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Some Aspects of Zinc Ion Involvement in Alcohol Dehydrogenase Catalysis

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(i) INTRODUCTION

Alcohol dehydrogenases from various sources catalyse the reaction:

$$R—CHO + NADH + H^+ \rightleftharpoons R—CH_2OH + NAD^+,$$

where NAD is nicotinamide adenine dinucleotide.

The first reports of the oxidation of alcohols by different animal tissues appeared toward the end of the nineteenth century, and since that time the dehydrogenases, the cofactors NADH and NAD^+ and their analogues, and the various substrates together constitute one of the most widely studied biochemical reactions. For this reason, the literature on the system is vast, and, therefore, this review does not pretend to be a comprehensive survey. It contains an account of the present state of thinking with specific reference to the metal ion involvement in the enzyme reaction, together with a résumé of the relevant earlier work and references to literature sources from which the original path of recognition, isolation, and investigation may be traced.

The lines of evidence which lead to a knowledge of the involvement of metal ion in an enzymic reaction are several: the effect of varying metal ion or metal ion content; the effect of metal-chelating agents; variation of cofactor, substrate, and inhibitors, together with X-ray, spectrophotometric, kinetic, and equilibrium studies are all possible tools. There is the added possibility of observing analogous nonenzymic reactions which may throw light on the possibilities open to the metal ion as it functions in the enzyme.

(ii) THE ENZYMES

Alcohol dehydrogenase activity occurs widely in natural systems (*1a, b*), for example, in the livers of humans, fishes, horses, and rats, in plant tissues, in microorganisms, and in yeasts. The enzymes do not possess pronounced substrate specificity and can react with a large number of normal and branched-chain aliphatic and aromatic primary and secondary alcohols and carbonyl compounds. A representative sample is contained in Table 1, where their activity with respect to alcohol dehydrogenase from equine liver (LADH) is tabulated. The transfer of the hydride is direct between coenzyme and substrate and is stereospecific.

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TABLE 1

SUBSTRATE SPECIFICITY OF LADH[a]

Alcohol oxidation		Carbonyl reduction	
Alcohol	Rate (relative v)	Carbonyl compound	Rate (relative v)
Ethanol	100	Acetaldehyde	100
Methanol	0	*n*-Butyraldehyde	1700
n-Propanol	108	Cinnamaldehyde	1160
n-Butanol	159	Formaldehyde	23
n-Hexanol	125	Acetone	0
Cyclohexanol	100	Cyclohexanone	16
Benzyl alcohol	87	Benzaldehyde	183

[a] Data were extracted from Ref. *1a*.

The first successful crystallization of a pure alcohol dehydrogenase was that from yeast (YADH) in 1937 (*2*), and that of LADH was reported in 1948 (*3*). Of all of the reported dehydrogenases (*1b*), LADH and YADH have been the most widely studied (the former somewhat more than the latter) and are the two with which this review is concerned. LADH has a molecular weight now thought to be about 80,000 (*4a*). Initial investigations, based on an incorrect molecular weight of ~73,000 (*5*), determined the number of gram atoms of zinc per mole of enzyme to be 2 (*6*, *7*). More recent studies, using purified enzyme, have shown the true value to be 4 (*8*).

The enzyme itself is a dimer (*9*) consisting of two identical subunits (*10*). Each subunit binds two zinc ions (*11*) and has one site on which to bind the coenzyme. The structure of the coenzyme is shown in Fig. 1.

The primary sequence of 374 amino acids in each residue has been reported (*10*) and

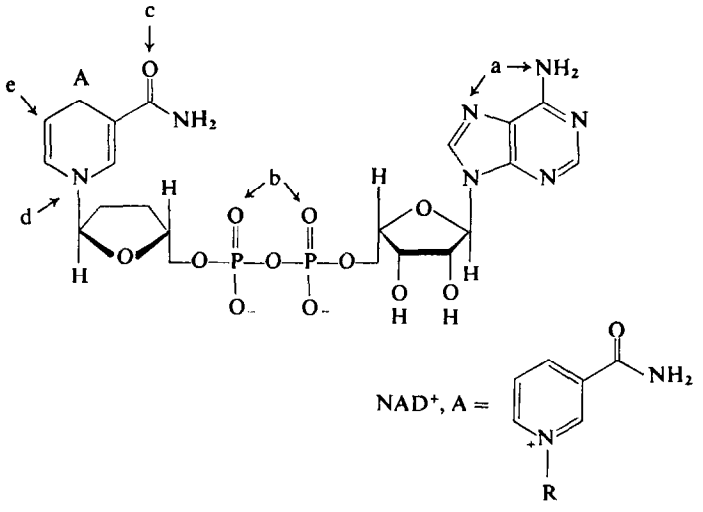


FIGURE 1

X-ray structural studies of the whole enzyme (*12*) at 2.9 Å resolution and of the active site at 2.4 Å (*13*) have confirmed the dimeric nature and the presence of two coenzyme binding sites and have identified the positions of the four zinc ions. The X-ray studies are discussed further below. Thus, the gross features of LADH are that it is a dimer, with four zinc ions and two active sites.

YADH, on the other hand, is a tetramer (*14*) of molecular weight ~140,000, which contains four zinc atoms (*15*). Initial work indicated that the enzyme bound 4 mol of cofactor per mole of enzyme (*16*), but more recent work by Dickinson (*17*) has suggested that the number is more probably 2.

(iii) METAL ION-REACTIVITY RELATIONSHIPS

For an enzyme to be unequivocally classed as a metalloenzyme, i.e., one which requires the presence of a certain metal ion for reactivity, the investigation of the relationship between metal ion and enzyme must be carried out with considerable care and with reference to the necessary criteria. Vallee (*18*) has pointed out that four such criteria for metals involved in catalytic function are (i) tight binding of metal ion to protein, (ii) the observation of an increasing metal:protein ratio as the specific activity of the enzyme increases during purification, (iii) the observation of an integral number of gram atoms of metal per mole of enzyme in the purified protein, and (iv) the observation of an integral molar ratio between metal content and cofactor binding. (This last criterion would, however, not apply if the metal served a purely structural role in a multichain enzyme with more than one active site.) Failure to apply such criteria with sufficient rigour has led to several enzymes [e.g., lactate dehydrogenase from rat liver (*19*)] being erroneously classed as metalloenzymes.

In the case of the alcohol dehydrogenases, the metal requirement is well documented; the major points of such documentation are presented below.

(*a*) *LADH*

The evidence for zinc ion involvement in both a catalytic and a structural role in LADH has been reviewed in Ref. *4*. The enzyme is inhibited by metal-chelating agents such as 1,10-phenanthroline and 2,2′-bipyridyl; the metal can be removed from the enzyme by dialysis against such metal-chelating agents, and the resulting apo- or 'half-apo'-enzyme depending on the system (*4a* and see below), is inactive. The activity can be regenerated by replacement of the metal ion using either zinc itself or cobalt.

The evidence for both a structural and a catalytic role for the metal ion arises from several sources. In phosphate buffer, for example, all of the zinc ions will exchange with $^{65}Zn^{2+}$ within 24 hr (*4a*), while in acetate buffer only two of the four do so, which indicates two different "types" of zinc. Inhibition by 1,10-phenanthroline is total at a ratio of 2 mol of 1,10-phenanthroline/mol of enzyme, and 1,10-phenanthroline binds to the 'catalytic' zinc (*12*). Dialysis of the enzyme against EDTA can produce the enzyme which contains only two zinc ions, and this half-apo enzyme is inactive. 1,10-Phenanthroline does not interact with the 'buried' zinc (i.e., noncatalytic or not easily dialysable) (*12*) which presumably plays a part in maintaining the enzyme structure (*4a*, *12*). It is the detailed function of the catalytic zinc which is of interest in this work.

(b) YADH

The inhibition of YADH by 1,10-phenanthroline differs from that of LADH in that the metal-free apo-enzyme cannot be prepared by dialysis: Rather, the tetramer dissociates into monomer when 1,10-phenanthroline is present in large excess (*15*). The dissociation may be represented as

$$((YADH)_4Zn_4) + 12\ 1,10\text{-phenanthroline} \rightarrow 4(YADH) + 4(Zn(1,10\text{-phenanthroline})_3)$$

and is irreversible.

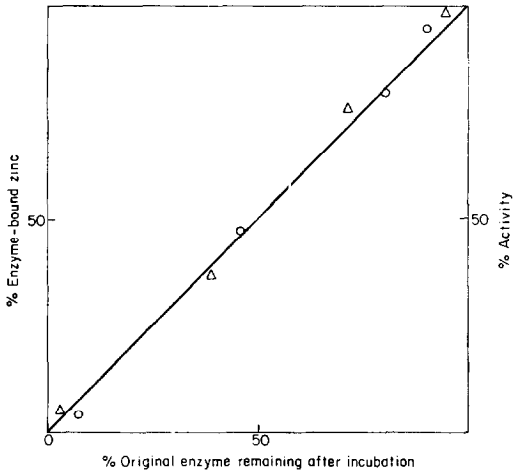


FIGURE 2

Progressive inhibition by nonexcess amounts of 1,10-phenanthroline (or 8-hydroxyquinoline-5-sulphonic acid) produces a linear dependence of the activity and structure of the enzyme on inhibitor concentration (*15*). The position is represented in Fig. 2.

Inhibition by these agents results in solutions which have uv spectra very similar to those of the ligand and free zinc salts in aqueous solution (*20*). The inhibition is prevented by NADH or NAD^+ (*15*). Thus, both ligands are competitive inhibitors and both bind at or near the metal site.

The foregoing is a very brief résumé of the evidence for metal ion involvement in the enzyme. More detailed reviews may be found in Refs. *1a–1e*, *4a*, and *19*. The specific point of interest in this instance is the question of whether the metal ion binds the coenzyme, the substrate, both simultaneously, or neither. The relevant literature is reviewed below.

(iv) COENZYME–ENZYME INTERACTIONS

In the binding of the coenzymes to the two dehydrogenases, there are two considerations: where the coenzyme binds the enzyme, and where the enzyme binds the coenzyme. The possible metal binding sites on NADH are illustrated in Fig. 1. The

metal ion may bind at the adenine (a), the pyrophosphate (b), the nicotinamide carbonyl (c), the pyridine ring nitrogen (d), or the nicotinamide C-5 position (e). In NAD^+, only (a), (b), and (c) are possible candidates.

The five possibilities in NADH have been reviewed by Mildvan (*21*), who points out that (a) and (b) are unlikely, since 1,10-phenanthroline and ADP–ribose (Fig. 3) (both NADH-competitive inhibitors) are not mutually competitive and, furthermore, ADP-R• [a paramagnetic analogue of NADH, see Fig. 9 and section (v) below] has been found to bind with the same stoichiometry to both native and apo-LADH (*22*). (This latter point must be treated with caution, however. As discussed below, that there is ligand–enzyme *and* ligand–apo-enzyme interaction does not necessarily say anything of metal–ligand binding.)

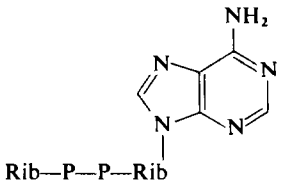


FIG. 3. ADP-R, an NADH-competitive inhibitor.

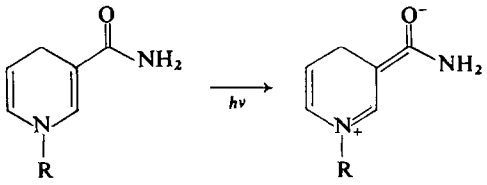


FIG. 4. Electronic transition of β-NADH (from Ref. *24*).

Mildvan (*21*) also points out that it is 'not apparent how coordination at "c" and "e" would facilitate hydride transfer, although coordination at "d" remains a possibility.' The evidence for the first part of the statement is not clear, since it is not explained how coordination at a and b would facilitate transfer. In terms of simultaneously coordinated substrate, there would seem to be no reason why coordination at c should not facilitate reaction [though there is evidence that this may occur in model systems (*74*)]; coordination at e (besides being intuitively unlikely) might be seen to raise certain steric difficulties. If a simultaneously coordinated substrate is *not* proposed, then it might be thought that each of a to e could be regarded as being equally unlikely.

Within this context, the uv spectrum of NADH is of interest. NADH in aqueous solution has $\lambda_{max} = 340$ nm, $\varepsilon = 6.2\ 10^3$ cm^2/mol (*23*). The transition is depicted in Fig. 4. On binding to LADH, the maximum is shifted to 325 nm. Kosower (*24*) has shown that such a shift may be explained by the interaction of a positively charged nitrogen (in the form of an ammonium ion) situated ~3 Å from the increasingly positive nitrogen of the pyridine ring.

It is of interest to note here that coordination of a metal ion to the amide carbonyl

would promote the transition and lower the energy, leading to a shift to longer wavelengths. Kosower, however, included in his assumptions that the zinc ion bound the NADH via the pyrophosphate and so could not constitute the positive charge responsible for the shift in absorbance. The zinc ion does not so coordinate (see above), and it may be argued that the zinc ion–dihydropyridine interaction [i.e., Mildvan's (*21*) d] is therefore possible. That this may not be the case is evidenced by differences and similarities between LADH and YADH. There is *no* shift in NADH absorbance on binding to YADH, and yet the metal ion function in both cases is very similar [section

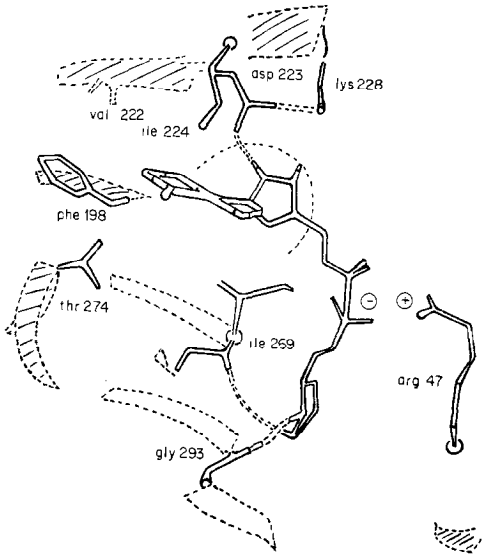


FIG. 5. Schematic representation of the interaction of LADH with ADP–ribose (from Ref. *13*).

(vi)], indicating that in the LADH active site there is a positive nitrogen moiety which is absent in YADH.

There is a further consideration regarding the conformation of NADH on binding. Several investigators utilising nmr (^1H, ^{13}C, and ^{31}P) spectroscopy have shown that NADH exists in a stacked conformation in aqueous solution at pH 4.0 and 30°C. The stacking is such that there is interaction between the base pairs (nicotinamide and adenine). Barrio *et al.* (*29*) have pointed out that such a stacked or folded conformation results in a quenching of the nucleotide fluorescence. The binding of NADH to LADH or YADH results in an enhancement of the coenzyme fluorescence (*30*), indicating that NADH is binding in an 'open' conformation. This is confirmed by the X-ray structural study on the enzyme–ADP–ribose complex (*12*) and by nmr (*57*).

In the detailed X-ray study of the active site (*13*), the binding of the coenzyme analogue is seen to be primarily a result of an interaction of the adenine with a hydrophobic pocket on the enzyme. The adenine NH_2 points away from the enzyme (*13*) and the O_2 hydroxyl of the adenine ribose is hydrogen-bonded to Asp-223. The situation is represented in Fig. 5, where it is seen that there is also an ionic interaction between the guanidinium group of Arg-47 and the pyrophosphate moiety.

An assumed position for the nicotinamide moiety can be deduced from the structure below (*12*, *13*), and the C-4 atom of the nicotinamide ring is ~4.5 Å from the catalytic zinc. It is also of interest that in all of the dehydrogenase structures reported, the residue Asp is invariant in the coenzyme binding site (i.e., in lactate dehydrogenase, LADH, and glyceraldehyde 3-phosphate dehydrogenase), indicating that the ribose oxygen–Asp hydrogen bond is an important feature of coenzyme binding (*13* and refs. therein).

That such a situation exists in the solid is, of course, no guarantee that the same is true in solution (see, e.g., Ref. *72*), since conformational changes may occur which alter the enzyme–coenzyme interaction. Luisi *et al.* (*31*) report, however, that the binding of the NAD^+ analogue nicotinamide $1,N^6$-ethenoadenine dinucleotide (Fig. 6) (ε-NAD^+) to various dehydrogenases results in an enhancement of the coenzyme fluorescence by a factor of 10–13 [indicating unfolding (*29*)] and in a blue shift of the fluorescence maximum, which the authors (*30*) interpret as the adenine 'sensing' a hydrophobic region. Thus, there is the indication that the solution enzyme–coenzyme interaction is the same as in the solid. (Indeed, the involvement of the adenine moiety in coenzyme binding may be inferred from the relative K_m's and V_{max}'s exhibited for

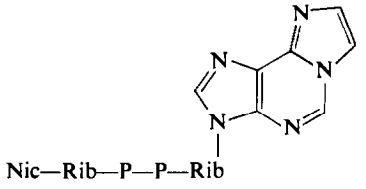


FIG. 6. The structure of ε-NAD^+.

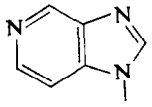
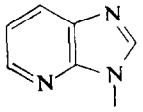
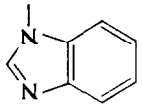
several NADH analogues with modified adenine substituents. A representative sample is contained in Table 2, where it is seen that modification drastically affects the interaction.)

Thus, the major binding factor is that of adenine in a hydrophobic region, which precludes adenine–metal ion interaction. The X-ray work puts the C-4 of the nicotinamide 4.5 Å from the zinc, a distance which may well be changed in solution, so that direct nicotinamide–metal ion interaction is possible. Shore and Santiago (*32*) have recently reported coenzyme interactions with totally cobalt-substituted LADH. Binding of NADH causes a shift in the 655-nm peak of the heteroenzyme to 670 nm, and similar perturbations were observed on binding pyrazole and trifluoroethanol with NAD^+, but not with NAD^+ alone. The authors interpret this as being metal ion involvement in binding alcohol and NADH, but not NAD^+.

On the other hand, Weiner *et al.* (*33*) and Iweibo and Weiner (*34*), in a continuing study, have shown by the techniques of fluorescence enhancement, quenching, and polarization that native zinc enzyme may bind as many as 6 or 8 mol of NADH/mol, that, of these, two are tightly bound, that apo-LADH binds 2 mol of NADH with the same affinity as the two which are tightly bound to the native enzyme, and that the apo-enzyme also binds substrate with the same affinity as the native enzyme (*34*). The work confirms earlier studies involving a spin-labelled analogue of NAD (Fig. 11)

TABLE 2

K_m AND V_{max} VALUES FOR SOME ADENINE-SUBSTITUTED NAD ANALOGUES[a]

Substituent (R)	YADH		LADH	
	$10^4 K_m$ (M)	V_{max}[b]	$10^4 K_m$ (M)	V_{max}
Adenine (NAD^+)	25	55,000	0.3	410
3-Deazapurine	40	15,000	0.6	260
				
1-Deazapurine	29	4,000	3.3	250
				
Benzimidazole	38	13,000	3.7	100
				
Phenyloxy (PhO)	50	7,000	6	240
HO–	100	1,000	40	100

[a] Nicotinamide–ribose–phosphate–phosphate–ribose-R; data are from Ref. *73*.
[b] Units of V_{max} are not given in Ref. *73* but are presumably min^{-1}.

(*22*) which was found to bind to both native and apo-LADH with the same stoichiometry as that reported above (i.e., 8 to the native and 2 to the apo enzyme) although the binding of the analogue to apo-LADH was slightly weaker than to the native enzyme. Thus, there is an indication that zinc is involved in interaction with neither substrate nor coenzyme.

There is, however, a further point to consider, in that it is not necessarily the case that the binding of the coenzyme (or the substrate) to apo-LADH occurs in the same way as to LADH. Indeed, Weiner *et al.* (*33*) report that they were prompted to use fluorescence polarization to search for additional binding sites for NADH on LADH since such binding sites may not enhance the coenzyme fluorescence *as was evidenced by the binding of NADH to the apo-enzyme*. Since (as was discussed above) the coenzyme binds to the enzyme in an open as opposed to a folded conformation, and the opening of the coenzyme enhances the fluorescence (*29–31*), the coenzyme *cannot* be binding to the apo-enzyme in the same way as to the active enzyme. A comparison of binding strengths between native and apo-enzyme therefore becomes invalid in considerations of metal ion involvement; rather, the *mode* of binding is of importance, and this is seen to be different. One possibility is that, in both cases, NADH binds via the adenine, but the metal promotes opening of the coenzyme (and fluorescence enhancement), either by conformational changes in the enzyme *or* by nicotinamide–metal interaction.

The use of ^{35}Cl-nmr line-broadening experiments has yielded valuable information with regard to zinc ion function in the enzymes alkaline phosphatase (AP) (*37*) and carbonic anhydrase (CA) (*36*). The addition of LADH to KCl solutions causes the ^{35}Cl resonance to broaden (*35*), and the subsequent addition of NADH decreases the broadening, which is consistent with the displacement of bound Cl^- (*35–37*). The metal ion in a metalloenzyme may be regarded as a possible chloride binding site [in AP and CA it is the actual site (*36*, *37*)], but the addition of 1,10-phenanthroline [which binds to the zinc in LADH (*12*)], cyanide, or 8-hydroxyquinoline derivatives has no effect on the ^{35}Cl line width of an LADH/KCl solution (*35*), indicating that such metal-ligating agents do not bind at the same place as NADH.

In contrast to the above, an analogous study involving ^{81}Br line widths (*38*) showed that binding of bromide to LADH was insensitive to the addition of NAD^+ or NADH, the most likely explanations for which being a stronger enzyme/bromide interaction or that the bromide ion binds at a different place from the chloride. Under these circumstances, it is perhaps a little surprising that these workers did not investigate the effect of chelating agents, which may have indicated whether the bromide was bound to the zinc ion.

In a study of the effects of chloride on coenzyme binding, Coleman and Weiner (*39*, *40*) established coenzyme-competitive inhibition at saturating substrate levels. With coenzyme saturating, however, the anion was found to be a noncompetitive inhibitor against either acetaldehyde reduction *or* isobutyramide binding (*39*). The authors interpret this as evidence for the existence of at least two specific anion binding sites on the enzyme, since they observed the formation of an enzyme–NADH–aldehyde–Cl complex (*40*). They also report that the effect of chloride on the fluorescence of enzyme-bound NADH is to diminish it (*39*), while other spectroscopic techniques (uv, ord) show the coenzyme to be still bound.

It would seem from this latter observation that the addition of chloride is similar in effect to the removal of metal ion (see above) in that both cause a different mode of binding of coenzyme. Whether the chloride ion effects mimic those of metal ion removal by blocking a positive charge on the zinc ion is a matter of conjecture; the ^{35}Cl-nmr results (*35*) militate against the suggestion, but the indication of multisite anion binding (*39*, *40*), supported by the ^{81}Br-nmr results, suggests that there is some uncertainty about the actual mode of chloride binding.

The absence of any coenzyme–metal ion interaction has been proposed by Takahashi and Harvey (*41*) as a result of measurements of energy transfer between bound NADH, thionicotinamide NADH, or Rose Bengal and cobalt in a hybrid Co(II)/Zn(II)-LADH. Measurements based on the fact that fluorescence enhancement is less with the hybrid than with the native enzyme result in a Co(II)-nicotinamide ring distance of 19 ± 2 Å (*41*). This is in accord with the observation (*42*) that binding of azide, 1,10-phenanthroline, or pyrazole does not affect the absorption spectrum of a similar hybrid enzyme. Such hybrid enzymes are prepared by utilising the known (*4a*) (see above) rapid exchange of two of the four zinc ions in acetate buffer. Ulmer and Vallee (*4a* and refs. contained therein) originally equated the two rapidly exchanging zincs with catalytic activity, while the other two were equated with maintenance of structure. Therefore, the above observations indicate that the catalytic metal ion serves no substrate or coenzyme binding function.

There is, however, another explanation (*75*). If the two 'structural' zincs, in fact, are rapidly exchanging ones, then their removal must necessarily affect the enzyme structure and hence the activity.

A recent report by Sytkowski and Vallee (*4b*) is of particular interest in this connection: Using the ^{65}Zn and Co hybrid enzymes, they showed that the catalytic atoms are those which are reactive to 1,10-phenanthroline, while the noncatalytic pair are not affected by this reagent. But *metal–metal exchange* studies shows the converse to be true: The chemical reactivity of the *noncatalytic* atoms is much higher and they *exchange more rapidly*.

The X-ray studies of Brändén *et al.* (*12*) have shown the two different zincs to be ~20 Å apart. There is one on the enzyme surface (*12*) and one at the bottom of the cleft

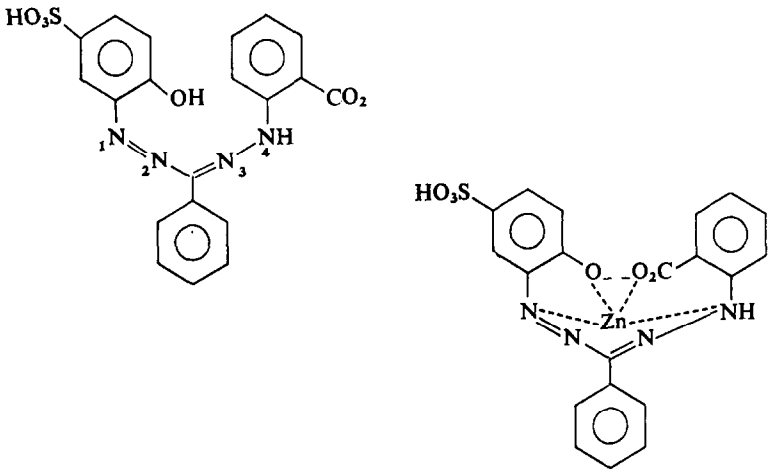


FIG. 7. The mode of binding of "Zincon" to zinc (from Ref. *64*).

that is the active site (*13*); 1,10-phenanthroline binds to this second, catalytic zinc (*12*), and 1,10-phenanthroline binding affects the absorption spectrum of the *totally* cobalt substituted enzyme (*32*). The surface zinc might possibly exchange more rapidly than the zinc at the bottom of the cleft. One would therefore expect no evidence of any coenzyme–cobalt (or substrate–cobalt) interaction in the hybrid enzyme (*41*) and no indication of a metal-inhibition interaction with 1,10-phenanthroline (*42*), and a distance of ~20 Å (*41*) between the nicotinamide ring and the cobalt ion is to be expected. It is also significant in this context that esr studies on the interaction of ADP-R$^{\bullet}$ [Fig. 11] with totally cobalt-substituted LADH (*62*) show the spin label to be ~6 Å away from the catalytic cobalt, although the nmr results of Mildvan *et al.* (*54*) on the same system (see below) indicate that this is the case in the hybrid enzyme, which does not agree with the work of Takahashi and Harvey (*41*) or the uv results on inhibitor binding (*42*). Thus, the work of Mildvan *et al.* (*54*) indicates that the two rapidly exchanging zincs are those at the bottom of the cleft, not the structural ones as argued above, although this offers no explanation of the other observations discussed above (*32, 41, 42*).

Some further evidence for a direct metal ion/NADH interaction has arisen from pH

effects on 1,10-phenanthroline binding (*63*) and resonance Raman investigations of binary complexation between 2-carboxy-2′-hydroxy-5′-sulfoformazyl benzene (Zincon) (Fig. 7) (*64*) and LADH or zinc.

1,10-Phenanthroline binds weakly to LADH at alkaline pH, and Reynolds *et al.* interpret this as resulting from displacement of zinc-bound hydroxide, as opposed to more readily displaced zinc-bound water at pH < 7, i.e., the 1,10-phenanthroline binds directly to the metal ion. Since 1,10-phenanthroline is competitive with NADH, there is an intimation that this may also be one mode of NADH–enzyme interaction.

Zincon is also strictly competitive with NADH (*64*) and binds zinc as shown. The resonance Raman study shows that in the enzyme the binding at pH 8.75 is to the

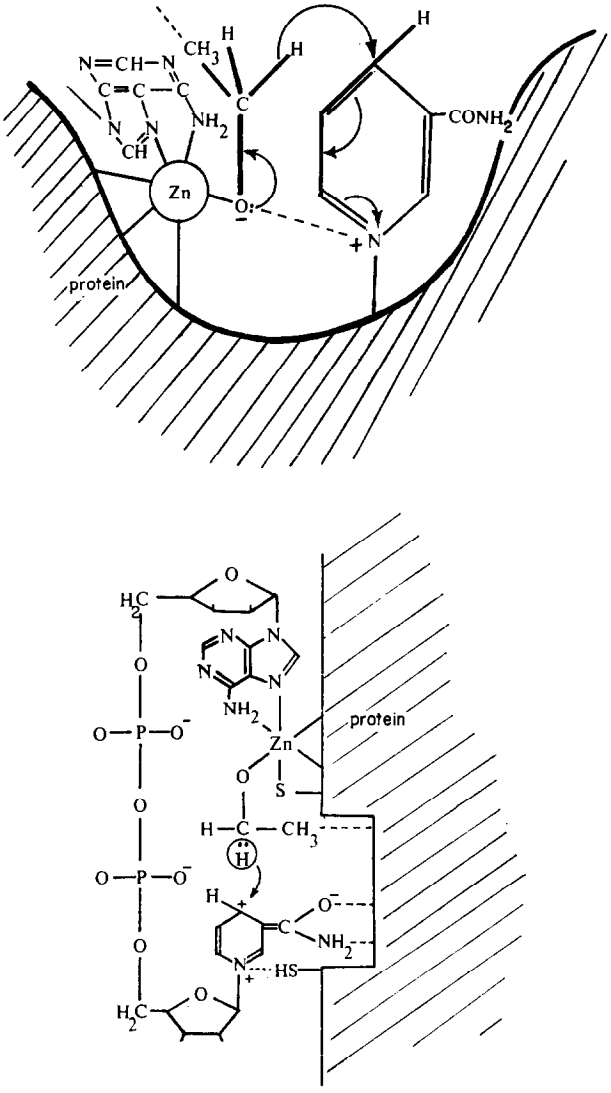


FIG. 8. Suggested mechanism of ADH reactions according to Sund and Theorell (*1a*).

zinc, but not via the carboxylate and N-4, only via the phenolate and N-1. Thus, there is a further indication of coenzyme–metal ion interaction.

A consideration of the foregoing discussion shows that, on the evidence available, no definite conclusion can be drawn regarding *direct* coenzyme–metal ion interaction: The inhibition results indicate an interaction, since chelating agents (*4*, *15*, *64*) are competitive; the X-ray data indicate the opposite (*12*, *13*); spectroscopic evidence is contradictory (*15*, *32*, *41*, *43*); nmr and the effect of addition of anion are inconclusive (*35*, *38–40*) (and see above), while resonance Raman is more positive (*64*).

(v) SUBSTRATE–ENZYME INTERACTIONS

The first suggestion that the function of the zinc ion in alcohol dehydrogenases was to act as a Lewis acid in polarising the carbonyl bond of coordinated aldehydes was made by Abeles *et al.* (*43*) following model reactions with NADH analogues and thioketones. Supporting evidence based on the stereospecificity of the transferred hydrogen and the deuterium isotope effect on the YADH-catalysed reaction led Sund and Theorell (*1a* and refs. contained therein) to suggest the mechanism depicted in Fig. 8.

A number of recent investigations have supported the suggestion of a direct carbonyl–zinc ion interaction. Jacobs *et al.* (*44*) have compared the rates of reduction of several benzaldehydes using both enzymic and nonenzymic conditions. The ratio of *p*-chloro to *p*-methoxy benzaldehyde was 100 for reduction by sodium borohydride, while for reduction by NADH with LADH as catalyst the ratio was 2. This lack of substituent effect in the enzymic reaction was concluded (*44*) to be a result of polarization of the carbonyl bond by zinc coordination. Dunn and Hutchinson (*45*), in a kinetic study of the stable intermediate formed between *trans*-4-*N*,*N*-dimethyl-amino cinnamaldehyde (Fig. 9), NADH, and LADH at pH $\geqslant$ 9.0, concluded that the zinc ion in the enzyme

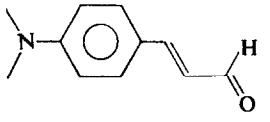


FIG. 9. *trans*-4-*N*,*N*-dimethyl-amino cinnamaldehyde.

played a Lewis acid role by binding through the carbonyl oxygen. The YADH-catalysed reduction of substituted benzaldehydes has been shown to involve a general acid in the active site (*46*) and, in subsequent work on the pH dependence of the reaction, Klinman (*47*) concluded that the ionising group (which has a pK of ~8.6) could be either an imidazole, a cysteine, a lysine, or a zinc-bound water molecule. In similar studies on the reduction of acetaldehyde and butyraldehyde (*48*) and the oxidation of butan-1-ol and propan-2-ol (*49*), Dickenson and Dickenson (*48*, *49*) reached the same conclusion.

Shore *et al.* (*50*), in a study of the liberation of protons during the steady-state turnover of LADH, concluded that there was on the enzyme a group with a pK of 9.6 and that this pK was perturbed by the binding of NAD^+ to 7.6 (*50*). The scheme is depicted in Fig. 10.

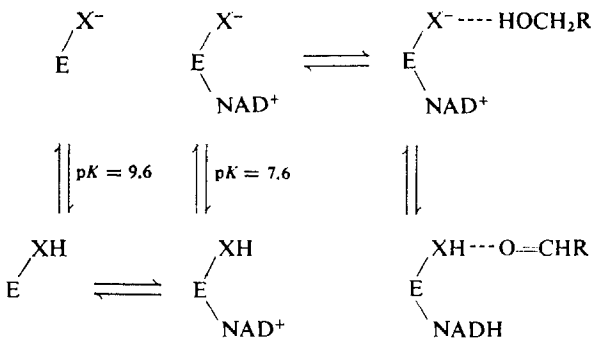


FIG. 10. Schematic representation of the mechanism of ADH (from Ref. *50*).

The group plays a significant part in the binding of the substrate and is concluded to be either an amino group or a zinc-bound water molecule (*50*).

McFarland and Chu (*51*), however, have shown that in the LADH-catalysed reduction of an aromatic aldehyde (β-naphthaldehyde or benzaldehyde) to an alcohol, or oxidation of alcohol to aldehyde, the only pH-dependent parameters are the kinetic binding constant for ternary complex formation and the rate of dissociation for alcohols. The other parameters (catalytic step and isotope effect) are pH independent. This is consistent only with *direct* carbonyl–zinc ion coordination. The same direct coordination has been concluded (*13*) from the X-ray studies.

Some of the most detailed solution studies on the nature of the metal ion–substrate interactions have been performed by Mildvan and Weiner (*52*, *53*) and Mildvan *et al.* (*54*) using nmr techniques involving spin-labelled ligands. The NAD^+ analogue adenosine-5′-diphosphate-4-(2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl) (ADP-R•)

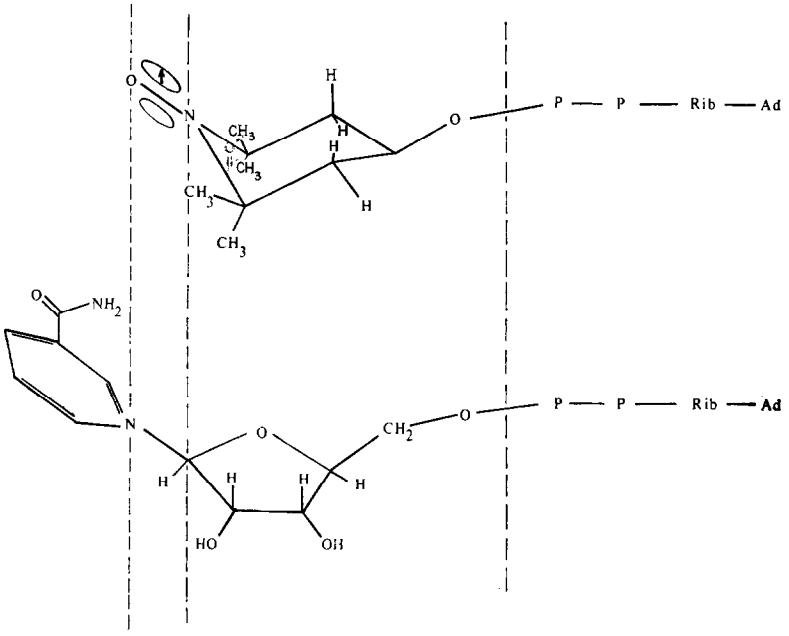


FIG. 11. The equivalence of ADP-R• and NAD^+ (from Ref. *52*).

[Fig. 11], first prepared by Weiner (*22*), has its unpaired electron located in a position equivalent to the nicotinamide nitrogen–ribose carbon bond in NAD^+ (Fig. 11) (*52*). The binding of ADP-R' to LADH is found to enhance the effect of the unpaired electron on the proton relaxation rate of water (*52*). The addition of ethanol to the

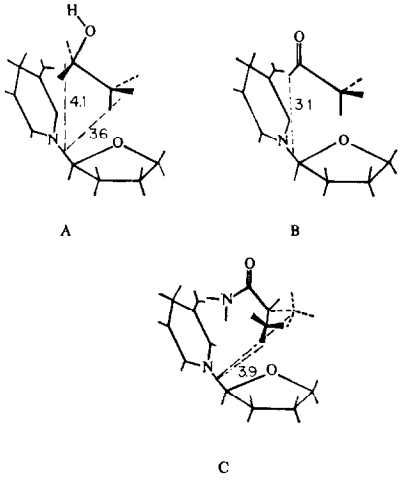


FIG. 12. Coenzyme–substrate distances in LADH. A, ethanol; B, acetaldehyde; C, isobutyramide.

binary ADP-R'/enzyme complex reduces the enhancement, leading to the suggestion that the ethanol binds to the solvent side of the bound coenzyme and overlies the ribosidic bond to pyridine (*52*). The relative positions of the appropriate coenzymes and ethanol, acetaldehyde, and isobutyramide (a substrate-competitive inhibitor) as calculated from enhancement of proton relaxation rates are shown in Fig. 12 (*53*).

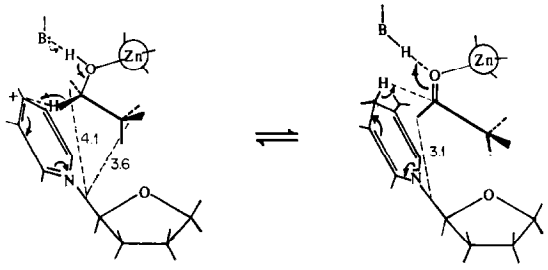


FIG. 13. Mechanism of LADH reaction based on calculated distances between the enzyme-bound substrates and enzyme-bound ADP-R' (from Ref. *54*).

A suggestion regarding the involvement of the metal ion is shown (*21*, *54*) in Fig. 13. The use of a spin-labelled iodoacetamide analogue [(4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidino oxyl)(X)] in similar studies on the yeast enzyme (*55*) resulted in the establishment of close similarities between zinc ion function in LADH and YADH through comparison with nmr (*54*) and X-ray (*12*, *13*) data. The calculated distances between the spin label, NADH, and isobutyramide (i–b) in the ternary complex EX(i-b) are shown in Fig. 14. [The locations of zinc and Cys-43 are taken from Ref. *13*

and the YADH primary acid sequence (*56*) which is discussed below.] The substrate is thought to be *directly* coordinated to the zinc (*55*).

In similar studies using the hybrid Co(II)/Zn(II)-LADH enzyme and the totally cobalt-substituted enzyme, Sloan *et al.* (*57*) have determined that binding of isobutyramide occurs with the methyl protons 9.1 Å from 'catalytic' Co(II). The distance

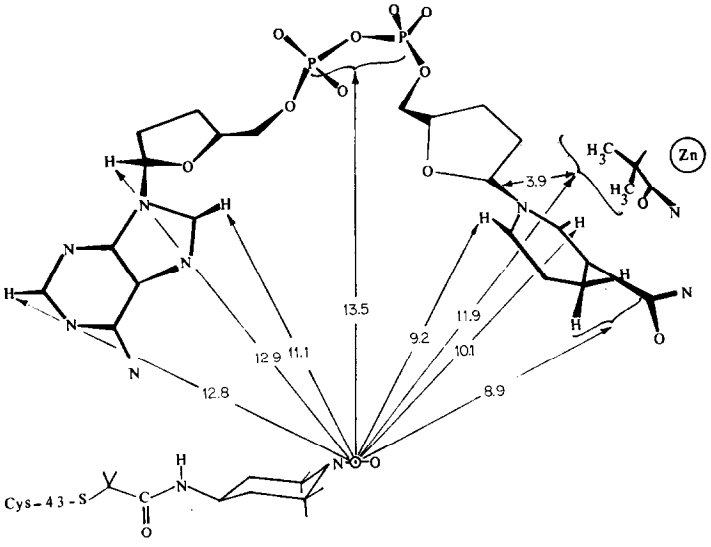


FIG. 14. Metal ion–substrate interaction in YADH (from Ref. *55*).

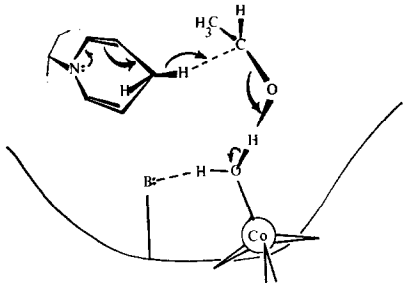


FIG. 15. Metal ion function in Co(II)-LADH (from Ref. *57*).

shortens to 6.9 Å on binding NADH, and the metal-methyne distance is 6.6 Å (*57*). The construction of the active site by consideration of Refs. *54*–*57* is shown in Fig. 15.

It is seen that in this instance a metal-bound water molecule is involved. In the work of Eklund *et al.* (*13a, b*), it was concluded that the water molecule bound to the catalytic zinc was displaced by substrate (see below). This seeming discrepancy may possibly be an effect of substituting Co(II) for Zn(II), which involves a contraction in metal ion size of 0.16 Å and may cause some conformational changes. When 1,10-phenanthroline binds, the water molecule is displaced (*1c*).

That there is *no* metal ion–substrate interaction has been suggested recently by Iweibo and Weiner (*34* and refs. contained therein) based on studies of apo-enzyme/substrate interactions, although there is, of course, no certainty that substrate binding to the apo-enzyme occurs at the same site as with the zinc enzyme (see above). In contrast, using spectrophotometric studies on the totally cobalt-substituted enzyme, Shore and Santiago (*32*) suggested direct metal–substrate interaction.

The binding of 4-(2′-imidazolylazo)benzaldehyde (Fig. 16) to LADH results in an unproductive binary complex (*58*, *59*) which has a uv spectrum very similar to the zinc(II)–azoaldehyde complexes (*59*).

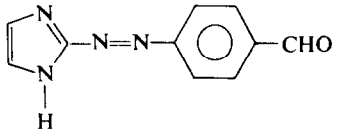


FIG. 16. 4-(2′-imidazolylazo)Benzaldehyde.

Both substrate- and coenzyme-competitive inhibitors displaced the aldehyde, indicating that it overlaps both binding sites. If the azo-aldehyde is added to an enzyme–NADH mixture, then reaction occurs (*58*, *59*), indicating that, under these circumstances, a productive ternary complex is produced which involves a different interaction with the azoaldehyde. It is not impossible that the initial unproductive binding is via the imidazole to the zinc, while with the productive complex the interaction is via the aldehyde. There are also indications that pyrazole, a substrate-competitive inhibitor (*60*), acts by binding directly to the zinc (*61*).

Thus, as with coenzyme binding, there is uncertainty about the mode of metal ion–substrate interaction: nmr indicates an involvement (*52–57*); some kinetics suggest direct coordination (*1*, *44*, *45*, *51*, *61*), and others suggest that there may be an alternative ionisable active site group involved in substrate binding (*46–49*); fluorescence studies and the apo-enzyme–substrate interaction suggest (*34*) no involvement, while spectrophotometric (*32*) and X-ray (*12*, *13*) studies indicate the opposite.

(vi) LADH AND YADH: METAL ION–ENZYME INTERACTION

Although there are certain differences between YADH and LADH [specifically, subunit structure, the effect of metal ion removal, and activity toward certain substrates (Table 3) (see section (iii))], the metal ion catalytic function has been treated in this review as virtually identical in the two enzymes. The basis for this is the following: Sloan and Mildvan (*55*) have established that the metal ion location and function in LADH are the same as in YADH; Jörnvall (*56*) has shown that, in the active site region, the principal amino acid residues are the same in YADH as in LADH. It is of interest here to speculate that it is a difference in amino acid residues that causes the difference in spectral shifts of NADH on binding to the two enzymes. As was discussed above [section (iv)], Kosower (*24*) has shown that a shift from 340 to 325 nm on NADH binding by LADH may result from an ammonium ion–pyridine nitrogen interaction. figure 5 shows (*13*) an interaction between the ammonium group of Arg-47 and the

TABLE 3

SOME DIFFERENCE IN SUBSTRATE SPECIFICITY BETWEEN LADH AND YADH[a]

LADH (relative rate)	Substrate	YADH (relative rate)
100	Ethanol	100
0	Methanol	0.8
108	*n*-Propanol	65
159	*n*-Butanol	60
125	*n*-Hexanol	30
135	*n*-Octanol	40

[a] Data were extracted from Ref. *1a*.

pyrophosphate of ADP–ribose in LADH. In YADH, the residue equivalent to Arg-47 is a histidine (*56*). Furthermore, the mechanism of the two enzymes is the same [ordered bi–bi in both cases (*65*, *66*); the order is NADH first and then aldehyde; alcohol leaves first and then NAD^+] and the inhibition patterns are identical [section (iii)].

In LADH, the metal ion is coordinated by two cysteine sulphurs and a histidine nitrogen (Cys-46 and -174 and His-67) with a water molecule or hydroxyl ion completing a distorted tetrahedron (*13*). In YADH, the equivalent residues in the active

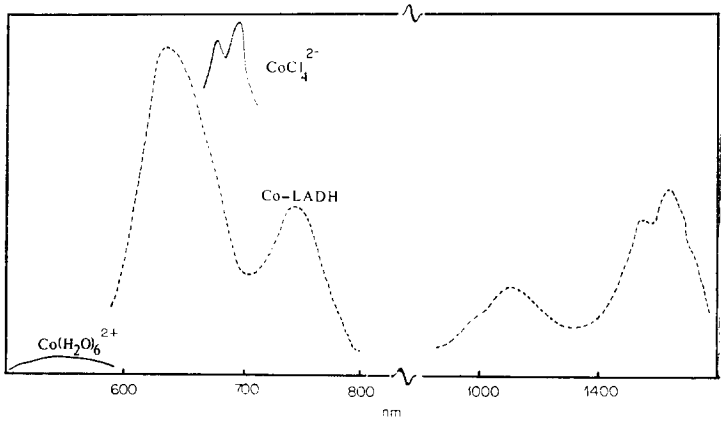


FIG. 17. Visible spectra of Co(II)-LADH (from Ref. *67*).

site are identical (*56*). It is felt, therefore, that the basic assumption of identical catalytic function of the metal ion is justified.

It is of interest that the visible spectrum of cobalt-LADH has been interpreted as possibly representing an 'entatic' state (*67*), i.e., the metal may be regarded as being in a distorted or 'strained' configuration. The spectrum is represented in Fig. 17. Such phenomena are common in cobalt-substituted zinc metalloenzymes as well as in others and have been reviewed by Williams (*68*). The question of whether such a strained configuration enhances catalysis has been the subject of debate (*67–69*), but it surely is

evidence of some distortion from the more stable octahedral or tetrahedral environment, and such distortion is commonly associated with catalytic activity.

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