Biochem. J. (1992) 287, 361–365 (Printed in Great Britain)

Importance of the structural zinc atom for the stability of yeast alcohol dehydrogenase

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Yeast alcohol dehydrogenase is a tetrameric enzyme containing zinc. Initially we confirmed the presence of two zinc atoms per subunit. Incubation of the enzyme with increasing concentrations of dithiothreitol, a method for partial chelation, allowed first the reduction of four disulphide bridges per enzyme, but eventually was sufficient to chelate the structural zinc atom without having any effect on the zinc located in the active site. The enzyme activity was not affected but the enzyme became very sensitive to heat denaturation. Chelation by EDTA was also performed. Given its location at an external position in the globular protein, protected in each subunit by one disulphide bridge, the results establish that the second zinc atom present on each enzymic subunit plays a prominent conformational role, probably by stabilizing the tertiary structure of yeast alcohol dehydrogenase. Recovery experiments were performed by incubation of the native enzyme, or the dithiothreitol-treated enzyme, with a small amount of Zn^{2+}. A stabilization effect was found when the structural zinc was re-incorporated after its removal by dithiothreitol. In all cases a large increase in activity was also observed, which was much greater than that expected based on the amount of re-incorporated zinc atom, suggesting the re-activation of some inactive commercial enzyme which had lost some of its original catalytic zinc atoms.

INTRODUCTION

Zinc is one of the peculiar metals having multiple co-ordination possibilities and various geometries, making it easily adaptable for various ligands (Vallee *et al.*, 1960; Cotton & Wilkinson, 1988; Vallee & Auld, 1989, 1990; Dent *et al.*, 1990). Zinc is found in many enzymes, where these properties of interaction with various donor groups lead to modifications in its affinity constant for the enzyme, and in its reactivity with substrate molecules.

Typical examples of such zinc enzymes are yeast alcohol dehydrogenase (YADH) and horse liver alcohol dehydrogenase (HLADH) (alcohol:NAD^+ oxidoreductase; EC 1.1.1.1). YADH is a tetrameric enzyme of molecular mass 150 kDa (Veillon & Sytkowski, 1975; Klinman & Welsh, 1976). The catalytic site contains one zinc atom which is absolutely necessary for enzyme activity (Coleman & Weiner, 1973). The catalytic reaction mechanism of the enzyme is known. However, since X-ray diffraction analyses of its three-dimensional structure have not yet been published, the rest of its structure and its quaternary structure are not known. Some theoretical attempts have been made to model the assembly of the subunits into tetramers (Harris, 1964; Jörnvall *et al.*, 1978), but a convincing solution has not yet been proposed.

The problem of the zinc content of YADH and its exact contribution to the enzyme's properties has been the subject of much controversy in the literature. The amount of zinc assayed in the enzyme suggested initially that only one atom was present in each subunit (Harris, 1964; Coleman & Weiner, 1973; Veillon & Sytkowski, 1975; Klinman & Welsh, 1976; Jörnvall *et al.*, 1978; Jörnvall, 1977*a*,*b*). However, other work has found that there are two zinc atoms per subunit (Klinman & Welsh, 1976).

There are also strong indications that the subunits of YADH have similar structures to HLADH, which is a dimer known to contain two zinc atoms per subunit (Coleman & Weiner, 1973; Bränden *et al.*, 1975; Jörnvall, 1977*a*,*b*; Jörnvall *et al.*, 1978; Vallee & Auld, 1989, 1990). In this enzyme, one zinc atom is found in the catalytic site of the enzyme and is bound to three ligands (Cys-46, His-67 and Cys-174). The sequence similarity around Cys-174 is notable for all members of the alcohol dehydrogenase (ADH) family. The position of the second zinc atom is also known for HLADH, where it is bound to four cysteine residues (97, 100, 103 and 111). These four cysteine residues are also present in YADH and in nearly all ADHs, so it is supposed that this zinc atom is also bound to these four cysteines (Harris, 1964; Vallee & Auld, 1990). In contrast, the other residues in the lobe, from 95 to 113, are not conserved in the different ADH enzymes.

If the presence of a second zinc atom is established, little is known about its role in YADH. By analogy with HLADH, a possible structural role has been suggested by Klinman & Welsh (1976) and Vallee & Auld (1990), but without any experimental evidence. We do not know the importance of this second zinc atom for the maintenance of the YADH quaternary or tertiary structure and concomitantly its influence on enzyme stability, especially at high temperatures, or its possible localization in the unknown three-dimensional structure of YADH. These questions are the purpose of this work, using a partial chelation of the eight zinc atoms of YADH.

EXPERIMENTAL

Materials

YADH (specific activity 360 units/mg of protein), NAD^+ and dithiothreitol (DTT) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Sephadex G-25, a dextran gel, was from LKB-Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were of the purest available grade and were obtained either from Janssen Chimica (Beerse, Belgium) or from Merck (Darmstadt, Germany). They were used without further purification.

Assay of YADH activity

The enzyme activity was assayed in a spectrophotometer

Abbreviations used: YADH, yeast alcohol dehydrogenase; HLADH, horse liver alcohol dehydrogenase; DTT, dithiothreitol.

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Table 1. Properties of the YADH preparations treated for 1 h at 4 °C with various amounts of DTT

The residual enzyme activity is expressed compared with the activity before treatment. The heat-labile fraction is calculated from the inactivation curves performed at 50 °C (Fig. 1) by extrapolation of the second slow rate of inactivation to zero time. The number of thiol groups are expressed as the molar ratio compared with the molar enzyme concentration, and the residual zinc as percentages compared with the enzyme preparation.

YADH properties [DTT] (mM)...	0	0.02	0.1	0.2	0.4	1	2	10	15	20	100
Residual activity (%)	99	100	101	97	99	99	102	100	96	98	95
Heat-labile enzyme (%)	22	0	0	3	6	9	16	35	40	58	88
Number of thiol groups	28	36	35	36	35	35	36	35	33	32	33
Residual zinc content (%)	100	98	96	94	96	97	81	79	73	67	52

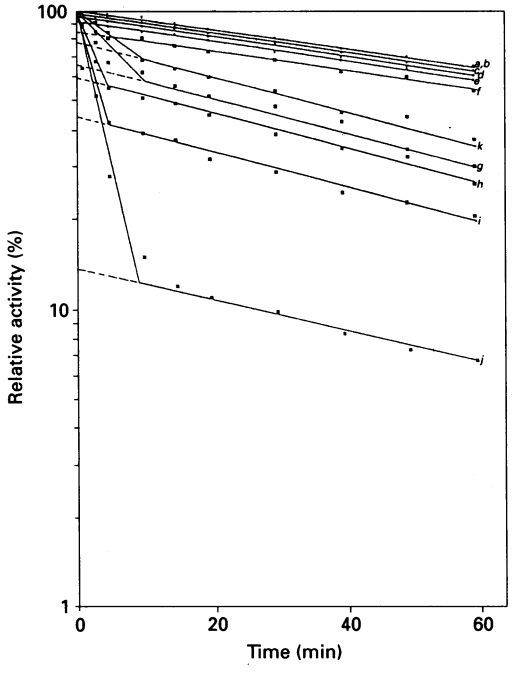

Fig. 1. Inactivation curves performed at 50 °C on various YADH preparations pre-incubated for 1 h at 4 °C with various amounts of DTT and purified by gel filtration

DTT concentrations were: 0.02 mM (*a*), 0.1 mM (*b*), 0.2 mM (*c*), 0.4 mM (*d*), 1 mM (*e*), 2 mM (*f*), 10 mM (*g*), 15 mM (*h*), 20 mM (*i*), and 100 mM (*j*). *k* is a control.

(Kontron Uvikon 930; Kontron, Milano, Italy) at 25 °C, using the method described by Bille & Remacle (1986).

The inactivation curves at 35 °C or 50 °C were obtained as explained by Bille *et al.* (1987).

DTT and EDTA treatments

The enzyme solution was incubated in a 0.1 M-Tris/HCl/0.1 M-NaCl buffer, pH 6.5, for 1 h at 4 °C with various amounts of DTT. DTT was removed by gel filtration on a Sephadex G-25 column (17 cm × 0.8 cm), and the enzyme was tested for its stability at 50 °C, for the amount of thiol groups and for its zinc content.

Inactivation by EDTA was performed in the same conditions, except a 2 h incubation at 30 °C (see Results section).

Other assays

The determination of the number of thiol groups on the enzyme was performed using the Ellman reagent (Ellman, 1959).

Protein concentration was measured by the method of Lowry *et al.* (1951). The fluorescamine method (Böhlen *et al.*, 1973) was used (as an independent check of the protein concentration) on the initial YADH preparations used for the determination of their zinc content. The precision of the Lowry method was within 2% below 0.5 mg/ml, whereas the presence of Tris buffer allows such precision to be obtained with the fluorescamine method only between 0.3 and 2 mg/ml

Quantitative determinations of the zinc content of YADH were performed by atomic absorption spectrophotometry on a Philips PU9200X absorption spectrophotometer ($\lambda = 214$ nm). Aliquots were aspirated directly into an air–acetylene flame, and three separate determinations were made on each sample. The buffer was also used as a control and its value was subtracted from the sample values. Standard curves were prepared both before and after each set of analyses.

Re-insertion of the zinc atoms in YADH

Zinc re-incorporation was tested on YADH, either treated or not treated with chelators. Preparations were incubated for the indicated time and temperature in the presence of 10 μM-$ZnCl_2$. Excess zinc was removed by gel filtration on a Sephadex G-25 column (17 cm × 0.8 cm), and the enzyme was tested for its activity, its stability at 50 °C, or its zinc content.

RESULTS

Effect of DTT on YADH

Data on the enzyme primary sequence showed that YADH contains nine cysteine residues (Harris, 1964). Two of them are linked to the catalytic zinc and four others to the second zinc atom. DTT is a powerful reducing agent with a standard redox potential of −0.33 V at pH 7.0 and 25 °C (Cleland, 1964), and will be able to reduce the remaining disulphide links if any are present. However, DTT is also known as a bidentate chelator with a high affinity for metal ions and for zinc cations in particular (Gracy & Noltmann, 1968; Cornell & Crivaro, 1972).

Activity of YADH after DTT treatment. YADH was pre-incubated for 1 h at 4 °C with various amounts of DTT and assayed for its residual enzymic activity. The results in Table 1 show that no loss of activity was observed over the entire range of concentrations. The zinc atom present in the catalytic site, which is essential for enzyme activity, is not affected by the presence of DTT over this range of concentrations.

Thermal inactivation studies. Using the same experimental protocol, YADH was pre-incubated with various amounts of DTT, purified and assayed for its stability at 50 °C. A dose-dependent increase in the thermal inactivation was observed as DTT concentrations increased (Fig. 1).

The inactivation curves clearly appeared to be the result of two denaturation curves: one which was very rapid and a second, slower, one. It can also be observed from Fig. 1 that the slopes of the second part of the curves in this semi-logarithmic presentation of the results are very similar for all of the curves, and identical to that of the native enzyme. Such behaviour is typical

Table 2. Properties of the YADH preparations treated with various concentrations of EDTA for 5 h at 4 °C

The residual enzyme activity is expressed as a percentage of the activity before the treatment. The heat-labile fraction is calculated from the inactivation curves performed at 50 °C (results not shown) by extrapolation of the second slow rate of inactivation to zero time. The residual zinc content is expressed as a percentage compared with the zinc content of the enzyme preparation.

YADH properties	[EDTA] (mM)...	0	10	100	250
Residual activity (%)		100	91	86	84
Heat-labile enzyme (%)		22	4	5	2
Residual zinc content (%)		99	93	69	72

Table 3. Properties of YADH preparations treated with various concentrations of EDTA for 2 h at 30 °C

The residual activity is expressed as a percentage of the activity before treatment. The heat-labile fraction is calculated from the inactivation curves performed at 50 °C (results not shown) by extrapolation of the second slow rate of inactivation to zero time. The residual zinc content is expressed as a percentage compared with the zinc content of the enzyme preparation.

YADH properties	[EDTA] (mM)...	0	0.1	1	10	100
Residual activity (%)		92	68	56	44	16
Heat-labile enzyme (%)		22	3	10	12	20
Residual zinc content (%)		96	87	79	57	32

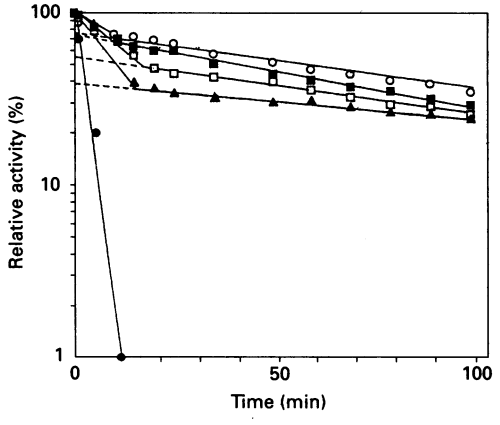

Fig. 2. Inactivation curves performed at 50°C on YADH preparations pre-incubated with various amounts of $ZnCl_2$ for 1 h at 25°C and then isolated by gel filtration

$ZnCl_2$ concentrations were: 1 μM (■), 10 μM (□), 100 μM (▲) and 1 mM (●). ○ indicates control.

of the presence of two populations of enzymes, one which is rather resistant to the temperature (which is seen in the second part of the denaturation curves), and one which is very quickly inactivated by heat (Sadana & Henley, 1986). This was confirmed by statistical analysis using a non-linear-regression computer program, which showed that the curves fitted well to the sum of two exponentials. The proportion of the heat-labile enzyme is easily found by extrapolation of the second part of the slope to zero time. These results are given in Table 1. Clearly, high concentrations of DTT lead to an increase in the proportion of heat-labile enzyme, with some also found in the native enzyme. However, the heat-labile fraction completely disappeared at low DTT concentrations.

Thiol group content. Determination of the content of thiol groups has been performed for the various preparations and the values are also given in Table 1. The number of free thiol groups has been found to be 28 in the native enzyme, which corresponds to seven free cysteines per subunit. However, a small amount of DTT leads to the appearance of eight new cysteine groups per YADH; we can conclude that in the native enzyme two cysteine residues are linked in a cystine bridge in each subunit. This is in agreement with reports already published (Sund & Theorell, 1963; Harris, 1964). A value of 36 thiol groups is obtained after treatment with DTT over a large range of concentrations, with a slight decrease found in a very high excess of DTT. This decrease could be caused by the attachment of some DTT molecules to some of the thiol groups of the enzyme at these unusually high concentrations, or to other non-specific effects such as a lack of accessibility of some thiol groups caused by conformational changes.

Zinc content. The absolute zinc content of the commercial YADH preparation was assayed by atomic absorption spectrophotometry. The assay was performed 23 times and we found a value of 7.59 ± 0.10 zinc atoms per YADH, suggesting the presence of two zinc atoms per subunit but also revealing a deficiency in the zinc content of the commercial YADH preparation. For comparison, HLADH, a dimeric enzyme, was also tested in the same way, and we found 4.07 ± 0.15 ($n = 6$) zinc atoms per enzyme.

The zinc content was also analysed in the various treated preparations and the results are given in Table 1. The zinc content of YADH incubated with low concentrations of DTT was similar to that of the native enzyme, but the content slowly decreased with increasing DTT concentrations to reach 52% at the highest excess of DTT.

Effect of EDTA on YADH

To discriminate between the chelating or reducing effects of DTT, we decided to test the effect of a classic chelating agent, EDTA. The enzyme was first treated with EDTA at 4 °C as for DTT but neither the activity nor the zinc content was affected (results not shown). Only the heat-labile fraction of the native enzyme was removed. The concentration of EDTA and the time of incubation were increased, and some modifications were then obtained as exemplified in Table 2. A loss of enzyme activity was obtained concomitantly with some removal of zinc. The heat-labile fraction of the native enzyme also disappeared. We then decided to perform the same experiment at 30 °C. The effects of YADH treatment by EDTA at this temperature are summarized in Table 3. A very strong inhibitory effect by EDTA on enzyme activity was obtained; this was correlated with a removal of zinc from the enzyme. The heat-labile fraction which was removed at the lowest concentration (0.1 mM-EDTA) gradually increased with the EDTA treatment, but remained much lower than with DTT treatment.

Effect of metal re-insertion on properties of YADH

First we studied the effect of zinc addition in the native enzyme preparation. The YADH preparation was incubated for 1 h at 25 °C with various concentrations of $ZnCl_2$ and the residual activity was then tested. Interestingly, an increase in YADH activity was obtained at low zinc concentrations, reaching 142% in the presence of 10 μM-$ZnCl_2$ (results not shown). The incubation with 10 μM-$MgCl_2$ did not lead to such re-activation of

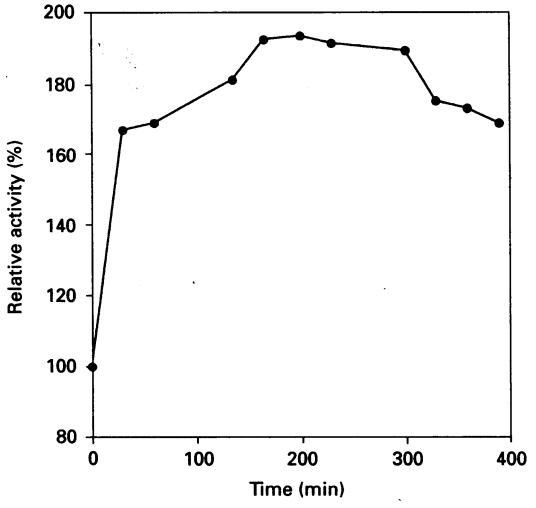

Fig. 3. Evolution of the activity of a YADH preparation first treated with 100 mM-DTT for 1 h at 4 °C and then incubated with 10 μM-$ZnCl_2$ for the indicated period of time

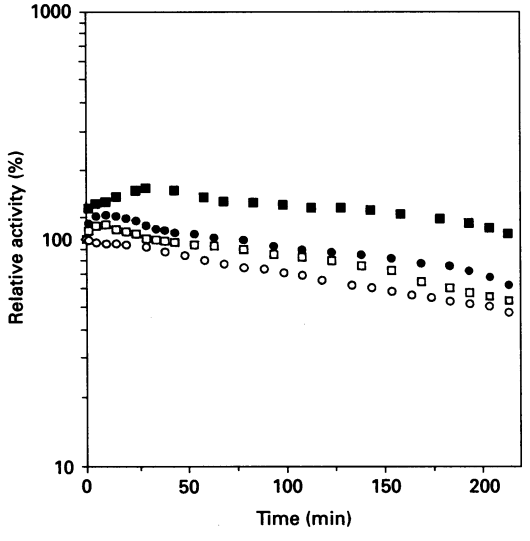

Fig. 4. Inactivation curves performed at 50 °C on YADH preparations first treated with 100 mM-DTT for 1 h at 4 °C and then incubated in the presence of 10 μM-$ZnCl_2$ at 35 °C for various periods

Periods of incubation at 35 °C were: 60 min (■), 90 min (●), 120 min (□) and 180 min (○). The excess of $ZnCl_2$ was removed by gel filtration before determination of the inactivation curve.

the enzyme (results not shown). Since YADH activity is directly dependent on the presence of a catalytic zinc, this result confirms the deficiency of the zinc content in the commercial preparation, especially of the catalytic zinc atoms. We assayed the zinc content of this commercial preparation and found an increase from 7.59 ± 0.10 ($n = 23$) zinc atoms per enzyme to 8.10 ± 0.08 ($n = 3$) after the incubation with $ZnCl_2$.

The effect of such zinc re-incorporation into the native enzyme was also tested on the heat stability of YADH (Fig. 2). There was no stabilization effect in the zinc-treated preparations, confirming the zinc depletion, in the catalytic sites only, of commercial YADH, and showing no effect on the structural zinc. Moreover, higher concentrations were found to be very destabilizing for the enzyme, with a total inactivation of all enzyme at 1 mM-$ZnCl_2$.

The effect of zinc re-insertion on YADH initially treated with 100 mM-DTT for 1 h at 4 °C was then tested by incubating the preparation with 10 μM-$ZnCl_2$, which was the optimal concentration found for the zinc re-insertion in the native enzyme. As with the native enzyme, we initially observed a sharp increase in activity which was temperature-dependent. Fig. 3 shows the time course of re-activation obtained at 35 °C in the presence of $ZnCl_2$. Maximum activity was obtained after 3 h. We chose this temperature for practical reasons, since at lower temperatures the recovery was very slow (probably because of the more rigid conformation of the protein which does not allow the zinc to migrate inside the enzyme pocket). This increase in activity caused by zinc treatment at high temperatures suggests that some zinc atoms are incorporated in the catalytic site.

The thermolability at 50 °C of some of these preparations is shown in Fig. 4. We observed an increased activity in the first few minutes of incubation in the preparations which were pre-incubated at 35 °C with 10 μM-$ZnCl_2$ for short periods of time before the assay at 50 °C, as is the case for the native enzyme. It is interesting to note that, in this situation, the $ZnCl_2$ has been removed by gel filtration after the indicated period of time before the thermostability assay. Thereafter, and in all the preparations, a very slow and constant rate of inactivation was observed at 50 °C. Zinc assays showed 7.59 ± 0.10 ($n = 23$) atoms zinc per YADH in the native enzyme, 3.80 ± 0.16 ($n = 4$) atoms after DTT treatment and 8.38 ± 0.26 ($n = 4$) atoms after the 3 h-long $ZnCl_2$ pre-incubation.

DISCUSSION

The presence of two zinc atoms per YADH subunit has been the subject of many publications, and was confirmed in this study. In fact, commercially available YADH preparations are usually dialysed during their purification in an EDTA solution, and this treatment may explain the lack of some of its catalytic zinc content that we also observed here.

The assays of the residual activity, of the zinc content, of the thiol content and of the percentages of heat-labile enzyme in the preparations treated with DTT clearly suggested that, at low concentrations, DTT reduces one disulphide bond per subunit without affecting enzyme stability or activity. At high DTT concentrations one of the two zinc atoms is selectively removed, leading to the appearance of a highly heat-sensitive enzyme, but with no loss of catalytic activity which requires the catalytic zinc atoms. The experiment is best interpreted by considering that the second zinc atom of each subunit was selectively removed by DTT and that this pseudo-apoenzyme is quickly inactivated at high temperatures, confirming the suggested conformational role of this second zinc atom. Indeed, the enzyme denaturation at 50 °C mainly involves an initial fast step due to the peptide chain unfolding, which can be followed by subsequent slow covalent modifications (Klibanov, 1983). We clearly observed that the appearance of the first step of inactivation was indicated by the fact that enzymes lost their conformational zinc without any effect on enzymic activity.

The results obtained with EDTA were concordant but more difficult to interpret, since EDTA removes both zinc atoms. The zinc removal is greater than 50% and a large loss of activity is observed. The behaviours of the two zinc atoms in the presence of these two chelating agents could therefore be different. The specific action of DTT on the conformational zinc atom at low temperature could be explained by the progressive substitution of the four cysteine thiol groups which chelate the zinc in the enzyme by the DTT thiol groups. Such substitution cannot occur with EDTA, which needs the simultaneous chelation of the four ligands to remove the zinc. However, the temperature-dependence of the EDTA effect compared with that of DTT and the very high concentrations required to remove the zinc also strongly suggest that the accessibility of EDTA to the zinc atom in the

enzyme is nil or very low at low temperatures; to remove the zinc some unfolding of the enyzyme, which is time- and strongly temperature-dependent, is then necessary. Such an explanation was confirmed by the observation that when the four disulphide bridges were first reduced by a very low concentration of DTT (0.02 mM), the removal of the conformational zinc atom by a further EDTA treatment was easier (results not shown). Such results show that the accessibility of EDTA to the conformational zinc atom can be partially lifted by the reduction of the four disulphide bridges. The reason for such increased accessibility is unknown, but it is possible to postulate an easier unfolding of the zinc-containing loop owing to the removal of such disulphide bridges.

The DTT effect on YADH is peculiar in that only the structural zinc has been removed, and that it can be re-inserted by incubation with $ZnCl_2$. These recovery experiments performed by incubation with Zn^{2+} showed that the removal of zinc was reversible, since all the enzyme population could recover their stability at high temperatures. This stabilization process of the protein structure by linkage of zinc to four cysteine residues is thus a property typical of the zinc, besides its well-known participation in the active site in enzyme catalysis. If replaced by manganese for example, the activity is conserved but this stabilization is lost (Coleman & Weiner, 1973). As described by Vallee & Auld (1989, 1990) and Block *et al.* (1990), cysteine residues around a zinc atom form tetradentate complexes with very high stability constants; this will ensure maintenance of both overall structure and local conformations akin to those provided by disulphide bridges. Such an organization of ligands would probably confer rigidity to that region of the molecule and, hence, lead to stabilization of the protein structure (Vallee & Auld, 1989, 1990). This kind of sulphur–zinc sphere is typical in several enzymes for maintaining enzymic stabilization (Dent *et al.*, 1990).

These re-insertion experiments also showed a greater re-activation of the DTT-treated enzyme than of native YADH, which could be explained by a higher zinc content, increasing from 7.59 to 8.1 atoms. The 200% re-activation obtained after DTT treatment could be explained if we postulate that a zinc deficiency in only one subunit could lead to a loss of tetrameric enzyme activity. In this case, an increase of 0.5 zinc atoms per enzyme could lead to the re-activation of the 50% inactive enzyme, explaining the 200% re-activation. However, at the present time there is no evidence for such subunit interactions.

Finally, another interesting observation was that an increase in enzyme activity was still obtained at high temperatures even after removal of the Zn^{2+} from the solution (Fig. 4). This suggests a two-phase process, first the binding of Zn^{2+} in the catalytic site and secondly a slow conformational change which occurs only at high temperatures (35°C or 50°C) and which leads to the formation of an active configuration. The re-activation would then depend on a conformational change of the tetrameric enzyme.

The disappearance of the heat-labile fraction observed in YADH after treatment with low concentrations of DTT or EDTA is difficult to explain in terms of their possible action on the zinc atoms since they were not removed at these low concentrations and the activity did not change. The removal of some contaminating metal from the free thiol groups, for example, is the most probable explanation for such an effect. Indeed, a rapid metal analysis showed that commercial YADH contains traces ($\leqslant 0.01\%$ wt. of enzyme protein) of copper (confirmed by Sigma Chemical Company), cobalt, cadmium and magnesium. Most of these atoms have a high affinity for the thiol groups (Gracy & Noltmann, 1968; Dawson *et al.*, 1986) and their binding to the cysteines could influence the stability of YADH.

All these results clearly establish that YADH contains one catalytic zinc atom and a second zinc atom which plays a prominent conformational role, probably by stabilization of the tertiary structure of YADH. Moreover, the results obtained with the combination of DTT and EDTA suggest an external localization of this structural zinc atom which is protected from chelation by disulphide bridges which probably strengthen the conformation. The external localization of this structural zinc affects local conformations of the enzyme, but could also easily affect such an exposed zinc atom, for example by its chelation. The protein seems to have decreased this risk by disulphide protection.

The crystal structure of YADH will be very helpful in confirming the position of the disulphide bridge and the localization of the structural zinc.

E. M. is supported by a grant from the S.P.P.S. (Services de la Programmation de la Politique Scientifique, Brussels), and D. D. is a fellow of I.R.S.I.A. (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture).

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Received 28 October 1991/16 March 1992; accepted 26 March 1992