzyme	Metal Ion Added	Aldolase Act. (units/mg)	%
Native		2.60	87
Apoenzyme		0.06	2
Apoenzyme	Zn^{2+}	3.00	100
Apoenzyme	Co^{2+}	2.55	85
Apoenzyme	Ni^{2+}	0.33	11
Apoenzyme	Mn^{2+}	0.45	15
Apoenzyme	Fe^{2+}	2.01	67
Apoenzyme	Cu^{2+}, Hg^{2+}, Cd^{2+} Mg^{2+}, Fe^{3+}	<0.06	<2

[a] Assays and metal restoration were carried out as described in the Materials and Methods. Final concentrations of metals were 16.6×10^{-7} M and final concentration of enzyme was 1.8×10^{-7} M.

The preparations of yeast aldolase employed in this study contained between 1.2 and 1.6 g-atoms of zinc per mole of protein. When assayed without precautions to prevent contamination by adventitious metals, the specific activity of all preparations was equally high. However, the activity of apoaldolase reconstituted with zinc is slightly higher than that of "native" aldolase, when assayed under the present conditions (Table I), suggesting that this may be due to the restoration of zinc lost during the preparation of the enzyme. Thus, native

[1] In the usual assay for aldolase activity, contamination by zinc of the order of magnitude of 1 ng/ml is sufficient to reconstitute the apoenzyme fully. This level of contamination is not detectable by presently available instrumentation, and difficult to achieve in practice. To reduce this problem of contamination, assays were performed at 15° in the absence of activating potassium. Under these conditions, the specific activity of native yeast aldolase is approximately 3–4% of that measured in the usual assay procedure.

yeast aldolase may have a higher metal content than found thus far.[2]

Divalent cobalt, nickel, manganese, and iron ions all restore significant activity to the apoenzyme, when added to the assay mixture in concentrations leading to a final molar ratio of metal to aldolase of 9:1. In contrast, cupric copper, mercury, cadmium, magnesium, and ferric iron do not restore function under the conditions employed (Table I).

Preliminary kinetic investigations of these various metalloaldolases suggest that the differences in activity observed for the differing metals arise from the variance in V_{max} for cleavage of fructose 1,6-diphosphate. The K_m's for this substrate for all the active metal species investigated are quite similar under the conditions employed.

The specific activation of yeast aldolase by potassium ion has been established (Richards and Rutter, 1961). The effect of this ion on the aldolase activities of the various metalloenzymes is detailed in Table II. In all cases, 0.1 M potassium acetate stimulates the specific activity of the active metalloaldolases from 5.5- to 7-fold, similar to its effect on the native enzyme.

TABLE II: Potassium Stimulation of Metalloaldolases.[a]

Metal	Aldolase Act. (units/mg)		
	No Potassium Acetate	0.1 M Potassium Acetate	-Fold Stimulation
Native	2.60	18.2	7.0
Metal-free apoenzyme	0.06	0.06	
Zn^{2+}	3.00	19.2	6.4
Co^{2+}	2.55	14.5	5.7
Ni^{2+}	0.33	1.8	5.5
Mn^{2+}	0.45	3.1	6.9
Fe^{2+}	2.01	12.1	6.0

[a] Assays were carried out as described in the Materials and Methods. Final concentrations used were 6.6×10^{-7} M metals, 0.7×10^{-7} M enzyme, and 0.1 M potassium acetate.

Discussion

The present experiments indicate the essentiality of zinc in the function of native yeast aldolase. While the enzyme is isolated containing stoichiometrically significant concentrations of zinc, the metal of the native enzyme can be removed, resulting in an inactive apoenzyme which, in turn, can be reconstituted to full activity by addition of Zn^{2+}. Thus, yeast aldolase fits the operational definition of a metalloenzyme: a metal species firmly bound to the enzyme in the native state has a specific relationship to the function and/or structure of an enzyme. The stoichiometry of this association is under study.

Although zinc is the only metal which has been found thus far in native yeast aldolase in significant concentrations (*vide supra*), the activity of the metal-free enzyme can be restored by certain ions of the first transition period, *i.e.*, Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+}. In contrast, Cu^{2+}, Hg^{2+}, Cd^{2+}, Mg^{2+}, and Fe^{3+} fail to restore activity to any measurable extent. The different metals restore the enzymatic activity of yeast aldolase to varying degrees; the Zn^{2+} protein, presumably the native enzyme, has the highest specific activity, followed by $Co^{2+} > Fe^{2+} > Mn^{2+} \geq Ni^{2+}$.

In contradistinction to the differences in the activities of the five enzymatically active metalloaldolases, these enzymes exhibit certain important functional similarities. Substitution of the metal ion does not alter significantly the K_m for fructose diphosphate; thus substrate binding is apparently not affected by the metal species present. Further, all these metalloenzymes are activated by potassium ion (Table II). Such activation is a characteristic feature of the class II aldolases (Richards and Rutter, 1961). These studies show that the specific metal atom at the active site is not critical to this phenomenon.

Zinc can be removed from several other metalloenzymes, and it can then be replaced by other group IIb or transition metals, resulting in enzymatically functional proteins (*cf.* Vallee and Wacker, 1969). Such replacements have, heretofore, been reported only for hydrolytic metalloenzymes. Yeast aldolase is a lyase and the postulated mechanism differs considerably from those thought to operate for the hydrolytic metalloenzymes.

The metalloenzyme nature of yeast aldolase is interesting from yet another point of view. The aldol cleavage of fructose diphosphate is catalyzed by two essentially different enzyme types. Yeast aldolase (and presumably other class II enzymes) employ a metal ion, as a functionally critical component, while muscle aldolase (and other class I aldolases) do not.

The mechanisms of action of these two types of enzymes must differ at least in some details. The aldolase reaction is thought to involve the formation of an enol form of dihydroxyacetone phosphate which adds to the polarized carbonyl of glyceraldehyde 3-phosphate to form the hexose diphosphate. With rabbit muscle aldolase, and probably all class I aldolases, the enolization of dihydroxyacetone phosphate is assisted by the formation of a Schiff base with a lysyl residue on the protein molecule. In the case of yeast aldolase and the other class II enzymes, the mechanism does not apparently involve such a Schiff base (Rutter, 1964). The metal atom may serve as an electrophile in the enolization of dihydroxyacetone phosphate.

The properties of the different metalloaldolases should allow examination of this hypothesis, and permit a wide range of other experimental approaches to the study of the mechanism of action of this enzyme.

[2] Such a loss of zinc during procedures involving crystallization from $(NH_4)_2SO_4$ has previously been noted (R. T. Simpson and B. L. Vallee, in preparation). The actual stoichiometric relation of zinc to protein in native yeast aldolase is currently under investigation.

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Alterations in the Structure and Function of *Escherichia coli* Alkaline Phosphatase Due to Zn^{2+} Binding*

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ABSTRACT: The enzymatic activity of alkaline phosphatase from *Escherichia coli* increases linearly with the mole ratio, Zn^{2+}/86,000 molecular weight of protein. There is one phosphate binding site per molecule of dimer containing from two to four bound zinc ions. The tertiary structure of alkaline phosphatase is altered by metal binding as evidenced by ultraviolet difference spectra and circular dichroism in the wavelength range 2600–3200 Å. In addition, spectrophotometric titration shows four tyrosine residues exposed to solvent in the native dimer containing three Zn^{2+} ions and ten such residues exposed to solvent in the metal-free apoprotein.

A refolded subunit of alkaline phosphatase obtained by neutralization of the acid-dissociated and unfolded protein has optical properties similar to those of the metal-free dimer. This subunit is capable of dimerization in the presence of chelating agent.

Alkaline phosphatase from *Escherichia coli* is a zinc metalloprotein containing identical subunits (Rothman and Byrne, 1963) each of molecular weight 43,000 (Schlesinger and Barrett, 1965). Previous studies from this laboratory (Schlesinger and Barrett, 1965; Schlesinger, 1965; Reynolds and Schlesinger, 1967, 1968) have been carried out with alkaline phosphatase in a dimeric state containing two to three Zn^{2+} per dimer. The protein in this state is globular and can be reversibly dissociated and unfolded by high positive charge or 6 M guanidine hydrochloride. The presence of bound zinc ions is essential for enzymatic activity (Garen and Levinthal, 1960; Plocke *et al.*, 1962), and at least one of the residues to which divalent metal ion is bound has been shown to be an imidazole group (Reynolds and Schlesinger, 1968).

However, the role of bound metal in the formation and maintenance of tertiary and quarternary structure of alkaline phosphatase has not been established. Reynolds and Schlesinger (1967) showed that refolding of the structureless monomer could take place in the absence of metal and that the refolded monomer had an optical rotatory dispersion spectrum identical with that of the native dimer. However, the refolded species had a tertiary structure different from that of the enzymatically active dimer in that 5 ± 1 tyrosine residues/subunit were accessible to solvent in the refolded state as opposed to 2 ± 1 tyrosines/subunit in the native state. At protein concentrations less than 10^{-4} g/ml the refolded state was shown to be monomeric.

The questions to which the present work is directed are as follows: (1) What is the relationship between bound metal ion and the enzyme function of alkaline phosphatase? (2) How does bound metal ion affect the tertiary structure of the protein moiety of alkaline phosphatase? (3) Is the binding of metal ion to the apoprotein necessary for formation and stability of quaternary structure?

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