OPTICAL PROPERTIES OF CATALYTICALLY ACTIVE COBALT AND CADMIUM LIVER ALCOHOL DEHYDROGENASES[+]

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Substitution of Co or Cd for the native Zn atoms of horse liver alcohol dehydrogenase (LADH) results in catalytically active metallodehydrogenases with characteristic enzymatic, chemical and optical properties. These metal substitutents generate extrinsic Cotton effects and, in turn, alter side chain Cotton effects in typical fashion. Further, the spectra generated by the metals and those arising from either the binding of NADH or of 1,10-phenanthroline to LADH cause mutual perturbations. These features of Co and Cd LADH provide new approaches to delineate the role of metal ions in this multichain enzyme.

INTRODUCTION: The essential role of Zn in the catalytic function of horse liver alcohol dehydrogenase (LADH) is well documented (1-4), but in contrast with many other metalloenzymes, it has not been possible thus far to remove Zn and restore it or some other metal with concomitant abolition and/or reappearance of activity (5). However, we have now succeeded in exchanging the native Zn atoms of LADH with Co or Cd (6). The resultant Co and Cd alcohol dehydrogenases are catalytically active, and the remarkable features of their absorption and circular dichroic (CD) spectra provide hitherto unavailable means by which to study their roles.

METHODS: The Zn atoms of LADH (Boehringer #7050334) were exchanged completely with $^{65}Zn^{2+}$ (7); the isotope subsequently constitutes both a gauge for Zn displacement and a check on contamination. Determination of

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enzymatic activity, molar absorptivity, metal content, radioactivity and the preparation and purification of reagents were performed as described (8,9). Solutions of Cd^{2+} and Co^{2+} were prepared by dissolving the pure metals (Johnson-Matthey Co., Ltd.) in metal-free HCl (Aristar-BDH) followed by removal of excess acid and dilution into Na acetate-acetic acid buffer with a final pH of 5.5. ^{65}Zn-LADH was dialyzed against 1×10^{-4} M Cd^{2+} or 0.2 M Co^{2+} in 0.2 M Na acetate, pH 5.5, until 95% or more of all the $^{65}Zn^{2+}$ was displaced. The samples were then dialyzed against 0.2 M Tris-acetate, pH 7, to remove free metal ions. All solutions were deaerated with and kept under nitrogen to minimize oxygen content. Absorption and CD spectra were obtained with a Cary 14R spectrophotometer and a Cary 60 spectropolarimeter equipped with a CD attachment.

RESULTS: Under carefully defined conditions, the Zn atoms of LADH undergo complete exchange with $^{65}Zn^{2+}$ to form an enzyme which in all other respects is identical to the native one (7). Co-LADH and Cd-LADH, obtained by dialysis of ^{65}Zn-LADH against Co^{2+} or Cd^{2+}, do not contain Zn, detectable either by isotopic or atomic absorption measurements (Table I). Both the specific enzymatic activities and the specific absorptivities of these two enzymes are characteristic of the respective metal substituents and differ from those of the Zn enzyme (Table I).

Table I: Properties of Liver Alcohol Metallodehydrogenases.*

	g-atoms Me/mole LADH				Activity (ΔA_{340})	Absorptivity (280 mμ)
	Zn	^{65}Zn	Co	Cd	min^{-1} mg^{-1}	mg^{-1} cm^2
Zn-LADH	3.7	---	---	---	14	0.43
^{65}Zn-LADH	3.7	3.6	---	---	14	0.43
Co-LADH	<0.1	<0.1	3.6	---	10	0.49
Cd-LADH	<0.1	<0.1	---	3.8	4	0.57

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*Details of measurement of these properties and the pertinent analytical considerations have been discussed (10, 11).

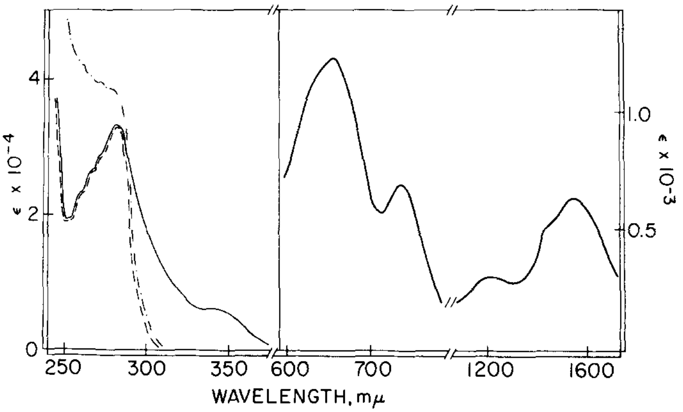

Fig. 1 Absorption Spectra of the Metallodehydrogenases. (---), Zn-LADH; (——), Co-LADH; (—·—), Cd-LADH. 0.1 M Tris acetate, pH 7.0.

Their optical properties afford further analytical evidence for the formation of Co-LADH and Cd-LADH. Co-LADH has a broad absorption band in the near-ultraviolet region centered at 340 mμ (ε = 6500) (Fig. 1) associated with two positive and three negative CD bands between 300 mμ and 450 mμ (Fig. 2). Absorption maxima also occur at 655 mμ (ε = 1330), 730 mμ (ε = 800) and in the near infrared between 1000 and 1800 mμ (ε = 270-540) (Fig. 1). A small negative ellipticity band is centered at 620 mμ; studies are in progress to delineate CD bands at longer wavelengths.

Cd-LADH exhibits an intense absorption band centered at 245 mμ (Fig. 1). This band may be pertinent to the identity of the metal ligands since its molar absorptivity (ε = 10,200) is close to that of 14,000 reported for the cadmium mercaptide chromophores of metallothionein (10).

As a consequence of these metal substitutions perturbations of the side chain Cotton effects of LADH are observed also (Fig. 2). Between 250 and 300 mμ the CD spectra of these metallodehydrogenases exhibit remarkable fine structure, characteristic of each. The CD difference spectrum of Cd-LADH *vs*. Zn-LADH below 270 mμ exhibits a positive ellipticity band which may well correspond to the maximum in the absorption spectrum at 245 mμ. In the

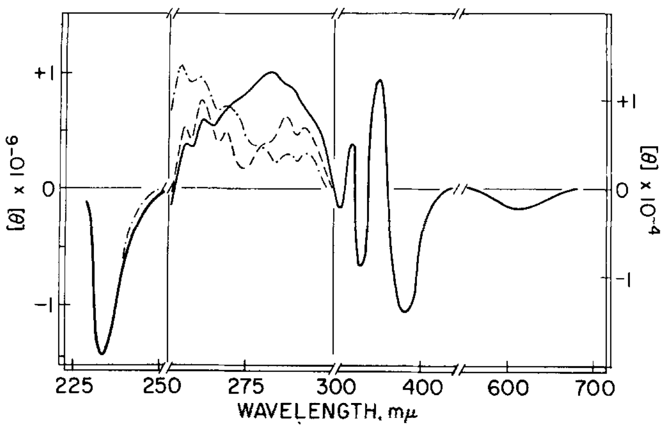

Fig. 2 Circular Dichroic Spectra of the Metallodehydrogenases. (---), Zn-LADH; (——), Co-LADH; (—·—), Cd-LADH. The symbol [Θ] denotes molecular ellipticity based on the concentration and Mol Wt of LADH (12). 0.1 M Tris acetate, pH 7.0.

region of maximum absorption due to aromatic amino acid residues, the ellipticity of the Co-LADH exceeds that of both Zn- and Cd-LADH.

Yet additional effects of the metal substitutions in LADH are reflected in the optical properties of extrinsic chromophores, bound asymmetrically. Both the reduced coenzyme, NADH, and 1,10-phenanthroline, OP, bind to Zn-, Co-, and Cd-LADH and generate extrinsic Cotton effects distinctive for each with magnitudes which relate quantitatively to binary complex formation (11, 12) (Table II). Based on molar ellipticities, the rotational strength of the NADH complex with Co-LADH is markedly greater than those with Cd- and Zn-LADH which are identical (Table II). In turn, binding of NADH alters the Co-LADH chromophore shifting the maximum at 655 mμ to 670 mμ and slightly diminishing absorptivity.

Similarly, the amplitude of the CD band of the Cd-LADH-OP complex, centered at 271 mμ, markedly exceeds that of Zn-LADH-OP, while that of the Co-LADH-OP complex is considerably smaller (Table II). Thus, the rotational strengths of these mixed complexes at 271 mμ afford sensitive probes of the identity of the metal substituent at the active site.

Table II: Properties of the Complexes of NADH and 1,10-Phenanthroline with Alcohol Dehydrogenase.*

	Extrinsic Chromophore			
	NADH		1,10-Phenanthroline	
	ε_{325}	$[\Theta_m]_{325}$†	ε_{271}	$[\Theta_m]_{271}$†
Zn-LADH	5800	-540	29,500	+2100
Co-LADH	6000	-930	28,000	+ 420
Cd-LADH	5800	-540	29,000	+3600

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*Spectra in 0.1 M Tris acetate, pH 7.0.

†In units of molar ellipticities, corrected for the measured dissociation constants (14).

DISCUSSION: The procedure here described allows complete exchange of Co^{2+} or Cd^{2+} for Zn in LADH, as assessed by multiple analytical procedures. The Co and Cd enzymes exhibit enzymatic properties characteristic of the specific metal and have distinctive spectral features not seen in the native enzyme. The absorption spectra, reflecting the presumable modes of metal binding to the apoenzyme, should ultimately aid in the delineation of features of the binding sites.

The CD spectrum of Co-LADH is richer in detail than that of any other comparable cobalt enzyme studied thus far (13). It may be analyzed on one hand in terms of protein structure, as reflected in the intrinsic and side chain Cotton effects of the protein, and of superimposed extrinsic Cotton effects resulting from NADH or OP binding on the other, as will be discussed in detail elsewhere (14). The mutual interaction of Co and NADH is of special interest in view of its potential relationship to the geometry and asymmetry of the metal binding site, not previously accessible to similar spectral approaches.

The presumable environment of the Co atoms and,hence, the geometry of its LADH complexes reflect in the visible and near-infrared bands. Low temperature, EPR and MCD studies, now underway, should further assist in their characterization. While Cd-mercaptide chromophores may account for the changes in the CD spectrum of Cd-LADH below 270 mμ, the dramatic effects of Co and Cd substitution may be susceptible to analysis by symmetry rules and the various mechanisms proposed for the generation of extrinsic and intrinsic Cotton effects (15). At least five bands of Co-LADH are optically active between 300 and 450 mμ, overlapping that of the Co-LADH-NADH complex at 325 mμ. Similarly, Cd-LADH exhibits an intense transition of higher energy at 246 mμ which lies close to the maximum of the mixed Cd-LADH-OP complex at 271 mμ providing possible bases for mechanistic interpretation.

It has become apparent that some of the metal cations of certain metalloenzymes are critical to the catalytic step while others seem to serve primarily in protein structure. In Zn-LADH the functional Zn atoms can be differentiated from those involved in structure (4). It would appear that these two discrete roles for metal atoms may be maintained in Co-LADH and Cd-LADH. Both the Co and Cd enzymes bind 2 moles of OP, suggesting that, like the native Zn enzyme, only two of the metal atoms are accessible to this agent. Selective replacement either of the "functional" or of the structural metal atoms with other metals, resulting in "metal-hybrid enzymes" (16), can now be documented by employing the unusual absorption and CD properties here presented, which augment the criteria available in the past (1-4, 7, 8, 12).

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