The Role of Zinc in Alcohol Dehydrogenase

V. THE EFFECT OF METAL-BINDING AGENTS ON THE STRUCTURE OF THE YEAST ALCOHOL DEHYDROGENASE MOLECULE

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The role of zinc in the catalytic action of yeast alcohol dehydrogenase has been studied through the kinetics of the inhibition of activity by chelating agents (1), particularly 1,10-phenanthroline (2-4). In aqueous solution the complexes of 1,10-phenanthroline with Zn²⁺ ions exhibit characteristic absorption spectra, as do the complexes of this agent with the zinc atoms of yeast alcohol dehydrogenase and of other zinc metalloenzymes (5). The molecular stoichiometry of the enzyme-inhibitor complexes, deduced from spectrophotometric measurements, is in agreement with that inferred from kinetic data (6).

Whereas the catalytic activity of the enzyme can thus be decreased by the localized attack of a chelating agent on a component of an "active site," alterations in the protein structure may have similar functional consequences, of course. Chelating agents are here shown to induce changes in enzymatic activity both through such local action and through subsequent alterations of the macromolecular structure of yeast alcohol dehydrogenase. As a function of the time of exposure to chelating agents and of their concentration, the apoenzyme, molecular weight 151,000, dissociates into four equal subunits, molecular weight 36,000, while the four zinc atoms are removed concomitantly. Thus, a direct correlation between the enzymatic activity, the zinc content, and the protein structure of the enzyme can be shown to exist. A preliminary report has been made (7).

EXPERIMENTAL PROCEDURE

Twice crystallized yeast alcohol dehydrogenase (C. F. Boehringer u. Soehne, Mannheim, West Germany) was stored in 0.6 saturated ammonium sulfate. Before its use, the enzyme was dialyzed for 5 days at 4° against 0.1 M sodium phosphate buffer, pH 7.5, to remove impurities of low molecular weight which absorb radiation at 280 mμ. The enzyme concentration was measured by the optical density at 280 mμ, based on an absorbancy index of 1.26 mg⁻¹ cm² (8). Some enzyme preparations which contained a second, more slowly sedimenting component were purified further by equilibrium centrifugation at 92,000 X g for 20 hours at 1° (Spinco ultracentrifuge, model L). Purified enzyme (20 mg per ml) was stable for several weeks in 0.1 M phosphate buffer, pH 7.5, 4°; during this time no change was demonstrated by analytical ultracentrifugation. The turnover numbers of different preparations varied from 36,000 to 40,000 moles of DPN per minute per mole of enzyme, as determined in a reaction mixture containing 4.5 X 10⁻⁶ M enzyme, 8.33 X 10⁻³ M DPN, and 0.33 M ethanol, in 0.016 M pyrophosphate buffer, pH 8.8, 25°.

Solutions of 1,10-phenanthroline hydrochloride, 1.25 X 10⁻² M (G. F. Smith Chemical Company), 8-hydroxyquinoline-5-sulfonic acid, 2 X 10⁻² M (Eastman Organic Chemicals), and Versene (disodium ethylenediaminetetraacetic acid), 3 X 10⁻⁵ M (Bersworth Chemical Company) were prepared in 0.1 M phosphate or 0.1 M Tris buffer at pH 7.5.

DPN (Fabel Laboratories) was 95% pure as estimated by the formation of the cyanide complex (9). Sodium phosphate and Tris (Sigma 121, Sigma Chemical Company) buffers, 0.1 M, were adjusted to pH 7.5, 0°, with NaOH and metal-free HCl, respectively (10). Ethanol (95%), Na₂HPO₄, Na₂H₂PO₄, and NaOH were reagent grade and were used without further purification. The purification of water and glassware has been described (1).

Yeast alcohol dehydrogenase was incubated with chelating agents in 0.1 M sodium phosphate, or Tris buffer, pH 7.5, at 0°. At predetermined intervals, samples were withdrawn for examination in the ultracentrifuge, for measurement of protein-bound zinc and of enzymatic activity.

Enzymatic activity was measured spectrophotometrically by determination of the rate of DPNH formation at 340 mμ, as previously described (1). Immediately before the assay, the enzyme sample used in sedimentation studies was diluted to a concentration of 10 μg per ml in 0.1 M phosphate buffer. The 3.0 ml reaction mixture contained 5 μmoles of DPN, 1000 μmoles of ethanol, and 0.5 ml of 0.1 M pyrophosphate buffer, pH 8.8, 25°. The reaction was initiated by the addition of 0.2 ml of enzyme solution to the reaction mixture. Activity was expressed as the change of optical density at 340 mμ per minute per milligram of enzyme.

After dry ashing of the samples in platinum crucibles, zinc determinations were performed by an extraction method (11, 12). Enzyme-bound and ionic zinc were measured after separation of the two moieties by dialysis of the enzyme against 10 ml of Tris buffer, pH 7.5, at 0° for 20 hours. Cellulose casings (Visking Company) were cleaned as described by Hughes and Klots (13).

Sedimentation measurements were carried out with the
standard analytical cell in a Spinco model E analytical ultracentrifuge. Experiments were conducted at a rotor speed of 59,780 r.p.m. (263,000 × g), at temperatures between 0 and 10°, maintained by means of the rotor temperature control unit. The sedimentation coefficients, reported as $s_\text{av}$ values, were corrected for the viscosity of water at 27° but not for the variation in the protein concentration, nor for changes of viscosity due to the addition of buffer or chelating agents. The sedimentation diagrams were enlarged by projection and the area of the boundaries was measured with a mechanical planimeter. Overlapping boundaries were resolved as described by Ogston (14). Correction for radial dilution during centrifugation was made by extrapolation of the boundary areas to zero time. The baseline of the Schlieren pattern was determined from separate controls containing only buffer and chelating agents.

Determinations of the molecular weight of the native protein and of the resultant fragments were performed by the method of Ehrenberg (15) with a 12-mm synthetic boundary cell with a rubber valve. Measurements were obtained on samples dialyzed for 20 hours against a solution of 1% sodium chloride and 0.05 M sodium phosphate, pH 7.5, 2°. A partial specific volume of 0.709 (8) was employed in all instances. The molecular weight of the new species was determined both from the weight average molecular weight, extrapolated to zero time, and from the condition of the boundary after centrifugation for 210 minutes (see Fig. 3).

RESULTS

The enzyme was incubated with 7.5 × 10⁻³ M OP for 24 hours and with 2 × 10⁻² M 8-OHQ5SA for 9 hours in 0.1 M phosphate buffer, pH 7.5, and sedimented in the analytical ultracentrifuge. These chelating reagents affect the sedimentation pattern markedly (Fig. 1). The single peak of the control both at 9 and at 24 hours has an average sedimentation value $s_\text{av}$ of 7.0, varying from 6.9 to 7.2 in different experiments. In the presence of either chelating agent, a second, more slowly moving peak appears with $s_\text{av}$ values of 2.7 and 2.9, respectively, the mean being 2.8 in different experiments. There is a concomitant decrease in the area under the original S-7 peak as that of the new S-2.8 peak increases. Both boundaries remain symmetrical throughout, indicating two homogeneously sedimenting species.

The transformation of the S-7 into the S-2.8 material is a function of time of contact of the enzyme with these chelating agents (Fig. 2). Thus, after 4 hours of incubation with OP, the area under the S-2.8 peak corresponds to only 20% of the total boundary area; after 15 hours, it comprises about 50%, and after 34 hours, about 85% of the original material.

At a constant concentration of enzyme, the rate of transformation of S-7 into S-2.8 increases as a function of an increase in the concentration of the chelating agent or in the temperature of incubation. The rate of transformation also varied somewhat with the batch of yeast alcohol dehydrogenase employed. The further disappearance of the original S-7 boundary is arrested by removal of OP by dialysis, or by adding concentrations of Zn⁺⁺ ions sufficient to combine with all OP present; however, these procedures do not reverse the changes in the sedimentation pattern which have already taken place.

The molecular weight of yeast alcohol dehydrogenase has been reported as 140,000 (16) or 150,000 (8). The reproducible appearance of a homogeneous peak with a much lower sedimentation constant than that observed for native yeast alcohol dehydrogenase suggests that the enzyme dissociates into discrete subunits in the presence of these chelating agents.

The molecular weight of the control, measured at seven successive intervals during centrifugation, remains constant, 151,000 ± 2,000, indicating the homogeneity of the native enzyme employed (Fig. 3A). The molecular weight of the fragment is 36,000, as determined by calculation from the weight average molecular weight extrapolated to zero time (76,200), and also from the appearance of the boundary after 210 minutes of centrifugation (Fig. 3B).

1 The abbreviations used are: OP, 1,10-phenanthroline; 8-OHQ5SA, 8-hydroxyquinoline-5-sulfonic acid; YADH, yeast alcohol dehydrogenase (in formulations only).

2 The sedimenting species of yeast alcohol dehydrogenase are henceforth referred to as S-7 and S-2.8, respectively.

![Ultracentrifuge patterns of yeast alcohol dehydrogenase in the presence of OP and 8-OHQ5SA.](http://www.jbc.org/)

![Effect of time of incubation with OP on the sedimentation pattern of yeast alcohol dehydrogenase.](http://www.jbc.org/)
The disintegration of the tetrameric alcohol dehydrogenase molecule into monomeric units on exposure to chelating agents is proportional to the loss both of its enzymatic activity and of its zinc content (Fig. 4).

Further information on the presumable mechanism of this depolymerization by OP was gained by simultaneous exposure

![Molecular weights](image)

**Fig. 3.** Determination of the molecular weights of native yeast alcohol dehydrogenase (A) and of the product resulting from exposure to OP (B) by approach to equilibrium centrifugation as described by Ehrenberg (15). Monodisperse yeast alcohol dehydrogenase 10 mg per ml, in 0.1 M sodium phosphate, pH 7.5, 2°, were dialyzed for 20 hours against the same buffer, 0.05 M sodium chloride, pH 7.5, 2°. The enzyme was sedimented at 10,880 r.p.m., and the molecular weight, 151,000, was determined at successive intervals, A. An aliquot was exposed to 1 X 10^{-3} M OP for 20 hours, dialyzed under identical conditions, and sedimented at 20,410 r.p.m. The molecular weights determined successively are plotted as B. The mixture, resulting from the exposure of the enzyme to OP, was shown to contain S-2.8 and S-7 material in a proportion of 65 to 35% determined in a separate velocity sedimentation run. From this proportion, the weight average molecular weight of the mixture, 76,200, obtained by extrapolation to zero time and from the molecular weight of the native enzyme, the S-2.8 fragment has a molecular weight of 36,000:

Molecular weight_{S-2.8} = \frac{76,200 - (0.35 \times 151,000)}{0.65} = 36,000

![Correlation](image)

**Fig. 4.** Correlation between the effects of OP and 8-OHQSSA on the activity, enzyme-bound zinc, and structure of yeast alcohol dehydrogenase. All measurements are expressed in percentages of the control in 0.1 M sodium phosphate buffer. The enzyme, 5 X 10^{-4} M, in 0.1 M sodium phosphate, pH 7.5, 0°, was incubated with 7.5 X 10^{-4} M OP or 2 X 10^{-3} M 8-OHQSSA. At various times, aliquots were analyzed for activity and protein-bound zinc and examined in the ultracentrifuge. The percentage of the original enzyme remaining (S-7) is plotted on the abscissa, enzymatic activity after incubation with OP (O), with 8-OHQSSA (●) or with 8-OHQSSA (△) on one ordinate and protein-bound zinc after incubation with OP (□) or with 8-OHQSSA (▲) on the other.

![Table](image)

**Fig. 5.** Comparison of the effects of OP on activity, zinc content and structure of yeast alcohol dehydrogenase in the presence and absence of DPN. The enzyme, 5.6 X 10^{-4} M, in 0.1 M sodium phosphate buffer, pH 7.5, 0°, was incubated for 24 hours with 7.5 X 10^{-4} M OP plus 0.3 M DPN (center) and with 7.5 X 10^{-4} M OP alone (bottom). Enzyme in buffer for 24 hours served as the control (top). Centrifugation was carried out at 59,780 r.p.m., 0°. The exposures were taken after 48 minutes of sedimentation. Enzyme activity and enzyme-bound zinc are expressed in percentages of the control. The arrow identifies the S-2.8 species which appears on exposure of the enzyme to OP.

of the enzyme to DPN, which competes with this chelating agent (2, 4). DPN, 0.3 M, prevents the loss of activity, the removal of zinc, and the changes in enzyme structure induced by 7.5 X 10^{-4} M OP to the same extent (Fig. 5). When DPN, which protects the enzyme, is first removed by dialysis, and the enzyme is then again exposed to OP, the correlated changes in the disintegration of the structure of the protein and the loss of both activity and zinc proceed to completion, as if OP had not originally been present.

Versene does not inhibit yeast alcohol dehydrogenase, nor does it remove protein-bound zinc atoms (17). After 24 hours of contact with 7.5 X 10^{-3} M Versene in 0.1 M phosphate buffer, there is no alteration of the sedimentation pattern of 5.9 X 10^{-4} M yeast alcohol dehydrogenase.

At acid pH, both activity and zinc of the enzyme are lost also (18, 19), but the effect of H⁺ ions on the structure of the enzyme differs markedly from that here described for chelating agents. Yeast alcohol dehydrogenase, 3.3 X 10^{-3} M, when dialyzed for 24 hours in 0.1 M sodium acetate, pH 4.0, 0°, becomes polydispersed and precipitates on increasing the temperature by only 4°. Apparently, H⁺ ions critically affect sensitive groups of this enzyme in addition to those involved in activity and zinc binding.

Although changes similar to those reported here for yeast alcohol dehydrogenase have been observed for glutamic dehydrogenase of beef liver in the presence of OP (20), such changes are not necessarily seen with all zinc enzymes. Under conditions similar to those described above, neither alcohol dehydrogenase of horse liver nor carboxypeptidase A of bovine pancreas form subunits in the presence of this chelating agent.

**DISCUSSION**

The monodisperse, highly active yeast alcohol dehydrogenase used in these experiments has a molecular weight of about 151,000 in agreement with previous data (8). On this basis, the enzyme has been calculated to contain 4 gram atoms of zinc (1) and to bind approximately 4 moles of coenzyme (8). The dissociation of yeast alcohol dehydrogenase into four subunits, coincident
with the removal of zinc by chelating agents, indicates the existence of quaternary structure, as defined by Bernal (21).

This finding extends the tetrad stoichiometry of zinc atoms and DPN-binding sites to the structural organization of the protein, implying an interrelationship. The empirical structural formula of this enzyme may now be written

\[
[(\text{YADH})_4\text{Zn}_4](\text{DPN})_4,
\]

where (YADH) represents a single unit of molecular weight 36,000 and (YADH), a tetramer of molecular weight 151,000.

The tetrad stoichiometry between the number of subunits, zinc atoms, and moles of DPN binding to the tetrameric apoenzyme, and its dissociation into monomers on exposure to chelating agents, all suggest that zinc atoms stabilize the quaternary structure of yeast alcohol dehydrogenase. It is attractive to view the zinc atoms as bridges between four identical monomers though alternate schemes are possible. The time-dependent, irreversible inhibition of yeast alcohol dehydrogenase by OP corresponds to the binding of 2 molecules of this agent to each zinc atom of yeast alcohol dehydrogenase (2):

\[
[(\text{YADH})_4\text{Zn}_4] + 4(2 \text{ OP}) \rightarrow [(\text{YADH})_4\text{Zn}_4](2 \text{ OP})_4 \quad (1)
\]

On the basis of the kinetics of inhibition and the time-dependent changes in the spectrum of the enzyme-inhibitor complex, it was postulated that the formation of this complex should lead to alterations in the structure of the apoenzyme (4). The present data confirm this hypothesis. When OP is present in excess, the overall reaction accounts for the simultaneous, irreversible loss in enzymatic activity, quaternary structure and zinc:

\[
[(\text{YADH})_4\text{Zn}_4] + 4(3 \text{ OP}) \rightarrow 4(\text{YADH}) + 4[\text{Zn OP}^{2+}] \quad (2)
\]

The depolymerization of the tetrameric apoenzyme into monomeric units with a molecular weight of 36,000 appears to be an obligatory consequence of the successful competition of OP, when in excess, for more than one pair of coordination sites of the zinc atom.

The competition of DPN with OP for the zinc atoms of yeast alcohol dehydrogenase, indicating that one molecule of coenzyme binds at or near each of its four zinc atoms (2), is also apparent in the prevention of the dissociation reaction by DPN (Equation 2). The placement of the zinc atoms as bridges between subunits, implied by the present findings, therefore simultaneously restricts the possible sites of binding of the coenzyme.

Some aspects of the topographic characteristics of this site may be inferred, in fact, by reasoning from the kinetics of inhibition of this enzyme: DPN reverses the instantaneous inhibition of the enzyme by 1 molecule of OP (3), whereas the tetrameric structure of the yeast alcohol dehydrogenase molecule is fully preserved. The coenzyme does not reverse the time-dependent inhibition of the enzyme attributed to the complexion of zinc by the second molecule of OP (4), which is here shown to be accompanied by the removal of the metal and by depolymerization. Thus, it would seem that the rupture of the zinc bridges destroys a critical constellation required for coenzyme binding. The proper configuration of the subunits, induced by zinc atoms, must be decisive for the ensuing catalytic action. DPN seems, therefore, to act in conjunction with each of the four zinc atoms, and also with each of two adjacent subunits, to conform to the requirements of stoichiometry. This could be accomplished readily if the coenzyme were to cover across the zinc-bridged gap between two subunits and interact at three points: one each with one of two adjoining monomers, the third, weakly (24), with zinc. One might even be led to suggest that DPN would thereby cooperate in reinforcing the metal linkages between the subunits of the holoenzyme, thus stabilizing the tetrameric structure while performing its stereospecific oxidation-reduction function. The removal of the zinc bridge, irreversible thus far in this enzyme, and the subsequent disintegration into monomers of the apoenzyme, would bring about the disorganization of the three-point interaction, manifested by the irreversible abolition of enzymatic activity. These conjectures would seem appropriate at this time, since such structural characteristics of this enzyme, which could not be taken into consideration previously, restrict the number of feasible mechanisms. Thus the kinetic, compositional, and structural data now available remove further handicaps in arriving at an internally consistent reaction scheme.

It has been emphasized previously that stability constants of metal chelates in aqueous solution are poor guides to the inhibition of this and other metalloenzymes (1). Steric hindrance, due to the quaternary structure of the enzyme, may well be reflected in the modification of the dissociation constant of OP with the enzyme-bound zinc, which is higher by \(5 \times 10^4\) than that with \(\text{Zn}^{2+}\) ions in aqueous solution (3, 25).

The failure of Versene to inhibit (17) and to dissociate the enzyme may be a specific example of steric hindrance.

Although the nature of the zinc-protein bond remains conjectural, the present data, together with others already on record (26), may illuminate the problem. Zinc shows a marked preference for sulfur or nitrogenous ligand groups, and the stability of such complexes has been emphasized (27). It has been suggested that zinc may be bound to yeast alcohol dehydrogenase as a mercaptide (1). Although the time-dependent displacement of zinc by \(\text{Ag}^+\) ions and \(p\)-chloromercuribenzoate, resulting in the formation of smaller fragments (26), cannot be employed as unequivocal support for the existence of zinc-mercapto, bonds maintaining the tetrameric structure, these observations are consistent with such a hypothesis.

Neither removal of OP by dialysis nor addition of zinc brings about polymerization and reactivation. Since studies of rotary dispersion have shown that the dissociation of yeast alcohol dehydrogenase by OP is not accompanied by changes in the helical conformation of the protein (28), the irreversible transformation of the tetramer into subunits may be attributable to a local configurational or chemical change at the zinc-binding site. The divergence in the rates of dissociation of different preparations of yeast alcohol dehydrogenase may well be due to variations in this rate-determining step. If zinc is bound to mercaptide groups, oxidation subsequent to the removal of zinc could readily account for these observations.
The irreversible, time-dependent inhibition of alcohol dehydrogenase of yeast by 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid is accompanied by the dissociation of its apoenzyme of molecular weight of 151,000 into four subunits with a molecular weight of 36,000. Hence, the empirical structure formula of yeast alcohol dehydrogenase may be written \( [(YADH)_rZn]_DPN \), where \( (YADH) \) represents a single unit of molecular weight 36,000. The rates of inactivation and of the loss of zinc from the enzyme, and the degree of dissociation of the apoenzyme are correlated directly. Zinc atoms are thought to stabilize the quaternary structure of the enzyme through the formation of bridges between the monomers to form the enzymatically active tetramer. A three-point attachment of DPN to two adjacent monomeric units of the apoenzyme and to the bridging zinc atom is postulated to account for interrelationships between the functional and structural features of the active holoenzyme and its zinc content.

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