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Effect of Zinc Ions on Conformational Stability of Yeast Alcohol Dehydrogenase

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Abstract—Yeast alcohol dehydrogenase preparations were prepared with the conformational zinc ion removed (Apo-I YADH) and with both the conformational and catalytic zinc ions removed (Apo-II YADH). The unfolding of Apo-I YADH and Apo-II YADH during denaturation in urea solutions was then followed by fluorescence emission, circular dichroism, and second-derivative optical spectroscopies. Compared with the native enzyme, Apo-I YADH incurred some slight unfolding, and its stability against urea was markedly decreased, while Apo-II YADH incurred marked unfolding but contained residual ordered structure even at high urea concentrations. The results show that native YADH is more conformationally stable against urea denaturation than Apo-I YADH, indicating that the conformational Zn^{2+} plays an important role in stabilizing the conformation of the YADH molecule. However, unfolding of the region around the conformational zinc ion is shown not to be the rate limited step in the unfolding of the molecule by the fact that the unfolding and inactivation rate constants of native and Apo-I YADH are the same. It is suggested that the catalytic zinc ion is more important in maintaining the structure of YADH. YADH lost its cooperative unfolding ability after the zinc ions were removed. The shape of the transition curves of Apo-I YADH suggests the existence of an unfolding intermediate. For both native and Apo-I YADH, inactivation occurs at much lower urea concentrations than that needed to produce significant conformational changes of the enzyme molecule. At urea concentration above 4 M, the inactivation rate constants are much higher than those of the fast phase of the reaction of unfolding. These results support the suggestion of flexibility at the active site of the enzyme (C. L. Tsou (1986) *Trends Biochem. Sci.*, **11**, 427-429; (1993) *Science*, **262**, 308-381).

Key words: alcohol dehydrogenase, zinc ion, conformational stability, yeast

Zinc is an essential component of many enzymes involved in virtually all aspects of metabolism [1]. It is found in many enzymes. Yeast alcohol dehydrogenase (YADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) is a typical example of such enzymes. It is a tetrameric enzyme with 8 zinc ions and a total molecular mass of 150 kD [2, 3]. The active site in each subunit contains one zinc ion that is absolutely necessary for enzyme activity [4]. The second zinc ion (conformational zinc) present in each subunit of the enzyme plays a prominent conformational role, probably by stabilizing the tertiary structure of the yeast alcohol dehydrogenase [5]. The catalytic reaction mechanism of the enzyme is known; however, its three dimensional structure is not yet available. Some theoretical attempts have been made to model the quaternary structure of YADH, but a convincing solution has not been proposed. Though the general subunit conformation of YADH is largely identical to that of horse liver alcohol dehydrogenase (HLADH) [6], YADH and HLADH are only distantly homologous, with many insertions/deletions, and with only 25% of all residues conserved [7]. Nonequivalent substitutions have also been suggested to result in stability and substrate specificity differences between the two dehydrogenases [6]. It is known that Cys-46, His-67, and Cys-174 of HLADH located in the active site are essential for enzyme activity and are ligands bound to the catalytic zinc ion [4]. Vallee and Williams [8] suggested that the presence of Zn^{2+} helps keep the conformation of the active site in a strained state that can be quantified by quantum-chemical and molecular mechanics calculations [9], this state being required for the catalysis by the enzyme. The position of the second Zn^{2+} is known for horse liver alcohol dehydrogenase, where it is bound to four cysteine residues (Cys-97, Cys-

Abbreviations: YADH) yeast alcohol dehydrogenase; Apo-I YADH) conformational Zn^{2+}-free enzyme; Apo-II YADH) both conformational and catalytic Zn^{2+}-free enzyme; DTT) dithiothreitol.

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100, Cys-103, and Cys-111). Site-directed mutagenesis of these Cys resulted in unstable enzymes, illustrating the important "structural" properties of the conformational zinc atom [10]. These four cysteine residues are also present in YADH, so it is supposed that this Zn^{2+} is also bound to these four cysteine residues [1, 11].

Extensive studies have been carried out on the denaturation of YADH [5, 12-18]. It was suggested that although the catalytic zinc ion is supposed to keep the conformation of the active site in a strained state, the active site is still more flexible than other regions of the enzyme molecule. The conformational zinc was shown to have effects on the stability of the enzyme. A slight unfolding of YADH occurred on removal of the conformational zinc, and marked unfolding occurred when both zinc ions were removed [17]. However, there is no report on how the catalytic and the conformational zinc ions affect the folding behavior of YADH.

In the present investigation, changes in the residual native structure of Apo-I YADH (with the conformational zinc ion removed) and Apo-II YADH (with both the conformational and catalytic zinc ions removed) during urea denaturation have been compared with the native enzyme to explore the relationship between the structural change of the enzyme and its activity as well as the effect of the presence of the zinc ions on this relationship.

MATERIALS AND METHODS

Yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase was obtained from Sigma (USA) and used without further purification. The YADH was homogeneous on polyacrylamide gel electrophoresis in the presence and the absence of SDS. DTT and the sodium salt of NAD^+ were also obtained from Sigma. The urea used was the ultra-pure grade product of Amresco (USA). Chelex-100 was from Bio-Rad (USA). All other reagents were local products of analytical grade.

Enzyme concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient $A^{1\%}_{1cm} = 12.6$ [19]. The enzyme activity was assayed with a Perkin Elmer Lambda Bio spectrophotometer using the method described in [20].

Preparation of Apo-I YADH (conformational zinc ion free) was carried out as previously described [5, 17]. Native enzyme (2-3 mg/ml) was dissolved in 0.1 M Tris-HCl buffer, pH 7.5. The enzyme solution was dialyzed against the same buffer containing 0.1 M NaCl and 60 mM DTT at 4°C for 24 h and then dialyzed against 0.001 M phosphate buffer (pH 7.0) to remove DTT-Zn^{2+} and excess DTT. Determination of the enzyme activity with the zinc ion content for Apo-I YADH decreased approximately 50% without any loss of activity, indicating that the zinc ion in the active site, which is essential for enzyme activity, is not affected by dialysis against DTT. The preparation of Apo-II YADH was the same as that of the preparation of Apo-I except that Cu-Reagent (sodium diethyldithiocarbamate) was used instead of DTT. The Apo-II YADH prepared retained approximately 10% of the original zinc content and less than 10% of the initial activity. Therefore, the Cu-Reagent removed both the catalytic and conformational zinc ions without any observable specificity [17]. The deionized water and all buffers used were treated with a Chelex-100 column. All containers were plastic to reduce the possibility of metal ion contamination.

The fluorescence emission spectra were measured with a Hitachi 850 (Japan) spectrofluorimeter. The measurements of the fluorescence of tryptophan in the protein molecules were made using excitation wavelength 295 nm. Circular dichroism spectra were recorded on a Jasco 500C (Japan) spectropolarimeter. The pathlength of the sample cell was 2 mm. Four scans between 200 and 250 nm were successively added to enhance the signal-to-noise ratio. The zinc contents of the YADH preparations were quantitatively determined by atomic absorption spectrophotometry using a ICAP/9000FP (Japan) spectrophotometer.

RESULTS

Fluorescence emission spectra of Apo-I and Apo-II YADH during denaturation in urea solutions. The fluorescence emission maximum near 335 nm of native yeast alcohol dehydrogenase at pH 7.5 may contain contributions from both tryptophan and tyrosine residues in the enzyme molecule [15]. The fluorescence emission spectra of Apo-I and Apo-II are shown in Fig. 1. It can be seen from Fig. 1a that, after removal of the conformational zinc ion, the emission maximum of Apo-I YADH was slightly red-shifted to 336.5 nm, suggesting that the enzyme was slightly unfolded or the environment around tryptophan was slightly changed. However, the emission maximum of Apo-II YADH with both the conformational and catalytic zinc ions removed was markedly red-shifted to 340 nm, indicating that the enzyme molecule markedly unfolded. These results are similar to those observed previously [17].

Figure 1a shows that the changes in the fluorescence emission spectra of Apo-I YADH after denaturation in urea solutions of different concentrations for 2 h. At low urea concentrations, less than 2.0 M, or at urea concentrations higher than 3.0 M, increasing the urea concentration caused the fluorescence emission intensity to markedly decrease; however, the fluorescence intensity increased at urea concentrations between 2.0 and 3.0 M. The changes in the fluorescence emission intensity of Apo-I YADH in urea solutions of different concentrations are similar to the changes of the native YADH reported previously [14]. For Apo-II YADH (Fig. 1b), at

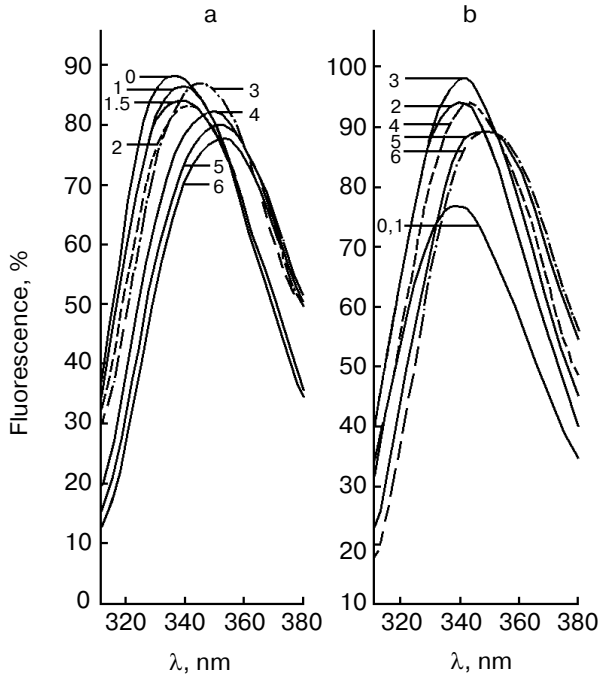

Fig. 1. Fluorescence emission spectra of Apo-I and Apo-II YADH during denaturation in urea solutions of different concentrations. a) Fluorescence emission spectra of Apo-I YADH; b) fluorescence emission spectra of Apo-II YADH. The enzyme was dissolved in 0.1 M sodium phosphate buffer, pH 7.5. The enzyme concentration was 2.0 μM. The enzyme solutions were incubated at 25°C for 2 h before measurements. The excitation wavelength was 295 nm. Numbers on each curve denote the molar concentrations of urea.

urea concentrations less than 3.0 M, the emission peak at 340 nm increased in intensity with very little if any red shift observed with increasing urea concentration. At high urea concentrations, greater than 3.0 M, increasing the urea concentration caused a marked red shift of the emission maximum from 335 to 352 nm together with a significant decrease in the fluorescence intensity.

Figure 2 compares the red shift of the emission maximum of native, Apo-I, and Apo-II YADH during denaturation in urea solutions of different concentrations. It can be seen that the conformation of the native YADH is more stable than that of Apo-I YADH against urea denaturation. The transition curve for Apo-I YADH shows a plateau between 1.0 and 1.5 M urea concentration, suggesting an unfolding intermediate.

Ultraviolet difference and second-derivative spectra of Apo-I and Apo-II YADH during urea denaturation. The effect of different concentrations of urea on the ultraviolet difference spectra of Apo-I YADH is shown in Fig. 3a. Urea-denatured Apo-I YADH showed two negative peaks, at 278 and 286 nm, as well as a small shoulder peak at 295 nm. With increasing urea concentration, both negative peaks and the shoulder increased in magnitude, reaching maximal values at 5.0 M urea. The negative magnitude of all peaks did not increase further when the urea concentration was increased beyond 5.0 M. The second-derivative spectra of Apo-I and Apo-II YADH after urea denaturation for 2 h are shown in Figs. 3b and 3c. Two maxima centered near 278 and 286 nm (positive peaks) and three minima at 275, 282, and 295 nm (negative peaks) were produced in urea solutions. With increasing urea concentration, all these peaks increased in magnitude. Figure 3d compares the second-derivative spectra of native, Apo-I, Apo-II, and 6 M urea-denatured holo-YADH. The second-derivative spectrum of the native enzyme is similar to that of Apo-I YADH, and the spectrum of Apo-II is situated between the native and urea-denatured enzyme.

Figure 4 compares the intensity changes of the positive peaks of the second-derivative spectra at 286 nm during denaturation in urea solutions of different concentrations. The results are similar to those obtained from the fluorescence except that the plateau in the transition curve of Apo-I YADH between 1.0 and 1.5 M urea disappears.

Far-ultraviolet CD spectra of Apo-I and Apo-II YADH during urea denaturation. Secondary structure changes of Apo-I and Apo-II YADH during urea denaturation were studied using the far-ultraviolet CD spectrum. Figures 5a and 5b show the far-ultraviolet CD spectra of Apo-I and Apo-II YADH denatured in urea solutions of different concentrations under conditions similar to those described above. The far-ultraviolet CD spectrum of native and Apo-I YADH are similar [14]. However, a marked decrease in the ellipticity occurred when the enzyme lost both of its zinc ions. It was previously reported that at low concentrations of urea, less than 2.0 M, no changes of the CD spectra of native YADH were observed [14]. However the CD spectrum of

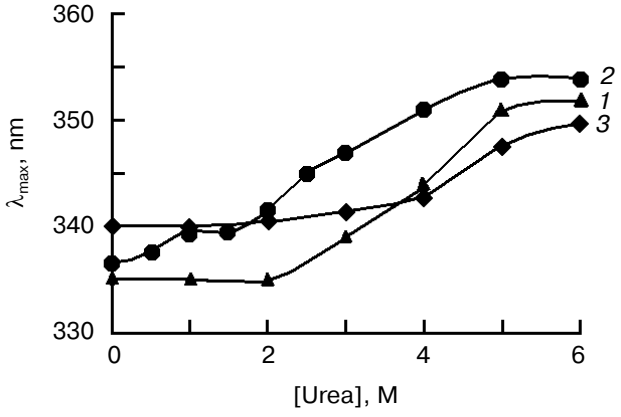

Fig. 2. Comparison of the red shifts of the fluorescence emission maxima of native (*1*), Apo-I (*2*), and Apo-II (*3*) YADH during denaturation in urea solutions of different concentrations. The data for native YADH were taken from [14].

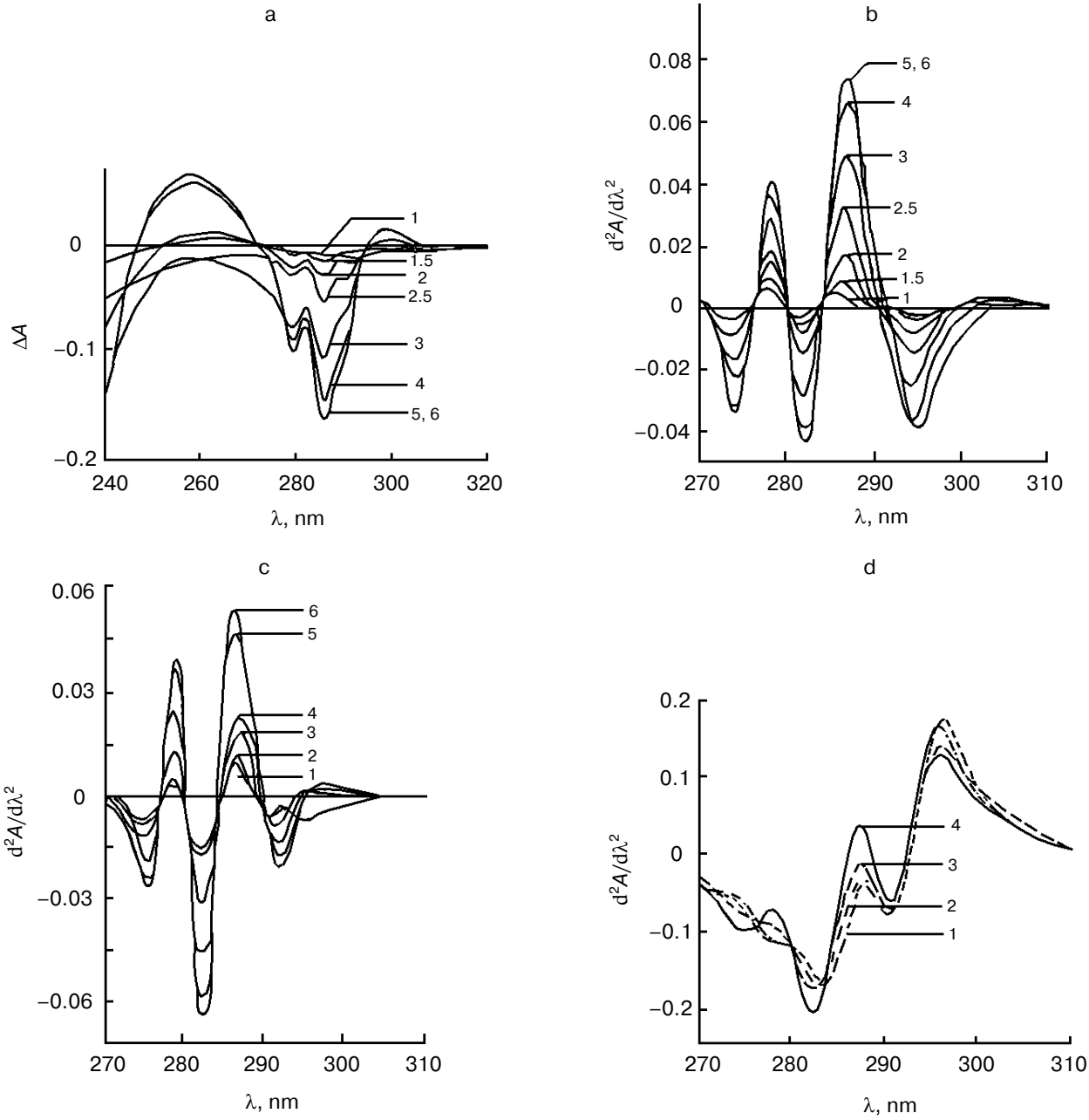

Fig. 3. Ultraviolet absorption spectra of Apo-I and Apo-II during urea denaturation. Experimental conditions were as for Fig. 1 except for the enzyme concentrations. The final concentrations of the native, Apo-I, and Apo-II YADH were 10.0, 10.0, and 9.0 μM, respectively. a) Ultraviolet difference spectra of Apo-I YADH denatured in urea solutions of different concentrations; b, c) the second-derivative difference absorption spectra of Apo-I and Apo-II YADH denatured in urea solutions of different concentrations. Numbers on each curve denote urea molar concentrations; d) curves *1-4* are the second-derivative absorption spectra of native, Apo-I, Apo-II, and 6 M urea-denatured native YADH.

Apo-I YADH at low concentrations of urea shows marked changes, but no changes were observed in the CD spectrum of partially unfolded Apo-II YADH at low concentrations of urea, less than 2.0 M. Further increases of the urea concentration resulted in marked changes in the CD spectra of both Apo-I and Apo-II YADH.

Figure 6 compares the changes in the α-helix content at different urea concentrations. The results show that native YADH was more stable than Apo-I and Apo-II YADH in urea solutions. These results are similar to those obtained by measuring the second derivative spectrum.

Comparison of inactivation and unfolding of native and Apo-I YADH during urea denaturation. Figure 7 relates the inactivation and emission maximum red shift of native and Apo-I YADH in urea solutions of different

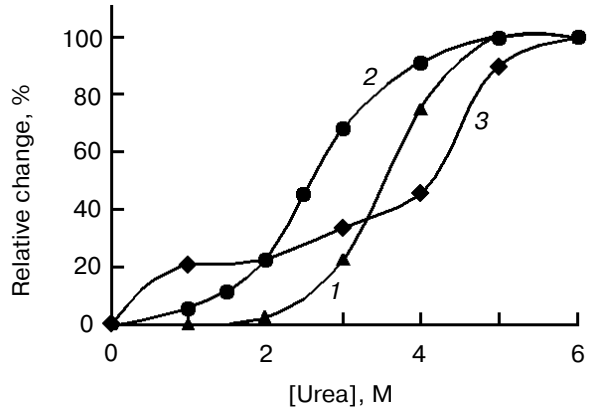

Fig. 4. Comparison of the intensity changes of the second-derivative spectra of native (*1*), Apo-I (*2*), and Apo-II (*3*) YADH at 286 nm during urea denaturation. The data for the native YADH were taken from [14].

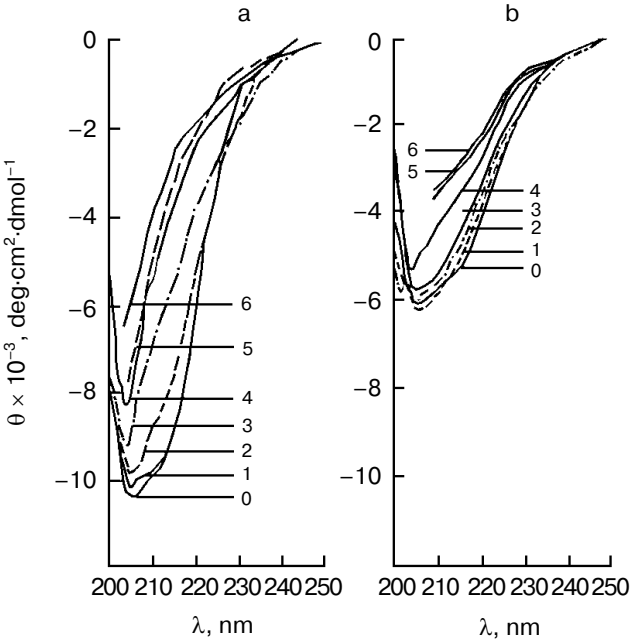

Fig. 5. Far-ultraviolet CD spectra of Apo-I and Apo-II YADH denatured in urea solutions of different concentrations. Experimental conditions were as for Fig. 1 except for the enzyme concentrations. a) Apo-I YADH, protein concentration was 10.0 μM. b) Apo-II YADH, protein concentration was 9.0 μM. Numbers on each curve denote urea molar concentrations.

concentrations. Comparing the extent of inactivation of native and Apo-I YADH shows that, after removal of the conformational zinc ion by DTT, the stability of both the conformation and the activity of yeast alcohol dehydrogenase against urea denaturation markedly decreased. For both native and Apo-I YADH, much lower concentrations of urea were required to bring about inactivation than were required to produce significant conformational changes of the enzyme molecules, indicating that the active site of this enzyme is more flexible than the enzyme molecule as a hole, as previously suggested by Tsou based on experimental data for other enzymes [21, 22]. It is also suggested that loss of catalytic activity in low urea concentrations is not due to the loss of the catalytic zinc, which may lead to marked unfolding.

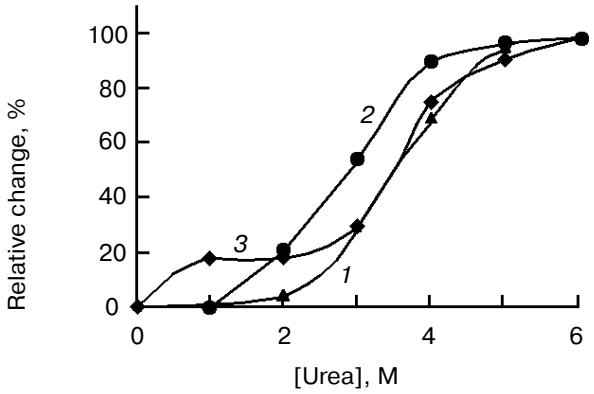

Fig. 6. Comparison of the relative changes of ellipticity at 220 nm during urea denaturation of native (*1*), Apo-I (*2*), and Apo-II (*3*) YADH. The data for the native YADH were taken from [14].

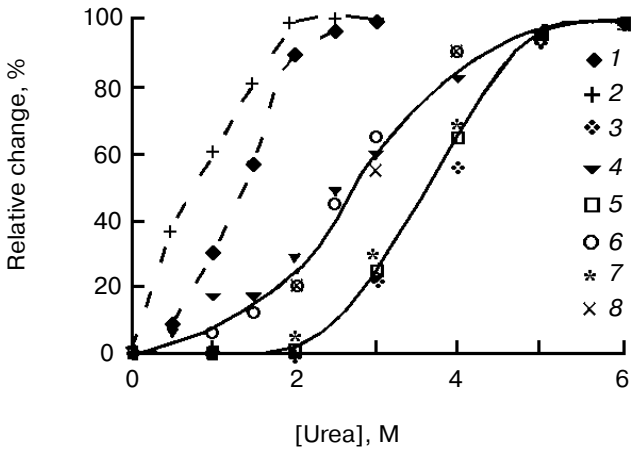

Fig. 7. Comparison of inactivation and conformational changes of native and Apo-I YADH during urea denaturation. The conformational change data were taken from Figs. 2, 4, and 6. Inactivation conditions were as for Fig. 1 except that, at the end of the incubation, small samples were taken for activity measurements. The enzyme was stable under the above conditions in the absence of urea. Remaining activity for native (*1*) and Apo-I (*2*) YADH; red shift the of fluorescence emission maxima for native (*3*) and Apo-I (*4*) YADH; intensity changes of the second-derivative spectra at 286 nm for native (*5*) and Apo-I (*6*) YADH, and α-helix content changes of for native (*7*) and Apo-I (*8*) YADH.

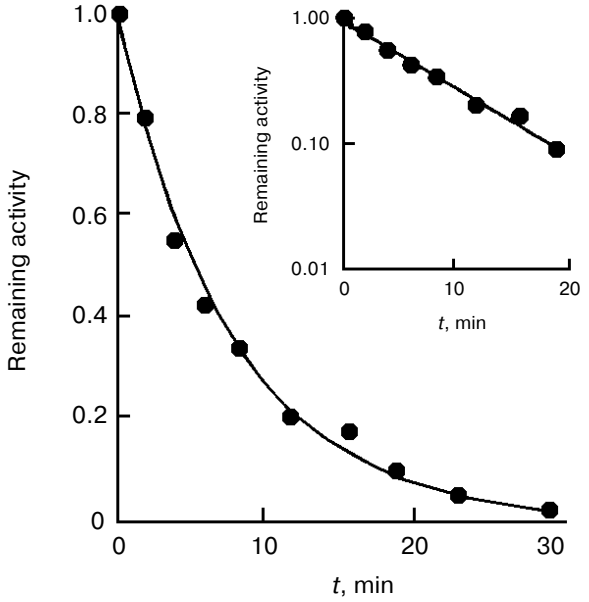

Fig. 8. Inactivation course of Apo-I YADH in 3.0 M urea solution. A mixture of Apo-I YADH (0.1 μM) and urea (3.0 M) in 0.1 M sodium phosphate buffer (pH 7.5) was incubated at 25°C. At the indicated times, 10 μl portions were taken for activity determination in 1.0 ml of reaction mixture. The inset shows a semilogarithmic plot of the results.

Rate of inactivation of native and Apo-I YADH during urea denaturation. The inactivation courses of native and Apo-I YADH were followed in urea solutions of different concentrations. Figure 8 shows the course of inactivation of Apo-I YADH in 3.0 M urea; the inset shows a semilogarithmic plot of the data. The inactivation rate constants of native and Apo-I YADH in 3.0 M urea solution were obtained from such data and are listed in the table. The inactivation rate constants in 4.0 M urea were too fast to be measured by the conventional method.

Rate constants of inactivation and unfolding of native, Apo-I, and Apo-II YADH during denaturation in urea solutions (k_1 and k_2 are the first-order rate constants for the fast and slow phases, respectively)

Enzyme	Concentration of urea, M	Unfolding rate constants		Inactivation rate constant ($k \times 10^3$, sec^{-1})
		$k_1 \times 10^3$, sec^{-1}	$k_2 \times 10^3$, sec^{-1}	
Native	3	3.3	0.7	2.16
	4	23.0	3.7	*
Apo-I	3	2.7	0.7	2.24
	4	22.0	3.5	*
Apo-II	3	*	0.6	—
	4	*	2.1	—

* Reaction rate was too fast to be measured by the conventional dynamic method.

Rate of conformational changes of native, Apo-I, and Apo-II YADH in urea solutions. The conformational change courses of native, Apo-I, and Apo-II YADH at different concentrations of urea solutions were followed by changes in the second-derivative spectra. Figure 9a shows the change course of the second-derivative spectra of Apo-I YADH in 4.0 M urea solution. Figure 9b shows the change course of the second-derivative value at 286 nm with a semilogarithmic plot of the data in the inset. The data can be resolved into two linear components, indicating that the unfolding process consists of two first-order reactions. The rate constants are shown in table. The rate constants of unfolding of native, Apo-I, and Apo-II YADH in urea solutions of different concentrations are also summarized in the table.

DISCUSSION

It is well known that enzyme activity is strongly dependent on conformational integrity. The native state and the fully denatured state have been well studied. In the native state, the protein molecule folds into a uniquely defined rigid and compact structure that has complete activity, while in the fully denatured state it unfolds into a flexible, swollen, and randomly coiled chain which is inactive [23]. Previous authors have largely concentrated on the unfolding and refolding of enzymes not containing prosthetic groups, with relatively few attempts made to explore the effect of metal ions on the unfolding of proteins. It is known that there are two zinc ions per YADH subunit [5]. One zinc ion is found in the active site of the enzyme and is essential for enzyme activity. The secondary zinc ion present on each subunit of the enzyme plays a prominent conformational role. Although X-ray diffraction analyses of the three-dimensional structure of this enzyme have not been published, evidence strongly indicates that the subunits of YADH have structures similar that of horse liver alcohol dehydrogenase (HLADH) [1, 6, 24]. HLADH is a dimeric enzyme with known three-dimensional structure. Comparison of the fluorescence emission spectra, far UV CD spectra, and second-derivative absorption spectra of native, Apo-I, and Apo-II YADH shows that the conformation of Apo-I YADH is changed little from the native enzyme, but complete inactivation and marked conformational changes were observed for Apo-II YADH, which indicated that partial unfolding occurred. The ligands of the conformational zinc are part of a lobe that projects out of the catalytic domain and has only a few-side chain interactions with the remainder of the subunit of HLADH [25]. Therefore, it is suggested that the conformational zinc primarily

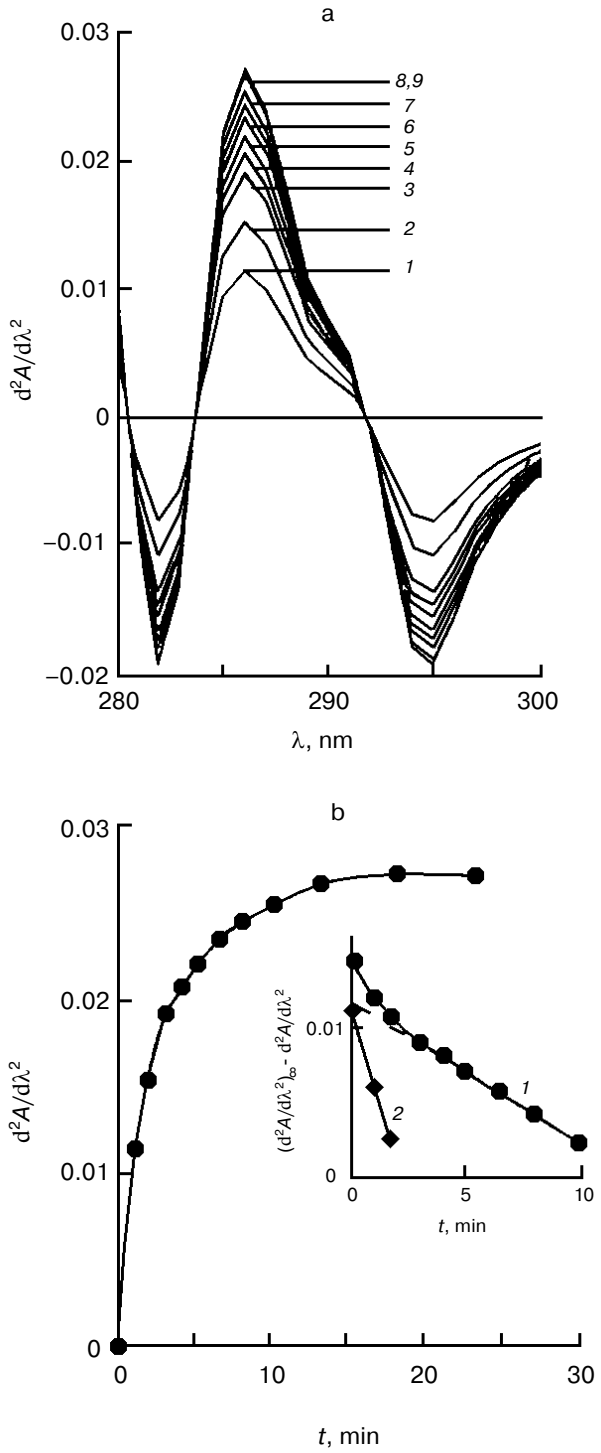

Fig. 9. Unfolding course of Apo-I YADH in 4.0 M urea followed by second-derivative spectroscopy. Experimental conditions were as for Fig. 2. a) Repeated scans of the ultraviolet second-derivative spectra after mixing the enzyme with the denaturant. The reaction times of enzyme denaturation for curves *1*-*9* were 1, 1.75, 3, 4, 6, 8, 13, 18, and 23 min, respectively. b) The intensity change course of the second-derivative difference absorption at 286 nm. The inset shows a semilogarithmic plot of the full experimental data (*1*) and points obtained by subtracting the contribution of the slow phase from the curve (*2*).

affects local structure and conformation [1]. Our results also suggest that the removal of the conformational zinc does not affect significantly the structure of YADH as a whole. However, the peptide backbones of all three ligands of catalytic zinc are firmly anchored in secondary structural elements and none is part of a flexible loop region [25], and Zn^{2+} helps keep the conformation of the active site in a strained state [8, 9]. It seems that partial unfolding of YADH that occurs when both the conformational and the catalytic zinc ions are were removed should be mainly attributed to the important role that the catalytic zinc plays in secondary structure stabilization in the YADH molecule. That unfolding of the region around the catalytic zinc ion is more important than the unfolding of the region around the conformational zinc ion is further suggested by the similarity of unfolding or inactivation rate constants for both native and Apo-I YADH under certain urea concentrations. Similar results were obtained in the thermal denaturation of YADH [17]. It is suggested that the unfolding of the region around the conformational zinc is not the rate-limiting step in the denaturation of YADH. When both the catalytic and conformational zinc ions were removed, the fast phase of unfolding disappeared, while the rate constant of the slow phase remained unchanged, suggesting that unfolding of the region around the catalytic zinc is related with the fast phase of unfolding, and the unfolding of the main chain is related to the slow phase of unfolding.

Comparison of the conformational changes of native and Apo-I YADH (Fig. 7) during denaturation in urea solutions of different concentrations by fluorescence, ultraviolet second-derivative spectra, and CD spectra shows that the conformational stability of the native YADH is much greater than that of Apo-I YADH during urea denaturation. These results suggest that the conformational Zn^{2+} not only affects local structure and conformation but also has some effect on the overall structure. There is evidence that the active site becomes more flexible after the conformational zinc is removed [18]. The conformational Zn^{2+} is suggested by the results here to play an important role in stabilization of the conformation of yeast alcohol dehydrogenase, coinciding with previously reported results of thermal [5, 17] and high pH [12] and low pH [13] denaturation. The stabilization effect may be caused by association of subunits as suggested in [26]. The shape of transition curve of native YADH suggests a coordinated unfolding process. However, it is interesting that the transition curve of Apo-I YADH indicated by the fluorescence emission maxima shows a plateau between 1.0 and 1.5 M urea concentration (Fig. 2), but such plateau does not exist in the curves measured by CD or second-derivative spectra. The cooperative folding ability of Apo-I YADH was reported to be lower than that of the holoenzyme [12, 13]. We proposed that the removing of the conformational zinc decreases the cooperative ability of the global structure of YADH and leads to flexibility of a

regional part around a tryptophan group that may be the interface of the subunit and therefore can be disturbed at urea concentrations less than 1.0 M, which was reflected by the intrinsic fluorescence. However, the overall structure of the enzyme remains unchanged at low urea concentrations, so there is no significant change in the CD and second-derivative spectra. The transition coordination of Apo-II YADH conformation in urea measured using fluorescence, ellipticity, and second-derivative spectra is much lower than that of native and Apo-I YADH. The residual native structure of Apo-II YADH is more sensitive to low urea concentrations (less than 1.0 M) than the native and Apo-I YADH. However, Apo-II YADH still possesses some residual ordered structure in urea solutions of high concentrations, which may be due to disulfide bonds formed by oxidation of the thiol groups during the removal of the zinc.

The result (Fig. 7) also shows that for both native and Apo-I YADH, inactivation occurs at much lower urea concentrations than significant conformational changes of the enzyme molecule. Similar results were observed during denaturation of other metalloenzymes, such as aminoacylase [27] and alkaline phosphatase [28, 29]. The kinetic results show that with increasing urea concentration the inactivation and unfolding rate constants markedly increased for both native and Apo-I YADH. Although at 3.0 M urea concentration the inactivation rate constants are similar to the unfolding rate constants of the fast phase reactions, at 4.0 M urea the inactivation rate constants are much higher than the rate constants of the fast phase unfolding. These results support the suggestion of flexibility at the active site of the enzyme as suggested in [21, 22].

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