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Biochemical and Biophysical Research Communications 326 (2005) 108–114
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Equilibrium unfolding of an oligomeric protein involves formation of a multimeric intermediate state(s)

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Received 18 October 2004
Available online 18 November 2004

Abstract

Superoxide dismutases (SODs) are important metalloenzymes which protect cells against oxidative stress by scavenging reactive superoxides. Missense mutations in SODs are known to lead to some familial cases of amyotrophic lateral sclerosis and several forms of cancers. In the present study, we investigate the guanidinium hydrochloride (GdnHCl)-induced equilibrium unfolding of apo-manganese superoxide dismutase (apo-MnSOD) isolated from *Vibrio alginolyticus* using a variety of biophysical techniques. GdnHCl-induced equilibrium unfolding of apo-MnSOD is non-cooperative and involves the accumulation of stable intermediate state(s). Results of 1-anilino-8-naphthalene sulfonate binding experiments suggest that the equilibrium intermediate state(s) accumulates maximally in 1.5 M GdnHCl. The intermediate state(s) appears to be obligatory and occurs both in the unfolding and refolding pathways. Size-exclusion chromatography and sedimentation velocity data reveal that the equilibrium intermediate state(s) is multimeric. To our knowledge, this is the first report of the identification of a multimeric intermediate in the unfolding pathway(s) of oligomeric proteins. The formation and dissociation of the multimeric intermediate state(s) appears to dictate the fate of the protein either to refold to its native conformation or misfold and form aggregates as observed in amyotrophic lateral sclerosis.

Keywords: Protein; Unfolding; Oligomeric; Intermediate

Protein folding is an intricately complicated process in which an ensemble of randomly disordered structures is rapidly guided to form a unique native ordered structure [1]. Our current understanding on the rules governing protein folding is largely based on the thermodynamic and kinetic studies on small, single domain proteins [2–5]. These model systems are chosen because the folding is reversible and they give a simplified view of folding [6]. Although the folding of individual polypeptide chains of oligomeric proteins might be similar to the single domain monomeric proteins, the role(s) of subunit–subunit interactions or interactions between domains on the overall stability and integrity could only be addressed in oligomeric proteins [7].

Manganese/copper/zinc (Mn/Cu/Zn) superoxide dismutases (SODs) are dimeric with identical subunits [8,9]. Superoxide dismutases (SODs) (EC 1.15.1.1) are important antioxidant metalloenzymes protecting cells against oxidative stress arising from reactive oxygen species [10]. Superoxide dismutases protect cells against oxidative damage by catalyzing the dismutation of two molecules of superoxide to oxygen and hydrogen peroxide [11]. SOD has been linked to some familial cases of amyotrophic lateral sclerosis (FALS), a fatal neurodegenerative disease that kills motor neurons [12,13]. There is significant evidence indicating that FALS may be another example of a protein conformational disorder such as prion diseases, Ig light chain disorders,

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.211

and *trans*-thyretin amyloidoses wherein naturally occurring proteins are altered or mutated, and the variant proteins misfold to form aggregates [14,15]. In FALS, inclusions (i.e., protein aggregates) that are intensely immunopositive for SOD are observed in motor neurons and astrocytes [16]. Therefore, investigation of the stability and folding/unfolding properties of SODs is important for understanding the molecular basis for misfolding/aggregation of these proteins leading to FALS. It is in this context, in the present study, we investigate the GdnHCl-induced equilibrium unfolding of a dimeric manganese superoxide dismutase (MnSOD) isolated from *Vibrio alginolyticus* [17]. Unlike the classical mechanism of unfolding described for oligomeric proteins, equilibrium unfolding of MnSOD is shown to proceed non-hierarchically involving the accumulation of a unique high molecular weight intermediate. To our knowledge, this is the first report of the identification of a multimeric intermediate(s) in the equilibrium folding/unfolding of oligomeric proteins. The formation and dissociation of the multimeric equilibrium intermediate appears to dictate the productive folding of the protein to its native state or the non-productive folding leading to misfolding/aggregation of the protein.

Materials and methods

Ni–NTA column was obtained from Qiagen (Germany). GdnHCl were purchased from AMRESCO, USA. All other chemicals used were of high quality analytical grade.

Protein expression and purification. A pET20b plasmid containing wild type Mn–SOD gene (from *V. alginolyticus*) with *Nde*I and *Xho*I restriction sites was transformed into competent *Escherichia coli BL21* (*DE3*) *pLys S*. The cells were grown at 300 K in minimal medium supplemented with 100 μg/mL ampicillin. Protein expression was induced by the addition of 0.4 mM IPTG when the culture medium absorbance at 600 nm reached 0.6. The cells were allowed to grow for an additional 3.5 h. The cells were harvested by centrifugation at 6000 rpm for 20 min. Cell pellets were resuspended in the binding buffer (25 mM phosphate buffer, pH 7.5, containing 500 mM NaCl). Cells were disrupted by French press at 1000 psi and the cell debris was cleared by centrifugation. The expression of the target protein was checked by SDS–PAGE.

Clear lysate was applied to Ni–NTA column and washed with 5 bed volumes (50 mL) of the binding buffer (as described above). Protein elution was monitored by 280 nm absorbance. After the absorbance reached the baseline, the Ni–NTA column was further washed sequentially with 2 bed volumes (20 mL) each of 20 mM imidazole and 35 mM imidazole dissolved in 25 mM phosphate buffer (pH 7.5). The bound protein (apo-MnSOD) was eluted out of the Ni–NTA column using 25 mM phosphate buffer (pH 7.5) containing 250 mM imidazole. The expression and purification of the target protein was checked by 15% SDS–PAGE. Buffer exchange was achieved by ultrafiltration using an Amicon set-up. The homogeneity of the protein was assessed by SDS–PAGE. The authenticity of the sample was further verified by electron spray (ES) mass spectrometry. The protein concentration was routinely determined using the molar extinction coefficient ($\varepsilon_{280} = 4.694 \times 10^4\ M^{-1}\ cm^{-1}$) of the protein.

Steady state fluorescence measurements. All fluorescence spectra were acquired on a Hitachi F-2500 spectrofluorimeter at room temperature (300 K). The intrinsic fluorescence measurements were carried out at a protein concentration of 100 μg/mL, using an excitation wavelength of 280 nm. Both the excitation and emission slits were set at 5 nm. ANS binding affinity to apo-MnSOD at various concentrations of GdnHCl was monitored with the excitation wavelength set at 397 nm. The concentrations of ANS and the protein used were 100 and 4.2 μM, respectively. All solutions for intrinsic and ANS fluorescence were prepared in 25 mM phosphate buffer (pH 5.0) containing 50 mM NaCl and 50 mM ammonium sulfate.

Circular dichroism measurements. Circular dichroism (CD) measurements were conducted at 298 K on a Jasco J-720 spectropolarimeter. The conformational change(s) in the protein (apo-MnSOD) at various molar concentrations of GdnHCl were monitored by ellipticity changes in the near (250–320 nm) and far UV (200–250 nm) regions using quartz cells of pathlengths, 1 and 0.2 mm, respectively. CD spectrum of each sample is an average of at least five scans. The concentration of the protein used was 1 mg/mL, dissolved in 25 mM phosphate buffer (pH 5.0) containing 50 mM NaCl and 50 mM ammonium sulfate.

GdnHCl-induced unfolding data of apo-MnSOD were analyzed using the Gibbs–Helmholtz equation assuming that the unfolding of the protein proceeds via a two-state mechanism (native state to unfolded state). The fraction of unfolded species (f_U) at each concentration of the denaturant was estimated using the equation

$$f_U = (Y_N - Y_X)/(Y_N - Y_U)$$

in which "Y" is the observed fluorescence or CD signal. "Y_N" and "Y_U" are the values of "Y" for the folded and unfolded forms, respectively. "Y_X" is the signal measured at various concentrations of the denaturant.

Size-exclusion chromatography. All gel filtration experiments were carried out at room temperature on a Superdex-200 column (HiLoadTM 16/60) using an AKTA FPLC device (Amersham–Pharmacia Biotech). The column was equilibrated with 1.5 bed volumes of 25 mM phosphate buffer (pH 5.0) containing 50 mM NaCl and 50 mM ammonium sulfate, and appropriate concentrations of GdnHCl. Protein peaks were detected by their 280 nm absorbance.

Analytical ultracentrifugation analysis. The molecular mass of the protein under various concentrations of GdnHCl was estimated by a Beckman-Coulter XL-A analytical ultracentrifuge with an An-60Ti rotor. Sedimentation velocity experiments were performed at 298 K and 35,000 rpm with standard double sector centerpieces. The UV absorption was scanned every 5 min for 18 h. The data were analyzed with the SedFit v.8.3 program.

Results and discussion

GdnHCl-induced equilibrium unfolding of apo-MnSOD is not cooperative

MnSOD isolated from *V. alginolyticus* is a 47 kDa dimeric protein and shares a high degree of amino acid sequence homology with other manganese SODs isolated from other sources [17]. MnSODs typically contain 3 β sheets and 10 α helices (Fig. 1). MnSOD from *V. alginolyticus* contains six tryptophan residues dispersed uniformly at various locations in the protein. Fluorescence spectrum of apo-MnSOD excited at 280 nm shows an emission maximum at 332 nm suggesting that the tryptophan residues in the protein are located in the interior of the protein (Fig. 2, inset). In the denatured state in 6 M GdnHCl, the wavelength of maximum emission shifts to 350 nm (Fig. 2, inset). These spectral

Fig. 1. Three-dimensional structure of apo-MnSOD from *Thermus thermophilus*.

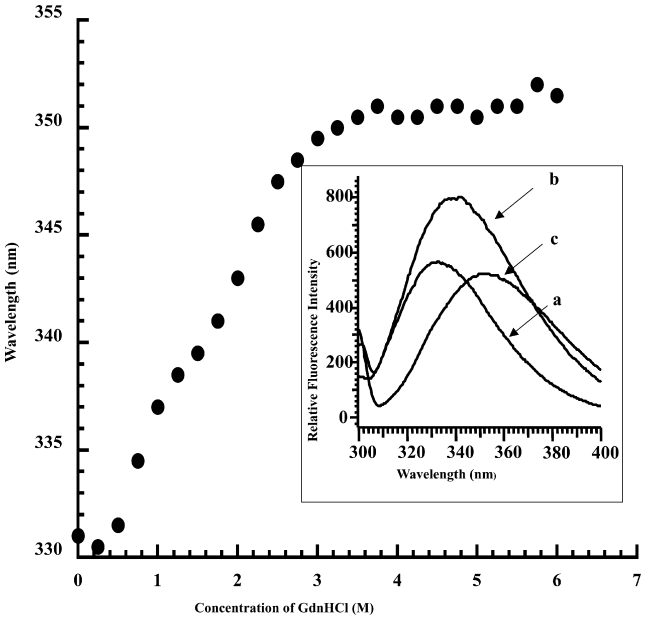

Fig. 2. GdnHCl-induced equilibrium unfolding profile of apo-MnSOD monitored by changes in the wavelength of maximum emission. The unfolding profile shows a plateau between 1.0 and 2.0 M GdnHCl, suggesting accumulation of an equilibrium intermediate state(s). The inset figure show the fluorescence spectra of apo-MnSOD in (a) 0 M GdnHCl, (b) 1.5 M GdnHCl, and (c) 3 M GdnHCl. The wavelength of maximum tryptophan emission shows a progressive red shift with the increase in the concentration of the denaturant (GdnHCl), suggesting that the changes in the intrinsic tryptophan fluorescence are a reliable probe to monitor the global structural changes accompanying unfolding of apo-MnSOD.

features suggest that the changes in fluorescence could be reliably used to characterize the global structural changes occurring during the unfolding of the protein.

GdnHCl-induced equilibrium unfolding of MnSOD in pH 7.0 (at 298 K) is monitored by changes in the emission intensity and shift in the wavelength of emission maximum. The protein begins to unfold beyond 0.3 M GdnHCl. Complete unfolding of the protein is achieved in 3 M GdnHCl and no significant changes in the unfolding profile are discerned beyond this concentration of the denaturant (Fig. 2). GdnHCl-induced equilibrium unfolding of apo-MnSOD is reversible (data not shown). Critical analysis of the GdnHCl-induced unfolding profile of MnSOD shows that the unfolding of the protein does not follow a two-state mechanism (native state–denatured state(s)). The unfolding profile shows a minor shoulder between 1.0 and 2.0 M GdnHCl suggesting the accumulation of stable equilibrium intermediate(s) in the GdnHCl-induced unfolding pathway(s) (Fig. 2).

To ensure that the changes in the tryptophan fluorescence reliably report the global conformational changes in the protein, we investigated the GdnHCl-induced unfolding of apo-MnSOD using far- and near-UV CD spectroscopy. Far-UV CD spectrum of apo-MnSOD shows two negative bands at 208 and 222 nm, implying that portion(s) of the backbone of the protein (apo-MnSOD) is predominantly in a helical conformation. This observation is consistent with the three-dimensional structure of apo-MnSOD which shows the presence of 10 helices [17]. The near UV CD spectrum, which signifies the tertiary structural interactions in the protein, shows a positive ellipticity centered at about 280 nm. GdnHCl-induced equilibrium unfolding profile of apo-MnSOD monitored by ellipticity changes at 222 and 285 nm shows a similar trend as shown by the one obtained using changes in the steady state tryptophan fluorescence (Fig. 3). However, the GdnHCl-induced

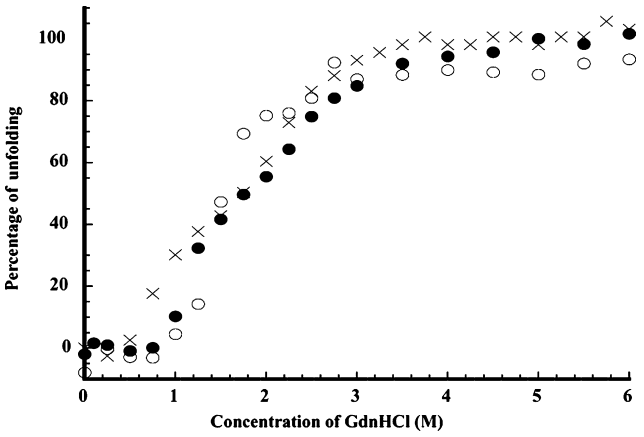

Fig. 3. GdnHCl-induced unfolding profile of apo-MnSOD monitored by changes in the intrinsic tryptophan fluorescence intensity at 350 nm (cross marks), ellipticity changes at 222 nm (closed circles), and at 280 nm (open circles). It could be seen that the unfolding profiles generated using three different spectral probes are non-superimposable, suggesting that the unfolding of the protein is non-cooperative and proceeds with the accumulation of stable equilibrium intermediate(s).

unfolding profiles of the protein generated using these three spectroscopic probes are not completely superimposable (Fig. 3). Such non-coincidence of unfolding profiles obtained using various spectroscopic techniques is indicative of accumulation of stable equilibrium intermediate(s) in the unfolding pathway of proteins [4]. Therefore, it appears that the GdnHCl-unfolding of apo-MnSOD is non-cooperative and proceeds via the formation of stable intermediate(s). It should be mentioned that GdnHCl-induced equilibrium unfolding of holo MnSOD (with the bound metal, Mn^{2+}) also does not follow a two-state mechanism (native state to denatured state(s)).

Unfolding of GdnHCl involves the formation of a stable intermediate(s)

1-Anilino-8-naphthalene sulfonate (ANS) is a fluorescent probe that is known to reliably report the accumulation of stable intermediate(s) with solvent-exposed non-polar surfaces [18,19]. This fluorescent probe has been immensely useful in the identification of equilibrium intermediates such as the molten globule states [20]. In this context, we examined the binding affinity of ANS to the protein (apo-MnSOD) as a function of GdnHCl concentration. The relative fluorescence intensity of the dye (ANS) at 510 nm does not show significant change(s) below a GdnHCl concentration of 1.0 M (Fig. 4). However, dramatic changes in the emission intensity at 510 nm could be discerned beyond 1.0 M GdnHCl and the fluorescence intensity (at 510 nm) reaches a maximum value at a denaturant concentration of about 1.5 M (Fig. 4). In fact, the emission intensity of the dye upon binding to the protein in 1.5 M GdnHCl is more than 5 times that observed when it binds to the native state conformation (in 0 M GdnHCl, Fig. 4, inset). In addition, the wavelength of maximum emission of the dye shows a 32 nm blue shift (from 510 to 478 nm), suggesting that a stable equilibrium intermediate(s), with solvent-exposed non-polar surfaces, accumulates maximally in 1.5 M GdnHCl (Fig. 4, inset). Beyond 1.5 M GdnHCl, the ANS emission intensity progressively decreases and reaches a value similar to the one obtained in the native state. As mentioned earlier, the unfolding of apo-MnSOD is reversible and ANS binding experiments reveal that the intermediate state(s) accumulates maximally in 1.5 M GdnHCl even in the refolding pathway(s) (data not shown). These results suggest that the equilibrium intermediate that populates maximally in 1.5 M GdnHCl is an obligatory folding intermediate which accumulates both in the refolding and unfolding pathways of the protein.

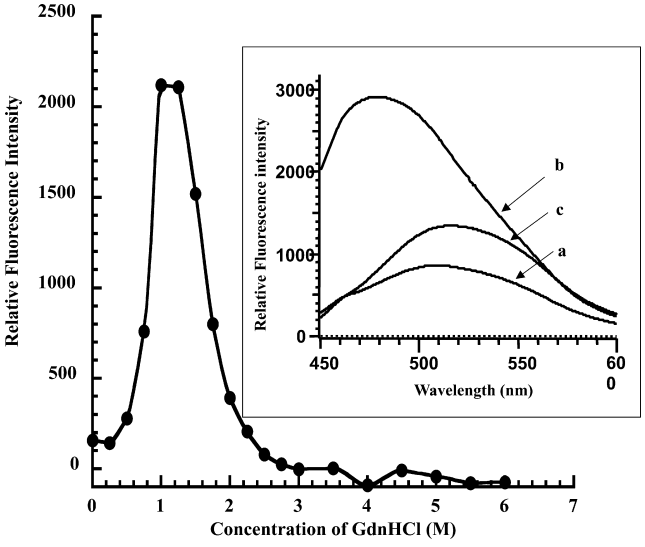

Fig. 4. ANS binding affinity of apo-MnSOD at various concentrations of GdnHCl. Inset figure shows that emission spectra of ANS in the presence of 10 μM of apo-MnSOD in (a) 0 M GdnHCl, (b) 1.5 M GdnHCl, and (c) 3.0 M GdnHCl. The ANS emission intensity (in the presence of the protein) at 510 nm in 1.5 M GdnHCl is at least 5 times of that observed in the absence of the denaturant. In addition, the dye (ANS) upon binding to the protein (apo-MnSOD) in 1.5 M GdnHCl shows a 32 nm blue shift (from 510 to 478 nm) indicating that an equilibrium intermediate with solvent-exposed, hydrophobic surface(s) accumulates maximally in 1.5 M GdnHCl.

The equilibrium intermediate is a multimeric species

Size-exclusion chromatography (SEC) is a useful technique to obtain information of integral changes in molecular dimensions under the influence of a denaturant [21]. This technique has been successfully used to identify and obtain hydrodynamic data on stable intermediates in the folding/unfolding pathway(s) of proteins [21,22]. In general, retention (elution) time of a protein from a size-exclusion column bears a linear and inverse relationship with its molecular mass. Under similar experimental conditions, proteins with larger molecular mass elute out with lower retention times than those with lower molecular mass. Apo-MnSOD in its native conformation, in the absence of the denaturant in 10 mM phosphate buffer (pH 5.0, containing 50 mm NaCl and 50 mM ammonium sulfate), elutes as a single peak with a retention time of 80.1 min (Fig. 5). The retention time of MnSOD extrapolated to the standard curve, which is constructed using 5 standard proteins (RNase A, 13.7 kDa; newt FGF, 16.1 kDa; carbonic anhydrase, 29 kDa; lactoglobulin A, 36.0 kDa, and bovine serum albumin, 66.2 kDa) under similar experimental conditions, corresponds to a homodimer with an estimated molecular weight of around 47 kDa. Interestingly, at around 1.5 M GdnHCl, the elution profile of the protein shows two peaks, one of which corresponds to the native state of the protein (77.5 min). The second peak elutes with a significantly lower retention time (45.4 min), implying the formation of a multimeric species with a molecular weight significantly larger than

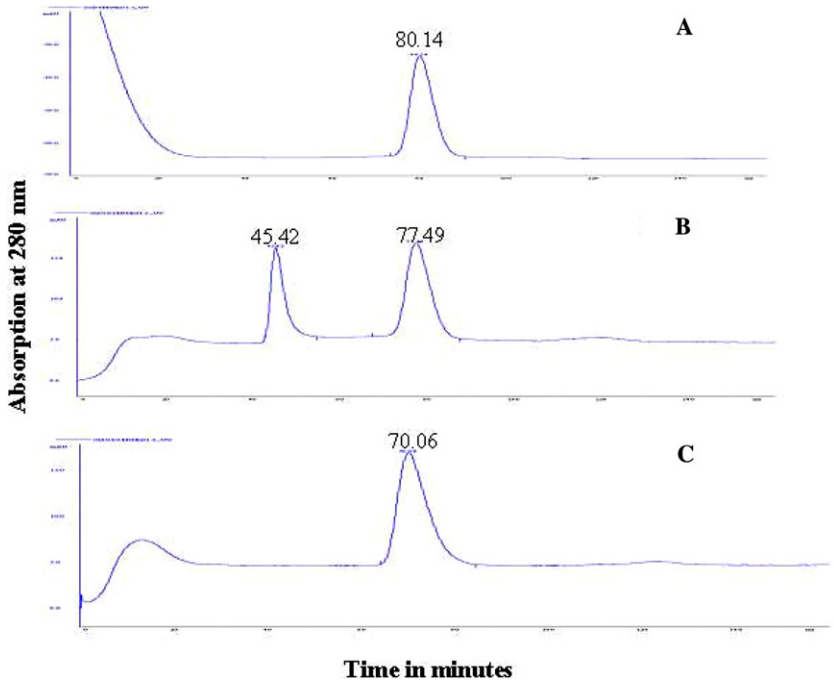

Fig. 5. Size-exclusion chromatography profiles of MnSOD in 0 M GdnHCl (A), 1.5 M GdnHCl (B), and 3 M GdnHCl (C). Elution of the protein was monitored by 280 nm absorbance. The numbers at the top of the peaks represent the retention times of the protein fractions. The retention times of peaks have not been corrected for changes in viscosity elution buffer (due to presence of GdnHCl). The peak which elutes with a retention time of 45.4 min represents the multimeric intermediate (B). It appears that the multimeric intermediate directly forms from the native state of apo-MnSOD because only two peaks corresponding to the native state (retention time 77.4 min) and the multimeric intermediate (45.4 min) could be observed in the elution profile obtained in 1.5 M GdnHCl.

that of the native protein (Fig. 5). At higher concentrations of GdnHCl (>3.0 M), wherein the apo-MnSOD exists in unfolded state(s), the elution profile shows a single peak with a retention time of about 70 min. This peak corresponds to the denatured state(s) of the protein. In summary, the results of the size-exclusion chromatography experiments clearly suggest that the multimeric intermediate state(s) is in equilibrium with the native dimeric state and the transition from the native state to the multimeric intermediate state appears to be cooperative. This aspect could be evidenced by the presence of only two peaks corresponding to the native state and the multimeric intermediate state in the elution profile of the protein in 1.5 M GdnHCl (Fig. 5).

Sedimentation velocity studies provide useful information on the molecular mass of macromolecules and help to assess the presence of heterogeneous molecular states (of macromolecules) in equilibrium with each other [23]. In this context, we performed sedimentation velocity experiments to further evaluate the quaternary structure of apo-MnSOD as a function of GdnHCl. Native apo-MnSOD has an apparent molecular mass of around 47 kDa and sediments as a single species at 3.4 S (Fig. 6). No significant change in the sedimentation value could be observed even in 1.0 M GdnHCl. Interestingly, in 1.5 M GdnHCl, the protein does not move as a discrete peak. Instead, there are species with 'S' values spread from 10 S to 40 S implying formation of an ensemble of multimeric intermediate states which possibly vary in both shape and size (Fig. 6). Accurate determination of the molecular weight of the multimeric intermediate state(s) is not possible because the sedimentation values are influenced by viscosity contributions from the denaturant and the possible changes in the shape of the protein molecule in the presence of the denaturant (1.5 M GdnHCl). Neglecting these contributions, the average molecular size of the multimeric intermediate is estimated to be in the range of 200–250 kDa. At higher concentrations of the denaturant (>3.0 M GdnHCl), the protein moves as a single peak with a sedimentation constant value of about 1.2 S suggesting the formation of unfolded monomeric species (Fig. 6). In summary, the size-exclusion chromatography and sedimentation velocity data corroborate with each other and unambiguously demonstrate that the GdnHCl-induced equilibrium unfolding of apo-MnSOD proceeds with the accumulation of an ensemble of multimeric intermediate state(s).

Significance of the multimeric intermediate state(s)

Non-cooperative equilibrium unfolding has been reported in a number of oligomeric proteins [24,25]. However, the equilibrium intermediate states that have been characterized to date, are mostly partially structured monomeric subunits that form after dissociation of the

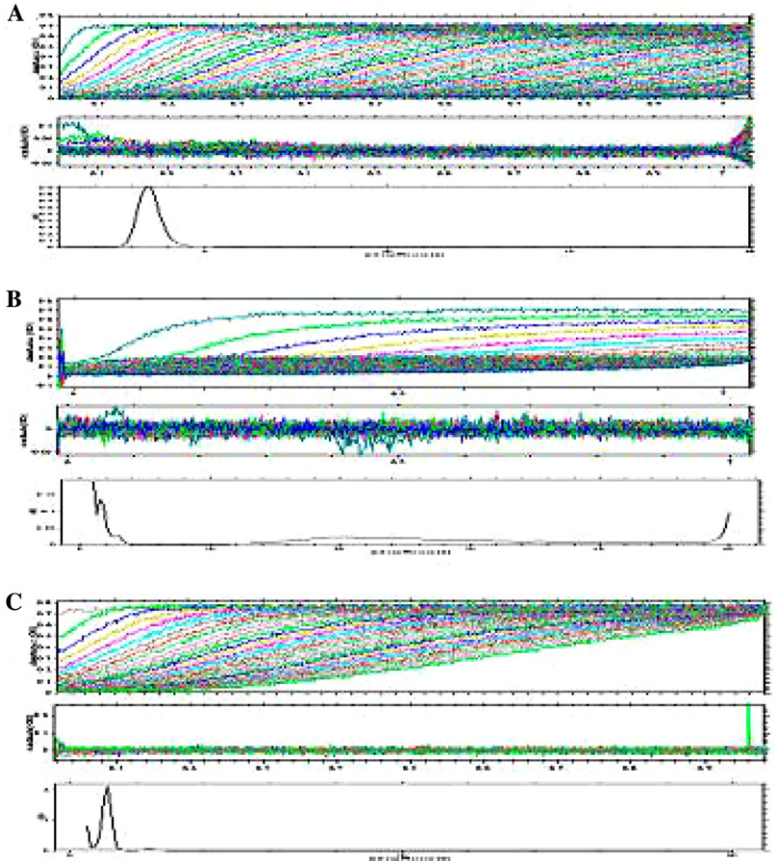

Fig. 6. Sedimentation velocity profiles of apo-MnSOD in 0 M GdnHCl (A), 1.5 M GdnHCl (B), and 3.0 M GdnHCl (C). The three subpanels in each main panel represent the trace of absorbance at 280 nm during the sedimentation (topmost subpanel), the residues of the model fitting (middle subpanel), and the sedimentation coefficient distribution of all species (lowermost subpanel). The broad distribution of the sedimentation coefficients in the range of 10 S to 40 S suggests that an ensemble of multimeric equilibrium intermediate states is formed in 1.5 M GdnHCl.

native oligomeric states. To our knowledge, this is the first report of the identification and characterization of a multimeric equilibrium intermediate in an oligomeric protein such as apo-MnSOD. The formation and dissociation of the obligatory multimeric equilibrium intermediate(s) appears to be a crucial step in governing the commitment of the protein to either refold productively to the native state or to misfold and form aggregates. Reversible dissociation of the multimeric intermediate appears to direct the protein to productively fold to the native state. However, stabilization/non-dissociation of the multimeric intermediate(s) possibly results in aggregation of the protein. In this context, it may not be far-fetched to believe that the missense mutations in SOD, as found in FALS patients, possibly stabilize the multimeric intermediate state and prevent its dissociation [11]. Undissociated multimeric intermediate state(s) possibly acts as a seed or template for aggregation of SOD leading to diseased conditions such as FALS [11,12]. At the present juncture, this proposal is speculative and the validity of the proposal needs to be verified by detailed structural characterization of the GdnHCl-induced equilibrium unfolding of the wild type and appropriate missense mutants of apo-MnSOD using dynamic light scattering and multidimensional NMR spectroscopy.

Acknowledgments

This study was supported financially by the National Institutes of Health Grants (NIH NCRR COBRE Grant 1 P20 RR15569), National Science Council, Taiwan, and the Arkansas Bioscience Institute.

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