Zinc Isotope Exchange in Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: $^{65}Zn^{2+}$ exchange has been employed to identify selectively the two zinc atoms involved in the catalytic activity of horse liver alcohol dehydrogenase of mol wt 80 × 10^3. At pH 5.5 in 0.1 M acetate only these two zinc atoms of the enzyme exchange with $^{65}Zn^{2+}$ in the buffer, whereas in phosphate buffer at pH 5.5 all of the zinc atoms exchange. During inactivation by either hydrogen ions or by diethyldithiocarbamate, enzymatic activity is directly proportional to the content of the two "free" zinc atoms of the enzyme exchangeable in 0.1 M acetate. The kinetics of the isotope-exchange reactions of horse liver alcohol dehydrogenase are consistent with two different populations of zinc atoms. At low horse liver alcohol dehydrogenase concentrations in 0.1 M phosphate buffer, two first-order exchange reactions are observed, corresponding to the exchange of "free" and "buried" zinc atoms, respectively. Unlike the exchange of the "buried," that of the "free" zinc atoms is retarded as a function of increasing horse liver alcohol dehydrogenase concentrations, in agreement with properties of the zinc atoms involved in the catalytic activity of horse liver alcohol dehydrogenase previously described.

Zinc has been known to be present in horse LADH[1] for over a decade (Theorell *et al.*, 1955; Vallee and Hoch, 1957) and its involvement in catalytic function has been well established (Vallee *et al.*, 1959b; Plane and Theorell, 1961; Yonetani, 1963). However, it has become apparent only recently that the element may also serve to stabilize the quaternary structure of the enzyme (Drum *et al.*, 1967). The zinc atoms in LADH which serve in catalytic function have been called "free" since they are exposed to the ambient environment; those involved in structure stabilization are much less accessible and have been designated "buried" by analogy with amino acid residues of proteins. Thus, the two zinc atoms that are catalytically essential are "free" to interact with 1,10-phenanthroline, 2,2′-bipyridine, and diethyldithiocarbamate, resulting in inhibition (Vallee and Hoch, 1957; Vallee *et al.*, 1959b; Sigman, 1967; Drum *et al.*, 1969). The remainder are "buried" in the interior; they are inaccessible to these chelating agents until exposed by modification of protein structure or by addition of excess hydrogen ions (Drum *et al.*, 1967, 1969).

The two catalytically essential zinc atoms also differ in physicochemical properties from those which are apparently largely involved in structural stabilization. In particular, the exchange of $^{65}Zn^{2+}$ for the intrinsic zinc atoms of LADH has proven an effective experimental means to differentiate the enzymatically active from the structural zinc atoms and for the selective labeling of either type of zinc atoms in LADH.

Methods

LADH was obtained from the Boehringer-Mannheim Corp. as a crystalline suspension in 10% ethanol–0.02 M sodium phosphate (pH 7). The two samples of LADH (lot no. 6076327 and 6256238) exhibited nearly identical specific enzymatic activities (13.8–14.5 ΔA_{340}/min per mg) and contained 3.4 g-atoms of zinc/mole of LADH, based on a molecular weight of 80 × 10^3 (Drum *et al.*, 1967) and the measured specific absorptivity (Drum *et al.*, 1969). Total zinc content of the enzyme was measured either by atomic absorption spectrometry (Fuwa *et al.*, 1964) or by diphenylthiocarbazone extraction (Vallee and Gibson, 1948).

The rate of exchange of $^{65}Zn^{2+}$ with the protein-bound zinc of LADH was measured during open dialysis at 4° using metal-free reagents, glassware, and dialysis tubing prepared as described elsewhere (Drum, 1967). Stock solutions of 3–5 × 10^{-4} M LADH in 0.1 M Tris-Cl (pH 7) were diluted to the desired concentrations with 0.1 M sodium acetate or phosphate buffer (pH 5.5) containing $^{65}Zn^{2+}$ and carrier Zn^{2+}. This mixture was placed into an open dialysis sac, permitting sampling, and dialyzed against 100 volumes of the same $^{65}Zn^{2+}$ containing acetate or phosphate buffer, constantly stirred by a magnetic mixer. A blank solution was prepared identically, using an aliquot of the 0.1 M Tris-Cl buffer in place of the enzyme solution. When correction for transport of Zn^{2+} across the dialysis membrane was necessary, an aliquot of the outside buffer was also sampled for measurement.

$^{65}Zn^{2+}$ was measured with a Tracerlab SC-76 spectrometer and SC-57A low-background well-type scintillation counter. The isotope (Iso/serve, Inc., Cambridge, Mass.) was of sufficiently high radiochemical purity (0.6 Ci/g) that its addition to carrier Zn^{2+} did not alter the absolute Zn^{2+} concentration significantly. Counting of enzyme and buffer blanks was performed on aliquots containing at least 10^4 cpm. The extent of zinc exchange was determined by

$$\text{g-atoms of } {}^{65}Zn^{2+}\text{/mole of LADH} = \frac{\text{net cpm/ml of LADH}}{\text{net cpm/ml of buffer}} \times \frac{(Zn^{2+})}{(\text{LADH})}$$

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[1] Abbreviations used are: LADH, horse liver alcohol dehydrogenase; $K_{E,I}$, dissociation constant for an enzyme–inhibitor complex.

TABLE I: $^{65}Zn^{2+}$ Exchange in LADH.[a]

Buffer (0.1 M, pH 5.5)	(LADH), M × 10^5	$^{65}Zn^{2+}$ Exchanged (g-atoms/mole)	Total Stable Zn (g-atoms/mole)	Sp Enzymatic Act.
Acetate	1.3	2.0	3.4	14.2
	7.5	2.0	3.4	14.5
	30	2.0	3.4	14.4
Phosphate	1.3	2.4	2.4	7.4
	8.2	3.4	3.4	14.2
	38	3.4	3.4	14.5

[a] The enzyme solutions were dialyzed against 1.3 × 10^{-4} M $^{65}Zn^{2+}$, in phosphate or acetate buffer (pH 5.5) until $^{65}Zn^{2+}$ incorporation was more than 95% complete. Buffer $^{65}Zn^{2+}$ was then removed by dialysis against 0.1 M Tris-Cl (pH 7.0) and the enzyme solutions were analyzed for specific enzymatic activity, $^{65}Zn^{2+}$, and total stable Zn content. The initial enzymatic activity was 14.4 and the Zn content was 3.4 g-atoms/mole.

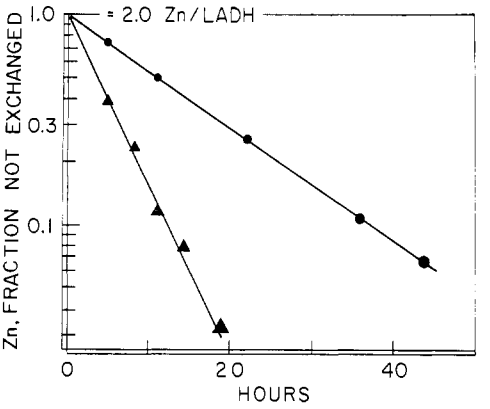

FIGURE 1: Zinc isotope exchange in LADH: acetate buffer. LADH, 30 × 10^{-5} M (●) and 7.5 × 10^{-5} M (▲), was dialyzed against $^{65}Zn^{2+}$ (1.3 × 10^{-4} M) in 0.1 M acetate buffer, pH 5.5, 4°. The course of the exchange reaction is reported in terms of the fraction of the 2.0 g-atoms of Zn/mole of LADH not exchanged, as discussed in the Appendix.

Analytical electrophoresis was performed on cellulose polyacetate (Drum *et al.*, 1969) and acrylamide gel (Canalco, Inc.). Sedimentation velocity measurements and inactivation of LADH by dialysis against sodium diethyldithiocarbamate were performed as described previously (Drum *et al.*, 1967, 1969).

Results

At neutral pH, the zinc atoms are firmly bound to LADH (Drum *et al.*, 1969), but they exchange with Zn^{2+} of the ambient medium at pH values below 6.5 (Druyan and Vallee, 1964). When Zn^{2+} is present in the buffer, the enzyme remains stable even at pH 5.5 (Drum *et al.*, 1969). In addition to pH, the nature of the buffer anions and their concentration and those of Zn^{2+} and LADH critically determine the course of the exchange reaction.

In 0.1 M acetate buffer (pH 5.5) dialysis of LADH containing 3.4 g-atoms of Zn/mole against $^{65}Zn^{2+}$ results in the incorporation of 2.0 g-atoms of ^{65}Zn/mole of enzyme of mol wt 80 × 10^3, at equilibrium. This end point is invariant over a wide range of enzyme concentration, although enzyme concentration does affect the *rate* of the exchange reaction (*vide infra*). After removal of excess $^{65}Zn^{2+}$ by dialysis against metal-free Tris-Cl buffer at pH 7.5, the total zinc content and the specific enzymatic activity of the ^{65}Zn-labeled enzyme are identical with those of the native enzyme (Table I). Thus, under these conditions, only 2.0 of the 3.4 zinc atoms of this preparation of LADH exchange with $^{65}Zn^{2+}$. Furthermore, in acetate, this end point of $^{65}Zn^{2+}$ isotope exchange is unaffected by the addition of 0.1 M phosphate (*vide infra*).

The uptake of $^{65}Zn^{2+}$ by 3.0 × 10^{-4} M LADH follows an exponential course (Figure 1), and the exchange is 95% complete in 50 hr. At lower enzyme concentrations, *i.e.*, 7.5 and 1.3 × 10^{-5} M LADH, the course of the reaction is similar, but the exchange is 95% complete in 16 and 9.5 hr, respectively. In each instance the ^{65}Zn-labeled LADH is fully active (Table I). Only at the lowest enzyme concentration (1.3 × 10^{-5} M), enzymatic activity begins to decline when dialysis is prolonged beyond the time required to complete exchange.

The selective nature of the exchange is confirmed by the reverse experiment. Enzyme prepared in the above manner and containing 2.0 g-atoms of ^{65}Zn/mole is dialyzed at pH 5.5 against 0.1 M acetate buffer containing stable Zn^{2+}. The 2.0 g-atoms of ^{65}Zn/mole incorporated previously are lost to the ambient environment and are now replaced by 2.0 g-atoms of stable zinc/mole. Thus, the exchange reaction is fully reversible and pertains to a particular class and number of zinc atoms; intramolecular exchange of the labeled with the unlabeled zinc atoms is not detected by this means.

However, in 0.1 M phosphate buffer (pH 5.5) *all* of the zinc atoms of LADH exchange at all the enzyme concentrations examined, contrasting with the results in 0.1 M acetate. Further, the stability of the enzyme and the time course of the reaction are altered. After dialysis to remove free $^{65}Zn^{2+}$, the specific enzymatic activity and *total* zinc content of solutions of 3.8 × 10^{-4} or 8.2 × 10^{-5} M LADH are identical with those of native LADH. At the lowest enzyme concentration examined (1.3 × 10^{-5} M), both enzymatic activity and zinc are partially lost (Table I) when the exchange reaction is allowed to proceed to completion.

In 0.1 M phosphate buffer the uptake of $^{65}Zn^{2+}$ by 3.8 × 10^{-4} M LADH increases exponentially and is 96% complete at 72 hr (Figure 2). At enzyme concentrations of 8.2 and 1.3 × 10^{-5} M, the exchange is more rapid and, significantly, two exchange rates become apparent (Figure 2). This is in contrast to the single rate of exchange observed for 3.8 × 10^{-4} M LADH in 0.1 M phosphate and for all concentrations of LADH in 0.1 M acetate.

Samples of LADH labeled either with 2.0 g-atoms of ^{65}Zn/mole by exchange in 0.1 M acetate or with 3.4 g-atoms of ^{65}Zn/mole by exchange in 0.1 M phosphate crystallize from 10% ethanol in 0.02 M phosphate (pH 6.8) as does the native enzyme (Bonnichsen and Brink, 1955). They are indistinguishable from each other and from the native enzyme by acrylamide gel and cellulose polyacetate electrophoresis, and all three enzymes

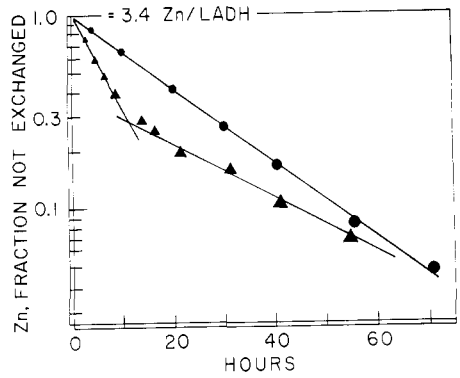

FIGURE 2: Zinc isotope exchange in LADH: phosphate buffer. LADH, 38×10^{-5} M (●) and 8.2×10^{-5} M (▲), was dialyzed against $^{65}Zn^{2+}$ (1.3×10^{-4} M) in 0.1 M phosphate buffer, pH 5.5, 4°. The course of the exchange is reported in terms of the fraction of the 3.4 g-atoms of Zn/mole of LADH not exchanged, as discussed in the Appendix.

sediment with $s_{20,w} = 4.8$ S. Thus, by these criteria, LADH isotopically labeled with ^{65}Zn through isotope exchange in either 0.1 M acetate or phosphate differs from the native enzyme only in the content of isotopic zinc.

Investigations of the effects of reactant concentrations on the rate constant of exchange, k_a, further reveal important features of the isotope exchange reaction in LADH. The apparent rate constants (see Appendix) for the zinc atoms that exchange rapidly in 0.1 M phosphate buffer, k_{a1}, and for those that exchange in 0.1 M acetate, k_a, are comparable in magnitude (Table II). When Zn^{2+} concentration is decreased tenfold the apparent rates decrease approximately threefold in either of the buffers. The bimolecular rate constants calculated from the apparent rate constants at both $^{65}Zn^{2+}$ concentrations show less than 15% variation, in accord with theory.

In contrast to the effect of Zn^{2+} concentration, a decrease of LADH concentration from 30 to 1.3×10^{-5} M *increases* the rate of exchange, in both these acetate and phosphate buffers (Table III). The computed bimolecular rate constants differ by as much as 30-fold, markedly at variance with the effect

TABLE II: Effect of Zn^{2+} Concentration on Isotope Exchange in LADH.

Buffer (0.1 M)	(LADH), M × 10^5	($^{65}Zn^{2+}$), M × 10^4	k_a (or k_{a1}) hr^{-1}	k (or k_1) M^{-1} hr^{-1} × 10^{-2}
Acetate	8.4	1.7	0.12	4.7
	8.4	0.17	0.04	4.0
Phosphate	8.2	1.3	0.11[a]	5.2[a]
	8.2	0.13	0.045[a]	4.8[a]

[a] In phosphate buffer there are two rates of $^{65}Zn^{2+}$ exchange at this enzyme concentration (Figure 2). The tabulated values are the apparent rate constants, k_{a1}, and the corresponding bimolecular rate constants, k_1, computed for the more rapidly exchanging zinc atoms.

TABLE III: Effect of LADH Concentration on Isotope Exchange in LADH.

Buffer (0.1 M)	(LADH), M × 10^5	($^{65}Zn^{2+}$), M × 10^4	k_a (or k_{a1}) hr^{-1}	k (or k_1) M^{-1} hr^{-1} × 10^{-2}
Acetate	1.3	1.3	0.31	22.0
	7.5	1.3	0.19	9.5
	30	1.3	0.060	1.4
Phosphate	1.3	1.3	0.38[a]	27.0[a]
	8.2	1.3	0.11[a]	5.2[a]
	38	1.3	0.043	0.85

[a] In phosphate buffer there are two rates of $^{65}Zn^{2+}$ exchange at this enzyme concentration (Figure 2). The tabulated values are the apparent rate constants, k_{a1}, and the corresponding bimolecular rate constants, k_1, computed for the more rapidly exchanging zinc atoms.

expected from reactant concentrations on simple isotope exchange. This effect was examined further employing 0.1 M acetate buffer and 1.3×10^{-4} M $^{65}Zn^{2+}$: retardation of isotope exchange with increasing LADH concentration was evident over the entire concentration range from 0.4 to 50 × 10^{-5} M LADH, although in no instance did the exchange of zinc exceed 2.0 g-atoms/mole. The requirements for maintaining full enzymatic activity, enzyme solubility, and the technical aspects of measuring isotope exchange place limitations on extending the range of concentration beyond those examined.

The effect of 0.1 M acetate on the end point of the $^{65}Zn^{2+}$ exchange suggested an approach for the identification of the enzymatically active zinc atoms and their differentiation from others. Diethyldithiocarbamate removes only two zinc atoms from LADH, resulting in loss of enzymatic activity (Drum *et al.*, 1969). When 1.2×10^{-5} M LADH is first differentially labeled with $^{65}Zn^{2+}$ in 0.1 M acetate buffer and then exposed to 0.01 M diethyldithiocarbamate in 0.1 M Tris-Cl (pH 7.5), only the two ^{65}Zn atoms but none of the unlabeled ones are removed. The loss of enzymatic activity correlates directly with the loss of radioactivity (Figure 3). Thus, the two metal atoms which exchange with $^{65}Zn^{2+}$ in 0.1 M acetate seem to be identical with those at the active centers (Drum *et al.*, 1969).

Additional support for this interpretation derives from the effect of H^+ on ^{65}Zn-labeled LADH. When LADH is dialyzed at low pH against *metal-free* buffer, both zinc and enzymatic activity are lost progressively and enzymatic *activity* declines more rapidly than the *total* zinc content (Drum *et al.*, 1969). This indicates that not all of the zinc atoms are directly related to catalysis. In contrast, when LADH containing 2.0 g-atoms of ^{65}Zn/mole, labeled by exchange in 0.1 M acetate buffer, is exposed to metal-free acetate buffer at low pH, the loss of enzymatic activity and loss of *radioactive* zinc atoms correlate closely at all pH values (Figure 4).[2]

[2] When enzyme labeled with $^{65}Zn^{2+}$ in 0.1 M acetate buffer is dialyzed against 0.1 M phosphate buffer at pH 5.0, 5.5, or 6.0, enzymatic activity is lost more rapidly than $^{65}Zn^{2+}$, but the relation between these parameters is closer than the divergence of enzymatic activity and *total* zinc content previously noted (Drum *et al.*, 1969).

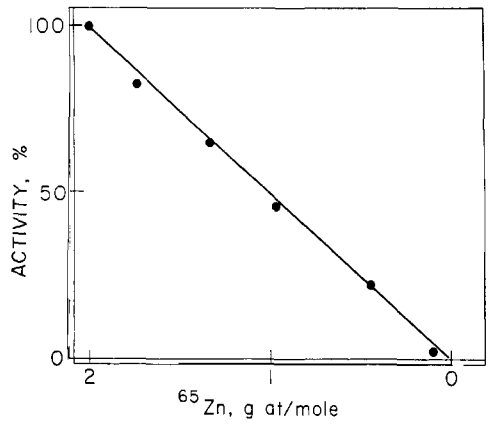

FIGURE 3: Enzymatic activity and ^{65}Zn content of ^{65}Zn-labeled LADH during inactivation by diethyldithiocarbamate. After differential labeling with $^{65}Zn^{2+}$ in 0.1 M acetate buffer, 1.2×10^{-5} M LADH, containing 2.0 g-atoms of ^{65}Zn/mole, was dialyzed against 0.01 M diethyldithiocarbamate in 0.1 M Tris-Cl (pH 7.5). During the subsequent inactivation, aliquots were removed and both specific enzymatic activity and ^{65}Zn content were measured.

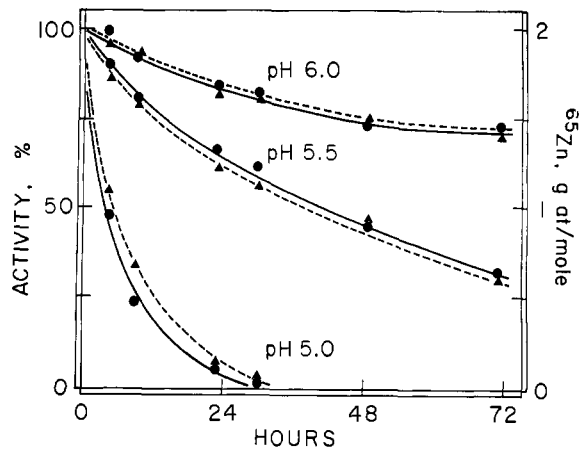

FIGURE 4: Correlation of loss of activity and labeled zinc atoms at low pH. LADH, labeled with $^{65}Zn^{2+}$ at the two active site zinc atoms by isotope exchange in 0.1 M acetate buffer, was dialyzed at a concentration of 1.2×10^{-5} M against metal-free 0.1 M acetate buffer, at pH 5.0, 5.5, and 6.0. Aliquots of each LADH solution were periodically removed from the dialysis sacs and analyzed for enzymatic activity (●) and $^{65}Zn^{2+}$ content (▲).

Discussion

Zinc may serve in the structure and function of metalloenzymes. The single zinc atoms in carboxypeptidase (Vallee and Neurath, 1954) and in carbonic anhydrase (Lindskog, 1960) are essential for the catalytic process, and the metal has not been found to affect tertiary structure. In multichain metalloenzymes, metals may serve also to stabilize quaternary structure. Thus, zinc functions solely to link the subunits of the α-amylase of *Bacillus subtilis;* the enzymatic activity of the zinc-free monomer is identical with that of the zinc-containing dimer (Vallee *et al.*, 1959a; Stein and Fischer, 1960). Zinc may play roles *both* in catalytic function and quaternary structure of an enzyme, as in yeast alcohol dehydrogenase, but here separate functional or structural roles for the individual zinc atoms of the enzyme have not been detected so far (Williams *et al.*, 1958; Kägi and Vallee, 1960).

The number and stoichiometry of metal atoms in multichain enzymes may vary depending upon the source and mode of preparation, the existence of isoenzymes, the conditions to which the enzyme is exposed prior to analysis, and the sophistication of the methods available for purification and characterization. Thus, the zinc content of fully active horse LADH can vary from 3.1 to 4.3 g-atoms of Zn per mol wt 80×10^3 (Drum *et al.*, 1969). Similar variations have been noted in yeast alcohol dehydrogenase (Vallee and Hoch, 1955; Sund and Theorell, 1963) and alkaline phosphatase of *E. coli* (Simpson and Vallee, 1968a,b; Simpson *et al.*, 1968; Harris and Coleman, 1968). While giving new insight concerning the role of metals in such proteins, this variability presents operational problems in relating the functional properties of metals to other parameters of the enzyme, such as the number of coenzyme binding sites or protein subunits.

In alkaline phosphatase of *E. coli*, metal atoms serving different roles can be identified and differentiated chemically (Simpson and Vallee, 1968b). Spectral properties characteristic of two functionally different species of metal, one catalytic and one structural, may be identified after substitution of cobalt for zinc. The zinc atoms in LADH can be differentiated similarly into chemically distinct classes; under appropriate conditions, they differ in their capacities for isotope exchange with $^{65}Zn^{2+}$.

LADH labeled with $^{65}Zn^{2+}$ by isotope exchange at pH 6.0 has been utilized previously to study the binding of coenzymes, substrates, and inhibitors which retard the isotope-exchange reaction (Druyan and Vallee, 1964). Subsequent experiments (Drum *et al.*, 1969) indicated that hydrogen ions compete with zinc of the enzyme and that different buffer anions critically affect this process, suggesting the present study. In order to accomplish more rapid exchange than that which occurs at pH 6.0 and to avoid the instability of LADH at pH 5.0, an intermediate pH, *i.e.*, 5.5, was chosen for the present study.

In the presence of 0.1 M acetate, only two of the zinc atoms of LADH are exchangeable, while after zinc exchange in 0.1 M phosphate buffer, LADH contains ^{65}Zn at *all* zinc binding sites (Table I).[3] It is somewhat surprising to find that the nature and concentration of the buffer ions can exert such a profound and specific effect on the end point of zinc exchange. Nevertheless, at equilibrium, ^{65}Zn-labeled LADH prepared in either buffer was identical with the native enzyme in all respects except for the content of ^{65}Zn.

The known characteristics of the enzyme together with this differential isotope exchange have permitted specific identification of the zinc atoms in terms of function. Activity correlates closely with the zinc atoms which exchange in 0.1 M acetate buffer (Figure 4). Moreover, these appear to be the zinc atoms responsible for the binding of 1,10-phenanthroline (Drum *et al.*, in preparation), and their removal by diethyldithiocarbamate is accompanied by loss of the characteristic Cotton effects of the enzyme–Zn–1,10-phenanthroline complex. Thus, the individual zinc atoms of LADH manifest chemically distinct properties that appear to be related to roles either in catalysis or to stabilization of quaternary structure of the enzyme (Drum *et al.*, 1967). The two zinc atoms which exchange in 0.1 M acetate are those which are catalytically es-

[3] For such experiments, the concentration and identity of buffer ions and the ionic strength are critical.

sential. The remainder which do not exchange in 0.1 M acetate apparently serve a structural role (Drum *et al.*, 1967). It would be anticipated that still other properties of these two classes of zinc atoms, which we have designated "free" and "buried," respectively, would vary on the basis of their differences in binding sites.

Since addition of sodium phosphate does not affect the isotope exchange in 0.1 M acetate buffer, acetate seems to prevent the exchange of the structural zinc atoms. There are indications for several modes of binding of acetate to LADH. The $K_{E,I}$ for acetate is 0.9–1.3 M, as measured by the displacement of 2,2′-bipyridine, which binds at the active-site zinc atoms (Sigman, 1967). However, the $K_{E,I}$ for acetate measured fluorimetrically by competition with NADH is 0.09 M (Winer and Theorell, 1960), suggesting that at lower concentrations acetate interacts with LADH largely in the vicinity of the coenzyme binding site. Acetate may bind even more firmly at yet other sites on the molecule, since significant quantities of it are bound to LADH when prepared by a modification of the Dalziel procedure (Jörnvall, 1967; Taniguchi *et al.*, 1967). It could well be that at certain concentrations acetate binds to LADH in such a manner as to prevent exchange of the buried zinc atoms. Based on these data, little, if any, acetate at the concentration here employed should be bound to zinc atoms involved directly in catalysis. The effect of acetate on zinc exchange appears to be related to its interaction at other sites, preventing access of the ambient medium to the structural zinc atoms. Such interactions may be quite strong (*vide supra*), yet not detectable by kinetic inhibition studies, since they occur in the vicinity of the structural zinc atoms.

Isotope-exchange reactions are known to follow first-order kinetics (McKay, 1938). In both acetate and phosphate buffers, the kinetics of the isotope-exchange reactions of LADH are first order. In 0.1 M acetate buffer, only a single rate process is observed at all of the enzyme concentrations examined. In 0.1 M phosphate buffer all of the zinc atoms appear to exchange at the same rate when high LADH concentrations are employed, but two exchange rates become apparent at low enzyme concentrations (Figure 2). In phosphate buffer and under these latter conditions, two zinc atoms exchange more rapidly than the remainder. The rate constant for their exchange is nearly equal to that of the two zinc atoms which exchange in acetate buffer (Table II), suggesting that these may be the "free" zinc atoms. The zinc atoms which exchange slowly in phosphate buffer would then correspond to those which do not exchange at all in acetate buffer, *i.e.*, the "buried" zinc atoms.

The effect of enzyme concentration on isotope exchange reveals an unexpected feature: an increase in the concentration of LADH decreases the rate of exchange of the "free" zinc ions, *i.e.*, those exchanging in acetate and rapidly in phosphate (Table III). This behavior differs from that expected for simple metal–ligand exchange reactions (Duffield and Calvin, 1946) and also from that observed in an enzyme system, *i.e.*, $^{64}Cu^{2+}$–ascorbic acid oxidase (Magee and Dawson, 1962). In contrast, the rate constant for the buried zinc atoms, *i.e.*, those exchanging slowly in 0.1 M phosphate, increases with increasing protein concentration (Figure 2) in accord with theory. It appears that, in combination, these two opposing rate effects in phosphate buffer yield an apparent single exchange rate at higher LADH concentrations.

These effects of enzyme concentration are further consistent with the existence of different populations of zinc atoms in LADH, and they may well relate to previous results showing that enzyme concentration also affects loss of zinc and activity during dialysis at acid pH (Drum *et al.*, 1969). Similarly, at high LADH concentrations diethyldithiocarbamate inactivates LADH and removes 2.0 g-atoms of Zn/mole (Drum *et al.*, 1967, 1969) much less rapidly (Drum, 1967).

These enzyme concentration-dependent features of the exchange are distinct from those which depend upon the concentration of zinc ions. At a constant protein concentration, increasing the concentration of Zn^{2+} in the ambient medium increases the apparent rate of isotope exchange (Table II), as would be expected (see Appendix).

The isotope-exchange data here presented indicate that concentration-dependent changes affect reactivity of the "free" zinc atoms of LADH. It might be conjectured that high enzyme concentrations may facilitate protein–protein interactions which could lower the rate of exchange; conversely, dilution could then increase it. A number of enzymes with quaternary structure are known to dissociate into subunits on dilution (Olson and Anfinsen, 1952; Fisher *et al.*, 1962; Bernfield *et al.*, 1965; Markert and Massaro, 1968), though none of these have been studied in the context of metal exchange as presented here for LADH. However, there is no evidence from either ultracentrifugation or molecular sieve filtration that LADH undergoes dissociation into subunits or polymerization under the conditions used in these experiments (D. E. Drum, unpublished observations). While experiments to evaluate other possible causes of the effect of LADH concentration on the rate of exchange of the active-site zinc atoms are currently in progress, a factual explanation for this phenomenon cannot be given at present.

In this regard, certain other properties of LADH have remained unexplained, *e.g.*, the failure of many chelating agents to remove zinc, the failure to restore zinc once removed from the enzyme, the asymmetric environment of chromophoric chelating agents binding to the zinc atoms and the poor correlation between the inhibition of LADH by many complexing agents on one hand and the stability constants of their ionic zinc complexes on the other. Conceivably, the parameters bearing on these features of the enzyme may also affect the accessibility and rate of exchange of zinc.

^{65}Zn exchange in LADH permits the specific labeling of either the two zinc atoms involved directly in catalysis or of the remaining, nonintegral number of zinc atoms but without causing other discernible alterations of the enzyme molecule. Thus, subsequent studies may focus more directly on the relation of either "free" or "buried" zinc atoms to the specific property of the protein under investigation, *e.g.*, the binding of chromophoric metal chelating agents, the side-chain moieties of the proteins responsible for zinc binding, or the functional significance of substitution of other metals for zinc. This general approach should prove valuable also in delineating the diverse functions of metals in other multichain metalloenzymes.

Appendix

The general equations for chemical isotope exchange (McKay, 1938, 1943; Duffield and Calvin, 1946) may be rewritten for exchange of a zinc atom bound to an enzyme

$$\underset{(a)}{(E\text{–}Zn)} + \underset{(d)}{{}^{65}Zn^{2+}} \rightleftharpoons \underset{(c)}{(E\text{–}{}^{65}Zn)} + \underset{(b)}{Zn^{2+}} \qquad (1)$$

where a, b, c, and d represent the initial molarities of the indicated species. At time, t, of exchange, the molarities are $(a - x)$, $(d - x)$, $(c + x)$, and $(b + x)$, respectively, where x is the molarity exchanged at time t. The maximum molar exchange possible is then x_∞, determined from the concentration of enzyme and the known zinc content.

The exchange rate is $d(E\text{-}^{65}Zn)/dt = d(c + x)/dt = dx/dt$, and integration of the rate law equation gives

$$\ln \frac{1}{1 - \frac{(a + b)}{(ab - bc)}x} = \ln \frac{1}{1 - x/x_\infty} = (a + b)kt \qquad (2)$$

or

$$1 - x/x_\infty = (1 - F) = \exp[-(a + b)kt] \qquad (3)$$

where F is the fraction exchanged at time t and k is the bimolecular rate constant for the isotope-exchange reaction.

Isotope-exchange experiments are described graphically by utilizing the logarithmic form of eq 3, which requires that a plot of the logarithm of the fraction of enzyme zinc not exchanged *vs.* time should be linear. In reporting these experiments. $^{65}Zn^{2+}$ refers to carrier Zn^{2+} in buffer containing uniformly distributed $^{65}Zn^{2+}$, and "apparent rate constant," k_a, refers to all terms in the exponent except the variable t. The true bimolecular rate constant, k, may be computed when reactant concentrations, (E–Zn) and (Zn^{2+}), are known.

If Zn^{2+} exchanges at two nonidentical sites, with different rate constants, the two general equations are

$$1 - F_1 = \exp(-k_{a1}t)$$

and

$$1 - F_2 = \exp(-k_{a2}t) \qquad (4)$$

and the observed fraction not labeled is given by

$$1 - F_{total} = {}^1/_2 \exp(-k_{a1}t) + {}^1/_2 \exp(-k_{a2}t) \qquad (5)$$

Under conditions where the apparent rate constants k_{a1} and k_{a2} are very different, two slopes should be apparent in appropriate plots of the experimental data.

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