Role of Quaternary Structure in the Stability of Dimeric Proteins: The Case of Ascorbate Oxidase

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ABSTRACT: Equilibrium denaturation experiments have been performed in order to study the dissociation into monomers and unfolding of the dimeric copper-containing enzyme ascorbate oxidase by urea and guanidine hydrochloride. The process has been followed by fluorescence intensity and anisotropy, by optical density, and by circular dichroism as a function of denaturant concentration. The noncoincidence of the unfolding curves obtained by different techniques suggests that a multiphasic process is occurring. The study of enzymatic activity and aromatic circular dichroism as a function of denaturant concentration shows that the first transition involves a change in the protein tertiary structure which is accompanied by the loss of biological function. Gel electrophoresis, ultracentrifugation, and protein dilution experiments demonstrate that a large fraction of protein molecules is still dimeric during this first transition with a stability which is strictly dependent on the denaturant used. The free energy change from the native form to this intermediate species was estimated to be ≈3.5 kcal/mol. The binding of 1-anilino-8-naphthalenesulfonic acid to the partially unfolded, inactive ascorbate oxidase dimer also suggests a large conformational change accompanied by copper release, allowing the probe to penetrate deep inside the protein structure. Further denaturation to give a fully unfolded form is protein concentration dependent, suggesting that dissociation into monomers is occurring. The monomers appear to be very unstable. No evidence for structured intermediates was in fact detected in the last step of the denaturation process. A three-state model has been used to fit the fluorescence data, and the fractions of different species have been calculated as a function of denaturant concentration. The total free energy change of the unfolding transition using either urea or guanidine hydrochloride is rather small (≈15−16 kcal/mol) and quite comparable to the value found for smaller proteins. The loss of secondary structure which occurs in the second part of the unfolding transition may be described by a simple two-state process which is characterized by a free energy change of 12−13 kcal/mol. These results suggest that the folding process of ascorbate oxidase follows a hierarchical model (Jaenicke, 1991). In this context, the assembly of monomers in a dimeric molecule plays a fundamental role by enhancing the protein stability and driving the final organization of the tertiary structure.

The characterization of the interactions which stabilize the structure of large macromolecules is a relevant topic in biophysics for several reasons. It is well-known that the biological function of a protein is strictly related to its three-dimensional shape which is assumed to depend, in principle, only on the sequence of amino acids, i.e., on the primary structure. Protein folding is therefore based on the interaction of relatively short stretches of the peptidic backbone to form complex domains. Nevertheless, the problem of protein folding *in vivo* is still far from well understood. Unfolding studies have shown that the free energy required for the stabilization of the native 3D structure is in the range 5−30 kcal/mol (Neet & Timm, 1994). The value of the unfolding free energy change, ΔG_{H_2O}, is correlated to the extension of the water-accessible surface and thus depends on the size and conformation of each protein. For several small globular proteins, simple two-state equilibrium transition curves have been successfully used to fit the data (Jaenicke, 1987). However, it has been recently pointed out that the two-state model is often a too simplified version of a more complex unfolding pathway which includes the formation of a metastable intermediate indicated as a "molten globule" (Kuwajima, 1989). The main features of this state are a native-like secondary structure and a fluctuating tertiary structure which leads to a slight increase of the protein volume and to a loosening of long-range intramolecular interactions (Barrick & Baldwin, 1993; Creighton et al., 1996). The presence of intermediates is rather important as they might explain the hierarchy and the high rate of protein folding (Baldwin, 1990). Assuming that the assembling mechanism may involve several intermediate structures, a kinetic hierarchical self-organization has been proposed for both unfolding and refolding processes (Jaenicke, 1991). Two general schemes have been suggested to describe these events (Baldwin, 1990). In the so-called "framework" model, the organization of the secondary structure is supposed to characterize the very first step of the folding transition which is then completed by progressive adjustments of the tertiary

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structure. In a second model, totally folded subdomains are independently formed with an intrinsic high number of tertiary interactions already stabilized. Both models have been found to describe rather well the folding of small globular proteins (Matouschek et al., 1990; Staley & Kim, 1990). However, a generalized theory of the assembling mechanism is not yet available. Quantitative equilibrium unfolding experiments on large polymeric structures are still scarce because of their complexity and low stability. The study of multimeric enzymes is crucial to understand the importance of quaternary structure in the folding process. Several studies clearly show that quaternary structure plays a fundamental role in the stabilization of the native protein form. In particular, it has been shown that in many cases the dissociation process immediately precedes the appearance of unfolded monomeric species (Kwon et al., 1993; Steif et al., 1993; Anil & Rao, 1996) or of collapsed monomers with the peculiar characteristics of a molten globule structure (Silva et al., 1992; Silva et al., 1993; Philo et al., 1993). On the other hand, folded monomeric species (Herold & Kirschner, 1990; Malecki & Wasylewski, 1997) and stable dimeric species (Blackburn & Noltmann, 1981; Ziegler et al., 1993; Clark et al., 1993; Couthon et al., 1995; Dutta et al., 1997) along the denaturation pathway have also been found.

In this paper, we report equilibrium denaturation experiments on ascorbate oxidase (AAO),[1] a dimeric copper-containing enzyme which catalyzes the redox reaction between ascorbate and oxygen. The protein has a molecular weight of about 140 000 and a rather complex structure. Each subunit is made of three distinct domains as shown by X-ray crystallography (Messerschmidt et al., 1989, 1992). We have investigated the interactions which stabilize the native protein form by spectroscopic and enzymatic measurements as a function of urea and GdHCl concentration. The data are consistent with the existence of a rather stable intermediate structure which has lost copper and is therefore inactive. Upon removal of denaturants by progressive dilution or dialysis, it was possible to restore all the spectroscopic features of the native enzyme. Activity was also recovered only after anaerobic readdition of copper, confirming a good reversibility of the unfolding process.

The data have been fitted using ad hoc equations describing the different processes. According to the best fit, a three-state model of unfolding has been proposed and the role of the intermediate structure discussed in detail.

EXPERIMENTAL PROCEDURES

Ultrapure urea and guanidine hydrochloride (GdHCl) were purchased from USB (United States Biochemicals). Stock solutions (9 M and 8 M, respectively) were prepared as previously described (Pace et al., 1989). Ascorbate oxidase from green zucchini was purchased from Boehringer Mannheim. The protein was dissolved in 80 mM potassium phosphate buffer, pH 6.0. Samples for the equilibrium unfolding experiments were prepared in Eppendorf tubes in the presence of increasing amounts of denaturant (yielding a final volume of 1.5 mL) and incubated at 10 °C for at least 15 h. Fully unfolded samples, at a protein concentration 5 times higher than that used in the unfolding experiments, were used for renaturation measurements. In particular, refolding was achieved by dilution in buffers containing decreasing amounts of urea or GdHCl. In order to test the activity of the refolded protein, the samples were furtherly incubated for at least 12 h at 4 °C, in anaerobic conditions, with $CuCl_2$ solution and ascorbate. A 2 h aerobic dialysis against phosphate buffer was then performed to remove the ascorbic acid and the copper in excess.

The enzymatic activity of AAO was measured at 20 °C by recording the decrease of ascorbate absorbance at 265 nm. The presence of copper was measured by atomic absorption on a Perkin Elmer 5000 atomic absorption spectrophotometer equipped with an HGA-500 graphite furnace. A 0.01 mg/mL $CuCl_2$ solution was used as a standard reference, and the measurements were repeated several times in order to get good statistics. Copper release was monitored spectrophotometrically using a Perkin Elmer Lambda-18 spectrophotometer and measuring the absorption at 330 and 610 nm through a 1 cm quartz cuvette.

Spectroscopic Assays. Fluorescence measurements were performed on a photon counting spectrofluorometer (ISS, Model K2, Champaign, IL) equipped with two Glan-Thompson polarizers. The steady-state spectra were corrected taking into account an instrument response curve calculated from standard fluorescent compounds. Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter using 0.5 cm quartz cuvettes in the peptidic and aromatic regions (protein concentration $\approx 4.6 \times 10^{-7}$ M). All experiments were carried out at 20 °C by thermostating the sample holder with an external water circulating bath. ANS binding was studied measuring the fluorescence emission spectra (from 450 to 550 nm, $\Delta\lambda = \pm 2$ nm) of the probe using a 350 ± 2 nm excitation wavelength. All spectra were corrected by blank subtraction taking into account the presence of the denaturant.

Electrophoresis and Ultracentrifugation Measurements. Electrophoresis experiments were performed at several urea concentrations and 10% acrylamide. The gel did not contain sodium dodecyl sulfate and 2-mercaptoethanol. Sedimentation velocity experiments were performed at 40 000 rpm and 20 °C using a Beckman Optima XL-A analytical ultracentrifuge. The movement of the protein toward the bottom of the cell was determined by absorption scans along the centrifugation radius at a wavelength of 280 nm. Sedimentation coefficients were corrected to $s_{20,w}$. For both techniques, bovine serum albumin (BSA) purchased from Sigma Chemicals was used as a standard compound.

Equilibrium Unfolding/Refolding Curves. The unfolding experiments were repeated several times, and the standard deviation was calculated and represented in each figure as an error bar. Since a simple two-state model failed to fit the fluorescence data, we used a three-state approach which was satisfactorily compatible with both GdHCl and urea transition curves. In particular, we assumed the existence of a dimeric intermediate species in equilibrium with both the native structure and the unfolded monomers according to the following scheme:

$$N_2 \overset{K_1}{\longleftrightarrow} I_2 \overset{K_2}{\longleftrightarrow} 2U$$

K_1 and K_2 are related to the respective free energy values

[1] Abbreviations: AAO, ascorbate oxidase; BSA, bovine serum albumin; ANS, 1-anilino-8-naphthalenesulfonic acid; CD, circular dichroism; GdHCl, guanidine hydrochloride.

$\Delta G^{(1)}$ and $\Delta G^{(2)}$ which are supposed to vary linearly with the denaturant concentration [D] (Pace et al., 1989):

$$\Delta G^{(i)} = \Delta G^{(i)}_{H_2O} - m_{(i)}[\mathrm{D}]$$

The measured spectroscopic signal, Y (i.e., steady-state fluorescence anisotropy and fluorescence spectrum peak), was then recalculated as a linear combination of each individual contribution, namely:

$$Y = f_N Y_N + f_I Y_I + f_U Y_U \quad (1)$$

where f_i are the corresponding fractional amplitudes which are related to the equilibrium constants as elsewhere described [see, for example, Clark et al. (1993)]. A nonlinear least-squares fit was used to evaluate the fundamental parameters which characterize the transition (i.e., $\Delta G^{(1)}_{H2O}$, $\Delta G^{(2)}_{H2O}$, m_1, m_2, and Y_I) according to the minimum χ^2 value. Steady-state fluorescence anisotropy data were used taking into account the correction due to the quantum yield of each molecular species (Eftink, 1994). A single transition model according to the equilibrium:

$$N_2 \overset{K_a}{\longleftrightarrow} 2U$$

was sufficient to fit the CD data.

RESULTS

Denaturation of AAO. The GdHCl- or urea-induced unfolding of AAO was followed by several spectroscopic techniques. The transition studied by steady state fluorescence and anisotropy clearly shows a rather complex behavior not compatible with a simple two-state unfolding mechanism (Figure 1a,b). The most relevant feature of these data, which is particularly evident in the case of steady-state anisotropy (panel b), is the presence of a plateau at intermediate concentrations of denaturant(s). This is evident in the presence of both GdHCl and urea, although the two unfolding pathways are different. In agreement with most literature data on other proteins [see, for instance, Gratton et al. (1992) and Couthon et al. (1995)], urea is definitely less efficient than GdHCl, as demonstrated by the higher concentration required to unfold the protein.

In order to ascertain to which extent the secondary structure is affected by the denaturant(s), circular dichroism spectra were measured in the peptidic region, and the intensity at 220 nm was reported in Figure 1c as a function of denaturant concentration. At variance with the fluorescence data, CD does not show any complexity in the unfolding pathway. Interestingly, a substantial decrease in the ellipticity signal may be detected only at the plateau regions, i.e., after ≈1.5 M GdHCl and ≈3 M urea, respectively.

Renaturation of AAO, by dilution of the fully unfolded samples, shows a complete recovery of all the native spectroscopic features (Figure 1 a,b,c). Enzymatic activity was achieved only after a further incubation in anaerobiosis of the refolded samples in the presence of cupric ions. Activity and atomic absorption measurements demonstrated that the recovered biological function was proportional to the amount of reincorporated copper.

Characterization of the Plateau Intermediate. The data reported in Figure 1a,b, as well as the noncoincidence

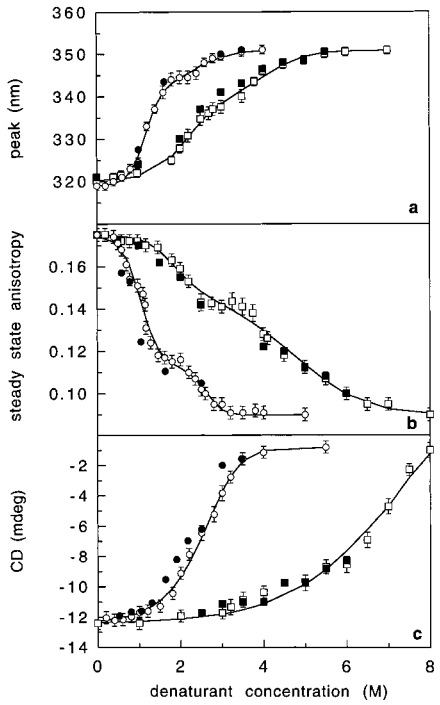

FIGURE 1: Dependence of the fluorescence emission maximum wavelength (panel a), steady-state fluorescence anisotropy (panel b), and circular dichroism at 220 nm (panel c) on urea (squares) and GdHCl (circles) concentration. Solid lines represent the best fits obtained using a three-state (panels a, b) or a two-state (panel c) denaturation model (see Table 1). Refolding curves are represented as filled symbols without error bars.

between the transition curves obtained by fluorescence and circular dichroism spectroscopy, point to the presence of at least one intermediate state. The properties of this intermediate have been analyzed in several ways. First, the unfolding of the three-dimensional structure has been studied by measuring the CD spectra in the aromatic region (250−300 nm). The CD intensity at 270 nm, which reflects the asymmetry of the aromatic side chain environment, is reported in Figure 2a for both denaturants. The data demonstrate that the first part of the denaturation curves, (0.0−1.3) M GdHCl and (0.0−3.5) M urea, respectively, is associated to a progressive loss of protein tertiary structure, rather than disruption of the secondary structure (see also Figure 1c). We checked if there was a relationship between the loss of enzymatic activity and that of tertiary structure. Interestingly, the biological function is totally lost at the plateau (Figure 2b) perhaps associated to the loss of copper.

The copper ions of AAO may be distinguished in different classes according to their specific spectroscopic features (Mondovi' & Avigliano, 1984). The absorptions at 330 and 610 nm, which are typical of the two different types of copper, were measured as a function of urea concentration (Figure 2c). The data show that copper-related absorptions fade away in parallel to the loss of enzymatic activity and the CD signal in the region of aromatic amino acids. Similar results were obtained in the case of GdHCl (data not shown). Again, a stronger effect of GdHCl was detected as the plateau region is reached at lower denaturant concentration.

Stability of the Dimeric Enzyme. With preliminary information on the intermediate structure at hand, we investigated the course of the quaternary structure loss upon denaturation. In order to test whether monomerization occurred, steady-state anisotropy and circular dichroism were

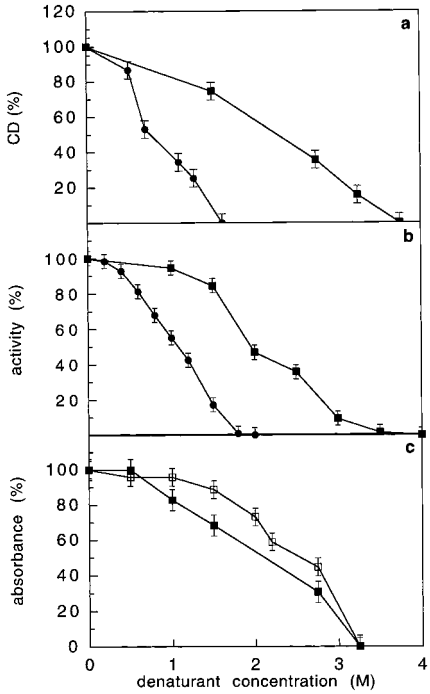

FIGURE 2: Panel a: relative circular dichroism of AAO at 270 nm as a function of urea (squares) and GdHCl (circles). Panel b: percentage of AAO biological activity in the presence of urea (squares) and GdHCl (circles). Panel c: relative absorbance of AAO at 610 nm (open squares) or 330 nm (filled squares) at increasing urea concentrations.

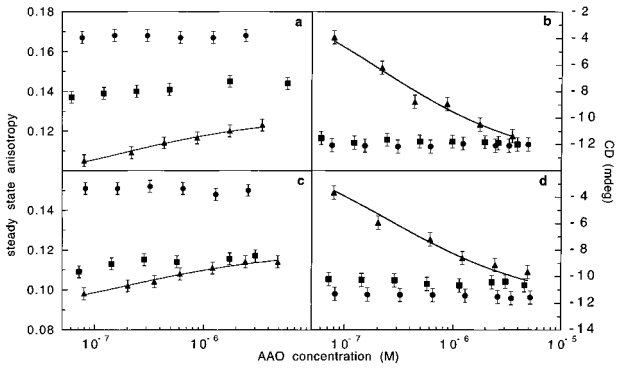

FIGURE 3: Steady-state anisotropy (panels a and c) and CD (panels b and d) of AAO in urea (a and b) or GdHCl (c and d) as a function of protein concentration. Dilution experiments were performed in the presence of different amounts of urea (1.6 M, circles; 3.0 M, squares; 4.5 M, triangles) and GdHCl (1.0 M, circles; 1.8 M, squares; 2.4 M, triangles). The fluorescence emission anisotropy was monitored at 337 nm while the CD signal was measured at 220 nm. Solid lines represent the best fits obtained using a two-state dissociation model, $I_2 \leftrightarrow 2$ U, with dissociation constants of the order of $\approx 0.6 \times 10^{-6}$ M.

measured in the presence of different amounts of denaturants, and at different protein concentrations (maximum dilution factor $\approx 10^2$). The data, reported in Figure 3, demonstrate that the measurements were independent of the enzyme concentration only below the plateau region, i.e., at 1.0 M GdHCl and 1.6 M urea. Instead, a relevant effect of protein dilution was detected at higher denaturant concentrations.

Electrophoresis experiments with AAO in the presence of increasing amounts of urea were also performed (Figure 4) running BSA simultaneously as a reference. BSA was well suited because of its stability at these urea concentrations and of the similarity of its molecular weight (≈68 000) to that of AAO subunits (≈70 000). Figure 4 indicates that

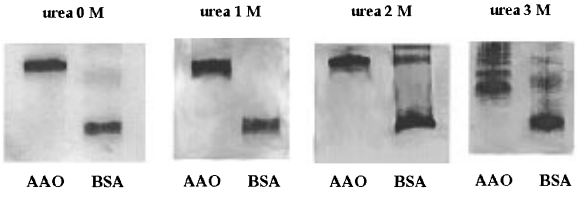

FIGURE 4: Electrophoresis in a native gel of AAO at increasing urea concentrations. BSA was used as a reference protein.

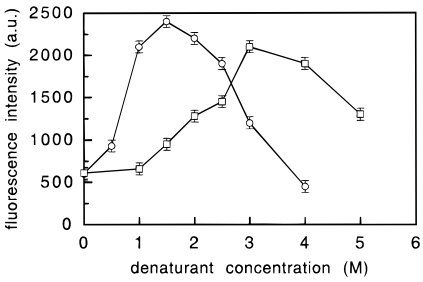

FIGURE 5: Dependence of ANS fluorescence intensity (measured at 475 nm in the presence of AAO) on the urea (squares) or GdHCl (circles) concentration. The excitation wavelength was 350 ± 2 nm.

the AAO mobility was unmodified before the plateau, i.e., at 1 or 2 M urea. Only at higher concentrations (Figure 4) was a significant appearance of faster moving species observed. Therefore, the first part of the unfolding transition is characterized by AAO molecules which migrate as dimers.

Finally, ultracentrifugation experiments were carried out in the presence of 1.2 M GdHCl and 2.2 M urea. The respective sedimentation coefficients were 6.7 and 7.4 S as compared with the value of 7.9 S for the native sample. This result suggests that at low urea concentration the majority of the protein molecules are still in the dimeric form while a small amount of monomers might be already present in GdHCl.

Ans Binding. ANS is a fluorophore which increases its fluorescence quantum yield upon binding noncovalently to hydrophobic regions of proteins. ANS was added to AAO at a final concentration ratio of 5:1 ([ANS] ≈5 μM, [AAO] ≈1 μM). When the protein was in its native conformation the fluorescence spectrum of ANS was similar to ANS in buffer (data not shown), indicating that native enzyme does not bind the fluorophore. However, upon increasing the concentration of urea or GdHCl the fluorescence intensity measured at 475 nm increased about ≈4 times (Figure 5). This effect may be related to the loosening of tertiary structure and to progressive exposure of hydrophobic patches on the protein surface. A peak of fluorescence intensity was observed around 3 M urea and 1.5 M GdHCl, but a further increase of denaturant concentration caused a decrease of ANS fluorescence (Figure 5). This may indicate that the protein is no more able to bind ANS at higher denaturant concentrations because of the progressive loosening of the surface's hydrophobic patch. The possibility that the denaturant itself decreases the affinity of ANS for hydrophobic surfaces seems to be ruled out as similar effects have been obtained when unfolding the protein by temperature. In particular, when the AAO sample was cooled down very quickly (in order to avoid the refolding process), the ANS added in solution did not show any significant fluorescence signal (data not shown). It is important to note that the

maximal ANS binding to AAO occurred in the first part of the unfolding transition, i.e., in the plateau region where the presence of intermediate species was observed.

DISCUSSION

In the past years, the number of papers dealing with protein folding has been growing exponentially thanks to new biochemical and biophysical techniques and to molecular engineering. The final goal of these experiments is the possibility of predicting the three-dimensional shape of a protein, once the complete sequence of its primary structure is known. More recently this problem attracted great interest also in clinical research because it has been found that wrong protein folding *in vivo* might induce the formation of aggregates responsible for important pathologies (Taubes, 1996). The understanding of the protein quaternary structure is therefore crucial for preventing uncontrolled polymerization followed *in vivo* by precipitation which may cause such diseases. Therefore, studying the stability of oligomeric proteins *in vitro* as a function of their environmental chemicophysical conditions is important.

Recently the unfolding of several dimeric proteins has been analyzed, and in most cases, a simple two-state denaturation model was found to satisfactorily fit the data (Neet & Timm, 1994). However, it should be noted that almost all the proteins studied had a relatively small molecular mass (30−40 kDa). Whenever the denaturation of larger enzymes (40−90 kDa) was considered in detail, the presence of stable intermediates increased the complexity of the unfolding pathway (Cheng et al., 1993; Clark et al., 1993; Sacchetta et al., 1993; Couthon et al., 1995). Ascorbate oxidase is a relatively large molecule (140 kDa) if compared with most globular proteins already studied in terms of the effect of denaturants. Differential scanning calorimetry (Savini et al., 1990) has demonstrated that thermal denaturation of AAO follows a transition which is more complicated than a single two-state equilibrium. A comparison of the data reported in this paper from several independent measurements, namely, steady-state fluorescence and CD (Figure 1), indicates that the denaturation pathway of AAO in urea as well as in GdHCl may not be interpreted by a simple two-state mechanism. Thus, a three-state process has been taken into account. Before the plateau region, the electrophoresis in urea (Figure 4) and the dilution experiments (Figure 3) support the hypothesis that the intermediate species of AAO still have a dimeric structure. Therefore, assuming that the dissociation is immediately followed by a collapse of the tertiary structure, the fluorescence data have been fitted according to the process:

$$N_2 \leftrightarrow I_2 \leftrightarrow 2U$$

and the main parameters of the model have been calculated (Table 1). The overall free energy change involved in the transition from the native dimer to the inactive intermediate (≈3.5 kcal) corresponds roughly to 20% of the total stabilization energy. A similar value has been also obtained for bacterial luciferase (Clark et al., 1993) which, however, is a heterodimer, much smaller in size than AAO. This finding suggests that dimeric intermediates in the unfolding of proteins might be common despite the different size and three-dimensional structure. The second part of the denaturation transition is characterized by larger values of

Table 1: Thermodynamic Parameters Characterizing the Biphasic Unfolding Transition of AAO[a]

	Y_I	m_1 [kcal/(mol·M)]	m_2 [kcal/(mol·M)]	$\Delta G^{(1)}_{H_2O}$ (kcal/mol)	$\Delta G^{(2)}_{H_2O}$ (kcal/mol)
			Urea		
$\langle r \rangle$	0.154 ± 0.003	1.8 ± 0.4	0.9 ± 0.2	3.8 ± 0.8	13.1 ± 1.2
λ_{max} (nm)	336 ± 3	1.5 ± 0.2	1.4 ± 0.2	3.2 ± 0.4	14.0 ± 0.9
CD			0.9 ± 0.1		13.6 ± 0.5
			GdHCl		
$\langle r \rangle$	0.120 ± 0.004	2.9 ± 0.3	1.8 ± 0.3	3.0 ± 0.3	12.8 ± 0.7
λ_{max} (nm)	340 ± 3	3.2 ± 0.3	1.5 ± 0.2	3.6 ± 0.4	11.8 ± 0.6
CD			1.8 ± 0.1		12.7 ± 0.2

[a] Parameters have been obtained by fitting the corrected anisotropy ($\langle r \rangle$) data, the CD, and the position of the spectrum peak (λ_{max}) as described under Experimental Procedures.

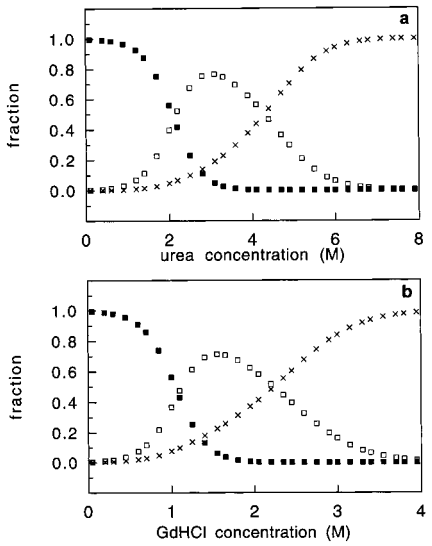

FIGURE 6: Percentage of native (filled squares), intermediate (open squares), and unfolded (crosses) molecules upon unfolding of AAO in urea (panel a) and GdHCl (panel b).

$\Delta G^{(2)}_{H_2O}$ (3−4 times $\Delta G^{(1)}_{H_2O}$) which are in good agreement with those obtained fitting the far-UV CD data by a simple two-state unfolding model (Table 1). The intermediate species is therefore energetically closer to the native dimer than to the unfolded monomers. According to the values of Table 1, it was also possible to evaluate the partition function representing the population of each molecular species (i.e., folded, intermediate, and unfolded) at different denaturant concentrations (Figure 6a,b). The percentage of intermediates midway in the unfolding transition is around 75%, while (15−20)% of the protein molecules were in a fully unfolded conformation. This result might explain the lower values of the sedimentation coefficients found in the presence of denaturants.

Recently it has been pointed out that for small dimeric proteins, which undergo a two-state denaturation process, a direct relationship exists between their molecular weight and the overall free energy change of unfolding, ΔG_{tot} (Neet & Timm, 1994). Interestingly, a rather small ΔG_{tot} value (≈16 kcal/mol) has been obtained for AAO, suggesting that this correlation may not hold for large dimeric structures. A possible role of copper ions in the overall stability of AAO might be envisaged on the basis of the X-ray structure (Messerschmidt et al., 1992) and of experiments on heat-induced denaturation (Savini et al., 1990). We have shown that ANS binds only to the intermediate dimeric species

(Figure 5). It is possible to argue that a loosening of tertiary structure may release (some) copper from the protein, leading to the loss of enzymatic activity and to the exposure of hydrophobic surfaces. Most of these features recall those of the molten globule state already described in the unfolding of smaller proteins (Kuwajima, 1989; Jaenicke, 1991). A similar structure, which may be in principle called the "molten dimer" state, could be proposed in the case of larger oligomeric proteins like AAO. It is well-known (Creighton, 1993) that both urea and GdHCl act as ligands, binding to the surface of folded proteins. Preferential interactions to the external regions of AAO might be the principal reason for a local unfolding of the protein structure, far from the dimeric interface. Thus, in contrast with the classical definition of a molten globule, the unfolding of the intermediate AAO species is only restricted to an outer layer of the dimer. In this context, the quaternary structure plays an important structural and functional role, keeping the two subunits together and stabilizing the two monomers. Furthermore, as the parameter m_1 is always greater than m_2 (Table 1), the degree of cooperativity of the folding mechanism is higher in the very last step of the AAO assembling process. In other words, once the quaternary structure is formed, it provides a suitable conformation for the progressive organization of the tertiary structure and the building up of the copper ligand fields. This schematic picture of the AAO folding mechanism seems in good accordance with the "framework" model already proposed for smaller proteins.

In conclusion, we have shown that the unfolding pathway of AAO is complex and that there is experimental evidence for, at least, one intermediate species. This intermediate, which may be regarded as a "molten dimer" state, is described by the following characteristics: (i) the presence of dimers associated to a native-like secondary structure; (ii) the absence of biological activity; (iii) the loss of tertiary structure which provides the scaffolding for the copper binding sites, responsible for the electron-transfer reaction; (iv) the partial exposure of hydrophobic surfaces which allows ANS binding; (v) a good reversibility to the native state which probably occurs with a cooperative mechanism once the denaturant is properly removed.

These features, which partially resemble those of a molten globule state, are all pointing to the role of quaternary structure in the overall stability of proteins.

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