J Fluoresc (2010) 20:827–836
DOI 10.1007/s10895-010-0625-1

ORIGINAL PAPER

Conformational and Functional Transitions in Class II α-mannosidase from *Aspergillus fischeri*

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Received: 5 June 2009 / Accepted: 8 February 2010 / Published online: 4 March 2010

Abstract The conformational transitions in an oligomeric and high molecular weight class II α-mannosidase from *Aspergillus fischeri* were examined using fluorescence and CD spectroscopy under chemical, thermal and acid denaturing conditions. The enzyme lost the activity first and then the overall folded conformation and secondary structure. The midpoint values of GdnHCl mediated changes measured by inactivation; fluorescence and negative ellipticity were 0.48 M, 1.5 M and 1.9 M, respectively. The protein almost completely unfolded in 4.0 M GdnHCl but not at 90 °C. The inactivation and unfolding were irreversible. At pH 2.0, the protein exhibited molten-globule like intermediate with rearranged secondary and tertiary structures and exposed hydrophobic amino acids on the surface. This species showed increased accessibility of Trp to the quenchers and got denatured with GdnHCl in a different manner. The insoluble aggregates of a thermally denatured protein could be detected only in the presence of 0.25–0.75 M GdnHCl.

Keywords α-mannosidase · Fluorescence · Circular dichroism · GdnHCl · Thermal denaturation · ANS binding · Hydrobicity · Aggregation

Introduction

α-Mannosidase (α-mannoside mannohydrolase, E.C. 3.2.1.24) is an exoglycosidase which catalyzes the hydrolysis of terminal nonreducing mannose residues in mannans. N-linked glycosylation is a posttranslational modification found in eukaryotes. Enzymes of this glycosylation pathway are therefore potential targets for the development of inhibitors for cancer treatments. In various tumor cell lines such as those from breast, colon, and skin cancer, the distribution of the cell surface N-linked sugars is altered [1]. In early clinical trials, the compound Swainsonine, a well-known inhibitor for α-mannosidase was found to reduce the tumor growth and metastasis when taken orally [2]. Also, the deficiency of α-mannosidase causes the fatal disease, mannosidosis in humans and cattle. The enzyme is also essential for total hydrolysis of plant polysaccharides and may have application in pulp and paper industry in order to enhance the traditional chemical delignification [3]. It is also important in the enzymatic saccahirification of hemicelluloses to monomeric sugars for further conversion to chemical and fuels [4]. The use of alpha-mannosidase and related enzymes in the synthesis of oligosaccharides for medical and other purpose is also interesting [5].

It has become increasingly evident that a polypeptide chain can adopt conformations different from the functional, native conformation of the protein. It is well established that protein folding goes through few kinetic intermediates, which can accumulate in the folding process. To understand the principles governing protein folding it is important to study these partly folded conformations [6–8]. The best way to do this is to study protein unfolding by strong denaturants such as urea or GdnHCl since these denaturants can really transform proteins in a more or less completely unfolded state [9]. The development of wide range of sensitive techniques has led to the identification and characterization of stable intermediates in several proteins [10]. By recording changes in intrinsic tryptophan fluorescence and the secondary and tertiary structural features of protein in response to tailored changes in surroundings, one can establish presence of interesting structural intermediates relevant to structure-function relationship of the protein.

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The aggregation of the protein is a phenomenon associated with many neurodegenerative diseases such as amyloidoses, prion disease and cataracts, that are caused by non-specific protein interactions [11]. In this regard, different agents such as temperature, pH, chaotropic agents and denaturants, either individually or in combination were used to study the conformational stability of the proteins. Denaturation is followed by monitoring the changes in any measurable physical property of the protein such as intrinsic fluorescence; CD spectra and corresponding change in the protein activity are checked.

The α-mannosidase from *A. fischeri* has already been characterized in detail. The enzyme is a Class II α-mannosidase as revealed by the substrate specificity and independency on the metal ion/s for activity [12]. The energetics of catalysis and inhibition of the enzyme has recently been studied by us [13]. The environment of tryptophans in the native and denatured states also has been studied by steady state and time-resolved fluorescence spectroscopy [14].

As a next step towards the understanding of a correctly folded structure and conformational stability of the α-mannosidase from *A. fischeri*, the protein was subjected to different denaturing conditions and transitions in the structure were monitored by biophysical techniques.

Material and methods

1-anilino-8-naphthalenesulfonate (ANS), Guanidine hydrochloride (GdnHCl), Urea were obtained from Sigma, USA. All other reagents used were of analytical grades. Solutions prepared for spectroscopic measurements were in MilliQ water.

Production and purification of α-mannosidase from *A. fischeri*

Production and purification of α-mannosidase was carried out as described earlier [12, 15]. Enzyme samples for spectroscopic measurement were centrifuged, filtered through a 0.45 μm filter and the exact pH and protein concentration was determined by Lowry's method before taking measurement.

Enzyme assay

α-mannosidase was assayed by incubating the 500 μl reaction mixture containing suitably diluted enzyme, 50 mM phospahte buffer of pH 6.5 and 500 μM substrate, at 50 °C for 15 min. The reaction was terminated by adding 1 ml of 1 M sodium carbonate and p-nitrophenol released was determined from absorbance at 405 nm. One unit (U) was expressed as amount of enzyme that liberates one μmole of p-nitrophenol per ml per min under standard assay conditions.

Zymogram of α-mannosidase

The enzyme (25 μg) was loaded on to the SDS gel (7.5%) and allowed to run for 2 h. The gel was removed and kept in water bath at 50 °C with 50 μM 4-methylumbelliferyl α-mannopyranoside for 20 min. The gel was washed with water to remove excess of 4-methylumbelliferylα-mannopyranoside and visualized under UV light.

Fluorescence measurement

Intrinsic fluorescence of the enzyme was measured using a Perkin–Elmer Luminescence spectrometer LS50B connected to a Julabo F20 water bath. The protein solution of 1.44 μM was excited at 280 nm and the emission was recorded in the range of wavelength 300–400 nm at 30 °C. At 280 nm, although major fluorescence is due to tryptophan residues, tyrosine fluorescence also contributes to it and the overall conformation can be monitored under different conditions. The slit widths for the excitation and emission were set at 7.0 nm, and the spectra were recorded at 100 nm/min. To eliminate the background emission the signal produced by either buffer solution or buffer containing the appropriate quantity of denaturants was subtracted.

Thermal inactivation of α-mannosidase

Effect of temperature on α-mannosidase was studied using a thermostatic cuvette holder connected to an external constant temperature circulation water bath. The protein sample (1.44 μM) was incubated for 10 min at specified temperature before taking the scan. For renaturation experiments the samples were cooled to 30 °C and left for 2 h before recording the spectra. Fluorescence spectra were recorded as described above. Time course of temperature effect on α-mannosidase was carried out by incubating the enzyme in the temperature range of 40–60 °C, for 2 h. The aliquots were removed after 20 min interval and the activity was checked under standard assay conditions. Activity at 0 min at 30 °C was taken as 100%.

Effect of pH

Samples of α-mannosidase (0.72 μM) were incubated in an appropriate buffer over the pH range of 1–12 for 3 h at 30 °C. The following buffers (50 mM) were used for these studies:

Glycine-HCl for pH 1–3, Glycine-NaOH for pH 10–12, acetate for pH 4–5, phosphate for pH 6–7 and Tris-HCl for pH 8–9. pH of the reaction remained stable till the end when it was checked. For refolding experiments, the pH of the each sample was adjusted back to pH 7 and incubated at 30 °C for 1 h before recording the spectral measurement. Fluorescence spectra were recorded as described above.

Quenching studies

Fluorescence quenching experiments were carried out by the addition of a small aliquot of acrylamide, KI or $CsCl_2$ stock solution (5 m) to the protein solution (0.90 μM) previously incubated at pH 2 or 6.5 at 25 °C for 2 h and the fluorescence intensities were determined. Protein was excited at 295 nm and emission spectra were recorded in the range of 300–400 nm. Slit widths of 7 nm each were set for excitation and emission monochromators.

Iodide stock solution contained 0.2 M sodium thiosulfate to prevent formation of tri-iodide (I^{-3}). For quenching studies with denatured protein, the protein was incubated with 6 M GdnHCl overnight at room temperature. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

The steady-state fluorescence quenching data obtained with different quenchers were analyzed by Stern–Volmer (Eq. 1) and modified Stern–Volmer (Eq. 2) equations in order to obtain quantitative quenching parameters [16].

$$\mathrm{Fo}/\mathrm{Fc} = 1 + \mathrm{Ksv}[\mathrm{Q}] \tag{1}$$

$$\mathrm{Fo}/\Delta\mathrm{F} = f_a^{-1} + 1/[\mathrm{K_a} f_a(\mathrm{Q})] \tag{2}$$

Where Fo and Fc are the relative fluorescence intensities in the absence and presence of the quencher, respectively, (Q) is the quencher concentration, Ksv is Stern–Volmer quenching constant, ΔF=Fo−Fc is the change in fluorescence intensity at any point in the quenching titration, K_a is the quenching constant and f_a is the fraction of the total fluorophore accessible to the quencher. Equation 2 shows that the slope of a plot of Fo/ΔF versus $(Q)^{-1}$ (modified Stern–Volmer plot) gives the value of $(K_a f_a)^{-}1$ and its Y-intercept gives the value of f_a^{-1} .

Guanidium hydrochloride mediated unfolding

Protein samples (1.44 μM) were incubated in 0–6 M denaturant solution at pH 6.5 for 3 h to attain the equilibrium. Fluorescence spectra were recorded as described above. After taking the scans suitable aliquots were removed from the samples to check for activity. Refolding experiments were conducted on samples diluted ten times to dilute the GdnHCl and incubated at 30 °C and left for 2 h before recording the spectra. Fluorescence spectra were recorded as described above.

ANS-binding assay

The intermediate states of denatured and native α-mannosidase under different denaturing conditions were analyzed by the hydrophobic dye (ANS) binding. The final ANS concentration used was 50 μM, excitation wavelength, 375 nm and total fluorescence emission was monitored between 400–550 nm. Reference spectrum of ANS in each buffer of respective pH and GdnHCl was subtracted from the spectrum of the sample.

Light scattering

Protein aggregation upon thermal, pH and GdnHCl denaturation was detected using light scattering method. The same experimental setup used for fluorescence studies was employed for this purpose. Both excitation and emission wavelengths were set at 400 nm. Excitation and emission slit widths were set at 5 nm and 2.5 nm respectively. Scattering was recorded for 120 s.

Circular dichroism measurements

The CD spectra were recorded on a J-175 Spectropolarimeter with a PTC343 Peltier unit (Jasco, Tokyo, Japan) at 25 °C in a quartz cuvette. Each CD spectrum was accumulated from eight scans at 50 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Far UV CD spectra (2.6 μM) were collected in the range of wavelengths of 200–250 nm using a cell path length 0.1 cm for monitoring secondary structure. All spectra were corrected for buffer contributions and observed values were converted to molar ellipticity. The tertiary structure of the enzyme (8.6 μM) was monitored with near UV CD spectra in the wavelength 250–300 nm using path length 1 cm. Results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as

$$\mathrm{MRE} = \mathrm{M}\,\theta_\lambda / 10\,\mathrm{d}\,\mathrm{c}\,\mathrm{r}$$

Where M is the molecular weight of the protein, θ_λ is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ml and r is the average number of amino acid residues in the protein. Helical content was

calculated from the MRE values at 222 nm using the following equation

$$\%\,\text{helix} = (\text{MRE}\,222\text{nm} - 2340)/30300*100.$$

Results and discussion

Thermal denaturation of α-mannosidase

Monitoring the activity of α-mannosidase from *Aspergillus fischer*i, incubated at different temperatures for different time intervals revealed the maximum stability of the enzyme to be at 50 °C for 40 min (Fig. 1a).The intrinsic fluorescence of the protein was monitored at different temperatures to correlate the thermal instability with conformation. The fluorescence spectrum of native enzyme showed a plateau in the range of 338–350 nm (Fig. 1b) indicating Trp residues present in differential environment [14]. The fluorescence intensity of the enzyme gradually decreased with increasing temperature which could be due to the deactivation of the single excited state by non-radiative process. At 70 °C, the spectrum showed a peak at 350 nm and at 90 °C, at 353 nm due to the exposure of buried Trp of partially unfolded protein to the solvent.

The Far UV CD spectra of the enzyme were recorded at different temperatures to monitor the changes in secondary structure (Fig. 1c). Gradual decrease in the ellipticity was observed with increase in temperature. Figure 1d shows slow decrease in the α-helix content of the protein on thermal denaturation. The α-helical content of the protein

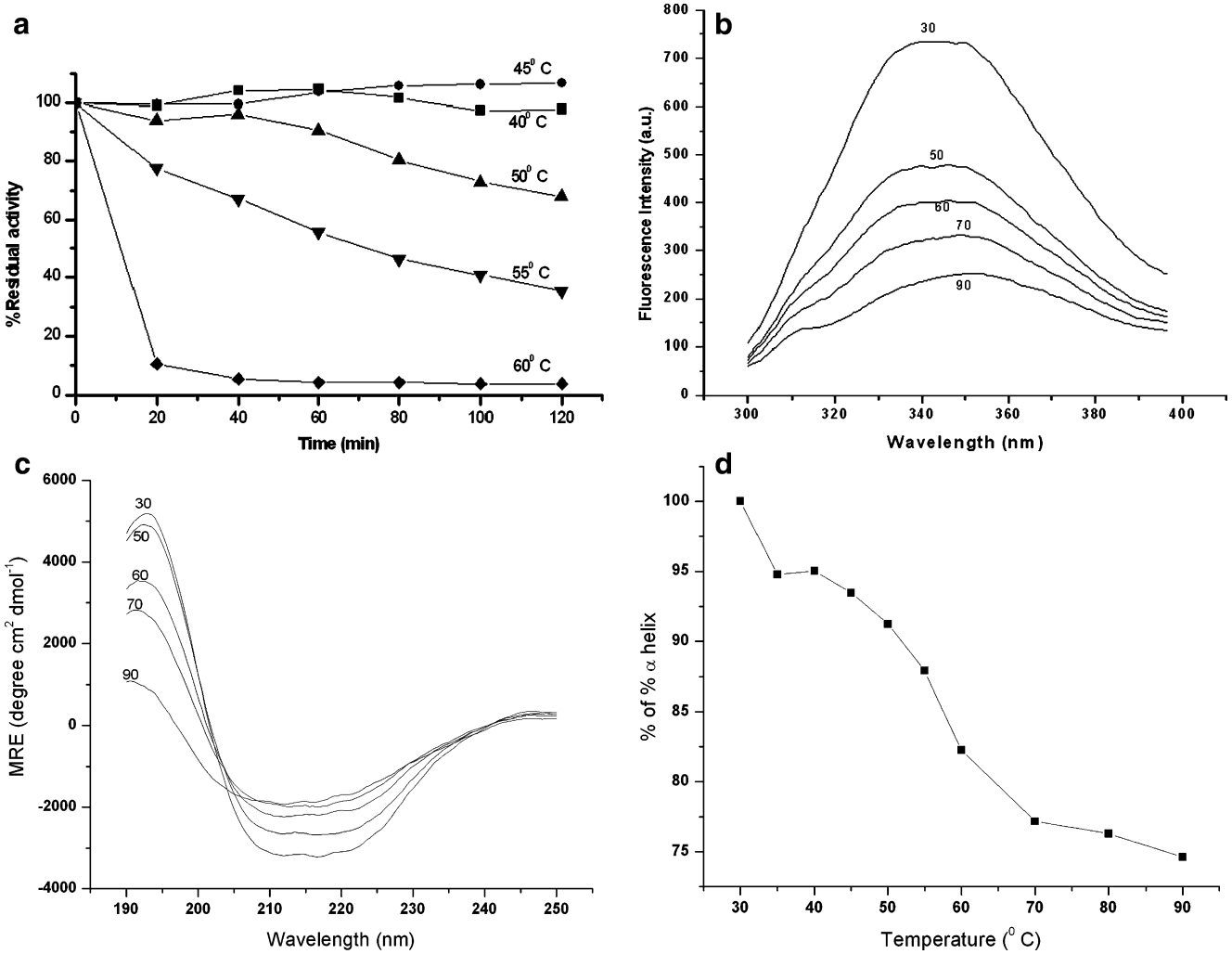

Fig. 1 Thermoinactivation and Denaturation of α-mannosidase: **a** Time course study of the enzyme (0.6 μg) at different temperature. **b** Fluorescence Scans of protein (1.44 μM) incubated at different temperature for 10 min. **c** Far UV CD spectra of α-mannosidase at (1) 30 °C, (2) 50 °C, (3) 60 °C and (4) 90 °C. 2.60 μM of protein was used, each sample was incubated for 10 min at the respective temperature and then scans were recorded. **d** The per cent α helical content of protein calculated by the formula given in "Material and methods"

was reduced by 8% at 50 °C while positive ellipticity at 190–195 nm was almost same in the temperature range of 35–50 °C. The activity is not affected in this range. At 60 °C, 18% and at 70 °C and above, 25% loss in the α-helical content of the protein was observed with significant decrease in the positive ellipticity. Although the λ_{max} of the fluorescence spectrum is at 355 nm indicating unfolding of the protein, major unfolding of the protein does not take place even at 90 °C, as evident from the far UV CD spectra.

No change in the light scattering intensity of the protein was observed till 90 °C, as there was no aggregation due to thermal denaturation. Renaturation or cooling of the heated protein samples to 30 °C did not lead to reactivation of enzyme indicating irreversibility of the thermal inactivation of α-mannosidase. Thermal inactivation of the enzyme taking place before any structural change has been reported in BSH [17].

Effect of pH on α-mannosidase

The enzyme shows maximum activity at pH of 6.0–6.5 and is most stable in range of 5.0–7.0. Inactivation of the enzyme is fast below pH 5.0 and slow above pH 8.0 and is irreversible.

The fluorescence scans at different pH show decrease in the fluorescence intensity at pH 1, 2 and 3 than at pH 6.5 (Fig. 2a) which could be due to the acid quenching and neutralization of COO^- groups on amino acid in the vicinity of tryptophan [18]. Under extreme pH conditions, red shift in the λ_{max} to 353 nm was observed indicating partial unfolding of the protein, which was further characterized by hydrophobic dye binding.

ANS binding for α-mannosidase was carried out at different pH. The protein could bind ANS only at extreme acidic pH, maximum at pH 2.0 showing blue shift in the λ_{max} from 520 nm to 480 nm and four times increase in the fluorescence intensity (Fig. 2b). Partial unfolding of the protein was observed at pH 2.0, as the λ_{max} of intrinsic fluorescence scan was red shifted to 353 nm. The binding of hydrophobic dye (ANS) to α-mannosidase indicated the exposure of hydrophobic patches in protein at extreme acidic pH.

Far UV CD-spectrum at pH 6.5 showed a trough with minima at 210 nm and 222 nm indicating presence of both α and β structures in the enzyme (Fig. 2c). The secondary structure elements calculated using the software CD Pro are 24% (Helix), 23.6% (Sheet), 21.2% Turn and 31.3% unordered structure (RMSD 0.028 and NRMSD 0.012). At pH 2.0, there is substantial rearrangement of the secondary structural elements as compared to pH 6.5.

Near UV CD spectrum of α-mannosidase (Fig. 2d) at pH 2.0 shows decrease in the overall ellipticity indicating change in the environment of the aromatic amino acid residues during which, the hydrophobic patches must be getting exposed on the surface of the protein. An inactive but compact and intermediate with rearranged secondary structure, altered tertiary structure, exposed hydrophobic amino acids on the surface of the protein together show that the molten-globule like intermediate exists at pH 2.0. Structural changes of α-mannosidase at extreme pH were found to be irreversible as indicated by unaltered λ_{max} of the fluorescence spectra of the protein samples under renaturing conditions.

Acids and bases are known to denature proteins by disrupting their electrostatic interactions. Protonation of all ionizable side chains below pH 3.0 leads to charge-charge repulsion and consequently protein unfolding. This induces loosening of side chain packing and the exposure of hydrophobic groups to solvent. Several proteins have been shown to exist in molten globule form at extremely acidic pH, Glucose/Xylose Isomerase [19], α-crystalin [20], Xylanase [21], glutathione transferase [22] and glucose oxidase [23]. Molten globule may be one of the first conformations embraced by the polypeptide chain in folding from the unfolded state [24].

The molten-globule like structure was further characterized by applying denaturation conditions and performing solute quenching studies. At pH 2.0, the enzyme treated with GdnHCl (0–6 M) showed gradual increase in the fluorescence intensity with increase in the GdnHCl concentration (Fig. 2e). The denaturation profile was different than that of protein at ph 6.5 which is presented in the section "Effect of GdnHCl on α-mannosidase". The tyrosine peak became prominent, the intensity of which increased with increase in the concentration of GdnHCl. This unusual pattern of fluorescence spectra could be due to the complex mechanism of energy transfer. There was no ANS binding with protein at pH 2.0 under these conditions.

Quenching of α-mannosidase at pH 2.0

Fluorescence quenching studies were carried out with quenchers like acrylamide, KI and CsCl at pH 2.0 (Table 1). Total fluorescence accessible to acrylamide was more (100%) at pH 2.0 while that under native condition accessibility was 73% [14]. For KI, fraction accessible at pH 2.0 was 67% which is double that of native protein. CsCl showed six times more accessibility as compared to native state which could be due to the redistribution of the charge density.

Ksv, the collisional quenching constant, for KI is more at pH 2.0 than native and denatured ones due to more positive charge and more exposure as compared to native protein. Ksv for KI is more than CsCl which can be again explained by the same property of protein at lower pH i.e., protein become more positively charged, so KI which is positive

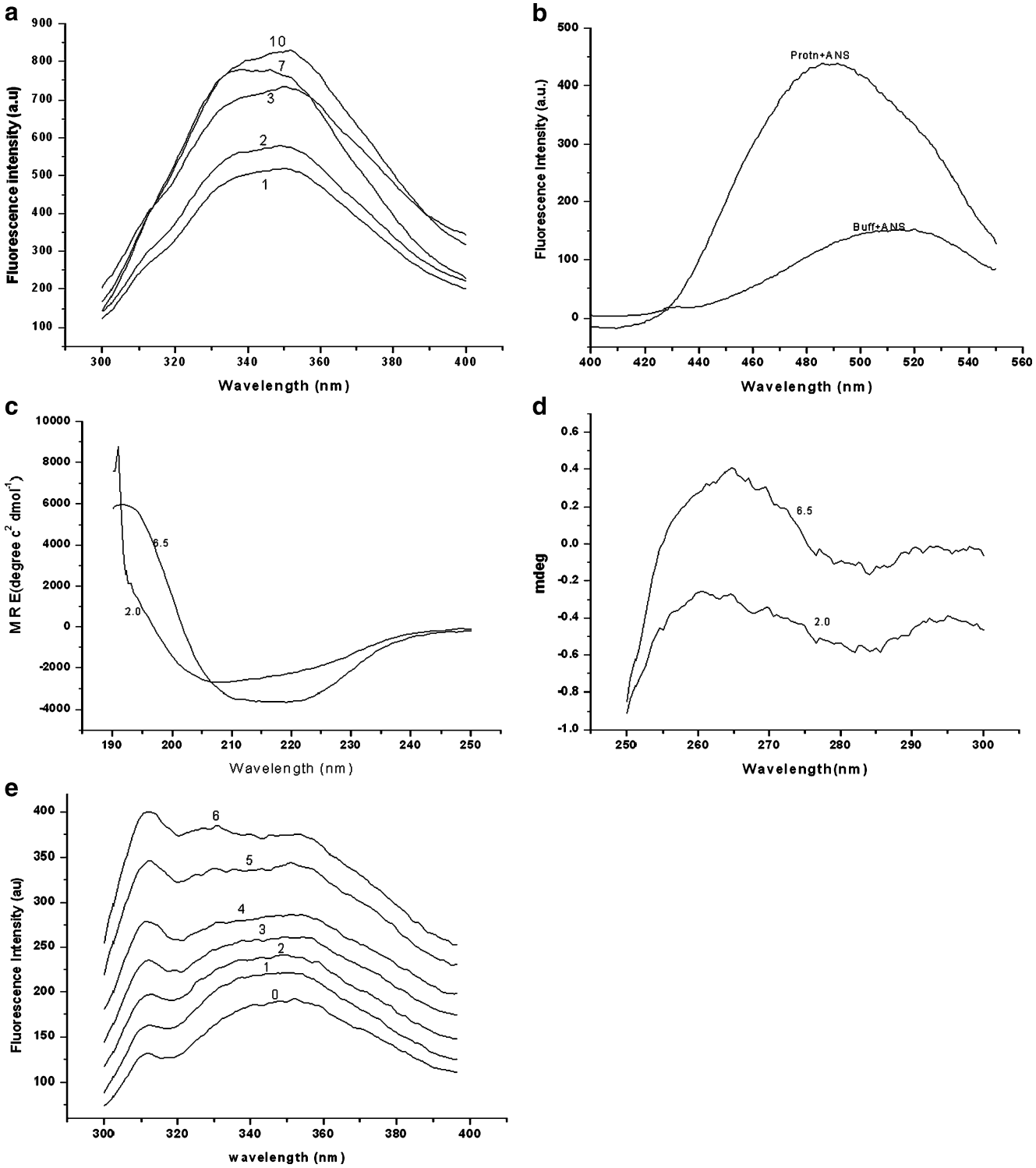

Fig. 2 pH dependent denaturation: **a** Fluorescence spectra of α-mannosidase (1.44 μM) at different pH. The numbers on the spectra indicate the respective pH of the sample. **b** Fluorescence spectra showing ANS binding of α-mannosidase (1.44 μM) at pH 2.0. **c** Far UV CD spectrum of the α-mannosidase (2.6 μM) at pH 6.5 and 2.0. Protein samples were incubated for 2 h at different pH. **d** Near UV CD spectrum of α-mannosidase (8.6 μM) at pH 6.5 and 2.0. The protein samples were incubated for 2 h at different pH. **e** Fluorescence spectra of α-mannosidase treated with different concentrations of GdnHCl (0–6 M) at pH 2.0. The protein (0.72 μM) was incubated with GdnHCl (0–6 M) for 2 h and fluorescence scans were recorded

Table 1 Summary of parameters obtained from the intrinsic fluorescence quenching of α-mannosidase at pH 2.0

Quencher and condition	K_{sv1} (M^{-1})	K_{sv2} (M^{-1})	*f*a	K_q
Acrylamide				
Native	3.414	–	0.73	6.72
Native + GdnHCl 6 M	10.634	–	1.00	8.57
pH 2.0	28.89	–	1.00	16.64
KI				
Native	1.821	0.835	0.33	9.65
Native + GdnHCl 6 M	3.670	–	0.71	7.90
pH 2.0	6.966	2.462	0.665	21.37
CsCl				
Native	1.017	0.311	0.124	10.32
Native + GdnHCl 6 M	1.050	–	0.380	5.84
pH 2.0	0.368	–	0.697	1.511

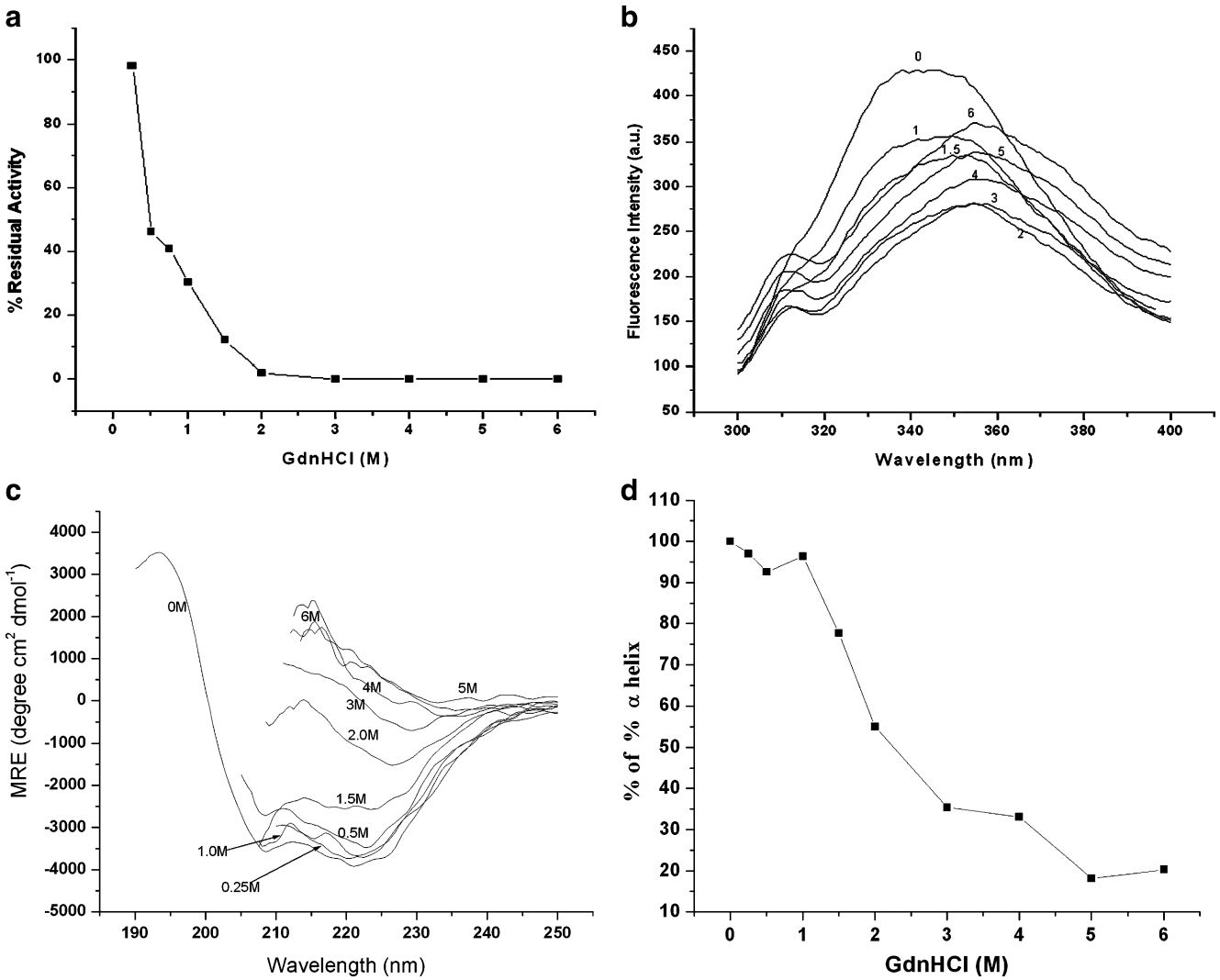

Fig. 3 Denaturation by GdnHCl: **a** Per cent residual activity of α-mannosidase at different concentrations of GdnHCl (0–6 M). **b** Fluorescence scans showing the spectra of α-mannosidase (1.44 μM) at different concentrations of GdnHCl (0–6 M). **c** Far UV CD spectra of α-mannosidase at different concentrations of GdnHCl (0–6 M). All the protein samples were kept in denaturant for 2 h and scans were recorded. **d** The per cent α helical content of protein calculated by the formula given in "Material and methods"

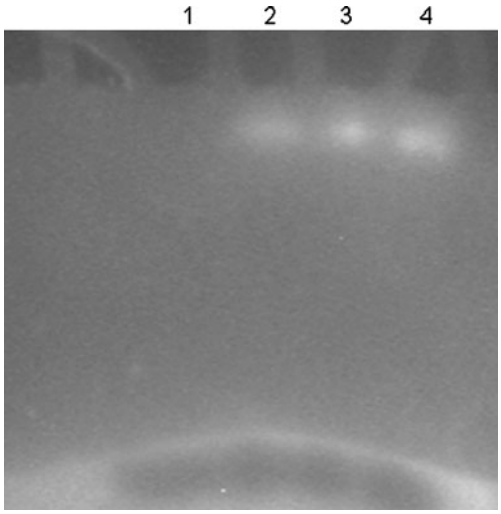

Fig. 4 Zymogram showing the activity of α-mannosidase under different conditions: (*1*) Enzyme + SDS (0.1%) boiled (*2*) Enzyme + SDS (0.1%) kept at 50 °C for 10 min. (*3*) Enzyme + SDS (0.1%). (*4*) Enzyme

quencher quenches more as compared to CsCl. Stern-Volmer plots were biphasic in case of KI at pH 2.0 which shows the heterogeneity in tryptophan environment. CsCl showed lesser Ksv values than native and denatured samples. This may be again due to lower pH of the protein environment.

Effect of GdnHCl on α-mannosidase

The enzyme lost 54% and 70% of the original activity in 0.5 M and 1.0 M GdnHCl, respectively (Fig. 3a) which could be due to the modification of carboxylate group at the active site of α-mannosidase. Specific chemical modification studies of the enzyme have revealed presence of one carboxylate group at the active site region of the protein (unpublished results). The positively charged guanidinium group electro statically interacts with the carboxylate group causing inactivation.

Fluorescence spectra of the enzyme treated with 0–6 M GdnHCl at pH 6.5 show decrease in the intensity up to 3 M GdnHCl (Fig. 3b), λ_{max} of the fluorescence of the inactivated protein in 2 M GdnHCl shifted to 352 nm indicating partial unfolding of the protein. The broad fluorescence spectrum of the protein was transformed into the sharper one with λ_{max} 352 nm, in presence of 1.5 M GdnHCl. The λ_{max} consequently shifted to 355 nm at higher concentration indicating the gradual unfolding of the protein. Increase in fluorescence intensity of the protein in presence of ≥ 4.0 M GdnHCl could be due to the change in the microenvironment of tryptophan of the unfolded protein.

The far UV CD spectra of GdnHCl treated samples (Fig. 3c and d) show that there is insignificant decrease in the α-helical content of the protein in presence of 1.0 M GdnHCl. Significant loss in the α-helical content of the protein in presence of 1.5 M and drastic loss at ≥ 2.0 M GdnHCl was observed. Major loss of enzyme activity in presence of 1.0 M GdnHCl takes place before any significant change in the secondary structure of the protein.

Under denaturing conditions the energy transfer from Tyr or Trp residues does not occur anymore and frequently results in increased tyrosine emission in the fluorescence spectra [25]. In the present studies, tyrosine peak becomes prominent and intensity increases with increase in GdnHCl concentration. There is initial decrease in fluorescence intensity and then increase in presence of higher concentrations of GdnHCl (> 4 M). The excited states of tryptophan interact with water molecules to form excited state complexes and such a process competes with the radiative relaxation and leads to diminution of the fluorescence intensity [26–28].

The enzyme did not get reactivated after dilution of 0.5 M or 1.0 M denaturant. After renaturation of the samples denatured with 3–6 M GdnHCl, the ratio of fluorescence intensity (336/356) increased from 0.85 to 0.91 indicating irreversibility of denaturation. Allowing the protein to renature for more time did not help in reconstituting the structure. The midpoint values of GdnHCl mediated changes measured by inactivation, fluorescence and negative ellipticity are 0.48 M, 1.5 M and 1.9 M, respectively.

The present native α-mannosidase has a molecular mass of 412 kDa and is composed of six subunits of 69 kDa [12]. The SDS-PAGE of the protein incubated at 50 °C in presence and absence of SDS shows fluorescence by activity staining method (Fig. 4) This protein species on the gel showed molecular mass of 210 kDa. The hexameric protein existing in the native state could be the dimer of

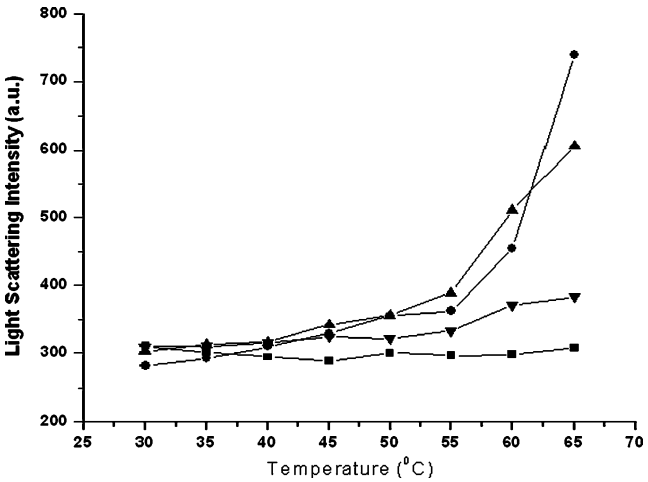

Fig. 5 Light scattering by α-mannosidase (1.44 μM) in the presence of GdnHCl at different temperatures: Protein in the absence of (■) and in presence of 0.25 M (●), 0.5 M (▲) and 0.75 M (▼) GdnHCl was incubated at different temperatures for 10 min

trimers. Trimer could be the ultimate active oligomeric structure of the enzyme. The gel filtration of native and 1.5 M GdnHCl treated enzyme on HPLC showed change in the retention time of the peak of the native enzyme indicating that the dissociation and unfolding of enzyme start simultaneously (data not shown).

Effect of GdnHCl at different temperature

Ray light scattering studies were carried out to monitor the formation of aggregates in the protein solution under different denaturing conditions. Exposure of the enzyme at 60–65 °C in presence of low concentrations of GdnHCl showed high light scattering intensity indicating the formation of insoluble aggregates (Fig. 5). In presence of 0.25 M and 0.5 M GdnHCl, the protein showed high light scattering intensity at 65 °C. No aggregation was observed at higher temperature in presence of ≥ 0.75 M concentration.

At 0.25–0.75 M GdnHCl, progressive inactivation and purterbance in the secondary structure of the enzyme was observed (Fig. 3a and c). No aggregation of protein was observed due to thermal denaturation in the absence of GdnHCl. At 55 °C and above, the loss in the secondary structure has also been observed (Fig. 1c). There is a cumulative effect of disturbance in the secondary structure at 60 °C and in presence of GdnHCl. Thus, due to distortion in the secondary structure, protein turns into insoluble aggregates on heating in presence of GdnHCl. The Trp environment of the α-mannosidase intermediate structure formed in the presence of 0.25–1 M GdnHCl showed decrease in fluorescence intensity. But the enzyme progressively lost catalytic activity and secondary structure. The protein under these conditions was susceptible to forming insoluble aggregates.

The aggregation process usually involves conformational change of the whole protein or of a specific domain and it is often attributed to the association of partially unfolded/folded molecules [29]. Aggregation process depends on temperature and it starts only after rearrangement of protein structure [30]. Irreversible unfolding of α-amylase was analyzed by unfolding kinetics, for all α-amylases the irreversible process was found to be fast and the preceding unfolding transition was the rate limiting step [31].

During the course of the unfolding studies, i)the partially unfolded states at 90 °C and ii) in presence of 1.5 M GdnHCl, iii) molten globule-like structure at pH 2.0 and iv) heat aggregated state only in presence of low concentration of the denaturant were the interesting intermediates observed for the high molecular weight oligomeric enzyme. Loss of enzyme activity due to thermal and chemical denaturant, before any significant change in the conformation was also observed. The irreversibility of the inactivation and denaturation of the enzyme could be correlated to the adverse effects of the deficiency of the enzyme.

Acknowledgments The authors are thankful to DBT, Department of Science and Technology, India, for the financial support. Financial support by the CSIR (India) to SKS is duly acknowledged. We thank Dr. V. Kumar, Organic chemistry Division, NCL, for allowing us to use the CD machine.

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