*STRUCTURAL AND FUNCTIONAL ZINC IN HORSE LIVER ALCOHOL DEHYDROGENASE**

By David E. Drum,† John H. Harrison, IV,‡ Ting-Kai Li,§ J. L. Bethune,** and Bert L. Vallee

BIOPHYSICS RESEARCH LABORATORY, DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON

Communicated by Hans Neurath, March 27, 1967

The location of amino acid side chains—whether at the surface or in the interior of protein molecules—and their immediate chemical environment are thought to be critical to their chemical reactivity toward modifying agents. Certain catalytically essential amino acid residues of enzymes have proven particularly reactive chemically. Similarly, metal atoms of metalloproteins can exhibit differential reactivity related to their location and roles. This is exemplified by recent studies of horse *liver alcohol dehydrogenase* (LADH).[1]

Materials and Methods.—Crystalline horse LADH isolated by a modification of the Dalziel procedure[2] and homogeneous by ultracentrifugation was obtained from Boehringer Mannheim Corporation. By chromatography on carboxymethyl cellulose, about 95 per cent of the protein and enzymatic activity eluted together in a single symmetrical peak in a position corresponding to the major component described by Dalziel[3] and contained 3.4–3.6 gm at. of Zn/mol wt 80,000. However, electrophoresis on cellulose acetate and starch gel at pH 8.5 revealed the presence of five bands, as demonstrated by staining techniques both for protein and enzymatic activity. About 75 per cent of the protein and the enzymatic activity were associated with the third and 25 per cent with the fourth cathodial bands, in accord with the findings of Pietruszko *et al.*[4, 5] Urea was recrystallized from ethanol and aqueous solutions prepared immediately before use. The control of metal contamination and the determinations of enzymatic and isotopic activities, protein concentration, and Zn content have been described.[6] A Cary model 60 spectropolarimeter was employed for the optical rotatory dispersion studies. Velocity sedimentation and sedimentation equilibrium were performed in a Spinco model E ultracentrifuge. The apparent partial specific volume[7] and density were determined pycnometrically.

Results and Discussion.—Zinc content: LADH contains two active enzymatic centers, each binding one molecule of coenzyme.[8] The Zn content of early preparations ranged from 1.7 to 2.8, with a mean of 2.1 gm at. of Zn based on a mol wt of 73,000.[9, 10] Both by spectral[11] and by kinetic[12] criteria, two moles of 1,10-phenanthroline (OP) competed with coenzyme and were thought to bind at or near the Zn atoms of the two active centers. However, the enzyme then employed contained substantial admixtures of impurities, largely eliminated by subsequent modifications of the isolation procedure as evidenced by specific enzymatic activity, sulfhydryl content, and chromatographic criteria.[2, 3] The enzyme prepared in this manner had a mol wt of 84,000.[13] The amount of Zn found earlier[9, 10] would be equivalent to 2–3.2 gm at. of Zn per mol wt 84,000. Carboxymethylation with C^{14}-iodoacetate inactivates LADH while labeling the cysteinyl residues of two identical peptides,[14, 15] suggesting two active sites and two identical subunits per mole.

The Zn content of more highly purified preparations has been found to be 4 gm at.

based on a mol wt of 84,000.[16, 17] The mol wt of the enzyme, redetermined independently in Dr. H. K. Schachman's laboratory and in our own, had been found to be 79,000[18] and 80,000 (*vide infra*), respectively. Atomic absorption, spectrographic, and chemical analyses in this laboratory of the Zn content of six different preparations (Boehringer Mannheim Corporation) varied from 3.1 to 3.9, with a mean of 3.5 gm at. of Zn per mol wt 80,000.[19] Our chromatographically purest preparations, which also exhibited the highest enzymatic activities, contained nonintegral values of 3.4–3.6 gm at. per mol wt 80,000. Similar, earlier findings in other metalloenzymes led to suggestions that different atoms of the same metal might play different roles in an enzyme: one primarily functional, the other structural but perhaps affecting function secondarily.[19]

The assignment of stoichiometry presumes precise knowledge both of the metal content and of the molecular weight of homogeneous molecular species. The discovery of LADH isoenzymes,[4, 20] and the presence of more than one isoenzyme in the preparations of LADH employed for these studies (*vide supra*) may be pertinent to the nonintegral number of Zn atoms that may find their explanation in the distribution, molecular weights, and metal contents of the subunits and in the characterization of their recombinants. The isoenzyme distribution in any given preparation may vary with the source and mode of isolation employed and should be known when stating its Zn content.

Differential reactivity of zinc atoms: The enzyme loses both activity and Zn when dialyzed at pH 5.5 over a period of 45 hours, but inactivation is more rapid than loss of Zn (Fig. 1). At 30 hours, over 75 per cent of the activity is lost, though more than half of the initial Zn content still remains. Apparently, only a fraction of the Zn is related primarily to enzymatic function. The addition of Zn^{2+} to the dialysate protects the enzyme against inactivation at low pH. All of the Zn atoms bound to LADH exchange with $^{65}Zn^{2+}$ in 0.1 *M* sodium phosphate buffer within 24 hours (Fig. 2), but with two distinct first-order rates of exchange: one with an apparent rate constant of 0.38 $hour^{-1}$, the other of 0.09 $hour^{-1}$. In contrast, in 0.1 *M* sodium acetate buffer, only two Zn atoms exchange with $^{65}Zn^{2+}$ (Fig. 2) and with a single rate constant of 0.31 $hour^{-1}$. Tris-chloride and ethylenediaminetetraacetate

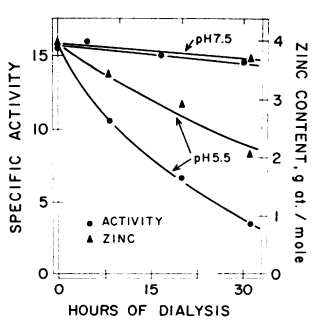

FIG. 1.—Effect of pH on specific enzymatic activity (●—●) and zinc content (▲—▲) of liver alcohol dehydrogenase. Enzyme (1 mg/ml) was dialyzed against 0.1 *M* sodium phosphate at pH 7.5 and 5.5, 4°C. The zinc content, expressed as gm at./mole enzyme is based on a mol wt of 80,000.

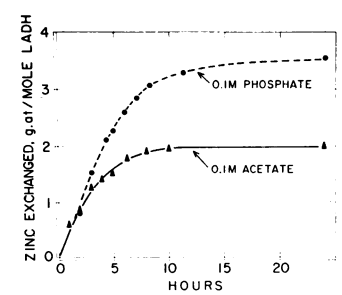

FIG. 2.—Exchange of ^{65}Zn in liver alcohol dehydrogenase. The enzyme (1 mg/ml) is dialyzed against 1.3×10^{-4} *M* $^{65}Zn^{2+}$ in 0.1 *M* sodium phosphate (●—●) and 0.1 *M* sodium acetate (▲—▲) pH 5.5, 4°C. The amount of zinc exchanged is expressed as in Fig. 1.

(EDTA) also affect the reactivity of Zn differentially.[16, 21] Using conditions derived from such experiments, all the native Zn atoms of LADH have now been replaced with Co^{2+}. The resultant cobalt LADH is catalytically active and exhibits both absorption and anomalous optical rotatory dispersion in the visible spectrum.[21]

LADH may be inhibited with chelating agents, either reversibly by their binding to the Zn atoms or irreversibly by removing the metal. OP inhibits *reversibly*, the first mechanism, through the formation of an LADH·Zn·OP-mixed complex,[11] thereby generating a characteristic absorption spectrum, a positive extrinsic Cotton effect,[22] and a circular dichroism band, centered at 271 mμ (Fig. 3). Both spectrophotometric[11] and rotatory dispersion titrations[23] demonstrate the binding of only two moles of OP per mole of enzyme (Fig. 3). Similarly, 2 moles of α,α′-bipyridyl bind to the Zn of the enzyme, as measured by rotatory dispersion titration.[21]

Sodium diethyldithiocarbamate (NaDDC), having greater affinity for LADH zinc than either OP or bipyridyl, inhibits the enzymes *irreversibly* by removing Zn, the second mechanism. When two Zn atoms of LADH are labeled with $^{65}Zn^{2+}$ in acetate buffer (*vide supra*) and the enzyme (1 mg/ml) is then exposed to 0.1 *M* NaDDC, pH 7.5, 4°, all enzymatic activity is lost in 72 hours. Only the two ^{65}Zn-labeled, but none of the unlabeled Zn atoms, are removed; further dialysis does not remove any additional Zn. Concomitantly, the OP Cotton effect is abolished, indicating that OP and NaDDC interact with the same two Zn atoms of the enzyme, i.e., those which exchange in acetate at pH 5.5 (Table 1). These two Zn atoms are chemically more reactive than the remainder and seem to be the ones involved directly in the catalytic function of the enzyme. Carboxymethylation of the active center cysteinyl residues also inactivates the enzyme, and renders the Zn atoms of LADH less stable.[6] Dialysis at pH 7.5 of carboxymethylated liver alcohol dehydrogenase (CM-LADH) labeled with ^{65}Zn in acetate buffer shows that only the *unlabeled* Zn atoms are lost. The two ^{65}Zn-labeled atoms remain bound and OP binding to CM-LADH is unimpaired (Table 1). Although carboxymethylation completely inactivates the enzyme, this is not due to the loss of its catalytically essential Zn atoms.

Depending on their location in the three-dimensional structure of proteins, amino acid side chains are known to react more or less readily with organic reagents; they are considered to be "free" and accessible to the ambient medium, or "buried" in the interior and inaccessible to reagents. Analogous considerations appear to pertain to the Zn atoms of LADH. The two Zn atoms that are catalytically essential are

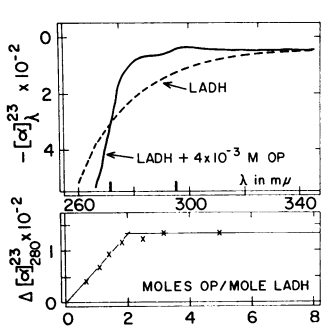

FIG. 3.—Extrinsic Cotton effects of enzyme·zinc·1,10-phenanthroline complex: rotatory dispersion titration. *Upper:* The rotatory dispersion of 67 mg/ml of enzyme (---) and enzyme plus 4×10^{-3} *M*, OP (——) in 0.1 *M* phosphate, pH 7.5, was measured in an 0.2-mm cell at 23°C. Extrinsic Cotton effects arise in the 297- and 271-mμ absorption bands of the LADH·Zn·OP complex. *Lower:* Increasing concentrations of OP were added to LADH and the optical rotatory dispersion of the solutions measured at 23°C. The change in specific rotation at 280 mμ ($\Delta[\alpha]^{23}_{280}$) due to binding of OP is plotted against the moles of OP added per mole of enzyme (×). Saturation of binding is attained at 2 moles of OP/mole LADH.

"free" to exchange with $^{65}Zn^{2+}$ in acetate buffer and to interact with OP, NaDDC, and bipyridyl, resulting in inhibition. The balance may be "buried" in the interior, and hence exchange slowly or not at all and are inaccessible to these chelating agents until exposed by modification of protein structure; they apparently serve a different function. The reactivity of the "buried" metal atoms seems sensitive to protein conformation which generates their chemical environment. Selective carboxymethylation of the active center cysteinyl residues apparently both exposes the "buried" Zn atoms—perhaps through a local conformation change—and renders them even more labile than the "free" Zn atoms. Thus, the conformation, tertiary and quaternary structure of the protein, the mode of binding of the metal atoms, and their location, environment, and accessibility may combine to account for the observed differences in the chemical and biological properties of the various Zn atoms.

Subunit structure: A role for Zn in the tertiary structure of LADH has been suggested,[16] and the role of metals in stabilizing the quaternary structure of other enzymes has been documented.[24, 25] The effects of 8 *M* urea, mercaptoethanol, and EDTA upon the sedimentation coefficient, molecular weight, and Zn content of the enzyme are shown in Table 2. Based on an apparent partial specific volume of 0.743 ml/gm,[26] the mol wt of the present material is 80,000, in good agreement with the results of Hamburg and Schachman.[18] In 8 *M* urea, the sedimentation coefficient of the enzyme decreases from 4.8*S* to 2.4*S* and the mol wt to 40,000, demonstrating dissociation into two subunits of approximately equal molecular weight which still contain all of the Zn initially present (Table 2*A*). Mercaptoethanol and EDTA do not remove Zn from the native enzyme but in 8 *M* urea, mercaptoethanol displaces 1.5 gm at. per mol wt 80,000, and EDTA removes all of the metal. In

TABLE 1

ZINC CONTENT AND 1,10-PHENANTHROLINE BINDING OF NATIVE AND MODIFIED LIVER ALCOHOL DEHYDROGENASE (MOL WT: 80,000)

	Zn total (gm at./mole)	^{65}Zn labeled* (gm at./mole)	Zn unlabeled (gm at./mole)	OP bound (mole/mole)
Native LADH	3.5	2.0	1.5	2.0
DDC-LADH†	1.5	0	1.5	0
CM-LADH‡	2.0	2.0	0	2.0

* By dialysis against $^{65}Zn^{2+}$ in 0.1 *M* acetate buffer pH 5.5, 4°.
† Inactivated with 0.1 *M* Na diethyldithiocarbamate, pH 7.5.
‡ Inactivated with 600 × molar excess of iodoacetate, pH 7.5.[27]

TABLE 2

EFFECT OF UREA, MERCAPTOETHANOL, AND EDTA* ON SEDIMENTATION COEFFICIENT, MOLECULAR WEIGHT, AND ZINC CONTENT OF NATIVE AND MODIFIED LIVER ALCOHOL DEHYDROGENASE

	$s_{20,w}$ (*S*)	Mol wt	Zn (μg/gm)	Zn (gm at./mol wt 80,000)
A. Native LADH	4.8	80,000	2900	3.5
In 8 *M* urea	2.4	40,000	2800	3.4
In 8 *M* urea and 0.1 *M* mercaptoethanol	1.5	20,000	1550	1.9
In 8 *M* urea and 0.01 *M* EDTA	1.5	20,000	0	0
B. DDC-LADH	4.8	—	1200	1.5
In 8 *M* urea	2.2	—	1200	1.5
In 8 *M* urea and 0.1 *M* mercaptoethanol	1.5	—	0	0
C. CM-LADH	4.8	—	1600	2.0
In 8 *M* urea	1.6	—	1550	1.9

* Mercaptoethanol, 0.1 *M*, and EDTA, 0.01 *M*, do not alter the sedimentation coefficient of LADH in 0.1 *M* phosphate buffer, pH 7.5.

both instances the protein then dissociates further into four subunits with an average mol wt of 20,000. Urea apparently unfolds and dissociates the native protein into dimeric units. This renders the Zn atoms accessible to mercaptoethanol and EDTA, and their removal is then accompanied by dissociation of the dimers into monomers. A fraction of the Zn atoms thus seems to maintain the subunit structure of the enzyme. Further observations are consistent with this interpretation.

NaDDC removes the two catalytically essential, "free" Zn atoms of LADH (Table 2*B*) but apparently without altering the quaternary structure: the sedimentation coefficient remains unchanged and the protein dimers are stabilized by the "buried" Zn atoms remaining in DDC-LADH. Since CM-LADH lacks these "buried" atoms, urea alone should dissociate the tetramer into monomers as is, indeed, observed (Table 2*C*).

Previous studies revealed two identical active center cysteinyl peptides[14, 15, 27] in LADH thought to reflect two identical polypeptide chains. A tetramer suggests two different monomeric forms, nonidentical in linear sequence; each dimeric subunit or protomer would then presumably contain one active enzymatic center. If the two different monomeric forms can aggregate randomly into active tetramers, similar to other multichain dehydrogenases, five active isoenzymes would be expected, in accord with results of recent electrophoretic studies.[4, 20] Furthermore, if the "buried" Zn atoms were to link together some, but not all, of the possible pairs of monomers, the individual isoenzymes could contain two, three, or four gm at. of Zn per mol wt 80,000. Hence, any given preparation containing mixtures of isoenzymes would have nonintegral values of Zn between two and four, depending on the proportions of its isoenzymes. The reversibility of the effect of urea was investigated to delineate this aspect of the problem further.

Reversible inactivation: Within 15 minutes, 8 *M* urea and 0.1 *M* mercaptoethanol inactivate LADH. On dilution and dialysis in 0.1 *M* phosphate buffer, pH 7.5, the enzyme is partially reactivated. The addition of either DPNH or Zn^{2+} to the diluent increases the per cent of control activity regained, and, jointly, they restore as much as 70 per cent of the original activity (Table 3). Coenzyme and mercaptoethanol are known to be required for the reactivation of other multichain DPN(H)-dependent dehydrogenases.[28] In the present instance, mercaptoethanol in conjunction with urea additionally removes Zn, perhaps accounting for the effect of Zn^{2+} in further restoring activity. The precise delineation of critical conditions, currently under investigation, should permit the reassociation of dissociated liver alcohol dehydrogenase into the enzymatically active tetramer.

TABLE 3

REVERSIBLE INACTIVATION OF LIVER ALCOHOL DEHYDROGENASE BY UREA AND MERCAPTOETHANOL

Reactivation (1:10 dilution)	Activity (% of control)
0.1 *M* phosphate, pH 7.5	30
+ DPNH, 6×10^{-4} *M*	50
+ Zn^{2+}, 6×10^{-6} *M*	35–40
+ DPNH, 6×10^{-4} *M*, and Zn^{2+}, 6×10^{-6} *M*	60–70

Enzyme, 0.5 mg/ml, in 0.1 *M* phosphate, pH 7.5, is inactivated by 8 *M* urea and 0.1 *M* mercaptoethanol in 10 min, 21°C. The solution is diluted with 10 vol of phosphate buffer containing DPNH, Zn^{2+}, or both and incubated at 21°C for 2 hr. Activity is measured after 16 hr of dialysis against 100 vol of 0.1 *M* phosphate, pH 7.5, 4°C.

Recent studies of metalloenzymes have focused upon the role of metals in their catalytic function and structure. The metal atoms of carboxypeptidase[29] and carbonic anhydrase,[30] both single polypeptide chains, each containing only one metal atom, have been shown to be essential to the catalytic process, apparently their primary function. It is possible, of course, that they concomitantly stabilize the tertiary structure of the active molecule, though this has not been documented as yet. The metal atom of some single-chain enzymes might serve largely to stabilize tertiary structure and hence affect catalytic activity indirectly.

In multichain metalloenzymes, metal atoms can have a third role by establishing cross-links between subunits or otherwise stabilizing *quaternary* structure. In these instances the interaction of metal atoms with proteins would presumably differ, depending upon whether a given metal atom would be involved in the catalytic process or in providing a coordination bridge between subunits. To realize these functions, some enzymes may employ two chemically different metal atoms to achieve catalytic function and/or stabilization of tertiary structure on the one hand and stabilization of quaternary structure on the other. This is well illustrated by α-amylase of *B. subtilis* where the catalytic role has been assigned to Ca^{2+}, while Zn^{2+} is thought to link together subunits, but without affecting activity.[25, 31] The effects of a pair of metals need not necessarily be that discrete in other systems, of course. It is conceivable, in fact, that two different metal atoms could serve as the active sites of two different catalytic activities in the same enzyme, though no such instance has been documented as yet with certainty. Furthermore, the same metal species may serve roles both in catalytic function and the quaternary structure of an enzyme as illustrated by yeast ADH, where it has not been possible to assign separate functional or structural roles to any one of the four Zn atoms of the enzyme. In that instance each Zn atom seems both to interact with one of the four molecules of NAD(H) and to stabilize quaternary structure.

It is of considerable interest that individual Zn atoms of LADH manifest chemically distinct properties that can be related to roles either in the catalytic function or in the quaternary structure of the enzyme. One might surmise that the thermodynamic and physical properties, characteristically different for these "free" and "buried" Zn atoms, could also imply different binding sites, stability constants, and other properties of the respective metal complexes.

Metal coordination complexes have not been considered, apparently, as significant factors in the stabilization of quaternary structure, which is essential to the catalytic function of some enzymes. The present data suggest that in addition to the energy provided by hydrophobic and electrostatic interactions, Van der Waals forces, and hydrogen bonding, that of metal coordination bonds should be considered in interaction of polypeptide subunits of multichain enzymes. They further indicate that metals may play a regulatory role in the formation and function of isoenzymes.

Summary.—Horse LADH, containing 3.4–3.6 gm at. of zinc/mol wt 80,000, dissociates into two subunits in 8 *M* urea and further into four subunits of mol wt 20,000 upon removal of a fraction of the zinc content. Two zinc atoms are essential for activity, "free" and accessible to the ambient medium as gauged by their reactivity toward certain chelating agents and by isotope exchange. They do not appear to participate in subunit interactions. The balance are unreactive or "buried" but participate in forming the quaternary structure of the enzyme. The

relationship of zinc content, reactivity, and modes of interaction with subunit and isoenzyme structure is discussed.

* This work was supported by grants-in-aid HE-07297 from the National Institutes of Health, Bethesda, Maryland.

† National Institutes of Health postdoctoral fellow.

‡ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

§ Fellow of the Medical Foundation.

** Career Development Awardee of the National Institutes of Health.

[1] Abbreviations: LADH, liver alcohol dehydrogenase; CM-LADH, carboxymethylated liver alcohol dehydrogenase; DDC-LADH, liver alcohol dehydrogenase inactivated with diethyldithiocarbamate; OP, 1,10-phenanthroline; NaDDC, sodium diethyldithiocarbamate; mol wt, molecular weight; gm at., gram atoms.

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