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State and Accessibility of Zinc in Yeast Alcohol Dehydrogenase

By VLADIMIR LESKOVAC, SVETLANA TRIVIĆ and MIRJANA LATKOVSKA
Department of Chemistry, University of Novi Sad, P.O. Box 187, 21000 *Novi Sad, Yugoslavia*

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1. Yeast alcohol dehydrogenase (EC 1.1.1.1) is inhibited in the presence of 1,10-phenanthroline. 2. A conformational change in the enzyme's structure is induced by 1,10-phenanthroline, and is abolished in the presence of NADH. 1,10-Phenanthroline binds to the enzyme competitively with respect to NADH, with a stoicheiometry of 2mol of 1,10-phenanthroline/144 000g of enzyme. 3. 1,10-Phenanthroline induces a time-dependent dissociation of Zn^{2+} from the enzyme, which is in correlation with its inhibition. 4. Spectrophotometric measurement indicates that the dissociation of half (2 zinc atoms/tetramer) of the total zinc content of the enzyme correlates with the full inhibition of its activity. Measurement of the tightly bound Zn^{2+} by atomic absorption photometry confirms this. 5. A proposition is advanced that the tetrameric molecule of yeast alcohol dehydrogenase possesses an inherent asymmetry, with four monomeric subunits being arranged in two mutually symmetrical pairs.

In our previous paper (Leskovac & Pavkov-Peričin, 1975) it was shown that the yeast alcohol dehydrogenase has one histidine and one cysteine residue in the substrate-binding site. The tertiary structure of horse liver alcohol dehydrogenase at 0.24nm resolution has been reported (Eklund *et al.*, 1974); the position of two Zn^{2+} atoms in the subunit of the liver enzyme has been established, and a plausible mechanism of action for the enzyme derived. In the present paper we have investigated with chemical methods the state and accessibility of Zn^{2+} atoms in yeast alcohol dehydrogenase. In doing so, we have applied some of the experimental approaches used by Vallee and co-workers (Hoch & Vallee, 1956; Hoch *et al.*, 1958; Williams *et al.*, 1958; Kägi & Vallee, 1960; Drum & Vallee, 1970); in particular, the ability of 1,10-phenanthroline to inhibit the enzyme and chelate its zinc was applied to the study of the state of Zn^{2+} in this enzyme. Our experimental results and conclusions about the state and accessibility of Zn^{2+} in yeast alcohol dehydrogenase are, in several important respects, essentially different from those obtained by Vallee and associates.

Experimental

Materials

Chemicals. 1,10-Phenanthroline was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., 8-anilinonaphthalene-1-sulphonic acid (magnesium salt) from Serva Feinbiochemica, Heidelberg, Germany and NADH (grade I) from Boehringer, Mannheim, Germany. All other chemicals were of analytical-grade purity, obtained from commercial sources. All solutions were prepared with glass-distilled water.

Enzyme. Yeast alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer Mannheim (catalogue no. 15420) (50mg of freeze-dried enzyme contained 30mg of enzyme, 15 mg of sucrose and 5mg of phosphate) or from Serva Feinbiochemica (catalogue no. 12081), which contained only freeze-dried enzyme. Specific activity of Boehringer preparations of the enzyme was the same as previously reported (Leskovac & Pavkov-Peričin, 1975). The zinc content of each enzyme preparation was determined as described under 'Methods'.

Methods

Spectrophotometric measurements were performed in a double-beam spectrophotometer (Carl Zeiss, Jena, model Specord UV VIS), and the spectrophotofluorimetric measurements in an Aminco–Bowman spectrophotofluorimeter, as previously described (Leskovac & Pavkov-Peričin, 1975; Leskovac, 1975). Unless otherwise specified, all experiments reported in this paper, and all spectrophotometric and fluorimetric measurements were performed at 23°C. Enzyme assay was performed as previously described (Leskovac & Pavkov-Peričin, 1975), and all calculations presented in this paper are based on the assumption that the molecule of yeast alcohol dehydrogenase consists of four very similar subunits with a molecular weight of 144 000 for the tetramer (Leskovac & Pavkov-Peričin, 1975). The concentration of alcohol dehydrogenase was determined spectrophotometrically, by using $E^{1\%}_{1cm} = 12.6$ at 280nm (Hayes & Velick, 1964).

Determination of zinc. Zinc content of enzyme samples was determined in a Unicam SP.90 atomic absorption spectrophotometer. Samples of alcohol dehydrogenase, dissolved in 0.1M-Tris/HCl buffer, pH6.5 (with 0.5mM-EDTA), were run through the spectrophotometer; simultaneously, a calibration curve was prepared for each experiment with solutions of $ZnSO_4,7H_2O$ in the same buffer as the enzyme, covering the appropriate concentration range. The concentration range for zinc used in our experiments was 0–30μM; the 'aging' of standard $ZnSO_4$ solutions was observed after several days, manifested as a decrease in readings in the spectrophotometer. Boehringer preparations of the enzyme (lot nos. 7204232 and 7085137) containing 1.05–1.19g-atoms of zinc/36000g of enzyme, and this Zn^{2+} content was not changed by dialysis in the cold, against 0.1M-Tris/HCl buffer, pH7.2, containing 0.5mM-EDTA. A typical calibration curve obtained with standard solutions of $ZnSO_4$, and the recorded readings obtained for several concentrations of the commercial Boehringer enzyme under identical conditions, are presented in Fig. 1. The zinc content of the Boehringer preparation of alcohol dehydrogenase, calculated in the experiment presented in Fig. 1 directly from the calibration curve, was 1.1g-atoms of zinc/36000g of enzyme. Zinc content of enzyme preparations partially depleted of their intrinsic zinc (see the Results section) was determined in the same way, with the aid of calibration curves. Since the protein was not ashed for the metal analysis, the influence of protein on the metal analysis was checked (Table 1). The experiment presented in Table 1 indicates that the photometric readings for Zn^{2+} are higher in the presence of the protein than in its absence, which indicates that the metal content of the enzyme samples, estimated by the present method, is approx. 10% too high.

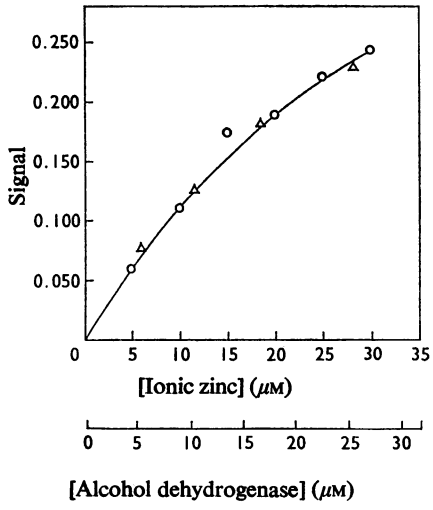

Fig. 1. *Calibration curve for the zinc content of alcohol dehydrogenase*

Solutions of $ZnSO_4,7H_2O$ in 0.1M-Tris/HCl buffer, pH6.5, containing 0.5mM-EDTA (○), and solutions of alcohol dehydrogenase in the same buffer (△), of indicated concentrations, were run through the atomic absorption spectrophotometer, and the signals (absorbance) registered (ordinate). Concentrations of $ZnSO_4$ solutions were determined gravimetrically (abscissa, above), and concentrations of alcohol dehydrogenase subunit (mol.wt. 36000) were determined spectrophotometrically (Hayes & Velick, 1964) (abscissa, below).

Table 1. *Influence of protein on Zn^{2+} determination by atomic absorption photometry*

Samples of alcohol dehydrogenase (mol.wt. 36000), $ZnSO_4,7H_2O$ and their equimolar mixtures, dissolved in 0.1M-Tris/HCl buffer, pH6.5, containing 0.5mM-EDTA, were run through the photometer at the indicated concentrations. Photometer readings are expressed in absorbance units.

Concentration (μM)	Alcohol dehydrogenase	Zn^{2+}	Alcohol dehydrogenase $+Zn^{2+}$
10.9	0.119	0.100	0.119
21.8	0.215	0.180	0.214

Since the protein was not ashed for the metal analysis, the influence of protein on the metal analysis was checked (Table 1). The experiment presented in Table 1 indicates that the photometric readings for Zn^{2+} are higher in the presence of the protein than in its absence, which indicates that the metal content of the enzyme samples, estimated by the present method, is approx. 10% too high.

Serva preparations of alcohol dehydrogenase contained 1.6–1.7g-atoms of zinc/36000g of enzyme, but 25–35% of the Zn^{2+} content of the commercial enzyme was removed by dialysis in the cold, against 0.1M-Tris/HCl buffer, pH7.2, containing 0.5mM-EDTA. Therefore all our experiments were performed with Boehringer preparations of alcohol dehydrogenase.

Repetition of experiments and statistical treatment. Unless otherwise stated, each experiment presented in the Figures and in the text was repeated. Maximum differences between experiments never exceeded 10%. For each experiment reported in this paper, unless otherwise specified, a result of a single determination only is given. Whenever a linear relationship for a set of experimental points was expected on theoretical grounds, the least-squares method was applied to draw straight lines for graphical representation.

Results

Inhibition of alcohol dehydrogenase by 1,10-phenanthroline

Yeast alcohol dehydrogenase rapidly loses its activity at room temperature in the presence of 1,10-phenanthroline in neutral solutions. The time-course of inhibition obeys the first-order reaction law, and is retarded in the presence of NADH.

Alcohol dehydrogenase (1.7μM, mol.wt. 144000) in the presence of 44μM-1,10-phenanthroline and 0.3M-acetamide in 0.1M-sodium phosphate buffer, pH7.5, was inhibited by 50% in 23min in the absence of NADH and in 53min in the presence of 13.7μM-NADH. Inhibition with 1,10-phenanthroline is irreversible, since the removal of 1,10-phenanthroline does not restore the activity. Removal of 1,10-phenanthroline by gel filtration immediately arrests the loss of enzymic activity (see below).

Conformational change of alcohol dehydrogenase induced by 1,10 *phenanthroline*

In the presence of 1,10-phenanthroline the enzyme undergoes a conformational change, which is initially in correlation with the loss of enzymic activity (see below). Protein fluorescence of the enzyme at 350nm (excitation at 280nm) does not change appreciably in the course of inactivation; therefore the fluorescence emission of the protein could not be used to monitor the conformational change of the enzyme.

8-Anilinonaphthalene-1-sulphonic acid binds specifically to yeast alcohol dehydrogenase with a stoicheiometry of 2mol of fluorescent dye/144000g of enzyme (Dickinson, 1971). Both 1,10-phenanthroline and 8-anilinonaphthalene-1-sulphonic acid induce the conformational change in the enzyme. The conformational change induced by 8-anilinonaphthalene-1-sulphonic acid or by a mixture of 1,10-phenanthroline and 8-anilinonaphthalene-1-sulphonic acid can be monitored by the increase in the fluorescence emission of the acid dye at 485nm (excitation at 410nm), assuming that the change in the fluorescence emission of the bound dye mirrors the conformational change in the enzyme's structure. The initial rate of the change in fluorescence of 1.1μM-alcohol dehydrogenase (mol.wt. 144000), 13.3μM-8-anilinonaphthalene-1-sulphonic acid and 0.3M-acetamide in 0.1M-sodium phosphate buffer, pH7.5, is three times lower in the absence than in the presence of 66μM-1,10-phenanthroline. The cause of the slow conformational change induced by 8-anilinonaphthalene-1-sulphonic acid alone is not clear; we cannot exclude the possibility that a number of other aromatic substances can induce a similar conformational change.

In the presence of increasing concentrations of NADH in the system enzyme+1,10-phenanthroline +8-anilinonaphthalene-1-sulphonic acid, the initial rate of the change of dye fluorescence decreases. At high concentrations of NADH the initial rate of the fluorescence change decreases to the rate of the fluorescence change of a mixture of enzyme+8-anilinonaphthalene-1-sulphonic acid. Thus NADH at high concentration prevents 1,10-phenanthroline, but does not prevent 8-anilinonaphthalene-1-sulphonic acid, from inducing the conformational change. Since 8-anilinonaphthalene-1-sulphonic acid binds to the enzyme non-competitively with respect to NADH, to the site distinct from the coenzyme-binding site (Dickinson, 1971), this indicates that the binding of 1,10-phenanthroline and NADH to the enzyme is mutually competitive. The dissociation constant of NADH from the ternary complex enzyme–NADH–acetamide (0.5μM) (Leskovac & Pavkov-Peričin, 1975) differs by three orders of magnitude from the dissociation constant of the binary complex enzyme–1,10-phenanthroline (2mM) (Hoch *et al.*, 1958) at neutral pH values. Therefore it is possible to determine the stoicheiometry of binding of 1,10-phenanthroline to the enzyme, by displacing 1,10-phenanthroline with NADH from the ternary complex 1,10-phenanthroline–enzyme–8-anilinonaphthalene-1-sulphonic acid, in the presence of acetamide (Fig. 2).

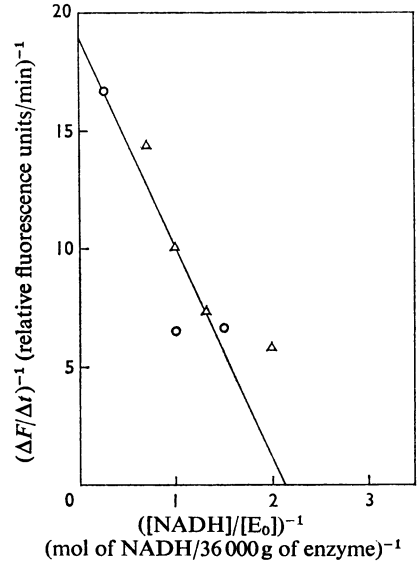

Fig. 2. *Influence of NADH on the rate of the* 1,10-*phenanthroline-induced conformational change*

Alcohol dehydrogenase (mol.wt. 36000) (4.3μM), 8-anilinonaphthalene-1-sulphonic acid (13μM), 1,10-phenanthroline (130μM) and acetamide (0.3M) were mixed in 0.1M-sodium phosphate buffer, pH7.5, in the presence of increasing concentrations of NADH. Reciprocals of the initial rates of the increase in fluorescence at 485nm (excitation at 410nm) of reaction mixtures (ordinate) were plotted against reciprocals of concentrations of NADH (abscissa). Rate of increase in fluorescence was measured over the 5–20min from the beginning of the reaction; the fluorescence change in the presence of 1,10-phenanthroline was corrected for the rate of the fluorescence change in the absence of 1,10-phenanthroline, so that the influence of 8-anilinonaphthalene-1-sulphonic acid on the rate of the conformational change was excluded. Different symbols represent different experiments.

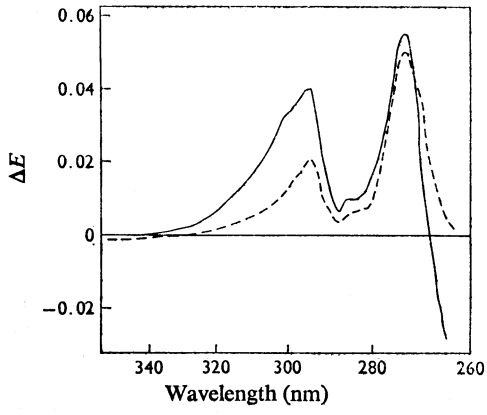

Fig. 3. *Perturbation spectra of* 1,10-*phenanthroline*

$ZnSO_4,7H_2O$ solution (2μM) was mixed with 1,10-phenanthroline (25μM) in 0.1M-Tris/HCl buffer, pH6.5, and alcohol dehydrogenase (mol.wt. 36000) (2μM) was mixed with 1,10-phenanthroline (25μM) in the same buffer. Difference spectra for Zn^{2+} (——) and the enzyme (– – – –) were recorded 50min after the mixing of ligands.

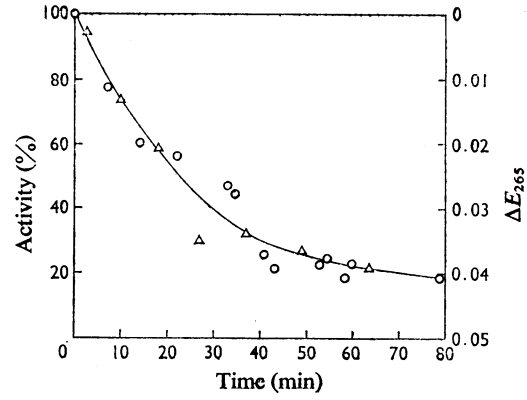

Fig. 4. *Correlation between zinc dissociation and inhibition of alcohol dehydrogenase*

Alcohol dehydrogenase (mol.wt. 36000) (3.5μM) was mixed with 1,10-phenanthroline (57μM) in 0.1M-Tris/HCl buffer, pH6.5. Enzyme activity (△) (left ordinate) and difference-spectral values at 295nm (○) (right ordinate) were measured at the indicated times.

A double-reciprocal plot of the initial rate of conformational change associated with the addition of increasing concentrations of NADH to the mixture enzyme + 1,10-phenanthroline + 8-anilinonaphthalene-1-sulphonic acid indicates that 2mol of 1,10-phenanthroline are bound/144000g of enzyme, competitively with respect to NADH.

Spectrophotometric measurement of the 1,10-*phenanthroline-induced dissociation of zinc from alcohol dehydrogenase*

In the presence of alcohol dehydrogenase, 1,10-phenanthroline develops a difference spectrum in the u.v. region, which is identical with the perturbation in the spectrum of 1,10-phenanthroline caused by its chelation with Zn^{2+} ions (Fig. 3).

The identity of two difference spectra, and the rate of the appearance of the perturbation spectrum of 1,10-phenanthroline, induced by alcohol dehydrogenase (see below), indicates that 1,10-phenanthroline induces a restricted but complete time-dependent dissociation of zinc from the enzyme. Native and fully active enzyme chelates 4 zinc atoms/tetramer (Kägi & Vallee, 1960), which could not be removed by dialysis (see the Experimental section). The extent of 1,10-phenanthroline-induced dissociation of zinc from the enzyme was monitored by the appearance of a 295nm-absorbing peak in the perturbation spectrum of 1,10-phenanthroline, caused by its chelation with zinc dissociated from the enzyme. The extent of zinc dissociation correlates with the inactivation of the enzyme (Fig. 4).

The perturbation in the 1,10-phenanthroline spectrum at 295nm, caused by Zn^{2+} or zinc dissociated from the enzyme, can be abolished by titration with stoicheiometric concentrations of EDTA in 0.1M-Tris/HCl buffer, pH6.5. This indicates that EDTA chelates zinc more strongly than does 1,10-phenanthroline under the conditions of our experiments.

With the increasing 1,10-phenanthroline/Zn^{2+} concentration ratio, in 0.1M-Tris/HCl buffer, pH6.5, at a constant concentration of Zn^{2+}, the perturbation of the 1.10-phenanthroline spectrum at 295nm increases, reaching a maximal value at saturating concentrations of 1,10-phenanthroline; the saturating concentrations were found to be at the 1,10-phenanthroline/Zn^{2+} concentration ratios above 5.

All experiments with alcohol dehydrogenase presented in this paper were conducted in 0.1M-Tris/HCl buffer, or in 0.1M-sodium phosphate buffer, pH6.5–7.5, with subunit concentrations of 2–12μM (mol.wt. 36000). Under these conditions, with the increasing 1,10-phenanthroline/enzyme concentration ratio, at a constant concentration of the enzyme, the perturbation of the 1,10-phenanthroline spectrum at 295nm is increasing, reaching a maximal value at the 1,10-phenanthroline/subunit concentration ratios above 5. These observations permit the calculation of molar extinction coefficients of the perturbation spectra of 1,10-phenanthroline due to the metal chelation with Zn^{2+} or a zinc dissociated from the enzyme (Fig. 5).

As indicated in Fig. 5, the perturbation of the 1,10-phenanthroline spectrum at 295nm (at saturating concentrations of 1,10-phenanthroline) increases

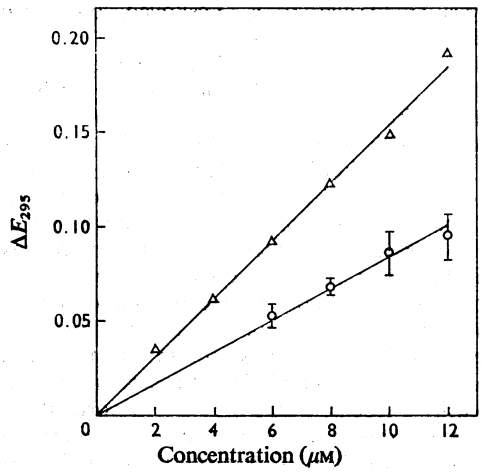

Fig. 5. *Stoicheiometry of zinc dissociation from alcohol dehydrogenase*

$ZnSO_4,7H_2O$ solutions (2–12μM) were mixed with 1,10-phenanthroline (125μM) in 0.1M-Tris/HCl buffer, pH6.5. Alcohol dehydrogenase (6–12μM) was mixed with 1,10-phenanthroline (125μM) in the same buffer. Difference-spectral values at 295nm were measured 30min after the mixing of ligands. Concentrations of Zn^{2+} were determined gravimetrically (△), and concentrations of enzyme subunit (mol.wt. 36000) spectrophotometrically (○) (Hayes & Velick, 1964). Experimental points for the enzyme are an average from four determinations, and the S.E.M. is represented for each concentration of the enzyme.

with increasing concentrations of both Zn^{2+} and the enzyme. From Fig. 5, molar extinction coefficients of the perturbation spectra of 1,10-phenanthroline at 295nm were calculated to be 15400 litre·mol^{-1}·cm^{-1} for Zn^{2+} and 8300 litre·mol^{-1}·cm^{-1} for alcohol dehydrogenase subunit (mol.wt. 36000). The perturbation caused by enzyme is 54% of the perturbation caused by Zn^{2+}, on a molar basis; this indicates that only half (2 atoms/tetramer) of the total zinc content of the enzyme has dissociated from the protein, as a consequence of the 1,10-phenanthroline-induced inactivation.

We were unable to obtain reliable difference-spectra values at low enzyme concentrations, e.g. at high 1,10-phenanthroline/enzyme ratios (Fig. 5). We assume that this finding might be due to the slow but complete dissociation of zinc from the protein at very high 1,10-phenanthroline/enzyme ratios; this may also explain the observation of Kägi & Vallee (1960) that 1,10-phenanthroline, after a long incubation time at 0°C, causes the dissociation of the tetrameric yeast alcohol dehydrogenase into dimeric and monomeric subunits, with a complete loss of tightly bound zinc. Therefore, in all our experiments, the 1,10-phenanthroline/subunit concentration ratio was kept between 5 and 20. In this concentration range, under specified conditions, the maximum in the difference spectrum of 1,10-phenanthroline develops immediately in the presence of Zn^{2+}; the appearance of the maximum is time-dependent (20–50min) in the presence of alcohol dehydrogenase, since some time is required for zinc to dissociate from the enzyme.

Measurement of zinc dissociated from alcohol dehydrogenase by atomic absorption photometry

In the preceding section, a spectrophotometric measurement of the zinc that had dissociated from the enzyme was described. Since the appearance of the 295nm peak in the perturbation spectrum of 1,10-phenanthroline correlates with the inactivation of the enzyme (Fig. 4), it is clear that the full inhibition of enzymic activity correlates with the dissociation of approximately half of the total zinc content of the enzyme. The other half of the zinc content remains bound to the protein, even after the full inhibition is obtained. To substantiate this conclusion, we have measured by atomic absorption spectrophotometry the correlation between the 1,10-

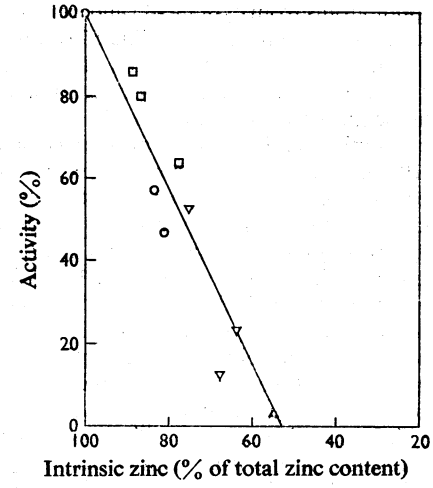

Fig. 6. *Correlation between the tightly bound zinc and the inhibition of alcohol dehydrogenase*

Alcohol dehydrogenase (mol.wt. 36000) (0.11mM) was mixed with 1,10-phenanthroline (3.65mM) in 0.1M-sodium phosphate buffer, pH7.0. Portions (0.5ml) were removed from the reaction mixture at short-time intervals (1–20min from the mixing of ligands), and immediately desalted over a Sephadex G-10 column (15cm×1cm), equilibrated with 0.1M-Tris/HCl buffer, pH7.2, containing 0.5mM-EDTA and 3mM-sucrose. Immediately after gel filtration, zinc content (atomic absorption photometry) and enzymic activity in eluates were determined. Different symbols represent different experiments.

phenanthroline-induced inhibition and the amount of zinc tightly bound to the enzyme (intrinsic zinc) (Fig. 6).

1,10-Phenanthroline-induced inactivation of alcohol dehydrogenase can be arrested at any time by a swift removal of 1,10-phenanthroline by means of gel filtration; in this way, the inactivation of enzyme is stopped, and dissociated (extrinsic) zinc is separated from the protein (Fig. 6). After the separation of the excess of 1,10-phenanthroline and extrinsic zinc by gel filtration, the enzymic activity does not change within 1h in the cold, in 0.1M-Tris/HCl buffer, pH7.2 (containing 0.5mM-EDTA and 3mM-sucrose). As shown in Fig. 6, there is a linear relationship between the tightly bound zinc and the remaining enzymic activity, which indicates that the full inhibition correlates with the removal of approximately half of the total zinc content of the enzyme.

Discussion

The tertiary structure of horse liver alcohol dehydrogenase at 0.24nm resolution has been reported (Eklund *et al.*, 1974). The catalytic zinc atom of the liver enzyme has three protein ligands: two sulphur atoms from cysteine-46 and cysteine-174, and one nitrogen atom from histidine-67 (numbering scheme is that for the liver enzyme). The sequence around cysteine-46 in the liver enzyme is homologous with the sequence around cysteine-43 in the yeast enzyme (numbering for yeast enzyme) (Holbrook *et al.*, 1967). The sequence around cysteine-174 in the liver enzyme is homologous with the Val-Leu-Cys-Ala-Gly sequence in the yeast enzyme (Jörnvall *et al.*, 1975). Crystallographic (Eklund *et al.*, 1974) and chemical evidence (Jörnvall *et al.*, 1975) for the liver enzyme and chemical evidence (Jörnvall *et al.*, 1975) for the yeast enzyme indicate that, in these two homologous proteins, both essential cysteine residues are close together in the tertiary structure, close to the coenzyme-binding regions at the active sites of the respective enzymes. Since two essential cysteine residues in the liver alcohol dehydrogenase chelate the catalytic zinc atom, it would be tempting to ascribe a similar role to the two homologous cysteine residues in the yeast enzyme. In this respect, our attempts to release zinc atoms from yeast alcohol dehydrogenase by the chemical modification of an essential cysteine residue have failed; a 5,5′-dithiobis-(2-nitrobenzoate)-modified alcohol dehydrogenase, with 0.9mol of inhibitor incorporated/36000g of subunit, which was still able to bind acetamide in the ternary complex enzyme–NADH–acetamide, was dialysed in the cold, against 0.1M-Tris/HCl buffer, pH7.2, containing 0.5mM-EDTA; only 10% of the total zinc content of the enzyme was removed by dialysis (S. Trivić & V. Leskovac, unpublished work).

The experiments reported in the present paper establish that 2 molecules of 1,10-phenanthroline interact with a tetrameric enzyme molecule, competitively with respect to NADH. This interaction brings about a full inhibition, which correlates with the dissociation of approximately half of the total zinc content of the enzyme. Thus it appears that 2 zinc atoms/tetramer dissociate much more easily from the apoenzyme than the other two, i.e. 2 zinc atoms/tetramer may be chelated in a different environment from the other two. It is not possible on the experimental evidence presented in this paper to ascribe an essential, catalytic, or a structural role to the easily dissociable zinc atoms. The correlation of the loss of activity with the removal of 2 zinc atoms/tetramer may indicate their essential or catalytic role in the mechanism of enzyme action. On the other hand, under the experimental conditions described by Kägi & Vallee (1960), 1,10-phenanthroline induces, after a long incubation time at 0°C, the dissociation of tetrameric yeast alcohol dehydrogenase into dimeric and monomeric subunits, and the complete removal of tightly bound zinc from the apoenzyme. We cannot entirely exclude the possibility that, under the conditions of our experiments, 1,10-phenanthroline dissociates the enzyme into subunits, together with the removal of less tightly bound zinc; in this context, a structural role might be ascribed to dissociable zinc atoms.

NAD^+-dependent dehydrogenases are usually made up of even numbers of subunits, with a number of active sites or specific binding sites corresponding to the number of subunits (Sund, 1968). Yeast alcohol dehydrogenase differs in this respect from other dehydrogenases. The enzyme binds specifically 2 molecules of NADH/tetramer (Dickinson, 1970) and 2 molecules of 8-anilinonaphthalene-1-sulphonic acid/tetramer (Dickinson, 1971). Reaction of 2 equiv. of iodoacetate with the enzyme molecule produces 90–95% loss of activity (Dickinson, 1974), and 2 zinc atoms/tetramer are easily dissociated from the enzyme through the influence of 1,10-phenanthroline (this study). It appears that, in comparison with other dehydrogenases, the molecule of yeast alcohol dehydrogenase possesses an additional inherent asymmetry, with four monomeric subunits being arranged in two mutually symmetrical pairs. Such arrangements of monomeric subunits would explain the appearance of two specific binding sites/tetramer, and two easily dissociable zinc atoms/tetramer.

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References

Dickinson, F. M. (1970) *Biochem. J.* **120**, 821–830

Dickinson, F. M. (1971) *FEBS Lett.* **15**, 17–20

Dickinson, F. M. (1974) *Eur. J. Biochem.* **41**, 31–36

Drum, D. E. & Vallee, B. L. (1970) *Biochemistry* **9**, 4078–4085

Eklund, H., Nornstöm, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. & Bränden, C.-I. (1974) *FEBS Lett.* **44**, 200–204

Hayes, J. E. & Velick, S. F. (1964) *J. Biol. Chem.* **207**, 225–232

Hoch, F. L. & Vallee, B. L. (1956) *J. Biol. Chem.* **121**, 491–500

Hoch, F. L., Williams, J. P. & Vallee, B. L. (1958) *J. Biol. Chem.* **232**, 453–464

Holbrook, J. J., Pfleiderer, G., Mella, K., Volz, M., Leskovac, V. & Jeckel, R. (1967) *Eur. J. Biochem.* **1**, 476–483

Jörnvall, H., Woenckhaus, C. & Jonscher, G. (1975) *Eur. J. Biochem.* **53**, 71–81

Kägi, J. H. R. & Vallee, B. L. (1960) *J. Biol. Chem.* **235**, 3188–3192

Leskovac, V. (1975) *Biochim. Biophys. Acta* **393**, 563–570

Leskovac, V. & Pavkov-Peričin, D. (1975) *Biochem. J.* **145**, 581–590

Sund, H. (1968) in *Biological Oxidations* (Singer, T. P., ed.), pp. 641–705, Interscience, New York

Williams, R. J. P., Vallee, B. L. & Hoch, F. L. (1958) *J. Biol. Chem.* **232**, 465–474