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Role of Metals in the Class II Aldolases. Spectral Studies of Cobalt Yeast Aldolase*

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ABSTRACT: The binding of 2 moles of cobalt(II) to each mole of yeast apoaldolase produces an active enzyme, exhibiting an absorption spectrum with maxima at 535 (155), 485 (125), 1290 (18), and 1640 mμ (18 M^{-1} cm^{-1}). The visible absorption bands are optically active, with circular dichroic bands of molecular ellipticities −2800 and −2500 (deg cm^2) per dmole, respectively. The addition of saturating concentrations of the substrate, fructose 1,6-diphosphate, results both in hypso- and hypochromic shifts of the main absorption band in the visible region. In contrast, neither K^+, an activator, nor phosphate ion, an inhibitor, affect these spectral properties of the cobalt enzyme. The optical asymmetry of the active-site cobalt atoms seems closely related to enzymatic activity, as is demonstrated by its correlation to the changes of the circular dichroic spectrum on titration to pH values of less than 8.

The present studies are discussed in the context of the proposal that the metal atom at the active site of yeast aldolase serves as an electrophile, aiding in bond polarization to facilitate the aldolytic cleavage of the substrate.

Cobalt(II) can substitute for zinc at the active site of fructose 1,6-diphosphate aldolase of *Saccharomyces cerevisiae* to form an active metalloenzyme (Kobes *et al.*, 1969b). Cobalt complexes, including cobalt enzymes, exhibit spectral properties which may reflect the environment of the metal atom. The state of cobalt at the active site of a metalloenzyme can be examined both in the presence and absence of inhibitors, activators, coenzymes and, under appropriate circumstances, in the enzyme–substrate complex. The information thus gained can contribute significantly to the elucidation of the role of the metal in the catalytic mechanism and/or substrate binding of the enzyme. Such mechanistic studies of yeast aldolase are of particular interest, since it has served as the prototype for the class II aldolases isolated from microbial sources. These aldolases are apparently metalloenzymes, usually containing zinc. They accomplish the same interconversion of substrates as the class I aldolases isolated from mammals and plants. Aldolases in the latter group, however, do not contain a metal but employ a reactive lysyl residue in catalysis (Grazi *et al.*, 1962; Rutter, 1964). Yet at least part of the mechanisms of the two classes of enzyme seem similar, as suggested by the formation in both of a catalytic intermediate with the characteristics of carbanions (Riordan and Christen, 1969). Definition of the basis for the catalytic role of the metal on the one hand, and of the lysyl residue on the other might augment significantly the understanding of the mechanism of enzymatic catalysis of aldol condensation reactions. Based on such considerations, the present study concerns the spectral characteristics of cobalt at the active site of yeast aldolase.

Two cobalt ions can bind to each mole of apoaldolase, generating an active enzyme exhibiting optically active visible absorption bands and a near-infrared absorption band. Interaction with substrate alters the spectral properties only slightly, as would be expected if the metal participates in catalysis as an electrophile, as postulated previously (Rutter, 1964; Kobes *et al.*, 1969b).

Experimental Section

Yeast aldolase was isolated from *Saccharomyces cerevisiae* by a modification of a procedure previously described (Rutter *et al.*, 1966). Enzyme prepared in this manner was physicochemically homogeneous by both ultracentrifugal and electrophoretic criteria. The sources of the reagents employed in enzyme assays have been detailed (Kobes *et al.*, 1969b). All other chemicals were of reagent grade, and all solutions were freed of trace metal contaminants either by extraction with dithizone in carbon tetrachloride (Thiers, 1957) or by passage over a Chelex-100 (Bio-Rad Corp.) column (Him-

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melhoch *et al.*, 1966). Cobalt solutions used for reconstitution of the apoenzyme were prepared by dissolving the spectrographically pure sulfate salt (Johnson Matthey, Ltd.) in metal-free distilled water. Precautions were taken to prevent contamination with adventitious metals (Thiers, 1957).

Protein concentrations were determined using 1.02 as the absorbancy of a 1-mg/ml solution of the enzyme at 280 mμ and 80,000 as the molecular weight of the enzyme (Harris *et al.*, 1969). Enzymatic activity was determined routinely at 15° in the absence of potassium ions, with 10^{-3} M fructose 1,6-diphosphate as substrate (Rutter *et al.*, 1966). Activities are expressed as micromoles of substrate cleaved per minute per milligram of enzyme. In determination of the pH dependence of the cobalt enzyme, assays were performed at 20° with 3×10^{-5} M substrate, using a 5-cm path-length cell and a Cary Model 14R spectrophotometer equipped with a 0–0.1-absorbance slide-wire.

Apoaldolase was prepared by incubation of high concentrations (*e.g.*, 120 mg/ml) of protein in 0.05 M EDTA at pH 8.0 for 1 hr at 0°. The protein was then separated from the chelating agent and metals by gel filtration, as described (Kobes *et al.*, 1969b). Apoenzyme prepared in this fashion had less than 2% of the original zinc content.

Zinc and cobalt were determined by atomic absorption spectroscopy (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; R. Erbe and B. L. Vallee, in preparation). Working standards were prepared daily from a dilution of a primary standard, using spectrographically pure zinc rod or cobalt sponge.

Absorption measurements at discrete wavelengths were performed with a Zeiss PMQII spectrophotometer and pH measurements with a Radiometer pH meter equipped with a general purpose combination electrode. Visible and near-infrared absorption spectra were obtained with a Cary Model 14R recording spectrophotometer equipped with a 0–0.1-absorbance slide-wire. In order to obtain the near-infrared absorption spectra, a solution of yeast aldolase was dialyzed *vs.* an equal volume of D_2O (Chelex treated to remove trace metals) in a dialysis cell of 1-ml capacity (Chemical Rubber Co.). Eight dialyses were carried out, the first six and the last for 2–3 hr, and the seventh for 16 hr. The final dialysate was employed as a reference for the spectral measurements. Circular dichroic spectra were obtained with a Cary Model 60 spectropolarimeter equipped with the Model 6001 circular dichroism accessory. The instrument was standardized with *d*-camphorsulfonic acid and was checked routinely for absorption artifacts by recording the spectrum of the standard in the presence of concentrations of an optically inactive substance (potassium dichromate) sufficient to give an optical density of over 2.0.

Results

Addition of stoichiometric amounts of cobalt(II) to apoaldolase results in a metalloenzyme exhibiting pink color. The visible absorption spectrum of cobalt aldolase, associated with d–d electronic transitions, extends from 450 to 650 mμ (Figure 1). The absorption maximum is at 535 mμ (18,700 cm^{-1}) with a shoulder at 485 mμ (20,600 cm^{-1}). The molar absorptivities at these two wavelengths are 155 and 105 M^{-1} cm^{-1}, respectively, when based upon concentration of protein. The cobalt enzyme has no additional absorption bands in the region from 450 to 300 mμ.

The cobalt enzyme has an absorption band in the near-infrared region in addition to those in the visible region.

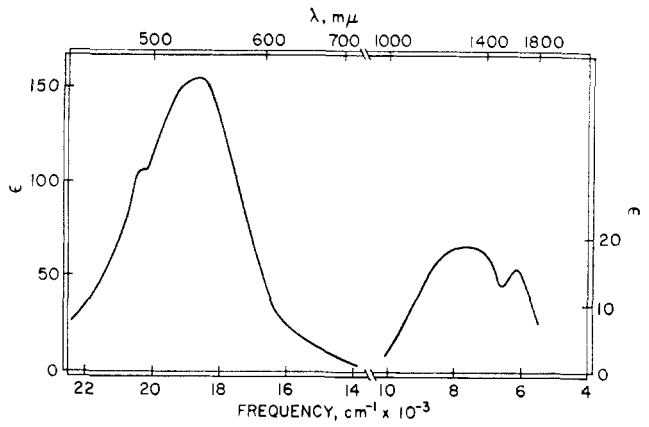

FIGURE 1: Visible (left) and near-infrared (right) absorption spectra of cobalt yeast aldolase. The spectrum in the visible region was obtained at a protein concentration of 40 mg/ml (5×10^{-4} M), while that in the near-infrared region was recorded at a protein concentration of 100 mg/ml. In both cases, cobalt was present in a two-fold molar excess over protein, and the apoenzyme was used as a blank. The molar absorptivities are based on the concentration of holoenzyme containing 2 g-atoms of cobalt/mole of enzyme, though imperfect balance of background as their cause cannot be excluded, since it occurs in the region of the first overtones of X–H vibrations.

The infrared band is broad, extending from about 1000 mμ to beyond 1800 mμ, the limit of spectral measurements in D_2O. There are two apparent peaks at 1290 mμ (7750 cm^{-1}) and 1640 mμ (6100 cm^{-1}).[1] Their absorptivities are approximately 18 M^{-1} cm^{-1}, much lower than those of the visible bands (Figure 1).

These spectra and their comparisons with simple cobalt(II) complex ions suggest that the basic geometry for the binding of cobalt to aldolase might be a distorted tetrahedral form (*vide infra*). In that case, the visible and infrared transitions described might represent the third ($^4A_2 \rightarrow {}^4T_1$ (F)) and second ($^4A_2 \rightarrow {}^4T_1$ (P)) transitions of the cobalt complex with the enzyme, and the values of the ligand field coulombic (D_q) and Racah interelectronic repulsion (B') parameters can be calculated (Carlin, 1965). Using visual judgment of the centers of the absorption bands, with the corresponding uncertainty, the values obtained are $D_q = 410$ cm^{-1} and $B' = 900$ cm^{-1}.

The incremental addition of 2 g-atoms of metal per mole of apoenzyme generates the visible absorption spectrum in Figure 2. Absorptivity at 535 mμ increases linearly on addition of cobalt up to 2 g-atoms of metal per mole of apoenzyme, but not beyond this amount. Similarly, over the range of 0–2 g-atoms, enzymatic activity also increases in proportion to the amount of cobalt added per mole of apoenzyme (Figure 2). Addition of cobalt in relatively small excess of this amount does not increase specific activity, although large excesses (*e.g.*, 50-fold), produce inhibition.

Saturating concentrations of fructose 1,6-diphosphate (10^{-2} M) induce small but reproducible changes in the absorption spectrum. The maximum at 535 mμ undergoes a hypsochromic and hypochromic shift to 528 mμ while the shoulder at 485 mμ remains unchanged (Figure 3). The infrared absorption maximum at 1290 mμ is also shifted to shorter wavelengths but its absorptivity remains unaltered. In

[1] The pertinent characteristics of the spectra of cobalt(II) model complexes have been reviewed recently (Carlin, 1965).

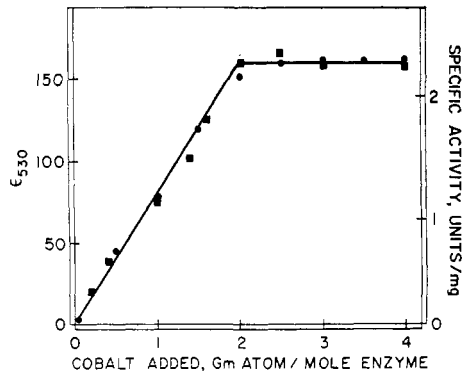

FIGURE 2: Dependence of enzymatic activity (●) and absorption spectrum (■) of cobalt aldolase on the molar excess of cobalt added to the apoenzyme. The indicated excesses of cobalt(II) were added to the apoenzyme and absorption at 530 mμ (■) or enzymatic activity (●) determined as described in the Experimental Section.

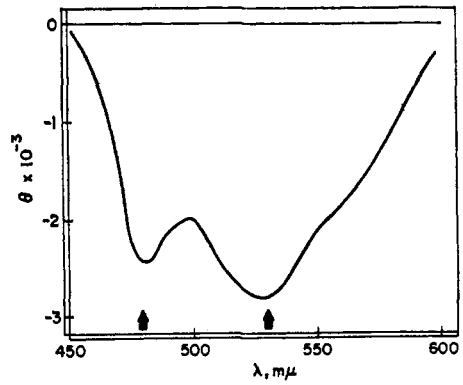

FIGURE 4: Circular dichroism of cobalt yeast aldolase. The molecular ellipticity of cobalt yeast aldolase is plotted as a function of wavelength. The spectrum was determined at a protein concentration of 40 mg/ml. The arrows indicate the wavelength positions of the visible absorption maximum and shoulder.

contrast, neither the addition of 0.1 M potassium (as the acetate), a specific activator of the class II aldolases, nor the addition of 0.01 M phosphate, an inhibitor of the enzyme (Richards and Rutter, 1961), significantly affects the visible absorption spectrum of cobalt aldolase.

The visible absorption bands of several cobalt metalloenzymes are optically active (Coleman, 1968; Simpson and Vallee, 1969; Vallee and Latt, 1969). The absorption bands of cobalt aldolase at 535 and 485 mμ are optically active also, as evidenced by the corresponding negative circular dichroic bands (Figure 4). Addition of potassium, phosphate, or fructose 1,6-diphosphate does not significantly affect the position or magnitude of the ellipticity bands of cobalt aldolase.

The asymmetry of the metal ions at the active sites of yeast aldolase, reflected by these circular dichroic bands, appears to be essential for enzymatic activity. Titration of cobalt aldolase to pH values of less than 8 concomitantly abolishes the circular dichroic spectrum and catalytic function (Figure 5). Since the enzymatic studies were performed at a substrate concentration 15-fold less than K_m, the observed activities represent k_{cat}/K_m, and ionizations detected in such an activity *vs.* pH profile should reflect ionizations of the enzyme itself, rather than those of the enzyme–substrate complex. Neither activity nor the alteration of the circular dichroic spectrum fits a theoretical titration curve, both being steeper than the titration of a single group. However, both change in parallel fashion, suggesting that the catalytic function of the enzyme correlates closely with the asymmetry of the metal ion at the active site of the enzyme.

In the presence of a 2.5-fold molar excess of cobalt over apoprotein, 2 g-atoms of cobalt are firmly bound to the enzyme over a pH range of from 5 to 8 when studied by equilibrium dialysis (Table I). This pH range encompasses the hydrogen ion concentrations which alter both specific enzymatic activity and the absorption properties of cobalt at the active sites (*cf.* Figure 5). The equilibrium metal binding data suggest that these physical and enzymatic alterations are not the consequence of loss of metal from the protein due to competition by hydrogen ion. Further, they are consistent with the titration data (Figure 2), suggesting that 2 moles of cobalt bind to two apparently indistinguishable active sites in yeast aldolase. A stoichiometry of 2 g-atoms of metal/mole of enzyme has also been suggested by studies of the manganese enzyme (Kobes *et al.*, 1969a).

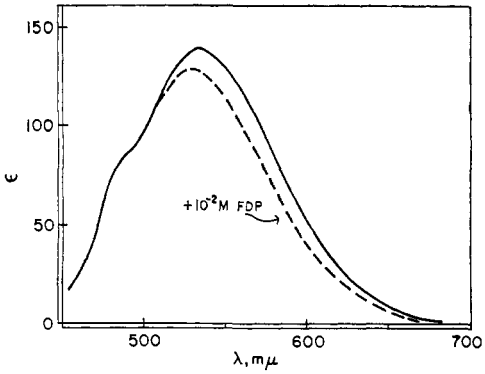

FIGURE 3: Effect of fructose 1,6-diphosphate on the absorption spectrum of cobalt yeast aldolase. The absorption spectrum of cobalt yeast aldolase (43 mg/ml) is shown in the absence (——) and presence (----) of 1×10^{-2} M fructose 1,6-diphosphate.

Discussion

A large number of metalloenzymes are now known to contain zinc, a diamagnetic metal whose complexes do not exhibit visible absorption spectra (Vallee and Wacker, 1970). These properties limit the suitability of zinc as a probe for the environment of the metal atom at the active sites of such enzymes. In contrast, cobalt is paramagnetic and gives rise to visible absorption spectra and, therefore, can be a good environmental probe. In complex ions, cobalt is known to share with zinc the ability to accept unusual coordination environments (Vallee and Williams, 1968a) and, further, cobalt can readily replace zinc to form functionally active derivatives of a large number of zinc enzymes (Vallee and Williams, 1968a,b; Vallee and Latt, 1970; Vallee and Wacker, 1970).

Detailed evaluation of the spectral and magnetic properties of complexes of cobalt with simple organic and inorganic ligands may allow assignments of their geometries. However, even in the case of these metal complex ions, referred to as "simple" as compared to the potential binding groups and geometries of a metalloenzyme, unambiguous assignment of the geometry of the complex has often proven difficult (*cf.* Carlin, 1965). Nevertheless, comparison of the spectra of cobalt(II) enzymes to those of cobalt(II) complex ions

TABLE I: Equilibrium Diaysis of Cobalt Aldolase.[a]

pH	g-atoms Co(II)/Mole
5.0	1.98
5.5	1.85
6.0	1.94
6.5	1.98
7.0	2.30
7.5	2.19

[a] Apoenzyme was dialyzed *vs.* a 2.5 molar excess of Co(II) at the indicated pH values in 0.01 M morpholinoethanesulfonic acid for pH 5.0–6.5 and 0.01 M hydroxyethylpiperazinesulfonic acid for pH 7.0 and 7.5. Dialysis was carried out for 36 hr at 4°.

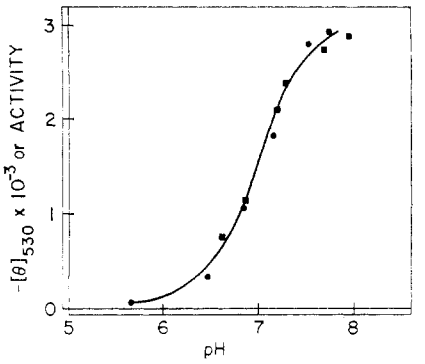

FIGURE 5: pH dependence of the enzymatic activity (●) and circular dichroism (■) of cobalt yeast aldolase. Circular dichroic spectra were determined as in the legend to Figure 4 at the indicated pH values, and $-\theta_{530}$ is plotted as a representation of the presence of the circular dichroic spectrum. Enzymatic activity was determined at 25° at a substrate concentration of 2×10^{-5} M, 15-fold less than the K_m for the enzyme. The activity data are plotted in arbitrary units.

might allow deductions concerning the nature and geometry of metal binding sites, and their environments in these enzymes. Such an approach is, of course, limited by the number and types of model systems available for comparison, as well as the difficulties noted above. Further, the possibility has been discussed that in some cases the spectral properties of metalloenzymes may differ significantly from those of metal complex ions currently known (Vallee and Williams, 1968a,b). Bearing these limitations in mind, we shall consider the possible implications of the present studies to the geometry of metal binding of the active site of yeast aldolase.

When compared to the absorption spectra of the three types of cobalt(II) complex ions, *i.e.*, octahedral, tetrahedral, and trigonal-bipyramidal, most commonly observed, the band positions, separations and degree of complexity for the spectrum of cobalt aldolase bear less resemblance to octahedral and trigonal-bipyramidal systems. The common tetrahedral complexes of cobalt(II), such as the tetrachloro complex, exhibit visible absorption maxima at about 700 mμ and molar absorptivities of about 1000 M^{-1} cm^{-1}; however they are relatively poor models for proteins. Complexes of cobalt with the second row donor atoms, such as nitrogen and oxygen, those most abundant in proteins, have electronic transitions at energies higher than those of tetrahedral complexes with lower row elements and are of lower intensity. Hence, neither the high-energy position of the visible band for the enzyme nor the low intensity is incompatible with a form of tetrahedral geometry, though a definitive assessment requires knowledge of the donor atoms.

The intensities of the visible absorption bands of tetrahedral cobalt complexes have been discussed in terms of covalency (Cotton and Soderberg, 1962). Assuming tetrahedral geometry, similarly the decrease of B, Racah interelectronic repulsion parameter, from the value for the free cobalt(II) ion 971 cm^{-1} to that in the complex has been related to the intensity of the third transition (Ballhausen and Liehr, 1958, 1960). This decrease for cobalt aldolase in B' to 900 cm^{-1} is quite small yet may be signifiant; hence the intensity of the visible band would also be small. Thus, the spectrum of cobalt aldolase resembles those reportend for distorted tetrahedral complexes with oxygen and nitrogen ligands (Cotton and Soderberg, 1962; Carlin, 1965; O'Connor *et al.*, 1968; Pignolet *et al.*, 1969). The intensities of the bands in these spectra are intermediate between those of the spectra of regular tetrahedral complexes and those normally associated with the spectra of octahedral complexes (Cotton and Soderberg 1962).

The asymmetry of metal binding implicit in the absorption band of cobalt aldolase has been observed for a number of other cobalt metalloenzymes (Coleman, 1968; Simpson and Vallee, 1968; Vallee and Latt, 1970). The correlation of the circular dichroic spectrum and activity as a function of pH strongly implies the association of the geometry, reflected by the spectra, with the presence of the catalytically functional configuration of the active site.

Both the absorption and the circular dichroic spectral measurements suggest that upon interaction of cobalt aldolase with its substrates there is little change in the geometry of metal binding. This contrasts with the more marked changes in absorption and circular dichroic spectra observed upon addition of inhibitors or substrates to a number of hydrolases, *e.g.*, cobalt carbonic anhydrase (Lindskog and Nyman, 1964; Coleman, 1965), cobalt alkaline phosphatase (Simpson and Vallee, 1968), or cobalt carboxypeptidase (Vallee and Latt, 1970).

The metal atom in yeast aldolase has been thought to serve as an electrophile, binding the oxygen function of C-2 of the substrate with activation of the C-3–C-4 bond for aldol cleavage. It is known that metal-bound water is displaced by substrate (Kobes *et al.*, 1969a). Given the proper geometric relationships at the active site, substrate might displace water and bind to the metal, so that catalysis would proceed. In the absence of a change in geometry on binding of substrate, major spectral changes would not be expected simply consequent to the replacement of water by a keto or hydroxyl group.

It would thus appear that the geometry enforced upon the metal at the active site of yeast aldolase by the protein ligand groups might place this Lewis acid in a state which allows its effective catalytic interaction with the substrate in a manner consistent with discussions concerning the entatic state of enzymes (Vallee and Williams, 1968a,b).

Acknowledgment

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Arsanilazochymotrypsinogen. The Extrinsic Cotton Effects of an Arsanilazotyrosyl Chromophore as a Conformation Probe of Zymogen Activation*

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ABSTRACT: Chymotrypsinogen A modified with diazotized *p*-arsanilic acid exhibits a characteristic circular dichroic spectrum with a small positive Cotton effect at 323 nm and a large negative one centered at 428 nm with a molecular ellipticity, $[\theta]^{26}_{428}$, of $-8000°$, characteristic of azo aromatic modification. Exposure of 6.4×10^{-5} M arsanilazochymotrypsinogen to concentrations of trypsin ranging from 1.9×10^{-7} to 7.6×10^{-7} M at pH 7.5, inverts the negative sign of the long-wavelength Cotton effect; the positive band, now centered at 448 nm, has a molecular ellipticity, $[\theta]^{26}_{448}$, of $+7000°$. The changes in sign, position, and magnitude are first order with respect to arsanilazochymotrypsinogen concentration and display pseudo-first-order rate constants directly proportional to the trypsin concentration. Both native and arsanilazochymotrypsinogens are activated by trypsin at similar rates, and the resultant enzymes display nearly identical activities toward *N*-benzoyl-L-tyrosine ethyl ester.

The variation in optical activity correlates closely with the induction of enzymatic activity, as gauged by the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester, and seemingly reflects the kinetics of structural rearrangements accompanying zymogen activation. The extrinsic Cotton effect of the arsanilazo aromatic chromophore provides a sensitive signal of changes in local conformation which accompany activation of the modified chymotrypsinogen.

In the process of zymogen activation rearrangements of protein structure have been thought to result in the juxtaposition of amino acid side chains critical for effective substrate binding and catalysis (Neurath, 1964). However, the experimental demonstration of such postulated conformational changes has proven difficult. The activation of bovine chymotrypsinogen A has been examined in particular detail in this regard. Hydrolysis of the Arg-15-Ile-16 bond is both necessary and sufficient to generate enzymatic activity (Jacobsen, 1947; Dreyer and Neurath, 1955; Hartley and

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