Chemical reactivities of catalytic and noncatalytic zinc or cobalt atoms of horse liver alcohol dehydrogenase: Differentiation by their thermodynamic and kinetic properties

(spectroscopy/stability constants/kinetics of metal-assisted dechelation)

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ABSTRACT Horse liver alcohol dehydrogenase (EC 1.1.1.1) contains one catalytic and one noncatalytic pair of zinc atoms that can be replaced selectively with cobalt and/or 65Zn. We have now prepared "hybrid" metalloenzymes by specifically replacing one or both pairs of zinc atoms with 65Zn and/or cobalt. Their differential chemical reactivities serve to characterize the metal atoms at either site. The spectral and kinetic properties of the resultant 65Zn, cobalt, and hybrid enzymes, as well as those of their complexes with 1,10-phenanthroline, identify the metal atoms that are at the catalytic sites and differentiate them from those at the noncatalytic sites. All data are in complete agreement with the results of the x-ray crystal structure analysis. Remarkably, under the conditions used, chemical reactivity, as gauged by thermodynamic methods under equilibrium conditions, identifies the catalytic metal atoms as those which are reactive to 1,10-phenanthroline, while this reagent does not affect the noncatalytic pair. Under dynamic conditions the kinetics of the metal–metal exchange reveals the converse to be true: the chemical reactivity of the noncatalytic atoms is much higher and, hence, they exchange more rapidly. The results are examined in terms of thermodynamic and kinetic properties of metal complex ions which serve as the basis of possible mechanisms underlying these observations.

Chemical modifications of particular amino-acid side chains affect the activities of enzymes, linking their chemical to their catalytic properties and differentiating functional from other residues. Such differentiation may be the result of thermodynamic, kinetic, or both these features of the reactants. In this context, the characteristic properties of the metals of metalloenzymes enable their use as spectral, kinetic, and spectrokinetic probes (1). The realization that many enzymes may contain different classes of metal atoms allows an extension of these approaches to the delineation of different chemical reactivities of metals in terms of spectra or rates of metal–metal exchange, the interaction of metals with ligands, the environment of metals, and their modes of interaction with substrates or inhibitors.

One such enzyme, horse liver alcohol dehydrogenase, LADH, (EC 1.1.1.1) contains four zinc atoms (2, 3) two of which are "catalytic" and two of which are "noncatalytic" or structural, as judged by kinetic, spectroscopic, and chemical evidence (3–5). On this basis, we have designated these two classes of zinc as "C" and "N," respectively, and will refer to them in this manner throughout this report. X-ray crystal structure analysis reveals the details of the ligands binding to these pairs of zinc atoms: those of the C sites are two sulfurs and one nitrogen, while those of the N sites are four sulfurs (6). All four zinc atoms exchange freely with 65Zn, cobalt, or cadmium, and each of the resultant metallodehydrogenases has characteristic enzymatic activities (7).

We have now prepared "hybrid" metalloenzymes by selectively replacing one or the other pair of zinc atoms with 65Zn and/or cobalt. Similar attempts in the past led to interpretations of the results that are inconclusive (3–11) in the light of knowledge derived from the enzyme structure as deduced from x-ray crystallographic analysis (6). The spectral properties of the resultant 65Zn, cobalt, and hybrid enzymes, as well as those of their complexes with 1,10-phenanthroline, identify the metal atoms that are at the catalytic sites, differentiating them from those at the noncatalytic sites both by these properties and by differential rates of metal exchange. All data correlate well with the results of x-ray crystal structure analysis (6).

Remarkably, chemical reactivity, as gauged by thermodynamic methods, identifies the C site metal atoms as the more reactive ones, but the kinetics of metal exchange reveals the N site metal atoms to be more reactive.

MATERIALS AND METHODS

LADH (Boehringer Mannheim Corp.) was labeled with 65Zn(II) by equilibrium dialysis (4) to yield either [(LADH)65Zn265Zn2], by exchange of only the first (N) pair of zinc atoms, or [(LADH)65Zn265Zn2], by exchange of both the first (N) and the second (C) pair. An aliquot of [(LADH)65Zn265Zn2] was re-exchanged by dialysis against 0.2 mM Zn2+ in 0.1 M sodium acetate, pH 5.5, 4°, leaving 65Zn only at the C sites, resulting in [(LADH)Zn265Zn2]. These three species of alcohol dehydrogenase allowed precise monitoring of the kinetics of subsequent metal replacements both at the N and C sites. The determination of enzymatic activity, metal content, and radioactivity, and the preparation and purification of reagents have been described (5).

Samples of [(LADH)Zn2Zn2], [(LADH)65Zn265Zn2], and [(LADH)Zn265Zn2] were dialyzed against 0.1 M sodium acetate, 0.2 M CoCl2 (Johnson-Matthey Co., Ltd.), pH 5.9 or 5.4, 4°, to replace zinc with cobalt, monitored by total zinc content, loss of 65Zn, and incorporation of cobalt. Samples were subsequently dialyzed against 0.2 M Tris-acetate, pH 7.5, to terminate exchange and to remove free metal ions. Absorption spectra were obtained with a Cary 14 recording spectrophotometer, and circular di-
chroism was measured with a Cary 61 spectropolarimeter. 1,10-Phenanthroline-HCl (OP) was obtained from C. Frederick Smith Chem. Corp.

RESULTS

Alcohol dehydrogenases prepared by selectively replacing the stable zinc atoms with $^{65}$Zn either at the N or at the C sites result in [(LADH)$^{65}$Zn$_2$Zn$_2$] and [(LADH)Zn$_2^{65}$Zn$_2$], respectively. The chemical and structural characteristics of these derivatives are identical to those of the native enzyme with respect to all parameters examined (Table 1). Importantly, there is no evidence for intersite exchange of Zn = $^{65}$Zn. The radioactivity of these derivatives allows quantitative monitoring of site-specific replacement of zinc atoms by cobalt. Dialysis of [(LADH)$^{65}$Zn$_2$Zn$_2$] and [(LADH)Zn$_2^{65}$Zn$_2$] against cobalt replaces 98% of the $^{65}$Zn of [(LADH)$^{65}$Zn$_2$Zn$_2$], but none of the $^{65}$Zn of [(LADH)Zn$_2^{65}$Zn$_2$] exchanges with cobalt during the first 12 hr of dialysis. The resultant [(LADH)Co$_2$Zn$_2$] and [(LADH)Co$_2^{65}$Zn$_2$] contain 1.9 g-atoms of cobalt and 2.0 g-atoms of zinc per mole of enzyme. The specific activities of both these species, $\Delta A^{340} = 14$ min$^{-1}$ mg$^{-1}$, are identical to that of the native enzyme (Table 1). Subsequently, the zinc atoms at site C also exchange with cobalt though at a slower rate. Only after 120 hr of dialysis has more than 98% of the total zinc content of [(LADH)Zn$_2$Zn$_2$] exchanged with cobalt to yield [(LADH)Co$_2$Co$_2$], with a specific activity of $\Delta A^{340} = 9.0$ min$^{-1}$ mg$^{-1}$, i.e., 64% that of the native enzyme. Hence, in contrast to replacement of the zinc atoms at the N sites, replacement of the second pair of zinc atoms at the C sites with cobalt alters enzymatic activity.

Both [(LADH)Co$_2$Zn$_2$] and [(LADH)Co$_2^{65}$Zn$_2$] exhibit absorption maxima at 340, 655, and 740 nm, with molar absorptivities (ε) at λ$_{340}$(8000), λ$_{655}$(1050), λ$_{740}$(750), respectively. $\Delta A^{340}$ and $\Delta A^{655}$ are consistent with interpretation of absorption maxima (14) previously observed in the near infrared region (7). Fig. 1 shows the spectra of [(LADH)Co$_2$Zn$_2$] and [(LADH)Co$_2^{65}$Zn$_2$] and the molar absorptivities at 340, 655, and 740 nm as a function of g-atom of cobalt per mole of enzyme. The intensity of the bands at 340 and 655 nm increases linearly with incorporation of cobalt into the first, i.e., N, pair of sites and continues to increase as cobalt enters the second, i.e., C, sites. Hence, the 340- and 655-nm bands reflect the presence of cobalt at both sites. In contrast, the intensity of the 740-nm band increases linearly from 0 to 2 g-atom of cobalt per mole of enzyme but not thereafter. It therefore correlates only with the presence of cobalt at the N sites. As cobalt replaces zinc at the C sites, absorbance at 740 nm does not increase further. Although the absorption spectra suggest that the metal atoms are coordinated similarly to the ligands of the N and C sites, they also reveal differences between the resultant metal complexes.

1,10-Phenanthroline (OP), a competitive inhibitor, interacts with but does not remove the two C site zinc atoms of LADH (3, 5, 6, 15). The resultant [(LADH)Zn$_2$(Zn-OP)$_2$] complex is optically active and exhibits a maximal ellipticity, $[\theta]_{740}^{20} = 2.1 \times 10^4$ deg cm$^2$ dmol$^{-1}$, when 2 moles of OP are bound per mole of enzyme (5). OP also interacts with [(LADH)Co$_2$Zn$_2$] to generate a positive extremum centered at 741 nm (Table 2). The molar ellipticity, $[\theta]_{740}^{20} = 1.7 \times 10^4$ deg cm$^2$ dmol$^{-1}$, is nearly identical to that of the native zinc enzyme, and the 2:1 molar stoichiometry is the same. Thus, OP binds in the same manner to the catalytic metal atoms of [(LADH)Co$_2$Zn$_2$] and [(LADH)Zn$_2$Zn$_2$], forming [(LADH)Co$_2$(Zn-OP)$_2$] in both instances. Importantly, addition of OP to [(LADH)Co$_2$Zn$_2$] alters neither the absorption nor the electron paramagnetic resonance spectra generated by the cobalt atoms.

In contrast, the results of the interaction of OP with [(LADH)Co$_2$Co$_2$] differ markedly from those with the other two species: OP alters the absorption spectrum but does not generate a circular dichroic extremum. Moreover, while OP inhibits both [(LADH)Zn$_2$Zn$_2$] and [(LADH)Co$_2$Zn$_2$] instantaneously and reversibly (5, 6, 15), the inhibition of [(LADH)Co$_2$Co$_2$] is time-dependent and is not reversed either by dilution or by addition of excess zinc or other metals (Fig. 2).

Table 1. Properties of zinc and cobalt liver alcohol dehydrogenases

| Me/LADH (g-atom/mole) | ΔA$^{340}$ | [| (LADH)Zn$_2$Zn$_2$] | 4 | 0 | 14 |
|-----------------------|----------|-----------------|---|---|---|
| [(LADH)Zn$_2^{65}$Zn$_2$] | 4 | 2 | 14 |
| [(LADH)Zn$_2$Zn$_2^{65}$Zn$_2$] | 4 | 2 | 14 |
| [(LADH)Co$_2$Zn$_2$] | 2.0 | 0 | 1.9 | 14 |
| [(LADH)Co$_2^{65}$Zn$_2$] | 2.0 | 0 | 1.9 | 14 |
| [(LADH)Co$_2$Co$_2$] | <0.2 | <0.1 | 3.9 | 9 |

Table 2. Circular dichroism titrations of zinc and cobalt alcohol dehydrogenases with 1,10-phenanthroline

<table>
<thead>
<tr>
<th>Me/LADH (g-atom/mole)</th>
<th>[θ]$_{740}^{20}$ (deg cm$^2$ dmol$^{-1}$)</th>
<th>OP/LADH* (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(LADH)Zn$_2$Zn$_2$]</td>
<td>+2.1</td>
<td>2</td>
</tr>
<tr>
<td>[(LADH)Co$_2$Zn$_2$]</td>
<td>+1.7</td>
<td>2</td>
</tr>
<tr>
<td>[(LADH)Co$_2$Co$_2$]</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Circular dichroism titrations were performed by adding 10-μl aliquots of 10 mM OP to 1-ml samples of 1.6 × 10$^{-4}$ M enzyme, 0.2 M Tris-acetate, pH 7.5, 25°C. * Based on maximal circular dichroism at 271 nm.
In order to establish the exact metal content of [(LADH)Co2Co2] both before and subsequent to interaction with OP, solutions of [(LADH)65Zn65Zn2], [(LADH)Co65Zn2], and [(LADH)Co2Co2] were dialyzed against OP under conditions known to inhibit the fully substituted cobalt enzyme completely and irreversibly. 65Zn, zinc, and cobalt were measured before and after exposure to the agent. [(LADH)65Zn65Zn2] and [(LADH)Co65Zn2] are inhibited instantaneously and reversibly, and the isotope and metal content remain unaltered. In contrast, OP reduces the cobalt content of [(LADH)Co2Co2] to 2 g-atoms/mole, consistent with the removal of the catalytic pair of cobalt atoms and accounting for the time-dependent, irreversible inhibition of this species (Table 3). These experiments conclusively demonstrate that OP interacts with the second, C, pair of metal atoms, proving them to be the catalytic pair.

**DISCUSSION**

Both the chemical reactivities and the biological roles of the two zinc atoms in each subunit of LADH differ, and correlation of enzyme structure with function calls for their chemical localization, characterization, and differentiation.

Toward this end, we have used both thermodynamic—particularly spectroscopic—methods and the kinetics of metal–metal exchange as well as the time dependence of the effect of OP on the activity and metal content of 65Zn, cobalt, and 65Zn/cobalt hybrid LADH. The data identify the noncatalytic, N, pair of metal atoms as those which exchange secondly and more slowly under the present conditions. OP inhibits native LADH instantaneously and reversibly by forming a mixed complex with the C metal atoms, [(LADH)Me65Zn(65Zn-OP)]. This instantaneous inhibition contrasts with that of 8-hydroxyguanine, sodium diethylthiocarbamate, and EDTA, which inhibit this enzyme in a time-dependent, irreversible manner (15), a more typical mode observed with most other metalloenzymes, and thought to reflect metal removal resulting in an inactive apoenzyme (16).

The realization that metals constitute intrinsic probes of their protein environments has increasingly revealed that the thermodynamic properties of the intrinsic, catalytically active metals of metalloenzymes are quite unusual when compared to those of well-defined model coordination complexes and noncatalytic metal atoms in proteins (1, 16). The present findings extend this awareness to the kinetics of their metal–metal exchange.

Differential chemical reactivity is here used to characterize both sites. It is remarkable that under equilibrium conditions the two active site atoms display high reactivity with OP, while the structural metal atoms are unaffected by this reagent. However, in a dynamic situation, i.e., in metal exchange reactions, the opposite is true: the chemical reactivity of the structural atoms is much higher and, hence, they exchange more rapidly.

The pair of zinc atoms that exchange first under the present conditions are at site N, near the surface of the molecule. They are coordinated to four S groups donated by cysteines 97, 100, 103, and 111, and do not readily accommodate other ligands; hence, they do not bind water, substrate, coenzyme, or OP. The pair of zinc atoms that exchange second are coordinated to the thiol groups of Cys 46 and Cys 174 and the N-3 atom of His 67; water occupies a fourth coordination position. These zinc atoms located within the active site cleft are 25 Å from the surface of the molecule, yet are accessible to ligands, substrates, and inhibitors such as OP (6).

The rates of exchange of the zinc atoms of [(LADH)Zn65Zn2] with 65Zn(II) and Co(II) and the chemical and spectroscopic properties of the resultant enzyme, partially or fully substituted with 65Zn(II) or Co(II), have not been correlated thus far either with the consequences of the interactions of all of these species with OP or with the x-ray structure. This requires consideration of both the thermodynamics and the kinetics of zinc and cobalt coordination with the enzyme.

**Thermodynamic Considerations.** The thermodynamic features of both sets of atoms in the [(LADH)Zn65Zn2] system can be understood comparatively well. In model systems, coordination to two sulfurs and one nitrogen, the ligands at the catalytic site of LADH, would result in cumulative stability constants (log β3) of 31 and 22 for zinc and cobalt, respectively (17). Circular dichroism titrations of [(LADH)Zn65Zn2] and [(LADH)Co65Zn2] demonstrate that OP interacts with their catalytic metal atoms in an identical manner. The ligand alters neither the cobalt absorption spectrum nor the electron paramagnetic resonance spectrum of [(LADH)Co65Zn2]. Hence, the agent must bind to zinc in both cases. In contrast, OP perturbs the absorption and electron paramagnetic resonance spectra of [(LADH)Co65Zn2], which differs from the other enzyme derivatives by virtue of a cobalt atom at each catalytic site, and the time-dependent and irreversible OP inhibition of this derivative becomes a striking diagnostic change (Fig. 2). OP removes and, thus, competes successfully with the enzyme for these cobalt atoms (Table 3), presumably because they are bound several orders of magnitude more weakly than zinc (17). Such a reaction will be reversible only if the integrity of the metal-binding site of the resultant apoenzyme is preserved so that...
it can continue to function in binding metal atoms. It is quite likely that the sulffhydryl groups that participate in metal binding might oxidize on removal of the metal from LADH, thereby precluding restoration of the metal and manifesting as irreversible inactivation of the enzyme.

The enzyme would be expected to bind zinc more tightly than cobalt (16) while OP is known to bind cobalt more tightly than zinc (17), supporting such reasoning. 6 The stepwise stability constants of Co(OP)\textsubscript{1}, Co(OP)\textsubscript{2}, and Co(OP)\textsubscript{3} are log \(K_1 = 7.25\), log \(K_2 = 6.70\), and log \(K_3 = 5.95\), respectively, with a cumulative constant, log \(\beta_3 = 19.9\), whereas those for the respective zinc complexes are log \(K_1 = 6.36\), log \(K_2 = 5.64\), and log \(K_3 = 5.20\) and log \(\beta_3 = 17.2\) (17).

Hence, the interaction between cobalt and OP is 2.7 orders of magnitude more stable than that between zinc and OP. These magnitudes of the relative stabilities of the [LADH]\textsubscript{2}, [Co] and [OP]\textsubscript{3}[Co] complexes are consistent with the analytical and spectral data and the kinetics of inhibition (Tables 2 and 3, Figs. 1 and 2). Similarly, the much higher stabilities expected when four sulfur ligands are coordinated either to cobalt (log \(\beta_2 = 28\)) or zinc (log \(\beta_2 = 40\)) (17) mitigate against metal removal by OP from the noncatalytic sites in accord with the experimental results (Table 3). While data from model systems cannot accurately predict the stability constants for zinc and cobalt when interacting with the ligands at the active site of LADH, they allow the inference that such a zinc complex would be more stable than the corresponding cobalt complex.

Thus, the relative stabilities of the coordination complexes involved can account for the instantaneous, reversible inhibition of the zinc enzymes by OP, on the one hand, and the time-dependent, irreversible inhibition of the cobalt enzyme, on the other: OP does not compete successfully with native LADH for its catalytic zinc atoms but is effective on substitution of cobalt. Hence, the mode of inhibition becomes a functional probe for the identification of the pair of metal atoms at the catalytic sites. Details of the relevant absorption, circular dichroic, magnetic circular dichroic, and electron paramagnetic resonance spectra of the various cobalt derivatives and their correlation with coordination geometry, structure, and function of the enzyme will be reported elsewhere (A. J. Sytkowski and B. L. Vallee, in preparation).

Kinetic Considerations. Stabilities of metal coordination complexes may not be valid gauges of their rates of metal exchange (18, 19). Moreover, for metal-assisted dechelation, e.g., the exchange of \(\text{\textsuperscript{65}}\text{Zn}(II)\) or Co(II) with the zinc of (LADH)[Zn\textsubscript{2}Zn\textsubscript{3}], the magnitudes of exchange rates cannot be cited even for models. There are extensive data on rates of formation and dissociation of metal complex ions, but information on the rates of metal assisted dechelation in such complexes is limited. Moreover, we have found neither rates which specifically characterize the mutual exchange of Zn(II) and Co(II) nor data which allow suitable comparisons with other enzyme systems. Hence, chemical reactivities based on the rates of metal-metal exchange at different enzyme sites cannot now be predicted.

The nature of the intermediate mixed complex is generally agreed to critically determine the exchange rates of metals (18, 19). In different model systems, the size and rigidity of ligands and their accessibility to solvents significantly affect exchange rates to different degrees, but the stability of a metal complex may not be decisive in their control. The electronic configurations of the exchanging metal pair impose additional variability, as do auxiliary ligands (19) such as buffer ions, e.g., acetate, phosphate, or chloride, here used in metal-metal exchanges of LADH. The multiplicity of factors that generate the resultant complexity precludes prediction of metal exchange rates of an enzyme from model data, and the tertiary structure of LADH (6) does not directly account for differences in rates of Zn = \(\text{\textsuperscript{65}}\text{Zn}(II)\) or Co(II) exchange of the two pairs of zinc atoms in such terms. However, the reaction rates of Cu(II) with a series of Ni(II) polyamines increasing in dentate number could be pertinent. The greater the dentate number of polyamine coordination to Ni(II) ion, the more rapid the transfer of the ligand to copper, a course of events that has been interpreted in terms of a transition state energetically favorable to the entry of Cu(II) (19).

By analogy to the Cu(II) = Ni(II) polyamine system, a fourth S donor of cysteine to the noncatalytic zinc atom at the N site might accelerate exchange of \(\text{\textsuperscript{65}}\text{Zn}(II)\) or Co(II) = Zn(II) compared with that at the C site, where zinc is coordinated to but three ligands of the protein. This expansion of the coordination number at the N site relative to that at the C site could increase the transfer rate of the entering Me(II). Further, environmental and steric factors, e.g., the location of the noncatalytic site, coordinated in a more regular tetra-

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4 The cumulative stability constants of metal complex ions are from ref. 17. Since the corresponding values for LADH are unknown, the order and magnitudes of these constants must be considered to be of more qualitative than quantitative significance.
hedron and readily accessible to the solvent at the surface, might further contribute to faster exchange, compared with that at the distorted catalytic site 25 Å from the surface of the molecule. In this context, Leussing’s discovery of the formation of sulfur-bridged polynuclear mercaptide-zinc complexes (20) might provide the basis for a plausible exchange mechanism. Such intermediates could further account for differences in rates of $^{65}$Zn(II) or Co(II) $\leftrightarrow$ Zn(II) exchange of the C and N pair of zinc atoms, respectively.

In any one subunit, Cys 46 and 174, His 67, and one H$_2$O are arranged in an irregular tetrahedron around the catalytic zinc atom (6). Access of $^{65}$Zn(II) to and exit of the displaced zinc atom from the catalytic crevice would seem limited compared with the noncatalytic site (Cys 97, 100, 103, and 111 at the surface of the molecule), where access of $^{65}$Zn(II) or Co(II) and exit of Zn are apparently unhindered. Given suitable topology and local conformational juxtaposition of the amino acid side-chains, two $^{65}$Zn or cobalt atoms could enter into an intermediate bridged dimercaptide with the

\[
\begin{array}{c}
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\text{S} \\
\bigtriangleup \\
\text{S} \\
\bigtriangleup \\
\text{P}
\end{array}
\]

complex of the N site for any one atom which could form a bridged dimercaptide complex with the

\[
\begin{array}{c}
\text{Zn} \\
\bigtriangleup \\
\text{H}_2\text{O} \\
\bigtriangleup \\
\text{N} \\
\bigtriangleup \\
\text{S} \\
\bigtriangleup \\
\text{S}
\end{array}
\]

complex of the C site (where P is the protein).

The mechanisms that account for the difference in chemical reactivities of the catalytic and noncatalytic atoms, as gauged by thermodynamic and kinetics of metal exchange, respectively, clearly deserve further study. The above suggestions are made in efforts to identify models suitable for this purpose.

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