J. Biochem. 119, 674-679 (1996)

Acid-Induced Folding of Yeast Alcohol Dehydrogenase under Low pH Conditions[1]

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Received for publication, September 25, 1995

Under conditions of low pH, the conformational states of holo-YADH and apo-YADH were examined by protein intrinsic fluorescence, ANS fluorescence, and far-UV CD measurements. The results obtained show that at low ionic strength, with the addition of HCl, the holo- and apo-YADH denatured gradually to reach the ultimate unfolded conformation in the vicinity of pH 2.0 and 2.5, respectively. With the decrease of pH from 7.0 to 2.0, the fluorescence emission decreased markedly, with its emission maximum red-shifting from 335 to 355 nm, indicating complete exposure of the buried tryptophan residues to the solvent. The far-UV CD spectra show the loss of the arrayed secondary structure, though the acid-denatured enzyme still maintained a partially arrayed secondary structure. A further decrease in pH by increasing the concentration of $HClO_4$ induced a cooperative folding of the denatured enzyme to a compact conformation with the properties of a molten globule, described previously by Goto *et al.* [*Proc. Natl. Acad. Sci. USA* 87, 573-577 (1990)]. More extensive studies showed that although apo-YADH and holo-YADH exhibited similar behavior, the folding cooperative ability of apo-YADH was lower than that of the holo-enzyme. From the above results, it is suggested that the zinc ion plays an important role in the proper folding of YADH and in stabilizing its native conformation.

Key words: alcohol dehydrogenase, acidic denaturation, molten globule state, refolding.

It is well known that the acid-induced unfolding of proteins is often incomplete, and the acid-unfolded proteins assume conformations that are different from the fully unfolded ones observed in the presence of high concentrations of guanidinium chloride (*1*). Recently, the results of studies on several proteins showed that further addition of acid to lower the pH, or an increase of salt concentration, resulted in folding of the acid-denatured enzyme to a compact conformation with the properties of a molten globule (*2, 3*). The mechanism of cooperative transition to a molten globule state has been discussed (*2*). On decreasing the pH from neutrality to approximately pH 2, the protein becomes maximally positively charged. The large net positive charge on the protein minimizes both intramolecular interaction (which leads to unfolding) and intermolecular interaction (aggregation). The abolition of the electrostatic repulsive force by the binding of anions leads to a decrease in the intramolecular repulsion, causing the molecule to fold into a compact structure (*3*). When the concentration of $HClO_4$ or salts is increased, anion binding to the acid-unfolded proteins induces folding of the protein structure. The major factor responsible for the folding is believed to be the binding of the anions, which reduce the intramolecular charge repulsion that initially brought about the unfolding (*3*). For many denatured proteins, the molten globule state might be a normal intermediate conformation (rather than the random coil state) during the course of folding. However, its precise role in the folding of protein is not clearly known. In order to explore the mechanism of protein folding, it is necessary to make further investigations on the structure and thermodynamic properties of the molten globule state of more proteins. However, previous authors have largely concentrated on studies of monomeric enzymes which contain no prosthetic groups. The folding of acid-denatured enzymes containing metal prosthetic groups has not been well explored.

Yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase) [EC 1.1.1.1] is a tetrameric enzyme with 8 zinc ions and a total molecular mass of 150 kDa (*4, 5*). The active site at each subunit contains one zinc ion, which is absolutely necessary for enzyme activity (*6*). The second zinc ion (conformational zinc) present on each subunit of enzyme predominantly plays a conformational role, probably by stabilizing the tertiary structure (*7*). It is well known that Cys-46 and Cys-174 of YADH, located in the active site, are essential for enzyme activity, and are ligands bound to the catalytic zinc ion (*6*). In the present study, the acid-induced

[1] The present investigation was supported in part by the Pandeng project of the China Commission for Science and Technology and by Grant 39570180 of the China Natural Science Foundation.

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Abbreviations: YADH, yeast alcohol dehydrogenase; holo-YADH, native, Zn^{2+}-containing enzyme; apo-YADH, structure zinc-free enzyme; NAD^+, nicotinamide adenine dinucleotide; CD, circular dichroism; UV, ultraviolet; DTT, dithiothreitol; ANS, 1-anilino-8-naphthalenesulfonate.

unfolding of YADH and the folding induced by increasing concentrations of anions under a low pH condition have been explored.

MATERIALS AND METHODS

The sodium salt of NAD^+ was obtained from Sigma. Coenzyme purity was found by enzymatic assay (*8*) to be 99.9%. Chelex 100 was a Bio-Rad product; DTT was from Promega; hemimagnesium salt of ANS was from BDH. All other reagents were local products of analytical grade.

Yeast alcohol dehydrogenase was obtained from Sigma and used without further purification. Apo-YADH was prepared as described by Magonet *et al.* (*7*). 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 in the presence of 0.1 M NaCl and 60 mM DTT at 4°C for 18 h, and then dialyzed against deionized water under the same condition for 24 h to remove DTT-Zn^{2+} and excess DTT. The apo-enzyme thus obtained was lyophilized. The deionized water and all buffers were treated with a Chelex 100 column. All containers were plastic.

Enzyme concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient $A^{1\%}_{1cm} = 12.6$ (*9*). The enzyme activity was assayed in a Kontron UVikon 860 spectrophotometer using the method described by Bille and Remacle (*10*).

The fluorescence emission spectra were obtained with a Hitachi 850 spectrofluorimeter. For the measurements of the fluorescence of tryptophan in protein molecules, an excitation wavelength of 295 nm was used. The final concentration of enzyme was 0.33 μM.

Circular dichroism (CD) spectra were recorded on a Jasco 500C spectropolarimeter. The path length of the sample cell used was 2 mm. Eight scans between 190 and 250 nm were successively added to ensure a good signal-to-noise ratio. The enzyme concentration was 0.33 μM. The results of CD were represented as ellipticity per residue.

Acidic Unfolding of YADH—apo- and holo-YADH were dialyzed against deionized water, then the dialyzed enzymes were divided into batches, added to HCl-water solutions of different concentrations (pH values), and incubated for 1 h.

Conformational Transitions of Acid-Denatured YADH—Acid-induced conformational transitions were measured by using tryptophan fluorescence in the absence and presence of $HClO_4$. The apo- and holo-YADH were unfolded in 6 mM HCl solution (pH 2.0), and the solution was mixed with the same solution containing $HClO_4$ of different concentrations. The acid-induced folding rates were very fast, and the measurements were made right after mixing to minimize the effects of aggregation at low pH.

ANS was added to the protein solution with different concentrations of $HClO_4$ at low pH or neutral pH to study the interaction with ANS. Since the fluorescence of ANS showed substantial time-dependent changes at acidic pH, the measurements were made after mixing and incubation for 20 min.

The measurements of pH were made on an Orion model 720A with a microcombination electrode. All measurements were carried out at 20°C.

RESULTS

Acid-Unfolding of Holo- and Apo-YADH—Figure 1, A and B, shows the fluorescence emission spectra of holo- and apo-YADH during acidic unfolding. It can be seen that at pH 7.5, the fluorescence emission peaks of the native YADH and apo-YADH excited at 295 nm are at 335 and 336.5 nm, respectively, indicating that the removal of Zn^{2+} led to a small conformational extension of the enzyme. Each subunit of YADH contains five Trp residues (*11*). The fluorescence emission spectrum of native YADH has a peak value of 335 nm and a width at half the peak height of 55 nm. These values are in accord with the fluorescence characteristics of proteins with half exposed and half buried Trp residues (*12*). At low ionic strength, the

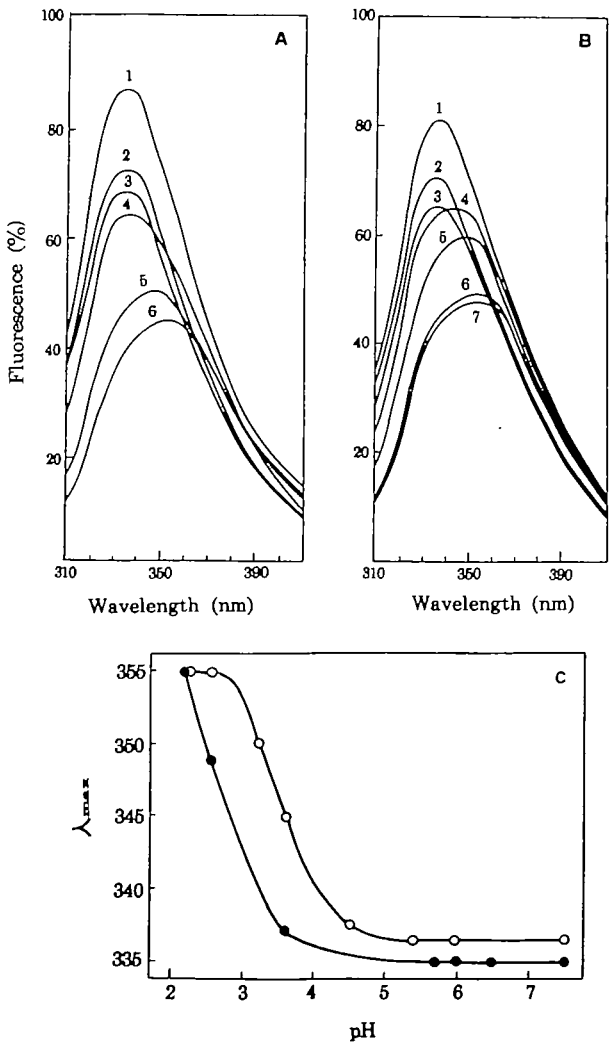

Fig. 1. **Fluorescence emission spectra of YADH during acid-denaturation at low ionic strength.** Enzyme concentrations of holo- and apo-YADH were 0.33 μM. Excitation wavelength was 295 nm. (A) Curves 1-6 represent the fluorescence emission spectra of holo-enzyme at pH 7.5, 6.0, 5.7, 3.7, 2.6, and 2.3, respectively. (B) Curves 1-7 represent the fluorescence emission spectra of apo-enzyme at pH 7.5, 6.0, 5.4, 3.7, 3.2, 2.6, and 2.1, respectively. (C) Relationship between the fluorescence emission maximum of YADH and pH value. Holo-enzyme (●); apo-enzyme (○).

fluorescence change occurs in two stages during acidic denaturation of the holo-enzyme, as can be seen from Fig. 1A. With decreasing pH from 7.5 to 5.5, a marked decrease in emission intensity takes place, but very little, if any, red shift of the emission maximum can be observed. With further decrease of pH, a marked red shift of the emission maximum (from 335 to 355 nm) occurs, together with a further decrease in emission intensity. Both the decrease in emission intensity and the red shift of the emission maximum become maximal in the vicinity of pH 2.0, indicating that the protein is in a relatively unfolded conformational state (U_A state). The fluorescence change of the apo-enzyme also occurs in two stages. However, it is different from that of the holo-enzyme: the apo-enzyme is more sensitive to acid denaturation, since at pH 3.7 the holo-enzyme has only a slight red shift, whereas the apo-enzyme shows a marked red shift. In addition, the red shift of the emission maximum reaches its maximal value (355 nm) in the vicinity of pH 2.5 (Fig. 1B), rather than pH 2.0 for the holo-enzyme. Figure 1C shows the relationship between the red shift of the emission maximum and the pH of the enzyme solution. It can be seen that the apo-enzyme is more sensitive than the holo-enzyme to acid denaturation.

The CD spectra and enzyme activity show that at pH between 7.5 and 5.5, although practically no sign of change is observed in the secondary structure of the enzyme, the activity of the enzyme is markedly decreased and then completely lost. Figure 2 shows the relationship between inactivation and unfolding of the enzyme. These results show that the inactivation of YADH is much more sensitive to pH decrease than the conformational changes of the enzyme molecule.

Acid-Induced Folding of YADH as Revealed by Fluorescence Emission Spectra—At low pH and low concentration of ClO_4^-, anions can induce refolding of acid-denatured proteins (*2*). Figure 3, A and B, shows the effects of different concentrations of ClO_4^- on folding of holo- and apo-YADH denatured in 6 mM HCl, followed in terms of the fluorescence emission. It can be seen that with increasing concentration of $HClO_4$, the fluorescence emission maximum of the acid-denatured enzyme is blue-shifted, from 355 to 335.5 nm for the holo-enzyme, and from 355 to 337 nm for the apo-enzyme. It appears from the results obtained that the tryptophan residues are buried in the hydrophobic interior. This is because the folded protein has a compact structure (hydrophobic core) with a decreased hydrophobic surface area. This conformational state with a compact structure is called the A state (acidic-folding state) (*13*). However, the final fluorescence emission spectrum of the A state is somewhat different from that of the native conformation. As compared with native conformation, the fluorescence intensity of the A state is slightly lower, indicating that the hydrophobic core formed is more mobile than in the native state.

Figure 3C shows the relationship between the blue shift of the fluorescence emission maximum of the acid-denatured enzyme and the concentration of the $HClO_4$. As shown in Fig. 3C, the transitions between the U_A (a

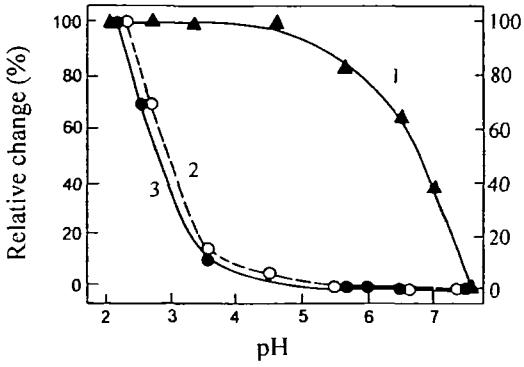

Fig. 2. **Comparison of inactivation and unfolding of YADH at different pH values.** Curves 1-3 represent relative changes in activity (▲), CD intensity at 220 nm (○) and red shift of fluorescence emission maximum (●) at different pH values.

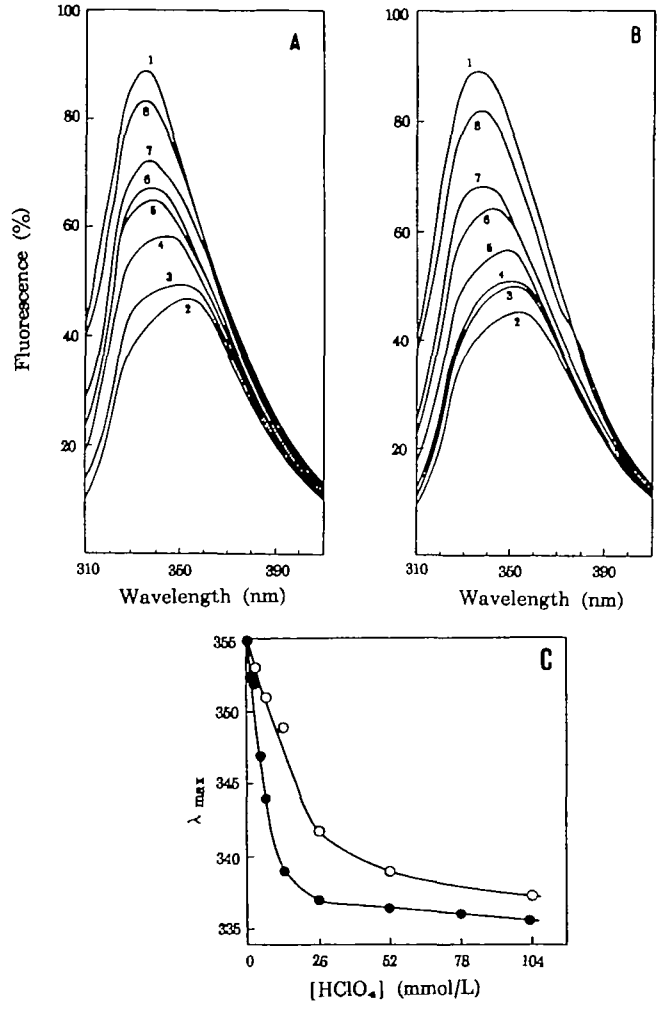

Fig. 3. **Changes in the fluorescence emission spectra during folding of acid-denatured YADH.** All solutions contained 6 mM HCl, except the native state of the enzyme at pH 7.5. Enzyme concentration was 0.33 μM. (A) Holo-enzyme: Curve 1 represents the native state at pH 7.5. For curves 2-8 the $HClO_4$ concentrations (mM) and pH values were 0, 2.3; 2.6, 2.0; 6.5, 1.9; 13, 1.8; 26, 1.5; 52, 1.3; and 104, 1.03, respectively. (B) Apo-enzyme: Curve 1 represents the native state at pH 7.5. For curves 2-8 the $HClO_4$ concentrations (mM) and pH values were 0, 2.2; 2.6, 2.0; 6.5, 1.9; 13, 1.8; 26, 1.5; 52, 1.3; and 104, 1.03, respectively. (C) Relationship between the fluorescence emission maximum and the $HClO_4$ concentration during acid-induced folding of YADH. Holo-enzyme (●); apo-enzyme (○).

relatively unfolded conformational state) and A states (compact structure) of holo- and apo-YADH are cooperative. The midpoints of the transitions of holo- and apo-enzymes are at about 6.5 and 15 mM, respectively. The results obtained suggest that the transition of the holo-enzyme induced by ClO_4^- is more cooperative than that of the apo-enzyme. It is known that under denaturing conditions, many denatured metalloproteins still have metal ion-binding sites. Khan *et al.* reported that the zinc ion was still bound to denatured thermolysin during denaturation at 90°C or by 6 M guanidinium chloride (*14*). It is possible that the acid-unfolded state and the molten globule state of YADH still contain a zinc ion-binding site, and the presence of Zn^{2+} may aid folding into the molten globule state from the acid-unfolded state.

ANS Binding—The fluorescence emission peak of ANS is known to increase when the dye binds to the hydrophobic regions of a protein (*15*). Figure 4 shows the fluorescence spectra of ANS in the presence of the native, acid-unfolded, and acid-induced folded states. As shown in Fig. 4, when ANS molecules are bound to protein in the U_A state or acid-induced refolded state, the fluorescence emission maximum of ANS shifted from 540 to 470 nm, indicating transfer of the ANS molecule to a more hydrophobic environment (*16*). The amounts of ANS bound to holo-enzyme of different folding extents are different. A small increase in fluorescence is observed in the U_A state. As the refolding increases, the fluorescence intensity increases markedly. The result observed suggests that A state is different from native state in terms of the ordered structure. The A state binds ANS strongly. This indicates the presence of hydrophobic regions at the surface of the A state, with its compact structure.

CD Spectra during Folding of Acid-Denatured YADH—Figure 5 shows the CD spectra in the far-UV region of YADH in different concentrations of $HClO_4$ solutions under a low pH condition. The CD spectra of holo- and apo-enzymes at pH 7.5 show a minimum at 208 nm and a maximum at 192 nm, respectively. The CD spectra of the acidic denatured enzyme show a marked negative peak at 198 nm, characteristic of unfolded proteins. As compared with apo- and holo-enzymes at pH 7.5, the secondary structure contents of the acidic denatured enzymes are decreased, but not completely lost, as in the case with 4 M guanidinium chloride. The addition of $HClO_4$ induced folding of the acid-denatured enzymes to form an ordered secondary structure. The CD spectra of refolded enzymes are similar to the spectra of the holo- and apo-enzymes at pH 7.5.

The α-helix contents under different conditions, evaluated from the CD spectra for the native, unfolding and folding enzymes according to the method of Chen *et al.* (*17*), are shown in Table I. It can be seen that the contents of α-helix structure in the folded holo- and apo-enzymes (A state) are similar to those of the same enzymes at pH 7.5. From Table

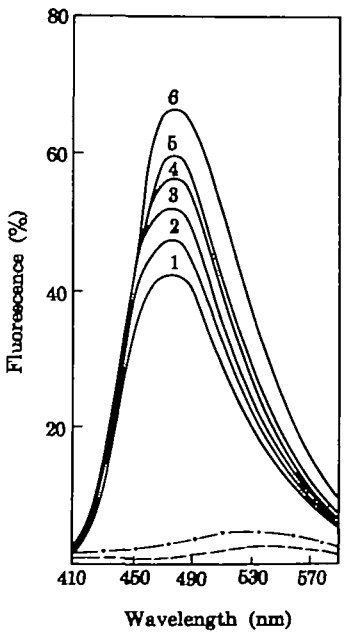

Fig. 4. **Fluorescence emission spectra of ANS bound with refolded holo-YADH.** Concentrations of enzyme and ANS were 0.33 and 10 μM, respectively. For curves 1-6 the $HClO_4$ concentrations were 0, 3.0, 13, 26, 52, and 102 mM, respectively, and the corresponding pH values were 2.2, 2.1, 1.9, 1.5, 1.3, and 1.2, respectively. Curve ---- represents the native state at pH 7.5, and curve —·— represents the spectrum of 10 μ NAS at pH 2.2 in the absence of YADH. The excitation wavelength was 400 nm.

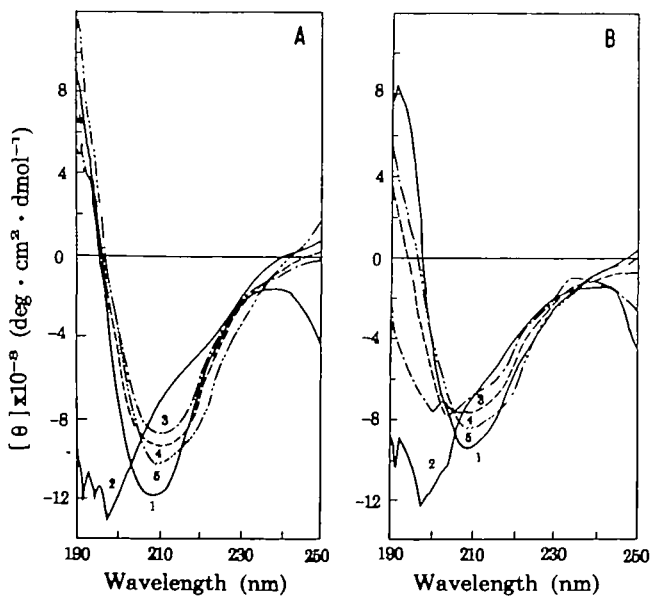

Fig. 5. **Changes of CD spectra during folding of acidic denatured YADH.** All solutions contained 6 mM HCl, except the native enzyme at pH 7.5. Enzyme concentration was 0.33 μM. (a) Holo-enzyme; (b) Apo-enzyme. Curve 1 represents the native state at pH 7.5. For curves 2-5 the $HClO_4$ concentrations were 0, 13, 26, and 52 mM, respectively.

TABLE I. **The α-helix contents of apo- and holo-YADH under different conditions (%).**

Condition	Holo-enzyme	Apo-enzyme
Native state (pH 7.5)	28.8	25.1
In 6 mM HCl (pH 2.2)	12.4	12.5
In 6 mM HCl, 13 mM $HClO_4$ (pH 1.9)	26.5	19.8
In 6 mM HCl, 26 mM $HClO_4$ (pH 1.5)	30.6	23.9
In 6 mM HCl, 52 mM $HClO_4$ (pH 1.3)	33.2	32.3

I, it is clearly seen that the transition from the U_A state to the A state of the holo-enzyme can be completed in 13 mM $HClO_4$, or 26 mM for the apo-enzyme. These results are similar to those observed in the measurements of fluorescence emission spectra. Although the high-resolution X-ray structure of YADH is not available, X-ray crystallographic analysis of horse liver alcohol dehydrogenase has shown that the helical content is 28% (*18, 19*). This result is similar to that obtained from the CD spectra of YADH in the present investigation. It is very interesting that at higher concentrations of $HClO_4$, the contents of α-helix structure in refolded enzymes are slightly higher than those in the native state at pH 7.5.

DISCUSSION

The acid-denatured U_A states of holo- and apo-enzymes, which exist under conditions of low ionic strength at the extremes of pH, have many similar properties. (i) Their intrinsic fluorescence emission spectra have maxima at 355 nm, indicating complete exposure of the tryptophan residues to the solvent. (ii) Their CD spectra show the loss of ordered secondary structure, but the acid-denatured enzymes still possess some residual ordered structure (see Table I). It is suggested that the acid-induced unfolding of YADH is not complete, as described previously (*20-22*).

The results obtained show that under low pH conditions, $HClO_4$ induces folding of acid-denatured YADH to a compact conformation which possesses the properties of molten globule (A state). These properties of the molten-globule state include the following. (i) In the compact conformation, the tryptophan residues are buried. (ii) Compared to the native state, this conformation has more ordered secondary structure. (iii) The fluctuation of the tertiary structure in A state is marked, and a lot of the hydrophobic surface of the molecule is exposed to hydrophilic solvent. The A states of both the holo-YADH and the apo-YADH are relatively stable, and the transitions from the U_A states to the A states show cooperative properties. These results suggest that the molten-globule state may occur during folding of the YADH molecule. It is believed that the molten-globule state may be a common intermediate state in the folding courses of many proteins (*23*).

It is well known that on decreasing the pH from neutrality to approximately pH 2.0, proteins become maximally positively charged, since the p*K*s of most carboxyl groups are greater than 3. The resulting intramolecular repulsion between the positively charged groups leads to unfolding and a relatively extended conformation. The addition of a stronger acid $HClO_4$ increases the concentrations of protons and anions ClO_4^- in the protein solution. Since the protein is already maximally protonated, the addition of more protons has, in principle, no effect on its ionization state. However, the addition of more anions will lead to electrostatic interactions with the positively charged centers on the protein, so as to shield the repulsive forces. The decrease of the internal repulsive forces, which favor unfolding, results in folding of the protein to a more compact structure.

From the present results, it is clear that at pH 7.5, as compared with the holo-enzyme, the fluorescence emission maximum of the apo-enzyme is slightly red-shifted, but the content of α-helix structure is increased. It is possible that after removal of conformational Zn^{2+} by DTT, the loss of disulfide bridges results in slight unfolding of the zinc-containing loop of the enzyme and formation of some ordered secondary structure. However, the apo-enzyme after the removal of conformational zinc is more sensitive to acidic denaturation than the holo-enzyme. The results obtained from fluorescence emission and CD measurements show that the cooperativity of the holo-enzyme is markedly stronger than that of the apo-enzyme, and both the compaction of the hydrophobic parts and the formation of ordered secondary structure of the holo-enzyme are faster than those of the apo-enzyme. It is well known that the presence of Zn^{2+} helps to keep the conformation in a strained state (*24*). The removal of Zn^{2+} leads to an increase in the mobility of the enzyme molecule and dynamic quenching of the fluorescence intensity. Although the apo-enzyme also forms a compact hydrophobic core, its extent of ordering is lower than that of the holo-enzyme. These results clearly demonstrate that the presence of the conformational zinc of YADH helps to fold the molecule and to maintain the native conformation of the enzyme molecule.

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