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ORIGINAL PAPER

Unfolding Studies of *Escherichia coli* Maltodextrin Glucosidase Monitored by Fluorescence Spectroscopy

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Abstract Equilibrium unfolding of a 69-kDa monomeric *Escherichia coli* maltodextrin glucosidase (MalZ) was studied using intrinsic and extrinsic fluorescence spectroscopy. The unfolding transition of MalZ followed a three-state process, involving the formation of a stable intermediate state having more exposed hydrophobic surface. It was found that the protein structure can be easily perturbed by low concentration of guanidium hydrochloride (GdnHCl) and, at a GdnHCl concentration of 2 M, MalZ was denatured completely. The active site of the protein also has been proved to be sensitive to a low concentration of GdnHCl since MalZ deactivated at 0.5 M GdnHCl completely. The surface hydrophobicity and ANS-binding site of the protein have been determined to be 150.7 and 0.24, respectively. Perhaps the formation of the stable unfolding intermediate, having higher surface hydrophobicity, may be one of the reasons for aggregation of MalZ and its recognition by chaperonin GroEL during the assisted folding pathway.

Keywords Equilibrium unfolding · Folding intermediate · Molten-globule state · Surface hydrophobicity · Fluorescence spectroscopy

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1 Introduction

Reversibility of protein folding/unfolding process was first demonstrated by Anfinsen [1] from the study of ribonuclease, who also suggested that the correctly folded conformation of a protein is dictated by its amino-acid sequence. However, there are instances in which a few proteins, such as subtilisin E [2], α-lytic protease [3], and carboxypeptidase Y [4], could not return to their natively folded conformation from their denatured state despite the removal of the denaturant, indicating that the refolding/unfolding processes of those proteins were not reversible. These observations have raised questions about the validity of earlier conclusion from the unfolding and refolding experiments of ribonuclease. Formation of misfolded and non-native species during the refolding process of various proteins has been reported in the literature [5, 6]. The conformation of the refolded protein depends not only on the refolding conditions, but also on the overall size and domain structure of the protein. Lower molecular mass proteins appear to exhibit reversible folding more consistently [7].

Here, we have reported equilibrium and thermal unfolding studies of maltodextrin glucosidase (MalZ), a 69-kDa monomeric protein responsible for degradation of maltodextrins to maltose by eliminating one glucose residue from the reducing end each time [8], monitored by intrinsic and extrinsic fluorescence measurement and spectrophotometric measurement of absorbance. Maltodextrin glucosidase was of particular interest since the protein is larger in size (69 kDa) and it has recently been reported that its spontaneous refolding efficiency is very small, and productive folding is possible only in presence of GroEL and GroES, either *in vivo* or *in vitro* [9]. Therefore, this series of reports raised the interest for studying its unfolding from a structural point of view.

It has already been reported that GroEL can stably bind with non-native MalZ. It is, therefore, significant to study the unfolding process of such a large protein which has a profound tendency to aggregate during the refolding process. The unfolding studies will provide information about the different non-native conformations of MalZ that accumulate during the process of equilibrium unfolding. It may give rise to an explanation of why MalZ irreversibly aggregates and associates with chaperonin GroEL in a non-native state.

In the present study, it has been reported that the GdnHCl-induced unfolding process of MalZ was a three-state event, involving the formation of a stable intermediate state having a higher degree of surface hydrophobicity. MalZ has higher value of calculated surface hydrophobicity which can be increased on unfolding, and this may be the probable reason for binding with GroEL in a non-native state.

2 Materials and Methods

2.1 Materials

BL21 *E. coli* strains were used for expression and purification of MalZ. The pCS19MalZ vector, containing $(His)_6$MalZ was a generous gift from Prof. Winfried Boos, Germany. IPTG, 8-anilino-1-naphthalene-sulfonic acid (ANS) and *p*-nitrophenyl α-D-maltoside were purchased from Sigma Chemical Company (USA). High purity grade guanidine hydrochloride (GdnHCl), imidazole, and sodium chloride were purchased from Merck, India. All other reagents used were of analytical grade.

2.2 Purification of MalZ

MalZ was overexpressed in TG1 *E. coli* cells. Plasmid pCS19MalZ was used for overexpression of MalZ. Cells were disintegrated in a French press and lysate was centrifuged at

45,000 rpm for 45 min. The supernatant was separated and placed in a Ni-chelating column (HisTrap HP) in the AKTA FPLC system (GE Health care). Ni^{+2}-bound His-tagged MalZ was eluted with buffer containing imidazole and, finally, the purity of the preparation was assessed using SDS gel electrophoresis.

2.3 Fluorescence Studies

Steady-state fluorescence was recorded on an LS 55 luminescence spectrofluorometer (PerkinElmer, New Delhi, India). Intrinsic tryptophan (Trp) fluorescence spectra were recorded by exciting the samples at 295 nm with excitation and emission slit widths set at 5.0 and 7.5 nm, respectively. The emission spectra were recorded in the range of 300–400 nm. Base-line corrections were carried out with buffer without protein in all cases.

3 Monitoring GdnHCl-induced Denaturation of MalZ by Intrinsic Fluorescence Measurement

Intrinsic fluorescence measurements were performed using a PerkinElmer LS 55 spectrofluorometer. MalZ (3.0 μM) was incubated with different concentrations of GdnHCl (0–6 M) at 25°C for 30 min in 20 mM sodium phosphate buffer containing 10 mM imidazole, pH 7. Tryptophan fluorescence measurements were performed using an excitation wavelength of 295 nm and recording the emission spectra from 300 to 400 nm. Relative fluorescence intensity values were plotted against molar concentration of GdnHCl to obtain the optimum denaturant concentration at which the change in fluorescence intensity was at a maximum.

The fraction of MalZ in denatured state (f_D) was expressed as:

$$f_D = (RF_c - RF_0) \big/ (RF_d - RF_0), \quad (1)$$

where RF_c is the relative fluorescence intensity at any concentration of GdnHCl, and RF_0 and RF_d are the fluorescence intensities in the native and denatured states, respectively.

4 Determination of Surface Hydrophobicity and Number of ANS-binding Sites of MalZ

ANS is an extrinsic fluorescence probe that binds to the hydrophobic patches present on a protein surface. Hence, protein-bound ANS fluorescence represents the surface hydrophobic properties of a protein molecule. MalZ-bound ANS fluorescence is the fluorescence intensity of the ANS-MalZ conjugate minus that of ANS alone. Varying final concentration of ANS was added (0, 20, 40, 60, 80, 100, 120, 140 μM) into 7.6 μM (0.5 mg/ml) MalZ solution in 20 mM phosphate buffer containing 10 mM imidazole, pH 7.0, and volume was made up to 1 ml. Solutions were incubated for 5 min at 25°C. Fluorescence spectra were recorded with a PerkinElmer LS 55 spectrofluorometer at 25°C at an excitation wavelength of 370 nm with slit width 5 nm each for excitation and emission bandpass and at a scan rate of 60 nm/min. The optimized ANS concentration, which produces maximum protein-bound ANS fluorescence intensity, was also measured.

MalZ solutions in the concentration range (0–7.5 μM) were denatured with different GdnHCl concentrations (ranging between 0–6 M final concentrations) for 30 min at 25°C

to reach the equilibrium state. Optimized concentration (50 μM) of ANS was added to each of the protein samples and incubated for 10 min at 25°C. MalZ-bound ANS fluorescence spectra were recorded for all protein samples at 25°C. The quantity "relative fluorescence" (F_R) was calculated according to (eqn. 2) Chaudhuri et al. [10],

$$F_R = (F - F_0)/F_0, \tag{2}$$

where, F is the fluorescence intensity of the protein–ANS conjugate and F_0 is that of aqueous ANS solution. F_R was plotted against the protein concentration. The surface hydrophobicity was obtained by measuring the slope of the curve. Since ANS is a relatively large molecule, it is unlikely to penetrate into the protein's inner hydrophobic groups and thus it reflects only hydrophobicity of the protein's outer surface.

4.1 Number of ANS-binding Sites

A range of concentrations of ANS (5–25 μM) was added to GdnHCl (0–6 M) and urea (0–8 M) 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 per micromole of ANS bound. The fluorescence measurements 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 per micromole of protein. By multiplying the amount of ANS bound per micromole of protein by the micromoles of protein, ν, i.e., the amount of ANS bound was derived. From "v", the concentration of free ANS was determined. The plot of $1/\upsilon$ vs. 1/[free ANS] was plotted. The y-axis intercept gave the number of binding sites [11].

5 Results

5.1 Equilibrium Unfolding of MalZ by Guanidium Hydrochloride

The GdnHCl-induced equilibrium unfolding transition of recombinant MalZ was studied by intrinsic tryptophan fluorescence spectroscopy. Denaturation of MalZ was monitored by measurement of intrinsic tryptophan fluorescence of the GdnHCl-treated samples. Two sets of samples were prepared; in one set, only GdnHCl was added as denaturant and in the second set, 2 mM DTT was added along with GdnHCl. It was observed that maximum change in fluorescence intensity took place at 2 M GdnHCl (Fig. 1). Hence, the complete unfolding of MalZ took place at 2 M GdnHCl since the activity was also completely lost at that denaturant concentration (Fig. 2). There was no visible change in the denaturation pattern of MalZ in presence of DTT (Fig. 1).

It was found that the activity of MalZ was lost completely at 0.5 M GdnHCl (Fig. 2). The fraction-denatured MalZ was calculated according to (eqn. 1) and plotted against GdnHCl concentration (Fig. 3). It was observed that 50% denaturation occurs at 0.5 M GdnHCl; 1.5 M GdnHCl can denature MalZ completely.

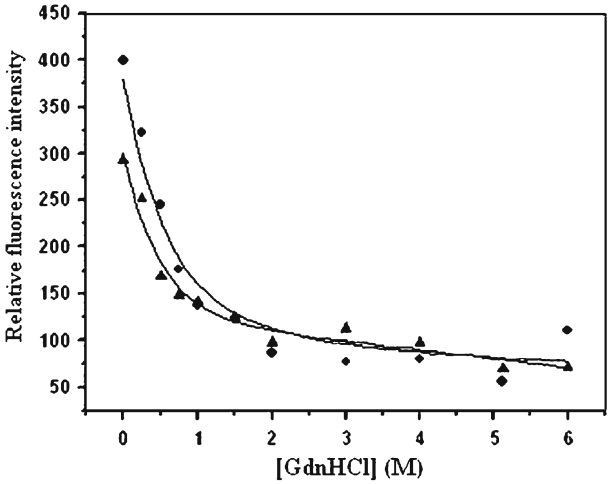

Fig. 1 GdnHCl-induced equilibrium unfolding of MalZ. Unfolding of MalZ (3 μM) was carried out by the addition of GdnHCl at pH 7.0 in 20 mM sodium phosphate buffer containing 10 mM imidazole. MalZ samples were incubated for 30 min at 25°C in GdnHCl alone (*filled circles*) and in 2 mM DTT along with GdnHCl (*filled triangles*). Different concentrations of GdnHCl were used for equilibrium unfolding of MalZ. Relative intrinsic fluorescence emission was measured at 346 nm for all the samples at the λ_{max}^{Ex} 295 nm. The excitation and emission bandpass were 5 nm and 7.5 nm, respectively, and the scan rate was 60 nm/min

5.2 MalZ-bound ANS Fluorescence

Fluorescence emission from the MalZ-ANS conjugate was used to study the changes in the surface hydrophobicity of MalZ during its unfolding process. MalZ-bound ANS fluorescence emission was measured with varying ANS concentration and the results are shown in Fig. 4. Optimum ANS concentration producing maximum fluorescence intensity from the MalZ-ANS conjugate was found to be 50 μM (Fig. 4), beyond which the relative ANS fluorescence intensity became constant. Using the optimum ANS concentration (i.e.,

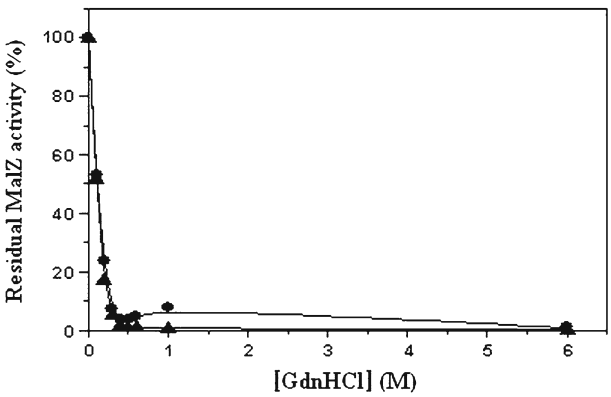

Fig. 2 Deactivation of MalZ by GdnHCl. Protein samples (0.3 μM) were incubated with different concentrations of GdnHCl in a buffer of 20 mM sodium phosphate, 10 mM imidazole and 12.5 mM NaCl final concentration. Titration was carried out in a range of 0.0 M to 6.0 M GdnHCl. Unfolding was performed using GdnHCl only (*filled circles*) and also by addition of 2 mM DTT along with GdnHCl (*filled triangles*)

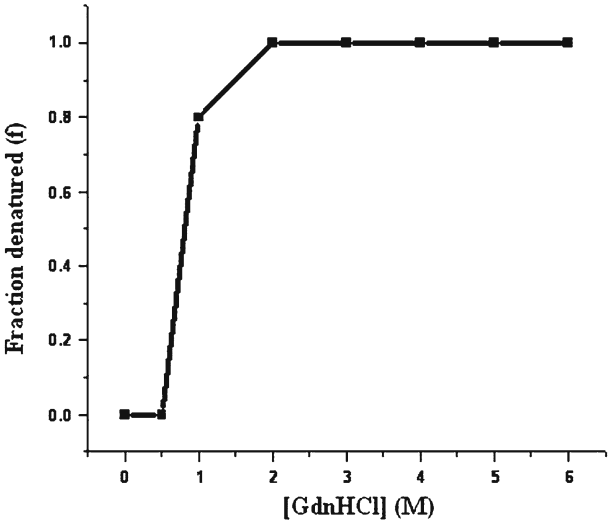

Fig. 3 GdnHCl-induced unfolding transition curve for MalZ. *Continuous* line indicates the theoretically drawn curve and *filled squares* are representing the experimental data

50 μM), two different sets of samples were prepared, taking 4.5 μM (0.3 mg/ml) of protein in 20 mM sodium phosphate buffer, pH 7.0. In one set of samples, varying concentrations of GdnHCl were added and in another set, 2 mM DTT was also added along with GdnHCl. Fluorescence emission at 460 nm were measured with a PerkinElmer LS 55

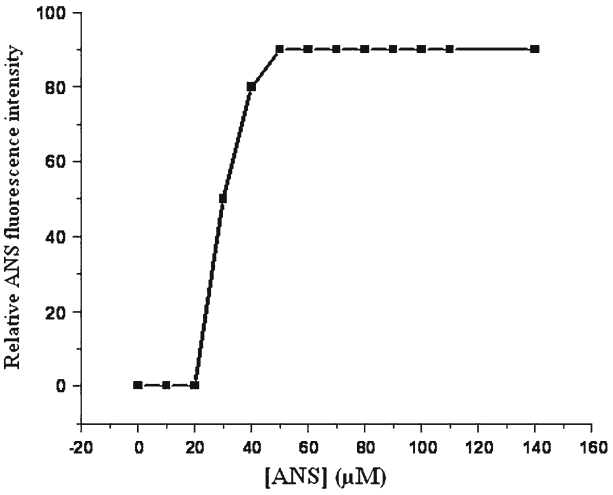

Fig. 4 Spectrophotometric titration of 0.5 mg/ml Maltodextrin glucosidase with ANS. Relative ANS fluorescence is the fluorescence intensity of the ANS–MalZ conjugate minus that of ANS at the same concentration as in ANS–MalZ conjugate. To 20 mM phosphate buffer containing 10 mM imidazole, pH 7.0, MalZ was added to a final concentration of 7.6 μM (0.5 mg/ml). ANS was added to a varying final concentration (0, 20, 40, 60, 80, 100, 120, 140 μM) and volume was made up to 1 ml. Solutions were incubated for 5 min. Fluorescence spectra were obtained with a PerkinElmer LS 55 spectrofluorometer at 25°C at a excitation wavelength of 370 nm with 5 nm each for excitation and emission bandpass; scan rate was 60 nm/min

spectrofluorometer at 25°C, keeping the excitation wavelength at 370 nm. Results are shown in Fig. 5a and b. It was observed that the ANS fluorescence emission reached the highest value at 0.25 M GdnHCl and then gradually decreased to a lowest value during unfolding of MalZ (Fig. 5a and b), indicating the formation of an equilibrium intermediate (molten-globule-like state) with solvent-exposed, hydrophobic surface(s) accumulating maximally at 0.25 M GdnHCl. DTT showed no discriminative effect on the formation of non-native intermediate states of MalZ.

5.3 Equilibrium Intermediate of MalZ Resembles Molten-globule-like State

ANS is a fluorescent dye that binds to hydrophobic regions of proteins. This fluorescent probe has been extensively used for the identification of equilibrium intermediates such as the molten globule (MG) state [12]. Molten-globule intermediate states often expose

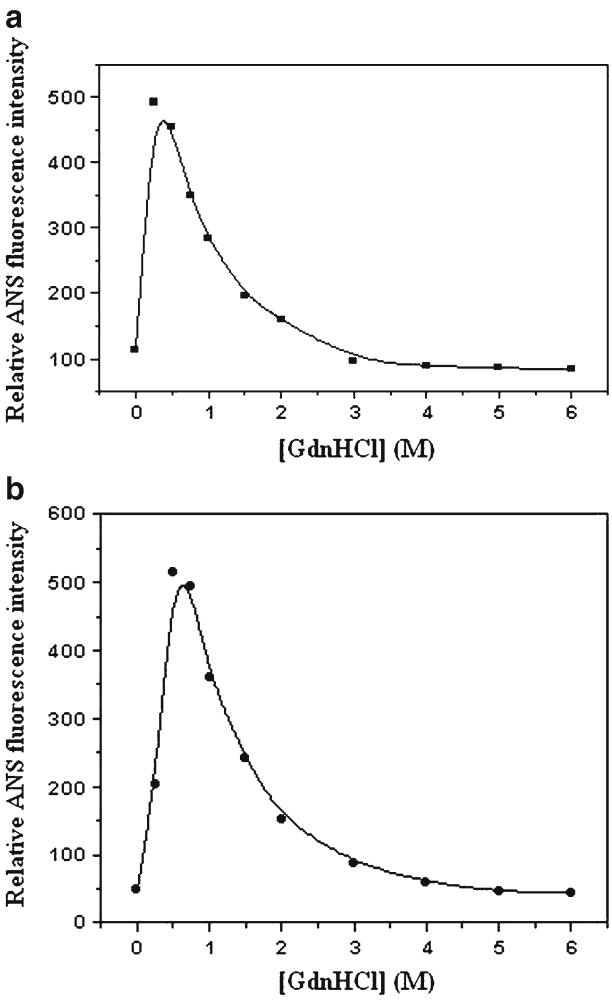

Fig. 5 Fluorescence intensity of MalZ–ANS complex as a function of GdnHCl concentration during GdnHCl-induced unfolding of maltodextrin glucosidase. MalZ 4.5 μM (0.3 mg/mL) in 20 mM phosphate buffer containing 10 mM imidazole, pH 7.0 was added to different concentrations of guanidium hydrochloride (0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6 M) and incubated for 30 min at 25°C. ANS was added into GdnHCl denatured MalZ solutions to a final concentration of 50 μM and the resulting mixtures were incubated for 5 min at 25°C. Fluorescence emission spectra at 460 nm were obtained with a PerkinElmer LS 55 spectrofluorometer at 25°C at an excitation wavelength of 370 nm with 5 nm each for excitation and emission bandpass and scan rate 60 nm/min. **a** Changes in emission intensity at 460 nm were maximum at 0.25 M GdnHCl (*filled squares*) in absence of any added DTT. **b** Maxima of fluorescence emission were observed at 0.5 M GdnHCl concentration when 2 mM DTT was used with GdnHCl (*filled circles*)

a significant portion of their hydrophobic cores to the solvent [13]. Hence, ANS binds strongly to the MG state of proteins and fluoresces intensely.

In our experiment, it was observed that the MalZ-bound ANS fluorescence emission measured at 460 nm reached a maximum at 0.25 M GdnHCl and gradually decreased to a minimum value during unfolding of MalZ (Fig. 5a and b), indicating the formation of an equilibrium intermediate (molten-globule-like state) with a maximum amount of solvent-exposed, hydrophobic surface(s).

5.4 Surface Hydrophobicity of MalZ

Fluorescence intensity of the MalZ–ANS conjugate increased with increased concentration of MalZ for a given concentration of ANS. Kato and Nakai [14] expressed surface hydrophobicity (SH) of a protein as the initial slope of the fluorescence intensity vs. protein concentration. Since fluorescence intensity is measured with arbitrary units, the initial slope determined from data obtained using various instruments may not be comparable. Thus, we have defined the quantity "relative fluorescence" according to (eqn. 2). Figure 6 shows a plot of relative fluorescence intensity of the MalZ-ANS system as a function of MalZ concentration.

Surface hydrophobicity has been calculated as 150.7 from the initial slope of the regression line in Fig. 6.

5.5 Determination of Number of ANS-binding Sites on MalZ

For each given ANS–protein interaction, a calibration factor was determined in order to relate the change in fluorescence to the amount of ANS bound to the protein. The MalZ

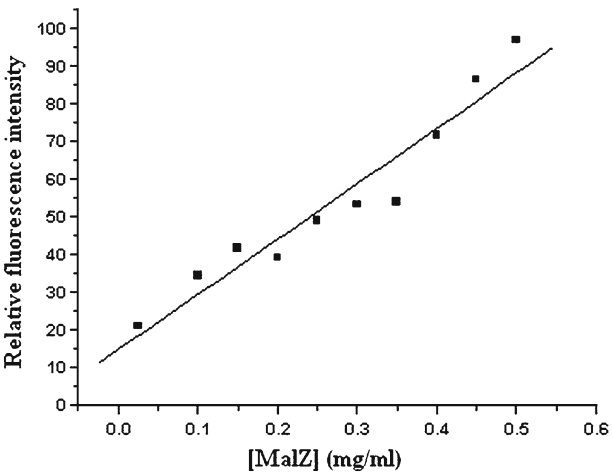

Fig. 6 Plot of relative fluorescence intensity vs. MalZ concentration. Optimized amount of ANS (50 μM) was added to different concentrations (0–7.6 μM or 0–0.5 mg/ml) of MalZ in 20 mM sodium phosphate buffer at pH 7.0 containing 10 mM imidazole and 200 mM NaCl. Emission spectra at 515 nm were obtained with a PerkinElmer LS 55 spectrofluorometer at 25°C at an excitation wavelength of 370 nm with a bandpass of 5 nm each for excitation and emission, respectively, and the scan rate was 60 nm/min. Relative fluorescence intensity values were plotted against MalZ concentration and the surface hydrophobicity value of the protein was calculated from the slope of the curve

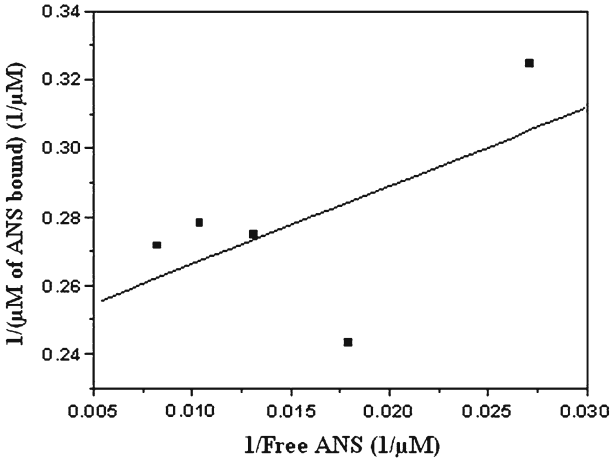

Fig. 7 Plot of 1/[amount of MalZ-bound ANS] vs. 1/[free ANS] in the absence of GdnHCl. Equation of the fitted curve: $y = 2.277x + 0.24341$. y intercept of the curve gives the number of ANS-binding sites: 0.24. The error in n is ±3%

concentration was increased up to 7.6 μM. The inverse y intercept of the curve $I/\Delta F$ vs. 1/[MalZ] resulted in a calibration factor of 80 (plot not shown). The number of ANS-binding sites on the MalZ molecule (n) was calculated from the y intercept of the 1/bound ANS vs. 1/free ANS curve (Fig. 7). The value of n was calculated to be 0.24 for native MalZ.

6 Discussion

In order to gain an idea of how MalZ unfolds in presence of GdnHCl, we monitored the denaturation process by intrinsic tryptophan fluorescence measurement of 3 μM MalZ solution after incubating with different GdnHCl concentrations (0–6 M). It was observed from Fig. 1 that there was a drastic change of fluorescence intensity in the range of 0.25 to 0.5 M GdnHCl concentration, which indeed indicated the denaturation of MalZ. Compared to other disulfide-containing proteins, this protein behaved very differently and unfolds at a very low concentration of GdnHCl. This also signifies that the structure of MalZ can be easily perturbed by very low GdnHCl concentration and the active site may perhaps also be destroyed, as evidenced from the activity vs. GdnHCl titration curve, where we observed that the protein lost its biological activity completely at 0.25 M GdnHCl (Fig. 2). The equilibrium unfolding transition curve, monitored by the change of intrinsic fluorescence, fits well with the two-state model, indicating that the transition could have taken place without the formation of any detectable intermediates, which would have altered the emission spectra. It is possible that the intermediate species might accumulate during the unfolding pathway; however, those non-native states were not distinct in terms of their microenvironment around the tryptophan residues. As a result, detectable intermediate species could not be traced while monitoring the GdnHCl-induced unfolding process of MalZ using intrinsic fluorescence spectroscopy. Hence, it is worthwhile following equilibrium unfolding process of MalZ using a different technique, which can monitor the changes of another intrinsic property of the native protein molecule.

Surface hydrophobicity (SH) is an inherent characteristic of a protein molecule, which is the measure of the hydrophobic character exhibited by the surface of a molecule in three-dimensional space [10]. On unfolding, the surface hydrophobic patches of proteins are

destroyed, and through refolding they become regenerated. Hence, measuring the changes of surface hydrophobicity can provide information about protein folding and unfolding process. 1-anilino-8-naphthalene sulfonate (ANS) is a unique surface-specific fluorescent probe, which gives characteristic fluorescence emission on binding with hydrophobic regions on the protein surface; however, when the dye is present in the polar solvent, it shows negligible emission of fluorescence. Hence, measuring protein-bound ANS fluorescence emission under different denaturation and renaturation conditions can give rise to an understanding of the folding and unfolding pathway of a protein molecule. ANS has been immensely useful in the identification of equilibrium intermediates such as the molten-globule state (MG). Molten-globule-like intermediates usually display a significant exposure of hydrophobic cores to the solvent [13]. Hence, ANS binds strongly to MG state and fluoresces intensely. An ANS-binding study was performed in detail to understand how the surface hydrophobicity of MalZ changes during unfolding process as well as to gain better insight into the mechanism of in vitro unfolding of the protein. ANS (1-anilino-8-naphthalenesulfonate) is a much-utilized fluorescent "hydrophobic probe" for examining the non-polar character of a protein [15]. It is generally assumed that if ANS becomes brilliantly fluorescent upon binding a host protein, the binding sites were non-polar and hydrophobic during association. Unfolding characteristics of MalZ was studied by monitoring MalZ-bound ANS fluorescence of GdnHCl denatured MalZ samples. It is evident from the Fig. 5 that, with increasing denaturant concentration, protein-bound ANS fluorescence increased rapidly within 0–0.25 M GdnHCl and it reached a maximum at 0.25 M GdnHCl and then decreased gradually to a minimum value at 6 M GdnHCl. This phenomenon emphasized the exposure of buried hydrophobic pockets of MalZ molecule during partial unfolding. The loss of MalZ-bound ANS fluorescence intensity might be due to the destruction of hydrophobic pockets during denaturation of the protein at higher concentration of denaturant. This observation clearly shows the formation of stable equilibrium intermediate species of MalZ, which accumulated at the early stage of GdnHCl-induced denaturation of MalZ. On the basis of this observation, a three-state unfolding pathway can be proposed for MalZ where the native protein (N) starts unfolding and the buried hydrophobic regions are exposed rapidly in presence of a relatively milder concentration of GdnHCl, which reaches the intermediate state (I) with maximum surface hydrophobicity; further unfolding of the protein (U) destroyed the hydrophobic pockets, thereby diminishing the binding of ANS to MalZ. Hence, the process of MalZ unfolding can be represented as:

$$\text{N (native)} \rightarrow \text{I (intermediate)} \rightarrow \text{U (unfolded)}\,.$$

Perhaps the formation of stable unfolding intermediates can be validated by recording and analyzing the far-UV CD spectra of different GdnHCl-denatured MalZ samples. It was not possible to perform CD spectroscopy of GdnHCl-treated MalZ samples because of the interference caused by the presence of imidazole in the MalZ solution.

Further calculations were carried out to determine the surface hydrophobicity of MalZ, according to Chaudhuri et al. [10], using protein–ANS-binding parameters. From the titration curve of ANS with MalZ (Fig. 6) the surface hydrophobicity of MalZ was calculated to be 150.7. The solubility and aggregation properties of a protein under physiological conditions are typically determined by the number and relative sizes of the surface hydrophobic sites [11, 16]. Earlier experimental results reported the surface hydrophobicity value of few proteins like BTSI, lysozyme, ovalbumin, and β-lactoglobulin [10] and among them BTSI has the lowest surface hydrophobicity value of 1.14, lysozyme

and ovalbumin have the values of 2.26 and 12, respectively, and β-lactoglobulin has the highest surface hydrophobicity value of 468. Hence, the surface hydrophobicity of MalZ is comparatively high with respect to other known proteins. Surface hydrophobicity was used in this present study for monitoring the unfolding process of MalZ because we observed that different states (N, I, and U) of the protein could be differentiated through their characteristic surface hydrophobicity values. Moreover, surface hydrophobicity is considered to be a reliable method for characterizing the non-native intermediate states of protein-like molten-globule states.

Further study on the determination of number of ANS-binding site (n) was performed according to the report described by Cardamon and Puri [11]. For MalZ, the n value was calculated to be 0.24. Chaudhuri et al. [10] have reported the number of ANS-binding sites of ovalbumin ($n = 0.9$), lysozyme ($n = 2.98$), and BTSI ($n = 0.6$). Hence, the native MalZ has a rather low ANS-binding sites value compared to other three proteins. But when denatured, the number of ANS-binding sites increased and the MalZ-bound ANS fluorescence for the non-native state was rather high compared to the native state of MalZ. Therefore, protein-bound ANS fluorescence measurements were successful in monitoring the unfolding process of MalZ.

A point worth mentioning here, in the context of using ANS as an external fluorescent probe for the elucidation of the unfolding pathway of MalZ as well as characterizing the nature of intermediate species accumulating during the unfolding process is that, despite the fact that ANS is routinely used as a reagent for determining the non-polar surface properties of protein molecules, not many studies on the unfolding process and characterization of the unfolding intermediates of large multi-domain proteins have been carried out over the years. In the present study, we have reported the unfolding pathway of a relatively large multi-domain protein MalZ by monitoring the change of surface hydrophobicity using the extrinsic fluorescence probe ANS. The unfolding intermediate species were also characterized on the basis of differential hydrophobic characters. Measurement of overall hydrophobicity of the native MalZ, as well as partially folded species using ANS, has provided sufficient information about the nature of the protein in different states. The idea of surface hydrophobicity of the native and partially unfolded MalZ may provide fruitful information about the potential cause of the aggregation-prone character of MalZ. We know that MalZ aggregates during the spontaneous refolding process. Considering the fact that the partially unfolded MalZ has substantially more hydrophobic surface than the native state, it may be anticipated that the intermediate species are susceptible to intermolecular aggregation. As a result, the denatured MalZ aggregates during the refolding process. Hence, it may be presumed that chaperonin GroEL binds with the unfolding intermediates through hydrophobic interaction during the chaperone-assisted folding of MalZ, as reported earlier [9].

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