Bivalent cations stabilize yeast alcohol dehydrogenase I

Xavier DE BOLLE*, Carla VINALS*, Jacques FASTREZ† and Ernest FEYTMANS *‡

*Laboratoire de Biologie Moléculaire Structurale, Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Rue de Bruxelles 61, B-5000 Namur, Belgium, and †Laboratoire de Biochimie Physique et des Biopolymères, Unité de Biochimie, Université Catholique de Louvain, Place Louis Pasteur 1/1B, B-1348 Louvain-La-Neuve, Belgium

The thermostability of yeast alcohol dehydrogenase (ADH) I is strongly dependent on the presence of NaCl, a salt that is almost neutral on the Hofmeister scale, which suggests that solvent-accessible electrostatic repulsion might play a role in the inactivation of the enzyme. Moreover, $CaCl_2$ and $MgCl_2$ are able to stabilize the enzyme at millimolar concentrations. Ca^{2+} stabilizes yeast ADH I by preventing the dissociation of the reduced form of the enzyme and by preventing the unfolding of the oxidized form of the enzyme. An analysis of several chimaeric ADHs suggests that Ca^{2+} is fixed by the Asp-236 and Glu-101 side chains in yeast ADH I, but that Ca^{2+} can be displaced by replacing Met-168 by an Arg residue, as suggested by a three-dimensional model of the enzyme structure. These results indicate that electrostatic repulsion can cause protein unfolding and/or dissociation. It is proposed that yeast ADH I binds Mg^{2+} *in vivo*.

INTRODUCTION

Several proteins have the ability to fix one or more cations, and structurally well-defined binding sites have been found to bind cations [1,2] (zinc fingers, for example [3,4]). These cations can have a strong effect on protein stability [5,6]. Yeast alcohol dehydrogenase (ADH) I from *Saccharomyces cerevisiae* (EC 1.1.1.1) is an interesting model for the study of cation–protein interaction because this tetrameric enzyme is able to fix two zinc ions per subunit [7–9], one in the active site and the other in a loop. Yeast ADH I exists in two different states of distinct stability: reduced (for example in the presence of 10 mM dithiothreitol) and oxidized [10,11]. Reduced ADH I, the form that is probably present *in vivo*, is rapidly inactivated by moderate heating, whereas oxidized ADH I is more stable. It has been proposed that reduced ADH I is inactivated by dissociation, whereas oxidized ADH I is inactivated by unfolding [10,11]. The analysis of native and chimaeric yeast ADHs suggested that unstable yeast ADHs contain an inter-subunit electrostatic repulsion between Glu-101 and Asp-236 [10]. Such an electrostatic interaction is potentially sensitive to the presence of cations, but it is often proposed that the electrostatic contribution to protein stability is negligible when potential ionic pairs are located near the solvent. Here we describe the effect of Na^+, Ca^{2+} and Mg^{2+} cations on the stability of yeast ADH I in its reduced and oxidized states. The effect of bivalent cations suggests that they participate in an inter-subunit binding, which strongly stabilizes reduced ADH I. It is thus suggested that electrostatic repulsion (1) can have a strong effect on thermostability, (2) can be predicted by three-dimensional models of proteins and (3) can be compensated for by Mg^{2+} *in vivo*.

MATERIALS AND METHODS

Unless otherwise indicated, all reagents were obtained from Merck and Sigma. Water used in this study was purified with the Milli-Q system (Millipore). The data on the reduced form of ADH I were obtained by performing the experiments in the presence of 10 mM dithiothreitol during the various incubations described below.

Preparation and analysis of enzymes

Enzymes used in this study were prepared from recombinant yeast overexpressing native and chimaeric ADH genes and were purified by fractionated precipitations and affinity chromatography as previously described [10]. The last chromatographic step [gel permeation on Superose 12HR (Pharmacia)] was performed in the presence of 50 mM Mops buffer, pH 7.0. Measurements of enzymic activity were performed at 25 °C, under the conditions described by Ganzhorn et al. [12].

Measurements of thermostability

The evaluation of the effect of salts on thermostability was performed by diluting purified enzyme preparations 10-fold in 50 mM Mops, pH 7.0, equilibrated at the appropriate temperature, and containing salts to final concentrations required by the analysis. The final enzyme concentration was 10 μg/ml. After 20 min of incubation the residual enzymic activity was measured in triplicate. The reference for residual activity (100 %) was obtained with enzyme kept on ice. The values for T_{50} (the temperature at which 50 % of the enzymic activity is lost) mentioned here were obtained under the same conditions but after incubations at various temperatures.

The kinetics of thermoinactivation was determined by diluting purified enzyme preparations 20-fold in 50 mM Mops, pH 7.0, equilibrated at the appropriate temperature. The final enzyme concentration was 10 μg/ml. When indicated, a final concentration of 5 mM $CaCl_2$ was included. The two determinations of kinetics of thermoinactivation (with and without $CaCl_2$) were performed simultaneously. Enzymic activity was measured every 3 min for each condition. Residual activity (expressed as $\log_{10}$ of the percentage of initial activity measured on ice) was plotted against time, and a linear fit was satisfactory in each case:

$$\log_{10}(\text{percentage of residual activity}) = \text{constant} - k_{\text{inact}} \times \text{time}$$

with time being expressed in min.

Abbreviations used: ADH, alcohol dehydrogenase; CADH, chimaeric ADH; T_{50}, temperature at which 50 % of the enzymic activity is lost.

‡ To whom correspondence should be addressed.

Measurement of dissociation propensity

Dissociation propensity was measured by analysis of the elution profiles of ADH after gel permeation. Freshly prepared tetrameric ADH (0.2 ml, 0.1 mg/ml) was injected (flow of 0.5 ml/min) on a Superose 12HR column, in a 50 mM Mops buffer, pH 7.0, containing 5 mM $CaCl_2$ and/or 2 M urea if indicated. Fresh urea was used instead of guanidinium chloride to avoid interference with the effect of salts examined in this study. All experiments with urea were performed with fresh urea stock solutions but urea was neither recrystallized nor treated with ion-exchange resins. The elution profile was recorded by measuring the absorbance at 280 nm. The final ADH concentration after chromatography was approx. 10 μg/ml.

Inactivation and unfolding by urea

Purified enzyme preparations (10 μg/ml final concentration) were incubated in the presence of several concentrations of urea, in a 50 mM Mops buffer, pH 7.0, at 25 °C. Fluorescence was used to quantify the unfolding. After a 1 min equilibration time, the sample was excited at 295 nm and the emission intensities measured at 332 and 355 nm (Luminescence Spectrometer LS-5B, Perkin-Elmer). The ratio between these two intensities (I_{332}/I_{355}) is an indicator of protein unfolding. The inactivation by increasing concentrations of urea was also measured after a 1 min equilibration time, in a 50 mM Mops buffer, pH 7.0, at 25 °C.

Three-dimensional model analysis

A three-dimensional model of yeast ADH I structure had been obtained in a previous study [10], using the known structure of horse liver ADH as template for homology modelling. The InsightII program (Biosym, San Diego, CA, U.S.A.) was used for the analysis of the modelled structure, on Indigo² workstations (Silicon Graphics). The Molscript program [13] was used for schematic representations.

RESULTS AND DISCUSSION

High concentrations of NaCl are able to stabilize reduced and oxidized ADH I (Figure 1A). High concentrations of NaCl have a larger effect on the reduced form of ADH I than on the oxidized form. NaCl is a salt that is almost neutral on the Hofmeister scale [14], suggesting that the large stabilizing effect observed on reduced ADH I is not due to a 'Hofmeister effect' but rather to the quenching of electrostatic repulsion. These results thus indicate that electrostatic repulsion can limit the thermostability of yeast ADH I, especially when it is reduced.

Experimental data and analysis of a predicted three-dimensional model of the yeast ADH I structure suggested that an electrostatic repulsion between Glu-101 of one subunit and Asp-236 of an another subunit is potentially responsible for the low thermostability ($T_{50} = 38$ °C) and the high dissociation propensity of reduced ADH I. It was thus tempting to predict that a bivalent cation such as Ca^{2+} or Mg^{2+} could stabilize reduced ADH I by removing an electrostatic repulsion:

$Glu^{101}\text{-}CO_2^- \cdots Ca^{2+} \cdots {}^-O_2C\text{-}Asp^{236}$

The effect of $CaCl_2$ and $MgCl_2$ (Figure 1B) was therefore examined. The bivalent cations have an effect at approx. 1/50 the concentration of NaCl, which is compatible with the prevention of electrostatic repulsion between two carboxy groups, as suggested by the analysis of the three-dimensional model of yeast ADH I. As observed with NaCl, the amplitude of the stabilizing effect is larger for the reduced form than for the oxidized form of ADH I, which suggests that stabilizing mechanisms for the two forms of the enzyme are not the same. The kinetics of thermoinactivation (Table 1) confirms that the stabilizing effect of Ca^{2+} is stronger for the reduced form (a 5-fold change in k_{inact}, at 45 °C) than for the oxidized form of ADH I (only a 2-fold change in k_{inact}, at 65 °C).

A 'Hofmeister effect' cannot explain stabilization by $CaCl_2$ and $MgCl_2$ because (1) these salts have a destabilizing effect according to the Hofmeister series and (2) the maximum effect is observed at very low concentrations compared with those expected for a classical Hofmeister effect. Because the reduced and oxidized forms of ADH I are potentially inactivated by

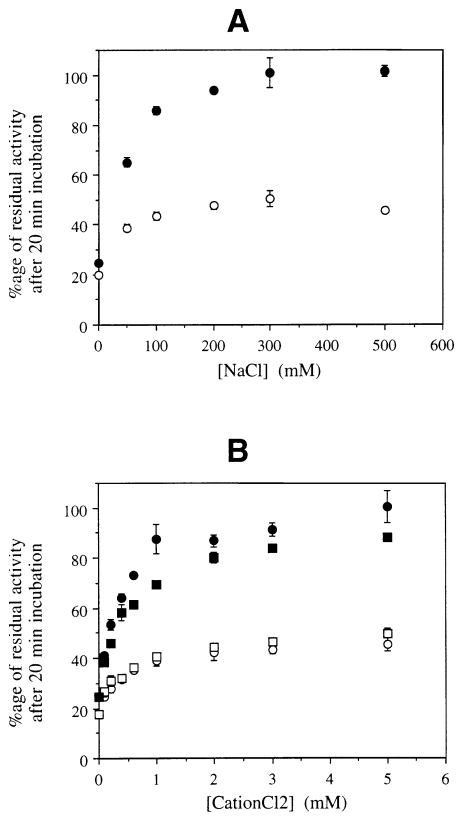

Figure 1 Effect of NaCl, $MgCl_2$ and $CaCl_2$ on the thermostability of reduced and oxidized ADH I

Reduced and oxidized forms of ADH I were incubated at 45 and 65 °C respectively. (**A**) Effect of the concentration of NaCl on the residual activity after heating, for reduced (●) and oxidized (○) ADH I. (**B**) Effect of the concentration of $CaCl_2$ and $MgCl_2$ on the residual activity after heating: reduced ADH I in the presence of $CaCl_2$ (●) and $MgCl_2$ (■), and oxidized ADH I in the presence of $CaCl_2$ (○) and $MgCl_2$ (□). The error bars indicate S.D. ($n = 3$).

Table 1 Parameters of the kinetics of thermoinactivation of the oxidized and the reduced forms of yeast ADH I

Form of ADH I	$[CaCl_2]$ (mM)	$10^3 k_{inact}$ (min^{-1})	Temperature (°C)
Oxidized	0	36.2	65
Oxidized	5	17.1	65
Reduced	0	47.1	45
Reduced	5	9.5	45

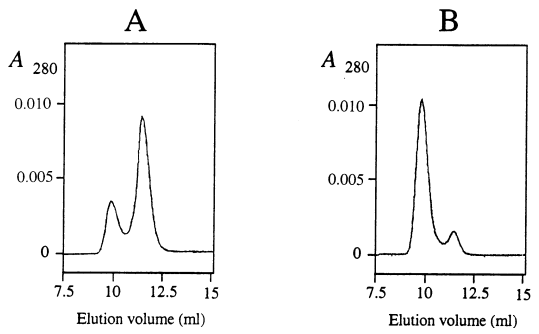

Figure 2 Effect of Ca^{2+} (5 mM $CaCl_2$) on the dissociation propensity of reduced ADH I

The proportion of tetramers (elution volume approx. 10 ml, subunits being eluted at approx. 11.5 ml) is lower in the absence (**A**) than in the presence (**B**) of Ca^{2+}.

Table 2 Substitutions between native and chimaeric ADHs

Positions correspond to the numbering of the yeast ADH I sequence without the initial methionine residue. Underlined residues are typical of the ADH II sequence.

Position	Enzyme... ADH I	CADH1	CADH2	CADH4	CADH5
168	Met	Met	Met	Met	Arg
173	Val	Val	Val	Val	Ala
204	Glu	Glu	Glu	Pro	Pro
211	Arg	Arg	Arg	Thr	Thr
213	Ile	Ile	Ile	Leu	Leu
229	Gly	Gly	Ser	Ser	Ser
232	Leu	Leu	Val	Val	Val
236	Asp	Asp	Asn	Asn	Asn
242	Val	Val	Ile	Ile	Ile
259	Val	Cys	Cys	Cys	Cys
265	Thr	Val	Val	Val	Val
270	Met	Leu	Leu	Leu	Leu
277	Cys	Ser	Ser	Ser	Ser
283	Gln	His	His	His	His
324	Thr	Ser	Ser	Ser	Ser
338	Val	Ala	Ala	Ala	Ala

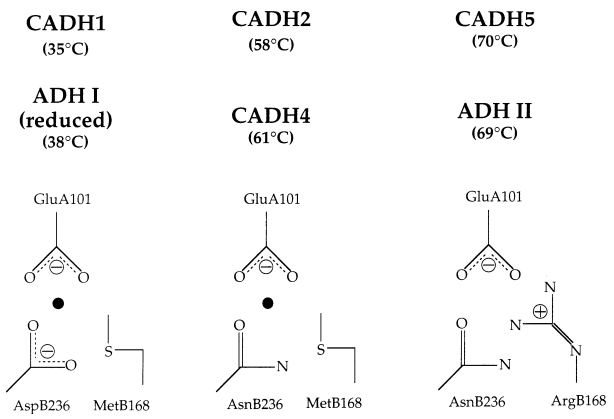

Figure 3 Schematic drawing of a part of the inter-subunit contact region

This schematic drawing is deduced from a three-dimensional model of the yeast ADH I structure. The names of the enzymes studied here are indicated. Residues indicated by A*n* and B*n* belong to subunits A and B of the model respectively. The hypothetical position of the Ca^{2+} ion is indicated by ●. The T_{50} values in 50 mM Mops, pH 7.0, are indicated below the enzyme names. The three groups of enzymes presented here thus correspond to three groups of distinct thermostabilities.

dissociation and unfolding respectively [10,11], it was proposed that 5 mM Ca^{2+} could act as an inhibitor of both the dissociation and unfolding processes. Ca^{2+} was chosen instead of Mg^{2+} because its interaction with proteins of known structure is better documented. The structures of Ca^{2+}-binding sites are well known and the effects of Ca^{2+} on the stability of proteins are often reported.

Because it was proposed that reduced ADH I is stabilized by removing an electrostatic repulsion between subunits, the dissociation propensity of reduced ADH I was tested in the presence and in the absence of $CaCl_2$. As shown in Figure 2, $CaCl_2$ promotes a marked increase of the proportion of tetramers during a gel-permeation assay. This suggested that Ca^{2+} stabilizes reduced ADH I by decreasing the dissociation propensity, and these results are thus in agreement with the predictions made from a three-dimensional model of yeast ADH I structure.

Several chimaeric ADHs are used to locate the Ca^{2+}-sensitive electrostatic repulsion responsible for the high dissociation propensity of reduced ADH I. Previous investigations led to the production of seven chimaeric ADHs (named CADH1 to CADH7), with an N-terminal sequence typical of ADH II and a C-terminal sequence typical of ADH I [10]. The ADH I and ADH II isoenzymes differ by 24 substitutions and ADH II presents a higher thermostability than ADH I [15,16]. Several ADH I–ADH II substitutions occur in a potential interface between subunits, including Asp-236 to Asn and Met-168 to Arg (Table 2). It was previously demonstrated that CADH2 is much more stable than CADH1 ($\Delta T_{50} = 23$ °C). A schematic representation of part of this potential interface between subunits is presented in Figure 3. CADH2 and CADH4 are more thermostable than CADH1 and the reduced form of ADH I, presumably because of the missing electrostatic repulsion between residues 101 and 236 in CADH2 and CADH4. CADH5 and ADH II are more thermostable than CADH2 and CADH4, and it was suggested that this difference comes from an additional inter-subunit salt bridge between residues Glu-101 and Arg-168 in CADH5 and ADH II. An analysis of the effect of Ca^{2+} on the stability and dissociation propensity of chimaeric ADHs can thus validate the hypothetical position of the Ca^{2+}-binding site in a three-dimensional model of the yeast ADH I structure.

In the presence of 2 M urea, CADH2 and CADH4 are largely dissociated on a Superose 12HR gel-permeation column (Figure 4A). However, the addition of 5 mM $CaCl_2$ strongly decreases the dissociation propensity of CADH2 and CADH4. This strong decrease was surprising, because the bivalent-cation-binding site was expected to be the result of electrostatic interactions with the two carboxy groups cited above. This stabilizing effect rather suggests either that the Ca^{2+} binding site does not involve residue 236 or that the interaction between the enzyme and Ca^{2+} is not only electrostatic. In the latter hypothesis, suppressing the negative charge of residue 236 does not prevent Ca^{2+} binding between subunits and the tetramer is still stabilized by Ca^{2+}. CADH5, which is proposed to contain a salt bridge between residues 101 and 168, remains tetrameric in the presence of 2 M urea (Figure 4A). This lower dissociation propensity of CADH5 compared with CADH4 is in agreement with the predictions made previously [10], which involve the presence of Arg-168 (instead of a Met residue) in CADH5, which was proposed to interact with Glu-101 to form a stabilizing inter-subunit salt

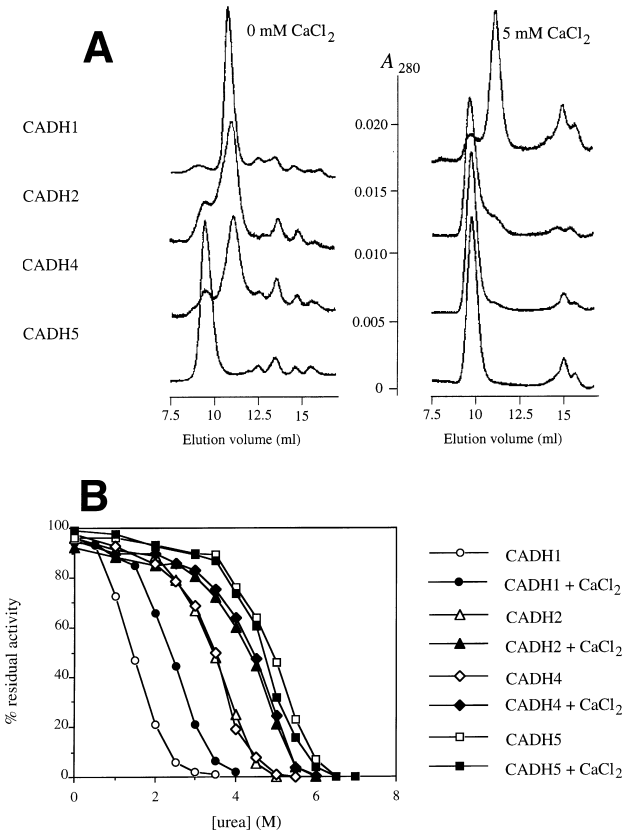

Figure 4 Effect of $CaCl_2$ on the dissociation propensity and stability of chimaeric ADHs

(**A**) Effect of $CaCl_2$ on the dissociation propensity of chimaeric ADHs. Gel permeation was performed in the presence of 2 M urea, yielding only subunits (elution volume approx. 11.5 ml) for CADH1 and only tetramers (elution volume approx. 10 ml) for CADH5. A large proportion (approx. 80%) of CADH2 and CADH4 was dissociated under these conditions. When the same experiment was performed in the presence of 5 mM $CaCl_2$, a large proportion (90–95%) of CADH2 and CADH4 remained tetrameric. Unidentified peaks were detected at higher elution volumes (more than 13 ml, corresponding to molecular masses below 30 kDa); these peaks were not detected in the absence of urea (results not shown), and could correspond to unfolded forms of the enzymes under study. (**B**) Effect of $CaCl_2$ on the stability of chimaeric ADHs. The chimaeric ADHs were inactivated with increasing concentrations of urea, in the absence and in the presence of $CaCl_2$. CADH1, CADH2 and CADH4 are stabilized by the presence of 5 mM $CaCl_2$. CADH5 is not stabilized by the presence of 5 mM $CaCl_2$; a slight but significant destabilizing effect of Ca^{2+} is observed at 5 M urea (results not shown).

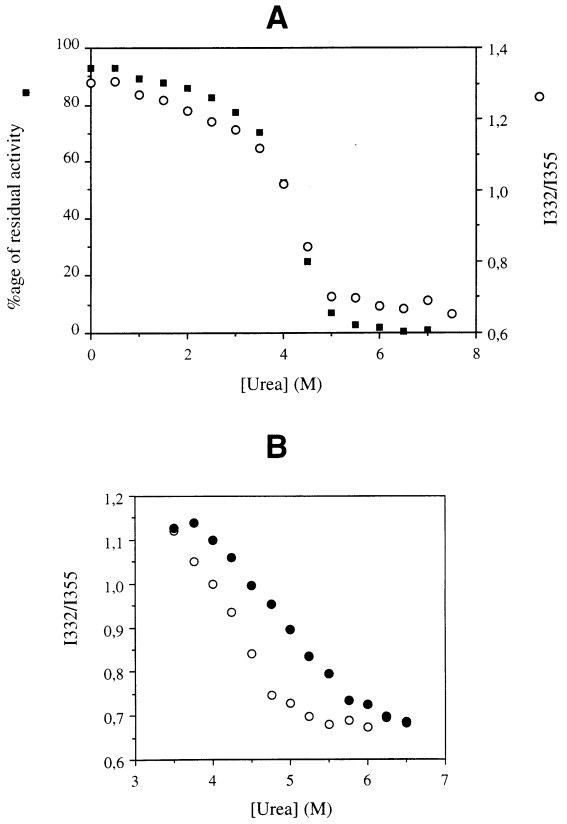

Figure 5 Analysis of the unfolding of oxidized ADH I

(**A**) Urea-induced inactivation (■) and denaturation (○) of oxidized ADH I. Denaturation is detected by a decrease in the ratio of fluorescence emissions, I_{332}/I_{355}. The main transition (at 3.5–5 M urea) is affected by the presence of Ca^{2+}. (**B**) Denaturation curves are shown in the absence (○) and presence (●) of 5 mM $CaCl_2$.

bridge. If these predictions were valid, no stabilizing effect of Ca^{2+} was expected for CADH5.

Analysis of the effect of Ca^{2+} on inactivation by urea clearly shows that the presence of 5 mM $CaCl_2$ is able to stabilize CADH1, CADH2 and CADH4 but not CADH5 (Figure 4B). This suggests that the binding of Ca^{2+} is prevented by the two substitutions occurring between CADH4 and CADH5: Met-168 to Arg, and Val-173 to Ala (Table 2). In a three-dimensional model of yeast ADH I structure, Val-173 is located in one of the hydrophobic cores of the coenzyme-binding domain, whereas Met-168 is located at the interface between subunits, near Glu-101 of another subunit. It is thus proposed that, in CADH5, Ca^{2+} cannot be fixed between Glu-101 and Asn-236 because of the presence of the guanidinium moiety of Arg-168. This result strongly suggests that Ca^{2+} is bound between Glu-101 and Asx-236 in reduced ADH I, CADH1, CADH2 and CADH4. This also suggests that electrostatic interaction between Ca^{2+} and the carboxy groups of Glu-101 and Asp-236 is not the only determinant of the stabilization observed with $CaCl_2$. An apparent contradiction between Figures 4A and 4B is observed: whereas CADH2 and CADH4 are almost completely dissociated in Figure 4A in the presence of 2 M urea, they remain fully active in Figure 4B at the same urea concentration, and it is well known that yeast ADH I monomers are inactive. Actually, it is probable that in the gel-permeation column the apparent equilibrium between tetramers and subunits is modified because subunits can be confined in the gel beads, apart from the tetramers that are moving more rapidly in the column.

Because oxidized ADH I has been proposed to be inactivated by unfolding, the stabilizing effect of Ca^{2+} on the thermostability of oxidized ADH I suggests that this cation could modify the unfolding characteristics of the enzyme. A denaturation curve in the presence of increasing concentrations of urea was first obtained (Figure 5A) by monitoring the decrease in the ratio of the intensity of fluorescence emissions at 332 and 355 nm (I_{332}/I_{355}). The superimposed inactivation and denaturation curves of oxidized ADH I in the presence of urea suggested that under these conditions oxidized ADH I is inactivated by denaturation. Because the denaturation by urea was irreversible (results not shown), no changes in free energies can be deduced from these data. In the presence of 5 mM $CaCl_2$ (Figure 5B), a shift is observed in the decrease of I_{332}/I_{355} towards higher urea

concentrations ($\Delta u_{50} \approx 0.5$–1 M, where u_{50} is the urea concentration at which 50% of the unfolding transition is observed). This suggests that the unfolding of yeast ADH I is decreased in the presence of Ca^{2+}. The position of the weak Ca^{2+}-binding site stabilizing yeast ADH I against unfolding is unclear because of the lack of a mutant ADH I insensitive to Ca^{2+} in the oxidized form and because pairs of Asp/Glu residues closely associated are found in all parts of a three-dimensional model of yeast ADH I structure.

In this study we suggest that Ca^{2+} could act as a general stabilizer of yeast ADH I because it protects the enzyme against both dissociation and unfolding. Because it is active at relatively low concentration, it is likely that Ca^{2+} binds to one or several sites that are solvent-accessible. Yeast ADH I seems to be a good model for the study of the stability of complex enzymes. Indeed, effects of the environment (e.g. buffer or salts) or structure (e.g. mutant enzymes) can easily be tested by measuring the inactivation of reduced and oxidized ADH I. A stabilizing effect on reduced ADH I indicates an effect on dissociation propensity, whereas a stabilizing effect on oxidized ADH I indicates an effect on unfolding. However, the irreversible nature of inactivation does not allow thermodynamic interpretation of the data. The reason for the irreversible inactivation by dissociation is probably that the entropic cost of associating monomers into tetramers would be too high. The irreversible nature of the inactivation by unfolding is possibly due to a phenomenon occurring after unfolding, such as dissociation or the loss of Zn^{2+} ions.

The effect of Ca^{2+} on both dissociation and unfolding of yeast ADH I is not in good agreement with the classical assumption that solvent-accessible electrostatic repulsion does not play a role in protein stability. As most of the studies on protein stability were performed in the presence of guanidinium chloride, which is a salt, solvent-accessible electrostatic interaction could have been underestimated in some cases. Because Ca^{2+} probably acts on stability by removing electrostatic repulsions near the protein surface, it is possible that electrostatic interactions might play a more important role than previously suggested.

The binding of a Ca^{2+} ion to an amide instead of a carboxylic acid (Asn-236 instead of Asp) seems to be quite common. Indeed, the analysis of 69 Ca^{2+}-binding sites in 33 proteins of known structure shows that 30% of them contain at least one amide group (Asn or Gln) near the Ca^{2+} ion, and 5.5% of the atoms co-ordinating Ca^{2+} are part of amide groups. A detailed study of the classical EF-hand sequences shows that Asn is a potential ligand for Ca^{2+} [17,18].

The potential binding site for Ca^{2+} was proposed after the collection of a set of experimental and theoretical data (including a three-dimensional model of the yeast ADH I structure) described elsewhere [10]. Attention must be paid to the low percentage of identities (25%) between yeast ADH I sequence and horse liver ADH sequence (the template used for homology modelling [7,8]). The successful prediction of the effect of Ca^{2+} on thermostability and dissociation propensity of reduced ADH I and CADH1 shows that, when experimental data are included, prediction at near-atomic resolution is possible even with a large divergence between the target model and the template structure. However, establishment of the exact position of the Ca^{2+} binding site(s) will be possible only by determination of the three-dimensional structure of ADH I by X-ray diffraction of crystals grown in the presence of Ca^{2+} salts. The structure of yeast ADH I will be available soon [19], giving a first opportunity to test the predictions made here.

The results presented here suggest that yeast ADH I *in vivo* is complexed to Mg^{2+}, which is probably at millimolar concentrations in the yeast cytosol. Abnormal migration patterns in zymograms (electrophoresis under non-denaturing conditions) of native and chimaeric ADH in yeast extracts have been observed that could be explained by Mg^{2+} binding (X. De Bolle, unpublished work). As Ca^{2+} is found only at nanomolar or micromolar concentrations *in vivo*, it is likely that the weak bivalent-cation-binding site proposed in this study has not been selected during evolution because of its Ca^{2+}-binding properties. The stabilizing effect of Mg^{2+} on reduced ADH I could explain the apparent contradiction between the very low stability of this enzyme *in vitro* and the presence of ADH activity *in vivo*. However, the evidence for the binding of Mg^{2+} to ADH I *in vivo* requires further experimental data.

We thank J. Vandenhaute, J.-Y. Paquet, J.-J. Letesson, P. Mertens, F. Melo, O. Laloux, P. Denoël, D. Delforge, L. Bertrand, G. Baudoux and members of URBM for critical, helpful and stimulating discussions.

REFERENCES

1 Ibers, J. and Holm, R. (1980) Science **209**, 223–235
2 Yamashita, M. M., Wesson, L., Eisenman, G. and Eisenberg, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5648–5652
3 Berg, J. (1990) Annu. Rev. Biophys. Biophys. Chem. **19**, 405–421
4 Vallee, B. L. and Auld, D. S. (1990) Biochemistry **29**, 5647–5659
5 Lowry, C. L., McGeehan, G. and LeVine, H. (1992) Proteins **12**, 42–48
6 Pantoliano, M., Whitlow, M., Wood, J., Rollence, M., Finzel, B., Gilliland, G., Poulos, T. and Bryan, P. (1988) Biochemistry **27**, 8311–8317
7 Jörnvall, H., Eklund, H. and Brändén, C.-I. (1978) J. Biol. Chem. **253**, 8414–8419
8 Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Södeberg, B. O., Tapia, O., Brändén, C. I. and Åkeson, Å. (1976) J. Mol. Biol. **102**, 27–59
9 Brändén, C.-I., Jörnvall, H., Eklund, H. and Furugen, B. (1975) in The Enzymes, 3rd edn. (Boyer P, ed.), vol. 11, pp. 103–190, Academic Press, New York
10 De Bolle, X., Vinals, C., Prozzi, D., Paquet, J., Leplae, R., Depiereux, E., Vandenhaute, J. and Feytmans, E. (1995) Eur. J. Biochem. **231**, 214–219
11 Hayen, P. (1995) Ph.D. Thesis, FUNDP, Namur, Belgium
12 Ganzhorn, A. J., Green, D. W., Hershey, A. D., Gould, R. M. and Plapp, B. V. (1987) J. Biol. Chem. **262**, 3754–3761
13 Kraulis, P. (1991) J. Appl. Crystallogr. **24**, 946–950
14 Jencks, W. P. (1969) Catalysis in Chemistry and Enzymology, McGraw-Hill, New York
15 Schimpfessel, L. (1968) Biochim. Biophys. Acta **151**, 317–329
16 Lutstorf, U. and Megnet, R. (1968) Arch. Biochem. Biophys. **126**, 933–944
17 Marsden, B., Shaw, G. and Sykes, B. (1990) Biochem. Cell Biol. **68**, 587–601
18 da Silva, A. and Reinach, F. (1991) Trends Biochem. Sci. **16**, 53–57
19 Ramaswamy, S., Kratzer, D. A., Hershey, A. D., Rogers, P. H., Arnone, A., Eklund, H. and Plapp, B. V. (1994) J. Mol. Biol. **235**, 777–779

Received 26 July 1996/18 November 1996; accepted 27 November 1996