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Monitoring Protein Folding and Unfolding Pathways through Surface Hydrophobicity Changes Using Fluorescence and Circular Dichroism Spectroscopy

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Abstract—In the present study we have investigated the characteristics of folding and unfolding pathways of two model proteins, ovalbumin and α-lactalbumin, monitored through the changes in surface hydrophobicity using fluorescence and circular dichroism spectroscopy. In the unfolding process, it was observed that ovalbumin and α-lactalbumin followed a three state transition pathway involving an intermediate state having high surface hydrophobicity. The intermediate state has also been characterized by circular dichroism spectroscopy, and it was found that the intermediate retained almost the same secondary structure as the native proteins, and therefore it can be referred to as molten globule state. The refolding process was monitored using fluorescence and circular dichroism spectroscopy, and it was observed that the refolding of α-lactalbumin was reversible and proceeded through the accumulation of similar type of intermediates as observed during its unfolding pathway. However, on refolding from the guanidine hydrochloride-denatured state, ovalbumin reached a different folded state.

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Key words: surface hydrophobicity, protein unfolding, folding intermediate, fluorescence and circular dichroism spectroscopy, extrinsic fluorescence spectroscopy

A protein must gain a stable and precisely ordered conformation to perform its biological function properly. Although much is known about their conformations and synthesis, little is known about their structures and folding intermediates that are generated during a protein folding process. Determination of the structural characteristics of partially folded intermediate states is crucial for understanding the mechanism of folding. Unfortunately, the cooperative nature of folding results in only minute amounts of these intermediates at equilibrium. Hence, kinetic means have been used to characterize transiently populated intermediates [1-3]. However, it has been shown that a few proteins adopt stable, partially folded equilibrium intermediates under unfolding conditions [4]. There is some evidence that these intermediates, termed "molten globules", also occur along the kinetic pathway [4]. However, the molecular details of even the equilibrium folding intermediates remain difficult to characterize experimentally.

Refolding of a protein from a denatured state to an ordered biologically active native state is considered to be a highly complex process because of the lack of information about the folding intermediates formed in the folding pathway. This process becomes more complex for multidomain proteins, where each domain may be capable of refolding independently.

In vitro protein unfolding and refolding experiments involve denaturing the protein with urea, guanidine hydrochloride, or heat and then refolding the protein by removing the denaturant, followed by monitoring the process using spectral methods. This technique has already been used for studying the native and completely denatured protein. Only a few reports are available so far where the folding intermediates have been studied using techniques like fluorescence and circular dichroism (CD) spectroscopy.

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; GdnHCl, guanidine hydrochloride; α-LA, α-lactalbumin; MRE, mean residue ellipticity; SH, surface hydrophobicity.

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Protein molecules comprise both hydrophobic and hydrophilic amino acid residues. When the protein folds to form the tertiary structure, some of the hydrophobic residues get buried in the interior, while others are exposed to the surface or located in crevices. Hydrophobic interactions in proteins play a major role in dictating conformation, solubility, ligand binding, aggregating properties, etc. Total hydrophobicity can be measured as a sum of the hydrophobicity of individual side chains of the constituent amino acid residues in the protein and hence, a more specific parameter has been coined: surface hydrophobicity (SH). Surface hydrophobicity is a measure of the hydrophobic character exhibited by the surface of a protein molecule in three-dimensional space [5]. On unfolding, the surface hydrophobicity is destroyed and through refolding, SH can be regenerated. Hence protein folding and unfolding processes can also be monitored through the changes of SH. 1-Anilino-8-naphthalene sulfonate (ANS) is a well known protein fluorescent probe, which produces characteristic fluorescence emission on binding with hydrophobic regions on the protein surface. Thus, the measurement of protein bound ANS fluorescence emission under different unfolding and refolding conditions can provide understanding of the folding and unfolding pathways of a protein molecule.

In the present investigation, our main aim was to monitor the protein folding pathway of ovalbumin and α-lactalbumin (α-LA) using SH as a probe. We observed that during the unfolding process, ovalbumin and α-LA follow a three state transition involving native (N), intermediate (I), and unfolded states (U). The refolding process of α-LA is reversible and proceeds through the accumulation of similar type of intermediates as observed during its unfolding pathway. However, on refolding from the guanidine hydrochloride (GdnHCl)-denatured state, ovalbumin does not follow a similar pathway as observed during unfolding and reaches partially folded states. Analyzing the secondary structural component, we conclude that the experimentally detected intermediates of the proteins are in molten globule state.

MATERIALS AND METHODS

Chemicals and reagents. Ovalbumin, α-LA, ANS, and GdnHCl were purchased from Sigma (USA); urea was purchased from Merck (Germany); sodium cacodylate ($(CH_3)_2AsO_2Na{\cdot}3H_2O$) was obtained from Central Drug House (P) Ltd. (India), and all other chemicals used were of analytical grade.

Protein and ANS estimation. Concentrations of ANS and the proteins were determined using a Beckman Coulter DU 800 spectrophotometer (USA) using a 10 mm cell. The protein concentrations were calculated using the absorbance values of 1% protein solution [6] at 280 nm which were as follows: $A^{1\%}_{1cm}$ (α-LA) = 20.3 and $A^{1\%}_{1cm}$ (ovalbumin) = 7.01. Concentration of ANS was determined using the molar extinction coefficient ε_m = 5000 $M^{-1}\cdot cm^{-1}$ at 350 nm [7]. The molecular masses of the proteins were taken as 14.2 kDa for α-LA and 45.9 kDa for ovalbumin, and for ANS it was taken as 328 Da. The number of amino acid residues was taken as 123 for α-LA and 385 for ovalbumin.

Fluorescence measurements. The protein solutions were prepared in 50 mM sodium cacodylate buffer, pH 7.0, containing 50 mM NaCl and 1 mM $CaCl_2$. GdnHCl solutions were prepared in the sodium cacodylate buffer at pH 7.0. All prepared solutions were filtered using a syringe top filter of pore size 0.45 μm. Fluorescence measurements were performed on a Perkin Elmer LS55 luminescence spectrometer equipped with FL WinLab Software at 25°C using a 10 mm cell. For ANS fluorescence measurements, the excitation wavelength was fixed at 370 nm and emission was monitored at the wavelength of maximum intensity, evaluated in each case from a variable wavelength scan (400-600 nm). For the unfolding experiments, the excitation and emission slits were set to 7.5 nm each. When ANS was bound to solvent exposed hydrophobic patches on protein surfaces, its quantum yield was enhanced and the maximum of the emission spectra was shifted from 510 to 467 nm.

Determination of SH and number of ANS binding sites of the proteins. *Surface hydrophobicity.* Protein samples of different concentrations (0.05-0.3 mg/ml) were denatured with different GdnHCl concentrations (ranging between 0-6 M final concentrations) and incubated for 2 h at 25°C. Optimized concentration of ANS was determined and added to each of the protein samples and incubated for 1 h. Fluorescence spectra were obtained for all samples at 25°C using a Perkin Elmer spectrofluorimeter. The quantity "relative fluorescence" (F_R) was calculated as defined by Chaudhuri et al. [5]:

$$F_R = (F - F_0)/F_0,$$

where F is the fluorescence intensity of the protein–ANS conjugate and F_0 is that of aqueous ANS solution. When F_R is plotted against the protein concentration, the slope of the curve gives the surface hydrophobicity.

Number of ANS binding sites. Number of ANS binding sites was calculated as described by Cardamone and Puri [8]. A range of concentrations of ANS (5-25 μM) was added to GdnHCl-induced denatured protein samples and incubated for 10 min at 25°C. For each given ANS–protein interaction, a calibration factor was determined. A double reciprocal plot of $1/\Delta F$ versus 1/[protein] was extrapolated to infinite protein concentration at 1/[protein] = 0 and $1/\Delta F = 1/\Delta F_{max}$. The inverse of the y-axis intercept gave the calibration factor in units of fluorescence increase/micromole of ANS bound. The fluorescence measurement for each concentration of ANS

was divided by the calibration factor to convert the difference in fluorescence for the ANS titration (ΔF) to the amount of ANS bound/micromole of protein. By multiplying the amount of ANS bound/micromole of protein with the micromoles of protein, "ν", i.e. the amount of protein bound ANS, was derived. From "ν", the concentration of free ANS was determined. The plot of 1/ν versus 1/[free ANS] was plotted. The *y*-axis intercept gives the number of binding sites [8, 9].

Refolding of proteins. Fixed amounts of proteins (0.2 mg) were incubated with 6 M GdnHCl solution for 2 h for attaining equilibrium condition. In this period, the proteins were fully denatured [7]. The solutions were then diluted to achieve a final GdnHCl concentration ranging from 0.6 to 5 M and the protein concentration of 0.2 mg/ml, to achieve protein refolding, by adding 50 mM sodium cacodylate buffer, pH 7.0. After allowing the protein to refold for a sufficient length of time, 50 μM ANS was added and incubated for 10 min at 25°C. We have monitored the change of SH and the number of ANS binding sites on the refolding proteins as a function of concentration of chemical denaturant, GdnHCl. We have performed same sets of experiments, as done before during the unfolding, for the determination of SH and number of ANS binding sites during the refolding process.

CD measurements. Far-UV circular dichroism measurements were performed on a Jasco J-810 spectropolarimeter using a 2 mm path length cell. The nitrogen flow was fixed at 10 liters/min. The CD spectrum was monitored over a variable wavelength scan (200-250 nm). The CD in millidegree obtained over the wavelength range of 200-250 nm was converted to mean residue ellipticity (MRE, θ) using the following conversion:

MRE = CD (in mdeg)/[molar concentration of protein × path length (in mm) × number of amino acid residues in the protein].

The unit of MRE is deg·cm^2·dmol^{-1}. The MRE for all the denatured states of proteins (0-6 M GdnHCl) were plotted against the wavelength. Since the MRE at 222 nm can be used to compare percentage of secondary structure retained during unfolding, it was plotted against the denaturant concentration.

RESULTS AND DISCUSSION

In the present study, our main aim was to characterize the protein folding and unfolding pathways of ovalbumin and α-LA using surface hydrophobicity (SH) as a probe. SH measurement using ANS (the external fluorescent probe) binding method is a rapid, non-destructive, and a rather simple technique to quantitatively assess the non-polar nature of different proteins. It is a quick diagnostic test to detect different conformational states of proteins during folding/unfolding and to judge whether the protein is in the native state or not.

Monitoring unfolding and refolding of proteins by extrinsic fluorescence measurement. Fluorescence emission spectra of protein–ANS conjugate for the two standard proteins, ovalbumin and α-lactalbumin, were obtained. Since it is known that the protein changes its surface hydrophobicity during the unfolding process, we wanted to observe the change in SH value of the proteins as a function of GdnHCl concentration using fluorescence and CD spectroscopy.

During the unfolding of ovalbumin by GdnHCl, it was observed that in the initial phase of unfolding (at lower denaturant concentration) the SH value increased and reached maximum value at 2 M GdnHCl (Fig. 1a). At this unfolding intermediate state, 2 M GdnHCl-induced conformation, the fluorescence intensity and number of ANS binding sites were also maximal as observed from Figs. 1a and 1c. This was the indicative of the fact that the protein undergoes a three-state transition during the unfolding process, forming an intermediate state between the native and unfolded states.

The refolding of the same protein was also studied. During the refolding of denatured ovalbumin, it was found that the SH value started increasing gradually until 3 M GdnHCl concentration but then increased rapidly after that and reached maximal SH value at a GdnHCl concentration of 1 M (Fig. 1b).

Fluorescence intensity and the number of ANS binding sites of ovalbumin were also maximal at the GdnHCl concentration of 1 M (Figs. 1a and 1c) indicating that the protein undergoes a three state transition with intermediate state occurring between the unfolded and refolded states. However, when ovalbumin was refolded, the denatured protein followed a different folding pathway, which can be observed by comparing the unfolding and refolding plots **(**Fig. 1, a-c**)** where the fluorescence intensity at each step during refolding was higher than that of unfolding. This observation could be attributed to the fact that when the protein was denatured completely and then refolded, some hydrophobic pockets, which were earlier not accessible, become now accessible. For this reason, ANS molecules bind in the hydrophobic pockets and generate fluorescence and hence produce a larger signal. It was also observed that with decreasing concentration of GdnHCl, the fluorescence intensity always increased, implying that the SH was continuously increasing while refolding.

When the unfolding process of α-LA was observed by monitoring the changes in SH using extrinsic fluorophore ANS, it was shown that initially when the protein was denatured, the SH value started increasing. However, the change occurred at a lower concentration of GdnHCl, with the SH value reaching a maximum at 1 M (Fig. 2b). Further increasing of the denaturant concentration

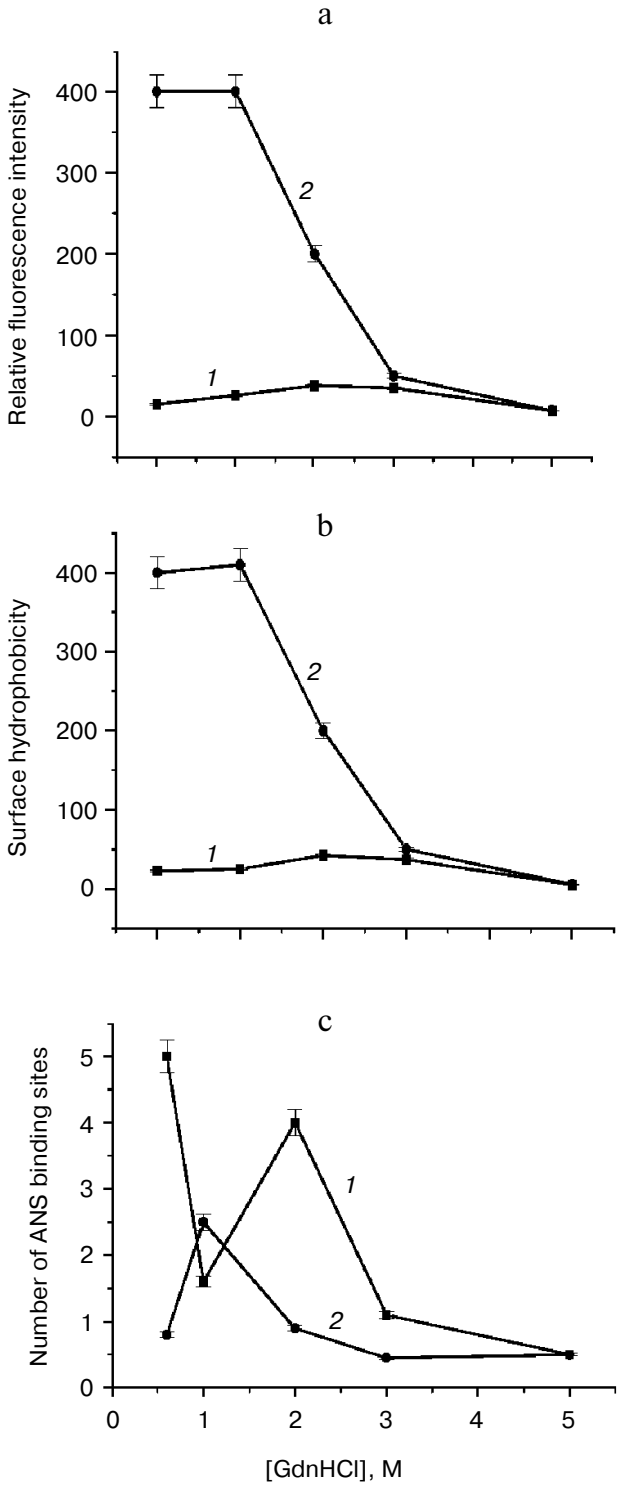

Fig. 1. Plot of unfolding (*1*) and refolding (*2*) of ovalbumin. Protein samples were unfolded by the addition of increasing concentrations of GdnHCl and refolding was initiated through dilution of the denaturant. a) Plot of relative fluorescence intensity versus GdnHCl concentration. b) Plot of surface hydrophobicity versus GdnHCl concentration. c) Plot of number of ANS binding sites versus GdnHCl concentration. Data represent the mean of triplicate results from at least three independent measurements ± S.E.M.

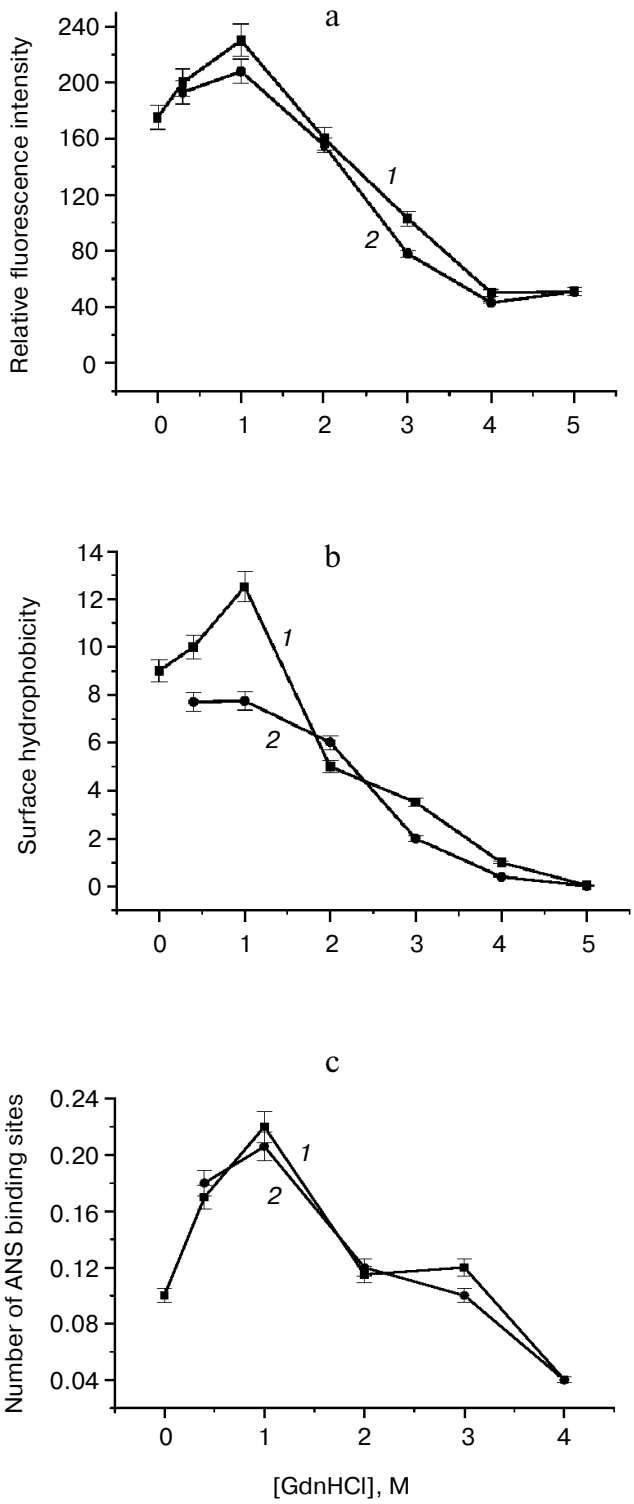

Fig. 2. Plot of unfolding (*1*) and refolding (*2*) of α-LA by GdnHCl. Protein samples were unfolded by the addition of increasing concentrations of GdnHCl and refolding was initiated through dilution of the denaturant. a) Plot of relative fluorescence intensity versus GdnHCl concentration. b) Plot of surface hydrophobicity versus GdnHCl concentration. c) Plot of number of ANS binding sites versus GdnHCl concentration. Results are given as means ± S.E.M. of at least three independent experiments done in triplicate.

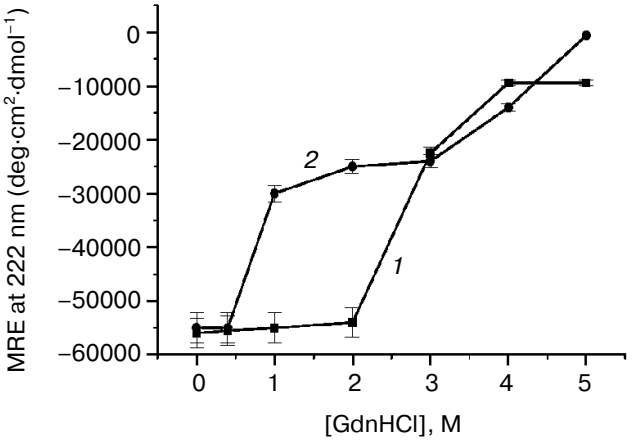

Fig. 3. Unfolding (*1*) and refolding (*2*) plot of MRE_{222} versus GdnHCl concentration for ovalbumin. Protein samples were unfolded by the addition of increasing concentrations of GdnHCl and refolding was initiated through dilution of the denaturant. Data represent the mean of triplicate results from at least three independent measurements ± S.E.M.

resulted in a decrease in the SH value. It was found that the unfolding intermediate state, which was formed at a GdnHCl concentration of 1 M, the fluorescence intensity and number of ANS binding sites were also maximal (Figs. 2a and 2c). This phenomenon again indicates that a three-state transition model was followed by the protein α-LA during the unfolding process with an intermediate state occurring between the native and unfolded states.

The initial rise in SH was indicative of the fact that there were newly exposed hydrophobic sites on the surface of the protein that were available for ANS binding. Through unfolding, a decrease in SH value was found that might be due to the destruction of the ANS holding capacity of the hydrophobic pockets during unfolding.

During refolding of GdnHCl-denatured α-LA, it was observed that the fluorescence intensities at different points were almost the same as compared to the values that were observed during unfolding **(**Fig. 2a**)**. This observation could be due to the fact that α-LA is a smaller-sized protein that is able to fold back in the same fashion as it unfolded and regain its native state. While refolding, the SH was found to be maximal at a GdnHCl concentration of 1 M (Fig. 2b) where the fluorescence intensity (Fig. 2a) and the number of ANS binding sites (Fig. 2c) were also maximal, and this indicates that the protein undergoes a three state refolding involving an intermediate state.

A question that might come to our mind is whether the partially folded conformation of the proteins was a molten-globule-like state. It has been observed that the equilibrium intermediates of different proteins during unfolding/refolding show common characteristics, such as absence of most of the native tertiary structure that occurs due to lack of the tight packing of side chains and loosely packed hydrophobic core, which increases the hydrophobic surface accessible to solvent. This equilibrium intermediate could be the molten globule state, and it has been proposed to be a general state of proteins. In the partially folded form of α-LA and ovalbumin, tertiary structure is lost as judged by ANS fluorescence, and it has enhanced solvent-exposed hydrophobic regions. Only CD spectroscopy could explain whether secondary structure is similar to the native state of the proteins, and that would prove whether the intermediate state is molten globule-like.

Monitoring unfolding and refolding of proteins using CD spectroscopy. When the unfolding of ovalbumin was monitored by CD spectroscopy, it was observed that the mean residue ellipticity (MRE) of ovalbumin at 222 nm remained almost constant until the GdnHCl concentration reached 2 M (Fig. 3). The MRE value then increased on further unfolding of the protein until 4 M GdnHCl concentration and finally became constant after that. Since MRE at 222 nm remained constant until 2 M GdnHCl concentration, it can be concluded that the protein did not lose its secondary structure until the GdnHCl concentration of 2 M (Fig. 3). This unfolding intermediate state during the denaturation of the protein may be referred to as the molten globule state as it retains its secondary structures. This shows that the unfolding of ovalbumin followed a three state transition with a molten globule-like structure at 2 M GdnHCl concentration, which was also comparable to the observations obtained with fluorescence spectroscopy (see Fig. 1).

This intermediate state during the denaturation of the proteins can be termed as the molten globule state as it retained the native like secondary structure, since the fact was confirmed by determining the percentage of α-helix, β-sheet, and random coil contents by online soft-

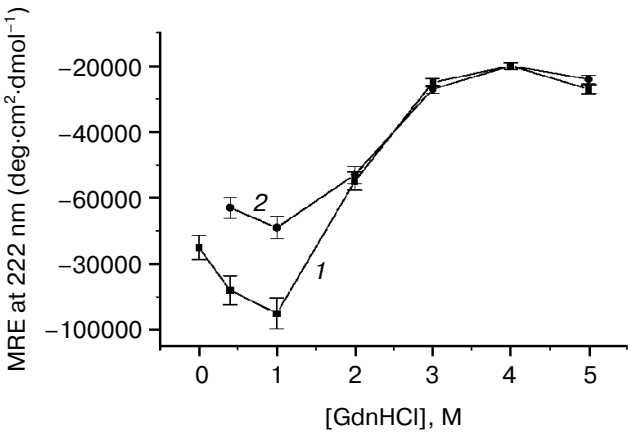

Fig. 4. Unfolding (*1*) and refolding (*2*) plot of MRE_{222} versus GdnHCl concentration for α-LA. Results are given as means ± S.E.M. of at least three independent experiments done in triplicate.

ware k2d at different concentrations of GdnHCl using the CD (millidegree) data (result not shown).

However, during the unfolding process of α-LA, the MRE_{222} versus GdnHCl concentration plot showed a different trend. Here the MRE_{222} value started decreasing with unfolding of the protein, and the value reached a minimum at a GdnHCl concentration of 1 M, then increased, and finally became almost constant at a GdnHCl concentration above 3 M (Fig. 4). This certainly showed that the unfolding of α-LA followed a three state transition with a molten globule-like structure forming at 1 M GdnHCl concentration, which was again comparable with the observations obtained from fluorescence spectroscopy (Fig. 2). However, it also provides additional information. As already mentioned, MRE_{222} is an index for the content of α-helical structure in a protein molecule. Therefore, in this case more negative value of MRE_{222} indicates that the denatured state at that concentration of GdnHCl has more α-helical content than the others. As the MRE attains a minimal value at a GdnHCl concentration of 1 M, it indicates that the α-helical content is maximal in this intermediate state. The protein showed similar characteristics during the fluorescence measurements, hence the results are comparable.

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