activate the enzyme but Mg^{2+} was not.

The only divalent cation which significantly stabilized the enzyme to denaturing forces was Mn^{2+}. The large effect demonstrated by Mn^{2+} may be related to its cooperative binding with the enzyme, but it could also simply be related to the greater binding ability of Mn^{2+} as compared to the binding of Mg^{2+} to the enzyme.

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Cadmium-109 as a Probe of the Metal Binding Sites in Horse Liver Alcohol Dehydrogenase†

Arthur J. Sytkowski* and Bert L. Vallee

ABSTRACT: The noncatalytic and catalytic zinc atoms of horse liver alcohol dehydrogenase, $[(LADH)Zn_2Zn_2]$ or LADH, have been replaced differentially with ^{109}Cd by equilibrium dialysis, resulting in two new enzymatically active species, $[(LADH)^{109}Cd_2Zn_2]$ and $[(LADH)^{109}Cd_2{}^{109}Cd_2]$. The UV difference spectra of the cadmium enzymes vs. native $[(LADH)Zn_2Zn_2]$ reveal maxima at 240 nm with molar absorptivities, $\Delta\epsilon_{240}$, of 1.6×10^4 M^{-1} cm^{-1} per noncatalytic ^{109}Cd atom and 0.9×10^4 M^{-1} cm^{-1} per catalytic ^{109}Cd atom, consistent with coordination of the metals by four and two thiolate ligands, respectively, strikingly similar to the 250-nm charge-transfer absorbance in metallothionein. Carboxymethylation of the Cys-46 ligand to the catalytic metal in LADH presumably lowers the overall stability constant of the coordination complex and results in loss of catalytic ^{109}Cd or catalytic cobalt but not catalytic zinc from the enzyme.

Twenty years ago horse liver alcohol dehydrogenase was found to be a zinc metalloenzyme (Vallee & Hoch, 1957). Some 10 years later its four zinc atoms were first shown to encompass one set each of two functional and two structural atoms as established in solution (Drum et al., 1967) and confirmed by the elegant X-ray crystallographic studies of Brändēn et al. (1975).

We have previously replaced the four zinc atoms of the enzyme with both cobalt and cadmium to facilitate spectroscopic and/or kinetic exchange experiments and ultimately to lead to mechanistic insights. More recently, it has proven possible to delineate conditions critical to the specific and selective replacement of zinc by cobalt and these have now led to an extension of earlier investigations with cadmium (Druyan & Vallee, 1962; Drum & Vallee, 1970b).

Comparison of the exchange properties, spectral characteristics, and inhibition kinetics of these cadmium derivatives with those of cobalt and zinc provides further insight regarding the metal coordination chemistry of LADH.[1]

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[1] Abbreviations used: $[(LADH)Zn_2Zn_2]$ or LADH, native horse liver alcohol dehydrogenase; CD, circular dichroism; OP, 1,10-phenanthroline. In order to differentiate and clarify presentation, we designated the first pair of exchangeable metal (Me) atoms in the standard formulation the "N" (noncatalytic) pair and the second the "C" (catalytic) pair, i.e., $[(LADH)N_2C_2]$. Hence, Zn and Co represent the N pair in $[(LADH)Zn_2Me_2]$ and $[(LADH)[Co_2Me_2]$, while they are the C pair in $[(LADH)Me_2Zn_2]$ and $[(LADH)Me_2Co_2]$.

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Table I: Properties of Zinc and Cadmium Horse Liver Alcohol Dehydrogenases

enzyme	metal content (g-atom/mol)				specific enzymatic activity (ΔA_{340}) (min^{-1} mg^{-1})
	Zn		Cd		
	atomic absorption	γ emission	atomic absorption	γ emission	
$[(LADH)^{65}Zn_2Zn_2]$	4.0	2.0			14
$[(LADH)Zn_2{}^{65}Zn_2]$	4.0	2.0			14
$[(LADH)^{109}Cd_2Zn_2]$	2.1	0.1	1.8	1.9	14
$[(LADH)^{109}Cd_2{}^{65}Zn_2]$	2.0	2.0	1.9	1.9	14
$[(LADH)^{109}Cd_2{}^{109}Cd_2]$	0.1	0.1	3.8	3.9	2

Materials and Methods

Enzyme. Horse liver alcohol dehydrogenase (90–95% ee isozyme) was obtained from Boehringer-Mannheim Corp. as a crystalline suspension with specific activity ΔA_{340} = 14–15 min^{-1} mg^{-1}. Alcohol dehydrogenase activity was determined at 25 °C with a Gilford Model 240 spectrophotometer equipped with a Heathkit IR-18M recorder. The rate of formation of NADH from NAD^+ (Grade III, Sigma Chemical Co.) was monitored at 340 nm as previously described (Drum & Vallee, 1970a). Protein concentration was determined spectrophotometrically at 280 nm by using molar absorptivities (ϵ) of 3.56 × 10^4 M^{-1} cm^{-1} ($A_{280}{}^{0.1\%}$ = 0.43) for native $[(LADH)Zn_2Zn_2]$ and $[(LADH)^{109}Cd_2Zn_2]$ and 3.83 × 10^4 M^{-1} cm^{-1} ($A_{280}{}^{0.1\%}$ = 0.46) for $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ (see Results).

Metal Exchange. Buffer solutions for metal exchange were prepared from reagent grade chemicals. They were rendered free of contaminating metal ions by extraction with 0.01% dithizone in CCl_4 (Thiers, 1957) and adjusted to the desired pH with isothermally distilled HCl or CH_3COOH. Specpure $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, and $CdSO_4$ were obtained from Johnson-Matthey, and ^{65}Zn (specific activity = 1.10 Ci/g) and ^{109}Cd (carrier-free) were from New England Nuclear. Zinc, cobalt, and cadmium were measured by atomic absorption spectrometry (Fuwa et al., 1964), and ^{65}Zn and ^{109}Cd were measured by γ-emission spectrometry (Model 1185, Searle Analytic). $[(LADH)^{65}Zn_2Zn_2]$, $[(LADH)Zn_2{}^{65}Zn_2]$, $[(LADH)Co_2Zn_2]$, and $[(LADH)Co_2Co_2]$ were prepared as described previously (Sytkowski & Vallee, 1978).

Absorption and Circular Dichroic Spectra. Absorption spectra were determined at 23 °C with a Cary 14 recording spectrophotometer equipped with 0–0.1 and 0–1.0 absorbance slide-wires using quartz cuvettes of 10-mm path length. Circular dichroism titrations with 1,10-phenanthroline (G. Frederick Smith Chemical Co.) were preformed at 23 °C with a Cary 61 spectropolarimeter using a quartz sample cell of 0.5-mm path length. Units of molar ellipticity, $[\theta]$, are in deg cm^2 $dmol^{-1}$.

1,10-Phenanthroline Inhibition and Carboxymethylation. Instantaneous, reversible inhibition of $[(LADH)Zn_2Zn_2]$, $[(LADH)Co_2Zn_2]$, $[(LADH)^{109}Cd_2Zn_2]$, and $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ by 1,10-phenanthroline was determined as previously described (Sytkowski & Vallee, 1976). The metallodehydrogenases were carboxymethylated with a 600-fold molar excess of twice recrystallized iodoacetic acid (Eastman Organic) and/or [^{14}C]iodacetic acid (New England Nuclear) in 0.1 M sodium phosphate, pH 7.5, 23 °C (Li & Vallee, 1965). A 1.0-mL aliquot of 5.8 × 10^{-5} M $[(LADH)Co_2Co_2]$ was carboxymethylated in an acid-cleaned quartz cuvette of 10-mm path length; the cuvette was kept at 23 °C in the thermostated cell holder of the Cary 14 spectrophotometer. To initiate carboxymethylation, 100 μL of 0.348 M [^{14}C]iodoacetic acid and 0.1 M sodium phosphate, pH 7.5, was added to the enzyme solution. At specified time intervals, 5-μL aliquots of enzyme–iodoacetate solution were removed and assayed for enzymatic activity and the absorption spectrum of the remaining solution was determined. Between spectral determinations the solution was shielded from the spectrophotometer light beam to prevent photodecomposition of iodoacetate and resultant formation of iodine. The reaction was stopped by gel filtration of the mixture through Bio-Gel P-4 (Bio-Rad), 100–200 mesh, equilibrated with 0.1 M sodium phosphate, pH 7.5, 23 °C.

Results

Cadmium Exchange in LADH. The rate, extent, and site specificity of cadmium exchange are determined by measuring the loss of ^{65}Zn from $[(LADH)^{65}Zn_2Zn_2]$ and $[(LADH)Zn_2{}^{65}Zn_2]$ radiolabeled at the noncatalytic and catalytic sites, respectively (Sytkowski & Vallee, 1976, 1978). In addition, the use of $^{109}Cd^{2+}$ permits the kinetics of cadmium incorporation to be measured directly. ^{65}Zn and ^{109}Cd were determined simultaneously in individual samples. The ^{65}Zn 1100-keV photopeak γ emissions were counted by using a 950–1250-keV window on channel B of the γ spectrometer. ^{109}Cd radioactivity was determined by counting the 88-keV photopeak emissions of the ^{109m}Ag intermediate using a 81–119-keV window on channel A. The lower energy ^{65}Zn γ photons which were counted in the ^{109}Cd channel (A) were determined experimentally to be 11.7% of ^{65}Zn counts in the ^{65}Zn channel (B). Hence, 11.7% of channel B counts were subtracted from the channel A counts of each sample.

Dialysis of 3-mL portions of 7 × 10^{-5} M $[(LADH)^{65}Zn_2Zn_2]$ and $[(LADH)Zn_2{}^{65}Zn_2]$ against 1000 mL of 1 × 10^{-5} M $^{109}Cd^{2+}$ and 0.1 M sodium *acetate*, pH 5.5, results in the exchange of the 2 g-atoms of noncatalytic ^{65}Zn but *not* the 2 g-atoms of catalytic ^{65}Zn with ^{109}Cd (Figure 1A). The resultant enzymes, $[(LADH)^{109}Cd_2Zn_2]$ and $[(LADH)^{109}Cd_2{}^{65}Zn_2]$, contain 1.8–1.9 g-atoms of noncatalytic ^{109}Cd and 2.0–2.1 g-atoms of catalytic Zn or ^{65}Zn per mol and exhibit specific enzymatic activities ΔA_{340} = 14 min^{-1} mg^{-1}, equal to those of the zinc enzymes (Table I). In contrast, dialysis of the zinc enzymes against 1 × 10^{-5} M $^{109}Cd^{2+}$ and 0.1 M sodium *phosphate*, pH 5.5, replaces both the noncatalytic and the catalytic zinc with ^{109}Cd (Figure 1B). The resultant $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ contains 3.8–3.9 g-atoms of ^{109}Cd and only 0.1 g-atom of zinc per mol and has a specific activity, ΔA_{340} = 2 min^{-1} mg^{-1}, 14% of that of the zinc and cadmium–zinc hybrid enzymes and consistent with replacement of the catalytic zinc with ^{109}Cd. In these exchange experiments the use of cadmium concentrations greater than 1 × 10^{-5} M results in a proportional loss of final product due to apparent denaturation and precipitation of the enzyme during dialysis. This contrasts with $^{65}Zn \rightleftharpoons Zn$ exchange, which may be accomplished at 1 × 10^{-4} M Zn^{2+}, and $Co \rightleftharpoons Zn$ exchange, the yield of which is maximal at 0.1–0.2 M Co^{2+} (Sytkowski & Vallee, 1976, 1978).

The molar absorptivities of the cadmium derivatives at 280 nm were ascertained by three independent procedures. The

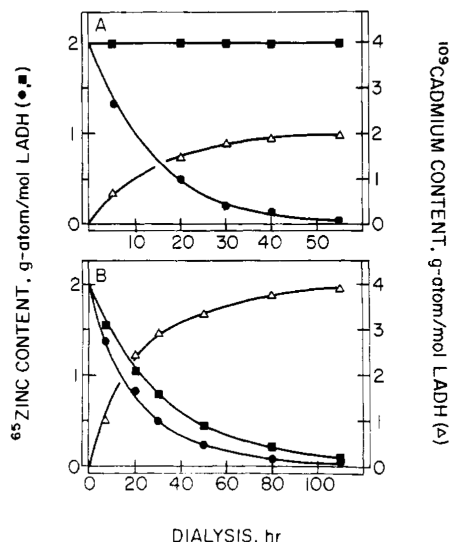

FIGURE 1: Preparation of $[(LADH)^{109}Cd_2Zn_2]$ and $[(LADH)^{109}Cd_2{}^{65}Zn_2]$ (panel A) and $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ (panel B). Aliquots of $[(LADH)^{65}Zn_2Zn_2]$ (●) and $[(LADH)Zn_2{}^{65}Zn_2]$ (■), 7×10^{-5} M, were dialyzed against 1×10^{-5} M $^{109}Cd^{2+}$ in 0.1 sodium acetate (panel A) or 0.1 M sodium phosphate (panel B), pH 5.5, 4 °C. Metal exchange was terminated by changing the dialysate to 0.1 M Tris-HCl, pH 7.5.

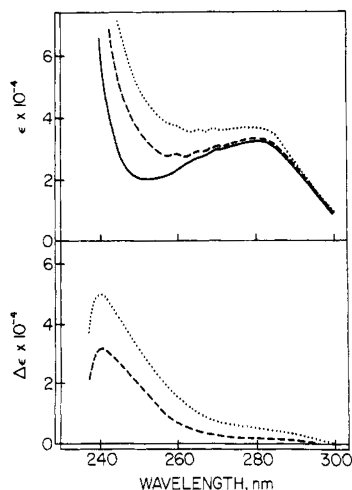

FIGURE 2: UV absorption spectra of cadmium and zinc metallohydrogenases. Upper panel: $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ (···), $[(LADH)^{109}Cd_2Zn_2]$ (- - -), and $[(LADH)Zn_2Zn_2]$ (—). Lower panel: difference spectra of $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ − $[(LADH)Zn_2Zn_2]$ (···) and of $[(LADH)^{109}Cd_2Zn_2]$ − $[(LADH)Zn_2Zn_2]$ (- - -).

method of Lowry et al. (1951) was employed by using native $[(LADH)Zn_2Zn_2]$ ($A_{280}^{0.1\%} = 0.43$) as a standard. The protein dry weight (Hoch & Vallee, 1953; Sytkowski, 1977) of duplicate samples of known A_{280} was assessed, and finally the ultracentrifugation method of Klainer & Kegeles (1955) was employed to determine the protein concentration of samples of known A_{280}. The mean and range of molar extinctions and the corresponding molar absorptivities obtained by the three methods are $A_{280}^{0.1\%} = 0.43 \pm 0.1$ ($\epsilon_{280} = 3.56 \times 10^4$ M^{-1} cm^{-1}) for $[(LADH)^{109}Cd_2Zn_2]$ and $A_{280}^{0.1\%} = 0.46 \pm 0.03$ (3.83×10^4 M^{-1} cm^{-1}) for $[(LADH)^{109}Cd_2{}^{109}Cd_2]$.

Absorption Spectra. The cadmium-substituted derivatives do not absorb radiation in the visible region of the spectrum. The near-UV spectrum reveals that, in addition to the 278-nm maximum generated by the aromatic amino acid residues of the protein, the difference spectra of cadmium enzyme − $[(LADH)Zn_2Zn_2]$ exhibit maxima at 240 nm (Figure 2). The molar absorptivities, $\Delta\epsilon_{240}$, are 3.2×10^4 M^{-1} cm^{-1} for $[(LADH)^{109}Cd_2Zn_2]$ − $[(LADH)Zn_2Zn_2]$ and 5.0×10^4 M^{-1} cm^{-1} for $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ − $[(LADH)Zn_2Zn_2]$. Hence, the absorptivities are 1.6×10^4 M^{-1} cm^{-1}/noncatalytic ^{109}Cd atom and 0.9×10^4 M^{-1} cm^{-1}/catalytic ^{109}Cd atom (see Discussion).

Table II: 1,10-Phenanthroline Inhibition of Horse Liver Alcohol Dehydrogenases

enzyme	pK_I^a
$[(LADH)Zn_2Zn_2]$	3.6
$[(LADH)Co_2Zn_2]$	3.6
$[(LADH)^{109}Cd_2Zn_2]$	3.6
$[(LADH)^{109}Cd_2{}^{109}Cd_2]$	4.6

^{a} $pK_I = -\log K_I$ where K_I is the OP concentration (M) at 50% inhibition.

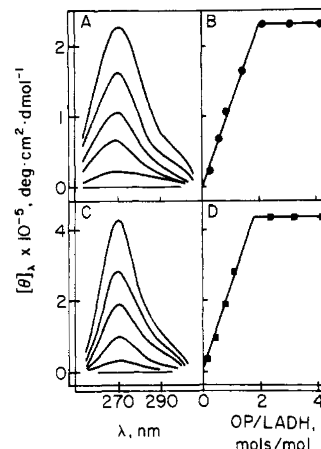

FIGURE 3: Circular dichroism titrations of 1,10-phenanthroline (OP) binding to the catalytic metal atoms of $[(LADH)^{109}Cd_2Zn_2]$ (panels A and B, ●) and of $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ (panels C and D, ■). Enzyme = 1×10^{-4} M; 0.1 M Tris-HCl, pH 7.5, 23 °C. OP, 2.5 or 10 mM, was added in 5–20-μL portions to 1.0 mL of enzyme.

Interaction with 1,10-Phenanthroline. The chelating agent 1,10-phenanthroline (OP) interacts with the catalytic metal atoms of LADH (Drum & Vallee, 1970a), displacing a water molecule from the inner coordination sphere. The noncatalytic metal atoms are fully coordinated by four S ligands and, thus, are inaccessible to OP (Brändén et al., 1975). Therefore, OP can serve to identify the active-site metal atoms of LADH either through its inhibition kinetics or through the optical activity of the mixed complex formed with the metal atom (Sytkowski & Vallee, 1978). Examination of the OP inhibition of the series of metallohydrogenases—$[(LADH)Zn_2Zn_2]$, $[(LADH)Co_2Zn_2]$, and $[(LADH)^{109}Cd_2Zn_2]$ in which the noncatalytic metal atoms are Zn, Co, or ^{109}Cd, respectively, but in all of which zinc is at the catalytic sites—reveals that all three enzymes are inhibited instantaneously and reversibly with an identical $pK_I = 3.6$ (Table II). In contrast, the inhibition of $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ in which the second, catalytic (C) pair of Zn atoms is replaced by ^{109}Cd displays a $pK_I = 4.6$, confirming replacement of the catalytic metal atom. Circular dichroism titrations with OP further support this assignment. Addition of aliquots of OP to $[(LADH)^{109}Cd_2Zn_2]$ generates positive ellipticity at 271 nm, reaching a maximum molar ellipticity $[\theta]_{271} = +2.3 \times 10^5$ deg cm^2 dmol^{-1} at a stoichiometry of OP/LADH = 2:1 (parts A and B of Figure 3). Virtually identical ellipticities and stoichiometries have been shown previously for $[(LADH)Zn_2Zn_2]$ and $[(LADH)Co_2Zn_2]$, viz., $+2.1 \times 10^5$ and $+1.7 \times 10^5$ deg cm^2 dmol^{-1}, respectively (Sytkowski & Vallee, 1976). In marked contrast, titration of $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ with OP exhibits the same stoichiometry, 2:1, but reaches this at a maximum molar ellipticity $[\theta]_{271} = +4.1 \times 10^5$ deg cm^2

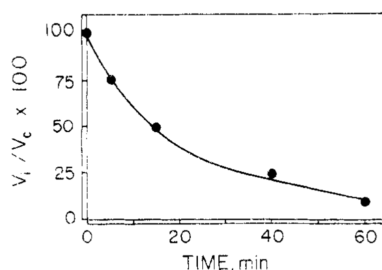

FIGURE 4: Carboxymethylation of Cys-46 in $[(LADH)^{109}Cd_2{}^{109}Cd_2]$. Enzyme = 4.6×10^{-5} M; iodoacetic acid = 28 mM (see Materials and Methods and Results).

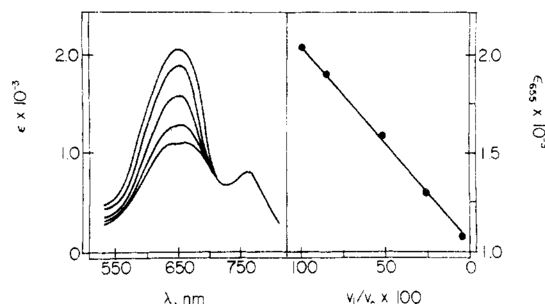

FIGURE 5: Carboxymethylation of Cys-46 in $[(LADH)Co_2Co_2]$. Left panel: time-dependent decrease in 655-nm maximum due to specific loss of the second, catalytic pair of Co atoms (from top to bottom: 0, 10, 25, 40, and 90 min). Right panel: ϵ_{655} vs. $V_i/V_c \times 100$. The molar absorptivity of resultant enzyme, ϵ_{655} = 1050 M^{-1}, is identical with the 655-nm absorption of cobalt in noncatalytic sites only, $[(LADH)Co_2-]$ (Sytkowski & Vallee, 1975). Enzyme = 5.3×10^{-5} M; $[^{14}C]$iodoacetic acid = 32 mM; 0.1 M sodium phosphate, pH 7.5, 23 °C (see Results).

dmol^{-1} (parts C and D of Figure 3), the altered maximum ellipticity indicating the replacement of the catalytic pair of zinc atoms with ^{109}Cd. The molar absorptivities of the $[Zn(OP)]^{2+}$ and $[Cd(OP)]^{2+}$ complexes are identical (Sone et al., 1955), as are those of [LADH·Zn(OP)] and [LADH·Cd(OP)] (Drum & Vallee, 1970b). Hence, the difference in the magnitude of the ellipticity of these two metalloenzyme mixed complexes (parts C and D of Figure 3) is not obvious.

Carboxymethylation of Cys-46 Ligands to the Catalytic Metal Atoms. In each subunit of horse liver alcohol dehydrogenase the catalytic metal atom is coordinated by the sulfur ligands of Cys-46 and Cys-174 and the N-3 of His-47 (Brändén et al., 1975). The fourth coordination position is open to water or other ligands such as OP. Iodoacetic acid selectively carboxymethylates Cys-46, thereby inactivating the enzyme (Li & Vallee, 1965). X-ray crystallographic analysis of carboxymethylated native $[(LADH)Zn_2Zn_2]$ demonstrates that the sulfur atoms of the resultant thioether remain coordinated to the catalytic zinc atoms (Brändén et al., 1975).

Incubation of 4.6×10^{-5} M $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ with 28 mM iodoacetic acid inactivates the enzyme to <10% of the control in 60 min (Figure 4). The difference spectrum of the resultant derivatives vs. $[(LADH)Zn_2Zn_2]$ exhibits a $\Delta\epsilon_{240}$ = 3.4×10^4 M^{-1} cm^{-1}, which is reduced from 5.0×10^4 M^{-1} cm^{-1} for that of $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ and virtually identical with that of $[(LADH)^{109}Cd_2Zn_2]$, 3.2×10^4 M^{-1} cm^{-1} (Figure 2). This suggests the loss of the catalytic ^{109}Cd atoms from $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ during inactivation. Metal analyses by atomic absorption and γ-emission spectrometry confirm that the resultant carboxymethylated enzyme contains only 2.2–2.4 g-atoms of ^{109}Cd/mol. These retained ^{109}Cd atoms thus have a $\Delta\epsilon_{240}$ = 1.6×10^4 M^{-1} cm^{-1}/^{109}Cd atom, identifying them as the noncatalytic pair (see above). Thus, the resultant enzyme is $[(LADH)^{109}Cd_2-]$, which has lost its catalytic atoms.

The results of carboxymethylation of the Cys-46 ligands to the catalytic metal atoms in $[(LADH)Co_2Co_2]$, a derivative with *cobalt* at the catalytic sites, are equally striking. Incubation of $[(LADH)Co_2Co_2]$ with a 600-fold molar excess of $[^{14}C]$iodoacetic acid inactivates the enzyme in a time-dependent manner and concomitantly decreases the absorption maximum at 655 nm. A plot of ϵ_{655} vs. V_i/V_c is linear (Figure 5). However, the absorption maximum at 740 nm, previously shown to reflect only the noncatalytic cobalt atoms (Sytkowski & Vallee, 1976), is unaffected. After 90 min the enzymatic activity is <10% of the control and the molar absorptivity (ε) at 655 nm is reduced to 1050 M^{-1} cm^{-1}, identical with that observed when 1,10-phenanthroline removes the catalytic cobalt atoms to yield $[(LADH)Co_2-]$ (Sytkowski & Vallee, 1975).

After gel filtration through Bio-Gel P-4 to remove excess $[^{14}C]$iodoacetic acid, liquid scintillation spectrometry reveals that 2 mol of $[^{14}C]$iodoacetate is incorporated per mol of enzyme, consistent with specific carboxymethylation of the Cys-46 residue in each subunit. The cobalt content of the carboxymethylated derivative is reduced from 3.9 to 2.0 g-atoms/mol, accounting for the reduction in the 655-nm maximum. Thus, as with $[(LADH)^{109}Cd_2{}^{109}Cd_2]$, carboxymethylation of the Cys-46 ligands to the catalytic cobalt atoms in $[(LADH)Co_2Co_2]$ is accompanied by loss of these metal atoms from the enzyme. In contrast, carboxymethylation of the Cys-46 residues of $[(LADH)Zn_2Zn_2]$, $[(LADH)Co_2Zn_2]$, and $[(LADH)^{109}Cd_2Zn_2]$ (data not shown), all of which have zinc at the catalytic sites, does not alter the metal content of these species, consistent with the results of Brändén et al. (1975) obtained by X-ray analysis of the native, crystalline enzyme.

Discussion

The present study describes the preparation of a series of enzymatically active cadmium–zinc hybrids and fully substituted cadmium derivatives of horse liver alcohol dehydrogenase. Importantly, the identification of the first (N) pair of metal atoms in the general formulation $[(LADH)N_2C_2]$ as the noncatalytic pair and the second (C) pair of atoms as the catalytic pair as established by studies of cobalt ⇌ zinc exchange in the enzyme (Sytkowski & Vallee, 1976, 1978) is in complete agreement with all of the results obtained in these studies of the cadmium enzymes. While the optimal concentration of cadmium for exchange with zinc is 1×10^{-5} M Cd^{2+} compared to 1×10^{-4} M Zn^{2+} for ^{65}Zn ⇌ Zn exchange, apparently due to the greater reactivity of cadmium toward the free sulfhydryl groups of the enzyme, ^{109}Cd ⇌ Zn exchange resembles the ^{65}Zn ⇌ Zn exchange closely in regard to the specific effects of the acetate and phosphate buffer anion: in acetate only the noncatalytic zinc atom pair exchanges with cadmium, whereas in phosphate both the noncatalytic and the catalytic pairs exchange (Drum et al., 1967; Sytkowski & Vallee, 1976, 1978).

Clearly, at this time the factors pertinent to the mechanism(s) of cadmium ⇌ zinc exchange in LADH cannot be known with any degree of certainty, but it seems possible that the information already reviewed for the Co ⇌ Zn exchange might also apply to that of cadmium (Sytkowski & Vallee, 1978). Indeed, studies of model systems have demonstrated that zinc, cadmium, and cobalt all readily form bridged dimercaptides (Leussing & Tischer, 1961; Jicha & Busch, 1962), a property likely of importance in the metal exchange of this enzyme (Sytkowski & Vallee, 1976).

The UV difference spectra of $[(LADH)^{109}Cd_2Zn_2]$ − $[(LADH)Zn_2Zn_2]$ and $[(LADH)^{109}Cd_2{}^{109}Cd_2]$ − $[(LADH)Zn_2Zn_2]$ exhibit maxima at 240 nm with molar absorptivities, $\Delta\epsilon_{240}$, of 3.2×10^4 and 5.0×10^4 M^{-1} cm^{-1}, respectively (Figure 2). Thus, the absorptivities per cadmium atom are 1.6×10^4 M^{-1} cm^{-1}/noncatalytic ^{109}Cd and 0.9×10^4 M^{-1} cm^{-1}/catalytic ^{109}Cd. These maxima are most consistent with charge transfer between cadmium and S ligands, and the ratio of their magnitudes (1.8:1) is striking in view of the coordination of the noncatalytic metal atom to *four* S ligands in contrast to the catalytic metal atom which is coordinated to only *two* S ligands. These absorptivities are also notable when compared to absorbance of metallothionein at 250 nm attributed to charge transfer. This protein contains 20 cysteinyl residues and up to a total of 6 to 7 g-atoms of cadmium and/or zinc (Kägi & Vallee, 1960, 1961). When containing a complement of 6 g-atoms of cadmium per mol, the molar absorptivity, ϵ_{250}, of this charge-transfer band in cadmium thionein is 1.45×10^4 M^{-1} cm^{-1}/Cd atom with an average of 3.2 S ligands per Cd atom. Hence, the magnitudes of the absorbance of the Cd mercaptide chromophores in the noncatalytic and catalytic metal binding sites of horse liver alcohol dehydrogenase and in metallothionein are quantitatively similar.

Carboxymethylation of the Cys-46 ligand to the catalytic metal atom in each subunit of LADH results in a thioether S ligand. When cadmium or cobalt occupies this catalytic site, carboxymethylation results in rapid loss of the metal atom from the enzyme (see Results). However, when zinc is at the catalytic site, either in the crystalline state (Brändén et al., 1975) or in solution (see above), the metal atom is retained, being coordinated to the thioether sulfur of Cys-46, the thiolate sulfur of Cys-174, the N-3 of His-47, and the iodide anion. The stability constants of thiolate ligands to the three metals under consideration are generally in the order Cd > Zn > Co (Sillén & Martell, 1964). Studies of transition-metal complexes of mercaptoamines, systems whose structures may be considered a first approximation of the coordination complex at the catalytic site of LADH, reveal that alkylating agents such as methyl iodide react with the coordinated mercaptide group, generating a thioether in situ (Busch et al., 1964). Analysis of the products of such reactions readily demonstrates that the stability of the thioether sulfur complexes is lower. In addition, the stabilities of zinc, cobalt, nickel, and copper complexes with amines containing thioether sulfur ligands are lower than those of the corresponding amines which do not contain sulfur, a finding interpreted in terms of the less favorable entropy changes accompanying the formation of the complexes with the sulfur-containing amines (Gonick et al., 1954). Although in part these data from model systems may explain the loss of cobalt but not of zinc from the catalytic site of LADH upon carboxymethylation, they do not in themselves appear to account for the rapid loss of cadmium under the same conditions. An explanation may be found in the steric restriction, ionic radii, and interatomic distances peculiar to the catalytic site coordination complex of LADH. These characteristics may be amenable to study by such methods as ^{113}Cd Fourier transform nuclear magnetic resonance and Raman spectroscopy (Haberkorn et al., 1976) but will require direct investigation of the enzyme itself by these or analogous approaches.

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