Eur Biophys J (2011) 40:923–935
DOI 10.1007/s00249-011-0708-1

ORIGINAL PAPER

Equilibrium unfolding of *A. niger* RNase: pH dependence of chemical and thermal denaturation

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Received: 13 December 2010 / Revised: 18 April 2011 / Accepted: 27 April 2011 / Published online: 25 May 2011

Abstract Equilibrium unfolding of *A. niger* RNase with chemical denaturants, for example GuHCl and urea, and thermal unfolding have been studied as a function of pH using fluorescence, far-UV, near-UV, and absorbance spectroscopy. Because of their ability to affect electrostatic interactions, pH and chemical denaturants have a marked effect on the stability, structure, and function of many globular proteins. ANS binding studies have been conducted to enable understanding of the folding mechanism of the protein in the presence of the denaturants. Spectroscopic studies by absorbance, fluorescence, and circular dichroism and use of K2D software revealed that the enzyme has $\alpha + \beta$ type secondary structure with approximately 29% α-helix, 24% β-sheet, and 47% random coil. Under neutral conditions the enzyme is stable in urea whereas GuHCl-induced equilibrium unfolding was cooperative. *A. niger* RNase has little ANS binding even under neutral conditions. Multiple intermediates were populated during the pH-induced unfolding of *A. niger* RNase. Urea and temperature-induced unfolding of *A. niger* RNase into the molten globule-like state is non-cooperative, in contrast to the cooperativity seen with the native protein, suggesting the presence of two parts/domains, in the molecular structure of *A. niger* RNase, with different stability that unfolds in steps. Interestingly, the GuHCl-induced unfolding of the **A** state (molten globule state) of *A. niger* RNase is unique, because a low concentration of denaturant not only induces structural change but also facilitates transition from one molten globule like state (A_{MG1}) into another (I_{MG2}).

Keywords ANS binding · Circular dichroism · Molten globule state · RNase T2 family · Unfolding

Abbreviations

ANS	8-Anilino-1-naphthalene-sulfonic acid
CD	Circular dichroism
GuHCl	Guanidine hydrochloride
RNase	Ribonuclease
UV	Ultraviolet

Introduction

The native conformation of a protein molecule, necessary for its biological activity, is stabilized by various non-covalent and covalent forces. Perturbation of these forces results in denaturation of the protein with either partial or complete loss of structure and biological activity. Despite accumulation of large number of experimental studies, solving the mechanism of protein folding is still a major challenge to modern structural biologists and biophysicists.

It is now well established that the folding of large globular proteins is characterized by the formation of intermediates between the native (N) and the fully unfolded (U) states. Characterization of folding intermediates is crucial for elucidation of the mechanism of folding of proteins to their biologically active conformations

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(Kim and Baldwin 1990; Matthews 1995; Privalov 1996; Dill and Chan 1997; Kuwajima and Arai 1999). Such intermediates are found, by spectroscopic and hydrodynamic measurements, to have special characteristics. For most proteins the mechanism of intermediate formation is poorly understood, because the rate of formation of these species is too rapid for conventional stopped-flow experiments (Radford 2000). An understanding of the structural and thermodynamic properties of such intermediates may provide insights into the factors involved in guiding the pathway of protein folding. These intermediates are well populated and stable at moderate denaturant concentrations, thus possibly enabling their detailed characterization using modern sensitive techniques. Chemical and thermal denaturation of proteins are standard techniques in protein biochemistry to determine protein folding and unfolding equilibrium and kinetics (Radford 2000; Jackson 1998; Eaton et al. 2000).

Among common equilibrium intermediates, the molten globule (MG) folding intermediates have been characterized for a number of proteins (Fink 1995; Ptitsyn 1995). The molten globule states are non-native intermediate states with an appreciable amount of substantial secondary structure but lacking the well defined rigid tertiary structure or side chain packing needed for biological activity (Kuwajima 1992; Ptitsyn 1987; Ptitsyn 1992; Barrick and Baldwin 1993). Several functions for MG intermediates have been proposed including chaperone-assisted refolding, transmembrane trafficking, and membrane insertion (Hartl et al. 1991). However, point mutation may convert the native protein into the molten globule-like state, and some of these mutations are correlated with genetic predisposition to human diseases (Booth et al. 1997; Matthews et al. 2000). Thus, exploring the structure and dynamics of the molten globule state of a protein is necessary not only to understand the mechanism of the protein folding but also to shed light on many natural or disease-related processes. The exact role of molten globule states is controversial (Udgaonakr 2008); consequently, it is important to study this aspect with new proteins to determine the generality of such states and their role in protein folding.

The small M_r ($\sim$12–14 kDa) RNases, namely, RNase A, RNase T1, and barnase, have proved to be excellent models for these studies. In contrast, there are very few reports of conformational stability studies on the high-M_r RNases constituting the RNase T2 family. *A. niger* RNase, which belongs to the RNase T2 family, has been purified and isolated from *Aspergillus niger* ATCC 26550 (Gundampati et al. 2011). This manuscript elaborates studies on conformational transitions of *A. niger* RNase under various conditions, for example pH, chemical denaturants, and temperature. Several intermediates with distinct spectroscopic properties are populated under different conditions. This manuscript may shed light on the folding-unfolding mechanism of *A. niger* RNase of T2 family.

Materials and methods

Materials

Aspergillus niger RNase was purified from *Aspergillus niger* using the method of Gundampati et al. (2011). The concentration of the enzyme was determined by spectrophotometry, by use of the Bradford assay. GuHCl and urea were purchased from Sigma Chemical, USA. The concentration of GuHCl was determined from the refractive index of the solution. All the solutions were prepared in double-distilled water. Samples for spectroscopic measurements were centrifuged and filtered through 0.45 μm filters and the exact concentration of the protein, and the pH, were determined before the measurements.

Absorbance spectroscopy

Absorbance measurements were carried out on a Beckman DU-640B spectrophotometer equipped with a constant-temperature cell holder. Protein concentration for all absorbance measurements was between 7 and 8 μM. Absorbance spectra were recorded between 240 and 320 nm.

Fluorescence spectroscopy

Fluorescence measurements were carried out on a Perkin-Elmer LS-50B spectrofluorimeter equipped with a constant-temperature cell holder. Temperature was controlled by Julabo F25 water bath. Tryptophan was excited at 292 nm whereas for both the tryptophan and tyrosine fluorescence of *A. niger* RNase the excitation wavelength of 278 nm was used. The emission was recorded between 300 and 400 nm with 10 nm and 5 nm slit widths for excitation and emission, respectively. The protein concentration was 2 μM for all fluorescence measurements.

Spectropolarimetry

CD measurements were done on a Jasco J 810 spectropolarimeter equipped with a constant-temperature cell holder. The instrument was calibrated using ammonium (+)-10-camphorsulfonate. The temperature of the cell holder was controlled by use of a Julabo F 25 water bath. Conformational changes in the secondary structure of the protein were monitored in the region between 200 and 260 nm with a protein concentration of 6 μM in a cuvette of 1 mm path length whereas changes in the tertiary structure were

observed in a cuvette with a path length of 10 mm in the region between 260 and 320 nm at a protein concentration of 36 μM. After subtracting appropriate blanks, mean residue ellipticities were calculated by use of the formula:

$$[\theta] = \theta_{obs} \times \mathrm{MRW}/10\mathrm{cl}$$

where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein (g/ml), and l is the path length in cm (Balasubramanian and Kumar 1976). A mean residue molecular weight 110 was used. Sensitivities of 1 and 2 m°/cm were used for far-UV and near-UV measurements, respectively. Five consecutive scans were accumulated and the average spectra stored. The far-UV CD spectrum was analyzed for the secondary structure content by use of K2D software (Andrade et al. 1993).

Assay for RNase activity

The RNase activity of *A. niger* RNase at different pH or in the presence of a chemical denaturant was monitored using the denatured natural substrate azoalbumin by following the procedure described in Gundampati et al. (2011). The protein was incubated overnight in denaturants before the enzyme assays.

pH denaturation of *A. niger* RNase

Acid denaturation of *A. niger* RNase was carried out as a function of pH using KCl–HCl (pH 0.5–1.5), Gly-HCl (pH 2.0–3.5), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.5–10.5), and Gly-NaOH (pH 11.0–12.5) buffers. Concentrations of all buffers were 50 mM. A stock solution of protein was added to the appropriate buffer and the mixture was incubated for 24 h at 25°C. The final pH and concentration of the protein in each sample were measured again.

ANS binding assay

Exposure of hydrophobic surfaces in the enzyme was measured by its ability to bind to the fluorescent dye ANS. A stock solution of ANS was prepared in methanol and the dye concentration was determined using an extinction coefficient of $\varepsilon = 5{,}000\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ at 350 nm (Khurana and Udgaonkar 1994). The protein was incubated with a 100-fold molar excess of ANS for more than 30 min at room temperature in the dark and ANS fluorescence was measured. The protein concentration was 2 μM. The excitation wavelength was 380 nm and emission spectra were collected between 400 and 600 nm. Slit widths for excitation and emission were 10 nm and 5 nm, respectively.

Thermal unfolding

Temperature-induced denaturation of the enzyme, under specified conditions, was performed as a function of increasing temperature. Protein samples were incubated at the desired temperature for 15 min before measurements were taken. The actual temperature of the sample in the cuvette was obtained with a thermocouple using a digital multimeter.

Data analysis

Denaturation curves were obtained by plotting the ratio of the fluorescence intensities at the emission k_{max} of the native protein and the denatured protein against the denaturant molarity or temperature and further analysis of the data was performed as described by Pace et al. (1989). From the denaturation curves, a two-state N⇆D unfolding mechanism was assumed, and, consequently, for any of the points, only the folded and unfolded conformations were present at significant concentrations. Thus, if F_F and F_U represent the fraction of protein present in the folded and unfolded conformations, respectively, $F_F + F_U = 1$. The observed value of Y at any point will be $Y = Y_F F_F + Y_U F_U$ and F_U was calculated by use of the equation:

$$F_U = (Y - (Y_F + m_F[D]))/(Y_U + m_U[D] - (Y_F + m_F[D])) \quad (1)$$

where Y is the value of the spectroscopic property measured at a concentration [D], Y_F and Y_U represent the intercepts, and m_f and m_u are the slopes of the folded and unfolded baselines of the data, respectively, which were obtained from linear least-squares fits to the baseline. For a two-state F⇆U unfolding mechanism, the equilibrium constant K and ΔG_U, the free energy of unfolding by denaturant at concentration [D], were calculated by use of Eqs. 2 and 3, respectively.

$$K = F_U/(1 - F_U) \quad (2)$$

$$\Delta G_U = -RT \ln K \quad (3)$$

where, R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature. It is assumed that the free energy of unfolding, ΔG_U, has a linear dependence on the concentration of the denaturant [D]:

$$\Delta G_U = \Delta G^{H_2O} + m[D] \quad (4)$$

ΔG^{H_2O} and m are, therefore, the intercept and the slope, respectively, of the plot of ΔG_U versus [D]. ΔG^{H_2O} corresponds to the free energy difference between the folded and unfolded states in the absence of any denaturant and m is a measure of the cooperativity of the unfolding reaction. The concentration of denaturant at which the

protein is half unfolded (when $\Delta G_U = 0$) is given by $C_{1/2}$ and from Eq. 4, $\Delta G^{H_2O} = -mC_{1/2}$.

Results

Biophysical characterization of *A. niger* RNase

The far UV (260–200 nm) CD spectra give information about the secondary structure of the protein. The native spectrum showed negative peaks at 222 and 208 nm with greater signal intensity at the latter wavelength (Fig. 1a), suggesting the protein belongs to $\alpha + \beta$ class (Manavalan and Johnson 1983). Ribonuclease in the native state retains all the secondary features with 29% α-helix, 24% β-sheet, and 47% random coil. The ellipticity at 222 nm was approximately -7.0×10^3 deg cm^2 dmol^{-1}.

The near UV CD spectra can be used to assess the tertiary structure of proteins. The shape of the spectra depends critically on the atomic environment and closeness of packing of the aromatic residues, including their accessibility to solvent (Sears and Beychok 1973). The CD spectrum of *A. niger* RNase at pH 7.0 (native) in the near UV region (320–260 nm) is characterized by one positive intense peak centered at 278–279 nm and a less intense negative peak centered at 298 nm (Fig. 1b). Probably tryptophan residues located in the asymmetric environment are responsible for negative spectrum at the latter wavelength whereas tyrosine and phenylalanine do not contribute to the CD spectrum at this wavelength (Strickland 1974). The significant amount of signal in the near UV region suggests that aromatic side chains are highly ordered and fixed in the native structure. In the presence of 6 M GuHCl, *A. niger* RNase loses all the CD spectral features, which confirms unfolding of protein (Fig. 1a, b).

Fluorescence spectra provide a sensitive means of characterizing proteins and their conformations. The spectrum depends on the polarity of the environment of tryptophan and tyrosine residues and on their specific interactions in the molecular architecture of the protein. The fluorescence spectrum of *A. niger* RNase in the native state contains an emission maximum at 339 nm upon excitation at 292 nm, indicating that Trp residues are buried inside the hydrophobic core of the tightly packed

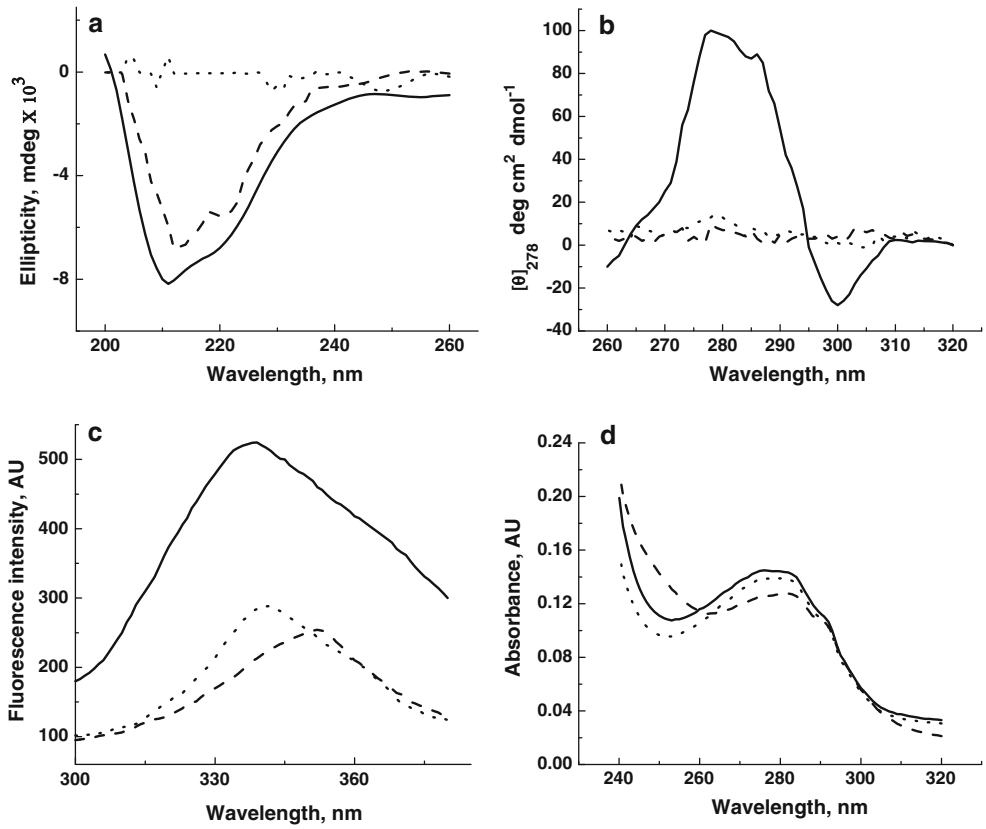

Fig. 1 Biophysical properties of *A. niger* RNase. **a** Far UV and **b** Near UV spectra, **c** Intrinsic fluorescence spectra, **d** Absorbance spectra of *A. niger* RNase at pH 7.0 (*solid line*), pH 1.0 (*dotted line*), and in the presence of 6 M GuHCl (*dashed line*). The protein concentration was 36.0 μM for near UV and 6.0 μM for far UV. The excitation wavelength was 292 nm with slit widths of 10 nm and 5 nm for excitation and emission, respectively. The protein concentration was 2.0 μM for fluorescence and absorbance spectra. The samples were incubated for 24 h at 25°C before the measurements

protein molecule (Fig. 1c). In the presence of 6 M GuHCl, the emission maximum of *A. niger* RNase suffers a red shift of 13 nm and the quantum yield diminishes by nearly 52% relative to the maximum emission intensity observed at 339 nm in the native state (Fig. 1c). The decrease in fluorescence intensity may be because of the reduced distance between tryptophan and specific quenching groups, for example protonated carboxyl, protonated imidazole, deprotonated amino groups, and tyrosinate, which consequently resulted in quenching of tryptophan fluorescence (Halfman and Nishida 1971).

The absorbance spectrum of *A. niger* RNase at neutral pH contains a peak at 280 nm, indicating significant contributions from tryptophan and tyrosine residues (Fig. 1d). A small inflection point on the declining shoulder of the main peak around 290 nm originates from tryptophan residues. The decrease in absorbance in 6 M GuHCl reveals significant disruption of tryptophan residues present on the surface of the denatured protein molecule. The possible explanation for this disruption is incomplete ionization of phenolic chromophores of tyrosine at extremes of pH. GuHCl, which is an ionic denaturant, causes a change in the spectrum of *A. niger* RNase similar to that caused by low pH, indicating disruption of the environment of the tyrosine residues. Because there was no significant difference in the absorbance spectra of the protein at different pH, absorbance was not used as a probe to follow the conformational change of the protein in this investigation.

pH-induced conformational changes in *A. niger* RNase

The structural and functional changes in *A. niger* RNase as a function of pH were followed by far UV CD, near UV CD, activity measurements, and fluorescence spectroscopy. Changes in the secondary structure of *A. niger* RNase at varying pH are shown in Fig. 2a. The pH-induced unfolding of *A. niger* RNase in terms of secondary structure seemed to occur through a multistep process of at least three transitions, although the final state of the third transition was not reached. The acid-induced unfolding of *A. niger* RNase is noncooperative and takes place with two transitions. The first transition from the native to the unfolded state was completed at approximately pH 2.0, along with a decrease in ellipticity at 222 nm. The second transition was completed at pH 1.0 along with increase in ellipticity at 222 nm. Increasing the pH from the native state, i.e., alkaline denaturation of *A. niger* RNase, led to an incomplete third transition.

Tertiary structural changes of *A. niger* RNase at different pH are shown in Fig. 2b. *A. niger* RNase is structurally stable up to pH 4.0–8.0 and loses tertiary structure below pH 4.0 and above pH 8.0. The overall spectral features remain the same between pH 4.0 and 8.0 with a positive peak centered at 278 nm and a negative peak centered at 298 nm. Below pH 4.0, loss of tertiary structure is manifested as loss of intensity of both the peaks without changing the shape of spectra. At and below pH 1.25 all the prominent peaks in near UV region were lost and the spectrum was identical with that in 6 M GuHCl (Fig. 1b). The change in RNase activity of the protein with pH also follows the trend similar to the ellipticity at 278 nm (Fig. 2b, inset).

pH denaturation of *A. niger* RNase was also studied by measurement of the intrinsic fluorescence emission maximum of tryptophan residues. This reveals information about solvent accessibility and the hydrophobicity of the environment of trp residues. The excitation wavelength of 292 nm was chosen to excite tryptophan residues. The fluorescence intensity and wavelength for maximum fluorescence intensity of *A. niger* RNase as a function of pH are shown in Fig. 2c, d. Here, also, the pH-induced transition is non-cooperative, and three transitions are observed. The first transition occurred between pH 7.0 and pH 2.0, with a red shift of 7 nm in the emission maximum and a decrease in the fluorescence intensity maximum. The second transition occurred between pH 2.0 and 0.5, with blue shift of 4 nm in wavelength maximum and an increase in the fluorescence intensity. The third transition, which was incomplete, occurred from 7.0 to 12.0, with a red shift of 4 nm and a slight decrease in the fluorescence intensity. The shape of the fluorescence intensity maxima in the spectra remains same at all pH values.

The hydrophobic regions are buried inside the enzyme in the native state and are exposed during unfolding of the protein. ANS, a polarity-sensitive extrinsic fluorescent probe binds to the hydrophobic surfaces of partially unfolded protein and helps in the detection of equilibrium folding intermediates, for example the molten globule state. Hence, exposure of any hydrophobic regions upon denaturation of the protein was monitored by ANS binding as a function of pH (Fig. 3a). Maximum ANS binding occurred at pH 1.0, because the ANS fluorescence intensity maximum increased approximately 9–10-fold and the wavelength emission maximum shifted from 510 to 480 nm, as compared with the native state. ANS binding decreased with increase or decrease of pH on either side of pH 1.0. ANS binding was much less in the native state (pH 7.0) and in the completely denatured state in 6 M GuHCl in comparison with the MG state as shown in Fig 3b. ANS fluorescence in the absence of protein gives a flat line at 0 in all buffers and was used as control.

GuHCl-induced unfolding of *A. niger* RNase

GuHCl-induced changes in *A. niger* RNase under different pH conditions were followed by ellipticity (278 and

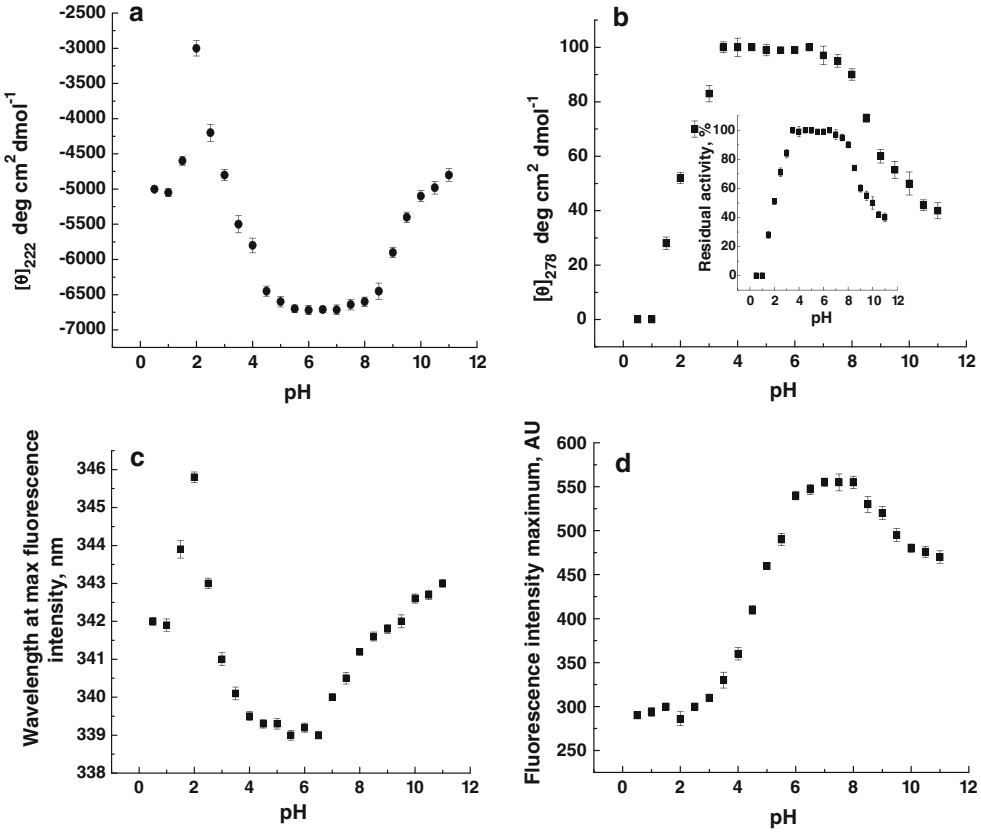

Fig. 2 pH-induced conformational changes of *A. niger* RNase. **a** Ellipticity at 222 nm, **b** Ellipticity at 278 nm (*inset* represents residual activity), **c** Fluorescence wavelength at maximum fluorescence intensity, and **d** fluorescence intensity maxima. The samples were incubated for 24 h at 25°C before the measurements. Each value in the figures represents mean ± SD ($n = 3$)

222 nm), fluorescence (wavelength maxima), ANS binding, and activity measurements. Under neutral conditions, GuHCl-induced unfolding of *A. niger* RNase, was cooperative and the transition curves were concurrent as seen by different methods (Fig. 4a). All the structural changes occurred between 1.5 and 4.5 M GuHCl with a transition midpoint of 2.80 ± 0.1 M GuHCl. Fluorescence wavelength maxima suffered a red shift of 13 nm from 339 to 352 upon complete unfolding of the *A. niger* RNase by GuHCl.

The loss in RNase activity coincides with loss in tertiary structure, reflecting good correlation between the activity and structural integrity of the enzyme. Because the enzyme is structurally stable with no loss of RNase activity even after prolonged exposure in the pH range 4.0–8.0, it is interesting to observe the structural integrity of the molecule at low pH. At pH 3.5, GuHCl-induced unfolding was cooperative but the transitions were non-coincidental when measured with different techniques (Fig. 4b). The RNase activity and tertiary structure were lost initially, followed by the loss of fluorescence and secondary structure. The transition midpoints (Cm) of the denaturation, followed by activity, and near UV, fluorescence, and far UV spectroscopy, were 0.81 ± 0.1, 0.80 ± 0.3, 1.34 ± 0.3, and 1.57 ± 0.2 M, respectively. The overall characteristics of GuHCl-induced unfolding of *A. niger* RNase at pH 2.5, were similar to those observed at pH 3.5 (Fig. 4c). The GuHCl-induced unfolding at pH 2.5 was also cooperative and non-coincidental. RNase activity, tertiary structure, intrinsic fluorescence, and secondary structure were lost in the same order, but with lower transition mid points of 0.52 ± 0.1, 0.48 ± 0.2, 1.12 ± 0.1, and 1.25 ± 0.2 M, respectively. Further, strong ANS binding in 1.5 M GuHCl and 1.38 M GuHCl was observed at pH 3.5 and at pH 2.5, respectively (inset of Fig. 4b, c).

The GuHCl-induced unfolding of *A. niger* RNase was also studied at pH 1.0, at which tertiary structure and activity were completely disrupted; hence GuHCl-induced unfolding was followed by monitoring intrinsic fluorescence maxima, far UV CD, and ANS fluorescence (Fig. 4d). The GuHCl unfolding of *A. niger* RNase at pH 1.0 was cooperative and transition curves were coincidental with the midpoint for 2.5 ± 0.1 M GuHCl. The higher transition midpoint for GuHCl-induced unfolding at pH 1.0, in comparison with that obtained for the states at pH 3.5 and 2.5, indicate the higher stability of the MG state.

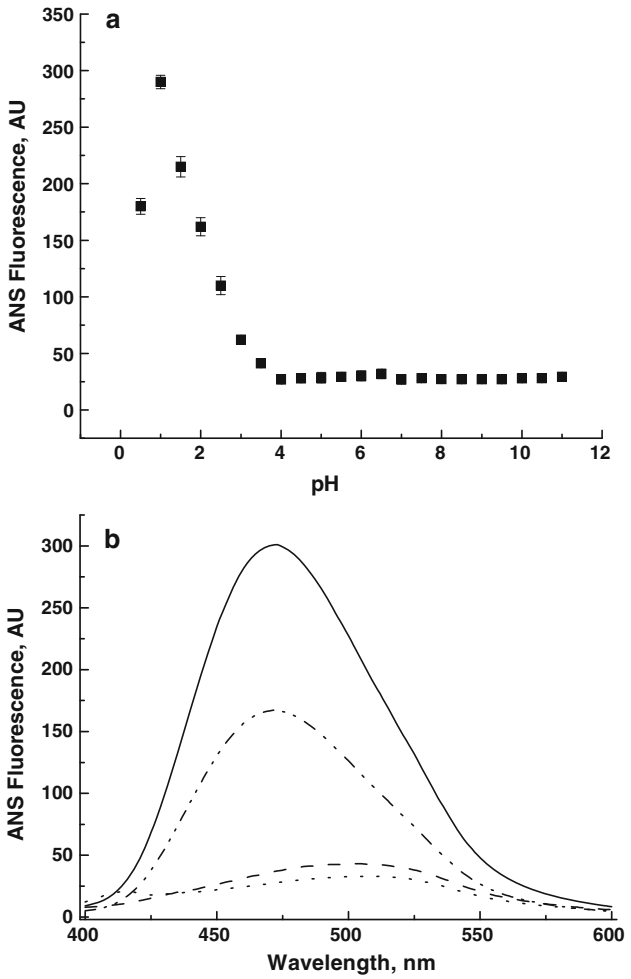

Fig. 3 ANS fluorescence of *A. niger* RNase at different pH. **a** Effect of pH on ANS fluorescence of *A. niger* RNase. **b** ANS fluorescence at pH 7.0 (*filled circles*), at pH 0.5 (*open triangles*), at pH 1.0 (*filled squares*), and in 6 M GuHCl (*open circles*). ANS emission spectra were recorded with excitation at 380 nm. ANS fluorescence intensity was measured at 480 nm within an hour of addition of ANS stock to the protein samples in the ratio of 1:100 of protein to ANS. ANS fluorescence without protein shows a flat line at 0 in all the buffers from 400 to 600 nm and was used as control. Protein concentration was 2 μM. Each value in Fig. 3a represents mean ± SD ($n = 3$)

The ANS fluorescence was also monitored, and decreased gradually in intensity with increasing denaturant concentration at pH 1.0 (inset of Fig. 4d), indicating the cooperative unfolding of the enzyme.

Urea-induced unfolding

Under neutral conditions, urea did not cause any structural perturbation—the enzyme suffered no loss of RNase activity even at higher concentrations of urea, for example 8 M. The enzyme was more susceptible to denaturants at lower pH. Therefore, urea-induced unfolding of *A. niger* RNase was studied at low pH, by monitoring secondary and tertiary structure, RNase activity, fluorescence emission maximum, and ANS binding. At pH 3.5, the unfolding transitions were cooperative and coincidental. At pH 3.5, all the structural changes occurred between 2.0 and 4.5 M urea with a transition midpoint at 3.25 ± 0.1 M urea (data not shown). No ANS binding was observed during urea denaturation of *A. niger* RNase at pH 3.5. *A. niger* RNase was structurally and functionally stable up to 2 M urea at pH 3.5, but at pH 2.5 unfolding started at less than 2 M urea. Unlike pH 3.5, at pH 2.5 the transition curves obtained by near UV, activity, far UV and fluorescence are non-coincidental with the transition midpoints of 1.63 ± 0.1, 1.58 ± 0.1, 2.47 ± 0.1, and 2.21 ± 0.2 M urea, respectively (Fig. 5a). The loss in tertiary structure and activity took place between 0.75 and 2.5 M urea whereas intrinsic fluorescence and secondary structural changes occurred between 1.25 and 3.75 M urea. ANS binding at pH 2.5 is shown in the inset of Fig. 5a. The maximum amount of ANS binding is seen at 2.5 ± 0.1 M urea. Interestingly, another small peak on ANS binding curve was observed at 1.58 ± 0.03 M urea.

At pH 1.0, urea-induced unfolding of *A. niger* RNase was followed by monitoring far UV CD and intrinsic fluorescence wavelength maxima. Urea denaturation was noncooperative and the transition was three-state equilibrium in nature, with two transition midpoints of 1.25 ± 0.1 and 3.25 ± 0.1 M urea (Fig. 4b). Moreover, ANS fluorescence intensity (which is highest at pH 1.0 in the absence of denaturant) concomitantly decreased with increasing urea concentration.

Temperature-induced unfolding

Temperature-induced unfolding of *A. niger* RNase is incomplete under neutral conditions. At least 45% of native structure of the protein was retained even at high temperature (90°C) as monitored by near UV, far UV CD, and fluorescence measurements. Thermal denaturation of *A. niger* RNase at pH 3.5 was also incomplete, because 20% of the native structure of the protein was retained up to 90°C. However, at pH 2.5 the temperature-induced unfolding transitions of *A. niger* RNase, as measured by near UV, far UV, activity, and fluorescence wavelength maxima, were cooperative and the curves were coincidental with a similar transition midpoint at approximately 52.4 ± 0.1°C (Fig. 6a). Interestingly, thermal unfolding of the protein at pH 1.0, was non-cooperative and three-state equilibrium in nature with two transition midpoints at 33 ± 0.5 and 49 ± 0.5°C (Fig. 6b).

Discussion

Conformational stability is studied by denaturation of proteins using different agents, for example temperature,

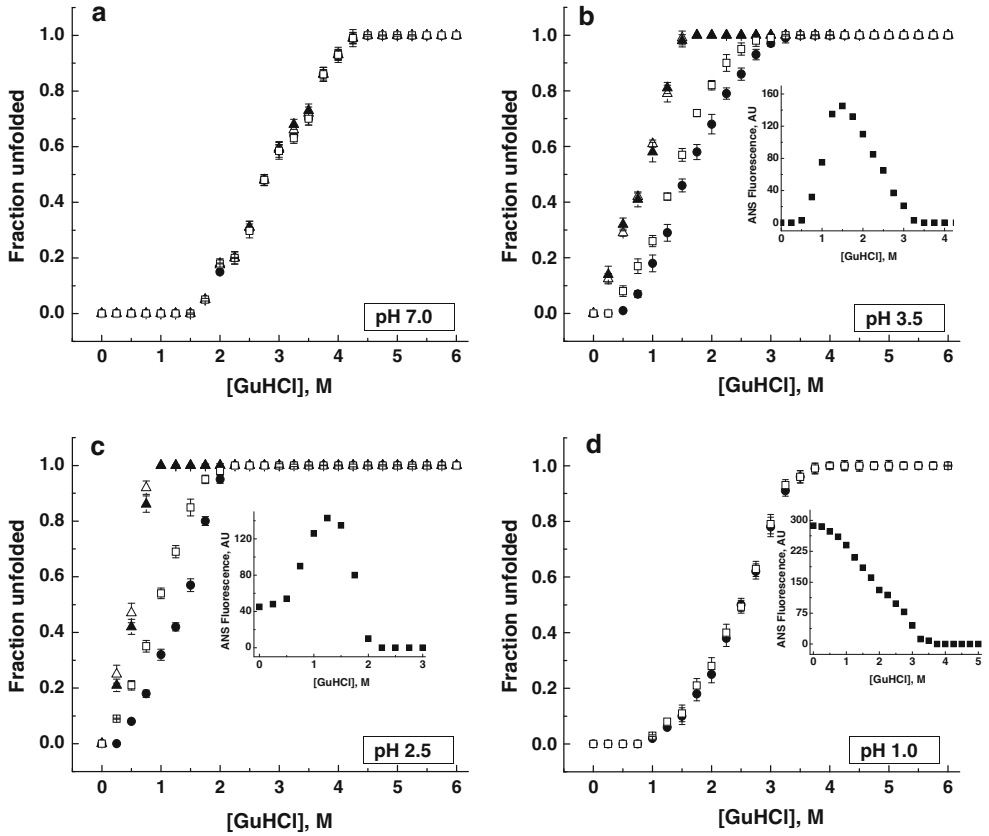

Fig. 4 Equilibrium GuHCl denaturation of *A. niger* RNase. The unfolding of the protein was monitored at **a** pH 7.0, **b** pH 3.5, **c** pH 2.5, **d** pH 1.0 by detecting changes in the near UV ellipticity at 278 nm (*open triangles*), far UV ellipticity at 215 nm (*filled circles*), RNase activity (*filled triangles*), and fluorescence wavelength emission maxima (*open squares*) arising from excitation at 292 nm. The resulting data were normalized to fractions of the unfolded protein and plotted. In **d**, RNase activity and tertiary structure curves are not shown because tertiary structure and activity was completely disrupted at pH 1. The samples were incubated in GuHCl for 24 h at 25°C before the measurements. Each value in all the figures represents mean ± SD ($n = 3$). *Insets* in (**b**), (**c**) and (**d**) show ANS binding as a function of GuHCl concentration

pH, chaotropic agents, and pressure, either individually or in combination. Denaturation is followed by monitoring the change in various spectroscopic properties, for example intrinsic fluorescence, optical rotation (CD), UV absorbance, etc.

The near UV spectrum of *A. niger* RNase under neutral conditions indicates that the aromatic side chains (Trp, Try, and Phe) are highly ordered and rigidly fixed in the well-packed native tertiary structure. The far UV spectrum under neutral condition shows that the secondary structure of *A. niger* RNase belongs to the $\alpha + \beta$ class.

The fluorescence emission maximum of the enzyme at neutral pH was 339 nm, indicating that the tryptophan residues are buried inside the tightly packed protein molecule, as is observed for some other proteins, for example Tra Y (Schildbach et al. 1995). In the denatured condition (e.g. in 6 M GuHCl) all the spectral features of the protein are completely lost. Fluorescence emission maxima show a red shift of 13 nm from 339 to 352 nm indicating complete exposure of the tryptophan residues to the aqueous environment and complete unfolding of the enzyme. The fluorescence intensity is quenched by protonation of tryptophan residues, by neighboring protonated acidic groups, or by high temperatures. The fluorescence of tryptophan is greatly affected by environmental factors such as polarity, pH, temperature, and the nature of the solvent (Freifelder 1982).

For most proteins denaturation curves have a single step and so are assumed to follow a two-state N⇆D mechanism in which only the native state and the denatured state are present at significant concentrations in the transition region (Deshpande et al. 2003). The transition region of denaturation curves is characterized by two properties, the transition midpoint ($C_{1/2}$ and T_m) and the steepness of the transition region (m). The free energy of unfolding (ΔG^{H_2O}) is used as a measure of the stability of the protein to denaturants, high ΔG^{H2O} indicating the protein to be more stable to denaturants. The conformational stability of almost all naturally occurring globular proteins has been observed to be between 5 and 15 kcal/mol (Pace 1990).

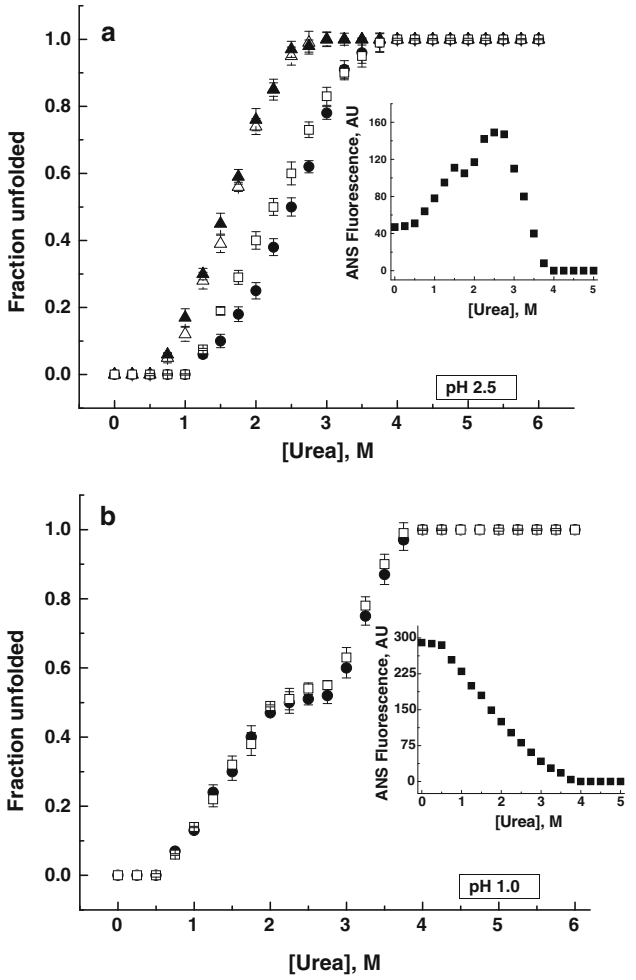

Fig. 5 Equilibrium urea denaturation of *A. niger* RNase. (**a**) pH 2.5. (**b**) pH 1.0. The unfolding of *A. niger* RNase was studied by monitoring changes in near UV ellipticity at 278 nm (*open triangles*), far UV ellipticity at 215 nm (*filled circles*), RNase activity (*filled triangles*), and fluorescence wavelength emission maxima (*open squares*) arising from excitation at 292 nm. The resulting data were normalized to fractions of unfolded protein and plotted. In Fig. 5b, RNase activity and tertiary structure curves are not shown because tertiary structure and activity were completely disrupted at pH 1. The samples were incubated in urea for 24 h at 25°C before the measurements. Each value in all the figures represents mean ± SD ($n = 3$). Insets in both figures show ANS binding as a function of urea concentration

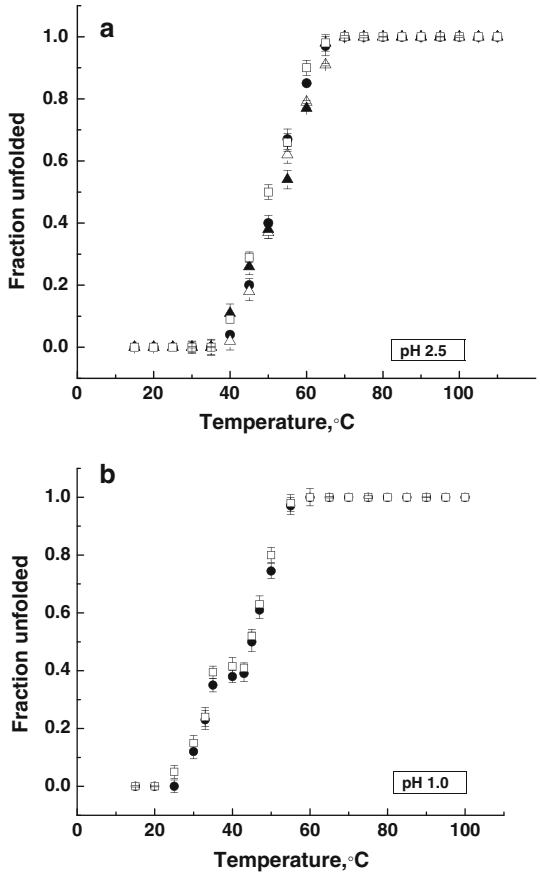

Fig. 6 Thermal unfolding of *A. niger* RNase. **a** pH 2.5. **b** pH 1.0. The unfolding of *A. niger* RNase was studied by monitoring changes in near UV ellipticity at 278 nm (*open triangles*), far UV ellipticity at 215 nm (*filled circles*), RNase activity (*filled triangles*), and fluorescence wavelength emission maxima (*open squares*) arising from excitation at 292 nm. In **b**, RNase activity and tertiary structure curves are not shown because tertiary structure and activity were completely disrupted at pH 1. The resulting data were normalized to fractions of unfolded protein and plotted. The samples were incubated in urea for 24 h at 25°C before the measurements. Each value in both figures represents mean ± SD ($n = 3$)

The $C_{1/2}$ (GuHCl) and ΔG^{H_2O} were observed at pH 7.0, indicating that the protein is most stable at this pH. ΔG^{H_2O} of -11.59 ± 0.2 kcal/mol falls in the range observed for other proteins (Deshpande et al. 2003). Denaturation with GuHCl at pH 2.5 yielded ΔG^{H2O} almost the same as that of urea denaturation, although GuHCl proved to be a more effective denaturant, with $C_{1/2}$ (GuHCl) nearly half that of $C_{1/2}$ (urea) at pH 2.5, as shown in Table 1.

Acid-induced changes of *A. niger* RNase as a function of pH indicate that the protein is structurally and functionally stable over the pH range 4.0–8.0, and slowly loses structural integrity on either side of this pH range. Moreover, pH denaturation of *A. niger* RNase is multistate, as seen by fluorescence and far UV CD studies, with population of different intermediates at different pH. On reducing the pH from neutral to acidic, two intermediates are found. The first intermediate state populates at pH 2.0 because of acid denaturation. Compared with the native state, this state has much reduced activity, substantial loss of tertiary contacts, little remaining secondary structure, and increased exposure of the tryptophan residues in the polar environment. Thus, this state is designated the "acid unfolded state" (**UA**). On further lowering the pH to 1.0 another intermediate populates with some secondary structure in comparison with the acid unfolded state, as seen from far UV CD spectra. Further, fluorescence emission maximum suffered a blue shift of 4 nm (340 nm)

Table 1 Results for unfolding of *A. niger* RNase

Condition	Denaturant	Method	$C_{1/2}$ (M)	ΔG^{H2O} (kcal/mol)	m (kcal mol^{-1} M^{-1})
pH 7.0, 25°C	GuHCl	CD $[\theta]_{222}$	2.88 ± 0.3	−11.59 ± 0.2	−4.1 ± 0.08
		CD $[\theta]_{278}$	2.86 ± 0.3	−12.2 ± 0.25	−4.5 ± 0.12
		Fluorescence	2.8 ± 0.3	−11.8 ± 0.24	−4.2 ± 0.14
		Activity	2.89 ± 0.3	−12 ± 0.25	−4.4 ± 0.2
pH 3.5, 25°C	GuHCl	CD $[\theta]_{222}$	1.57 ± 0.2	−5.1 ± 0.21	−3.3 ± 0.05
		CD $[\theta]_{278}$	0.80 ± 0.3	−2.3 ± 0.2	−2.9 ± 0.1
		Fluorescence	1.34 ± 0.3	−2.2 ± 0.1	−3.4 ± 0.15
		Activity	0.81 ± 0.1	−5.1 ± 0.13	−2.87 ± 0.1
pH 2.5, 25°C	GuHCl	CD $[\theta]_{222}$	1.25 ± 0.2	−3.1 ± 0.12	−2.3 ± 0.05
		CD $[\theta]_{278}$	0.48 ± 0.2	−2.2 ± 0.1	−2.1 ± 0.03
		Fluorescence	1.12 ± 0.1	−2.8 ± 0.1	−2.29 ± 0.02
		Activity	0.52 ± 0.11	−2.1 ± 0.11	−2.07 ± 0.04
	Urea	CD $[\theta]_{222}$	2.47 ± 0.12	−2.9 ± 0.1	−1.2 ± 0.06
		CD $[\theta]_{278}$	1.63 ± 0.14	−1.28 ± 0.24	−0.8 ± 0.1
		Fluorescence	2.21 ± 0.2	−2.6 ± 0.21	−1.2 ± 0.08
		Activity	1.58 ± 0.1	−1.24 ± 0.17	−0.8 ± 0.08
pH 1.0, 25°C	GuHCl	CD $[\theta]_{222}$	2.5 ± 0.1	−5.75 ± 0.1	−2.3 ± 0.05
		Fluorescence	2.51 ± 0.05	−5.6 ± 0.1	−2.23 ± 0.04
	Urea	CD $[\theta]_{222}$	1.25 ± 0.1	Not measured as unfolding is a three-state process	
			3.24 ± 0.09		
		Fluorescence	1.21 ± 0.04		
			3.23 ± 0.1		
	Temperature	CD $[\theta]_{222}$	33 ± 0.5°C		
			49 ± 0.5°C		
		Fluorescence	32.9 ± 0.3°C		
			48.7 ± 0.5°C		

The calculated thermodynamic data are approximate values, because they do not take into account some interactions present in native RNase that are irreversibly lost upon unfolding

Each value in the table represents mean ± SD ($n = 3$)

indicating tryptophan residues went into a non-polar environment again at pH 1. Thus, structure formation at pH 1.0 occurred possibly because of electrostatic stabilization by counter anions. This state is called the **A** state (acid unfolded refolded state). In the case of *A. niger* RNase, the **A** state is also the molten globule state, because it has significant amount of secondary structure and enormously high (maximum) ANS fluorescence intensity at 480 nm without having any tertiary structural content and activity. The **A** state of *A. niger* RNase at pH 1.0 is unique because it shares the properties of molten globule state. The alkaline denaturation of *A. niger* RNase is incomplete and leads to formation of the alkaline denatured state (**UB**) at pH 12.0, which has somewhat reduced tertiary structure and secondary exposed tryptophan residues in comparison with the native state. However, no ANS binding is observed for this state, possibly because of no exposure of the hydrophobic surfaces. All these features suggest the **UB** state is a distinct intermediate state. Denaturation studies against pH indicate that *A. niger* RNase is more susceptible to denaturation under acidic conditions than under alkaline conditions. Thus, the focus needs to be on the status of the enzyme at low pH. Therefore, urea, GuHCl and temperature-induced unfolding of *A. niger* RNase at low pH was carried out. *A. niger* RNase is stable towards the urea-induced unfolding under neutral conditions and all the structural and functional properties were preserved even in 8 M urea. However, urea unfolding studies at low pH yielded some interesting results. At pH 3.5, the urea-induced unfolding curves that were obtained by use of different techniques were sigmoidal and coincidental, indicating that the changes in the environment of the excitable tryptophan residues, and the changes in the secondary and tertiary structure, occurred concurrently. This suggests two-state unfolding (N→D) without any intermediates, substantiated by the lack of ANS binding.

However, at pH 2.5 the urea-induced unfolding characteristics are quite different from those at pH 3.5. The urea-induced unfolding at pH 3.5 is cooperative and transitions seen by use of different techniques are non-coincidental. RNase activity and tertiary structure are lost first, followed by the loss of fluorescence and secondary structure. The non-coincidental transitions suggest the presence of intermediate(s) in the unfolding pathway of *A. niger* RNase between the native and denatured states at pH 2.5. Further, the ANS binding curve showed two peaks at two different urea concentrations, one at the lower concentration of 1.25 M urea and the other at the higher concentration of 3.25 M, thus indicating the presence of two intermediate states during urea-induced unfolding at pH 2.5. One intermediate, at low urea concentration, had a high secondary structural content with some tertiary structure and activity whereas the other intermediate had less secondary structure with no tertiary contacts or activity.

Interestingly, in the molten globule **A** state, at pH 1.0, urea-induced unfolding of *A. niger* RNase is non-cooperative, as seen by three-state equilibrium apparent from both far UVCD and fluorescence measurements. Such a transition suggests the presence of two structural entities, possibly domains, in the molecular structure of the protein molecule which may be differently stabilized in the molten globule state, resulting in step-wise unfolding. Therefore, each transition corresponds to the unfolding of one structural entity/domain. The first transition corresponds to the unfolding of the less structured or less stable part of the protein molecule whereas the second transition corresponds to the unfolding of more structure, or the more stable parts in the protein molecule. Therefore, upon closer examination of the transition curve it can be concluded that in 2.25–2.75 M urea, the protein is present in a state in which one structural unit of the molten globule state is completely unfolded but the other has not yet started unfolding. Besides, ANS fluorescence at 480 nm almost ends with the first transition, suggesting that most of the hydrophobic surfaces exposed are in the domain that unfolds first and that the other part has few hydrophobic surfaces.

The remaining secondary structure content of the protein was analyzed by use of K2D software. At pH 1.0, either alone or with the increasing concentrations of urea, α-helices decrease by 80% from 0.5 M to 2.0 M urea and β-sheets decrease only by 30% in this region. Further, at higher concentration of urea both secondary structural contents (α and β) were disrupted. The same results were obtained at pH 2.5 alone and with increasing concentrations of urea from 0.5 to 2.0 M. These results indicate that the β-sheets are more resistant to pH and urea denaturation than the α-helices.

The temperature-induced denaturation of *A. niger* RNase is incomplete under neutral conditions, because ~45% of the native structure of the protein is retained even at 90°C. Thermal denaturation of *A. niger* RNase at pH 3.5 was also incomplete, because 20% of the native structure of protein was retained up to 90°C. At pH 2.5, temperature-induced unfolding is cooperative and transitions are coincidental, suggesting two states unfolding at pH 2.5. However, in the molten globule state at pH 1.0, temperature-induced unfolding is a three-state equilibrium, with the presence of two structural entities/parts of different stability which unfold sequentially. In the molten globule state at 38–43°C *A. niger* RNase has one unfolded and one folded entity/part. Hence, both temperature and urea unfolding in the molten globule state strongly suggest the presence of two structural domains in the molecular architecture of the *A. niger* RNase.

GuHCl-induced unfolding at neutral pH is cooperative and the transitions are coincidental. At pH 3.5 and pH 2.5, transitions monitored by use of different techniques are non-coincidental, indicating the appearance of states different from the native and the denatured states. Strong ANS binding was observed in 1.58 M GuHCl and 1.38 M GuHCl at pH 3.5 and pH 2.5, respectively, indicating the presence of intermediates at those particular GuHCl concentrations. Interestingly, the secondary structural content, fluorescence properties, and ANS fluorescence properties of these two intermediates were very similar, suggesting that they are the same intermediate populating under different conditions. Moreover, the secondary structure and fluorescence properties of the intermediate state identified in 2.5 M urea at pH 2.5 are closely similar to those of the intermediate states observed during GuHCl-induced unfolding at pH 3.5 and 2.5.

The GuHCl-induced unfolding of the molten globule state at pH 1.0 is cooperative with a transition midpoint higher than at pH 3.5 and 2.5. Because of the ionic nature of GuHCl, it interacts with stabilizing/destabilizing properties of molten globule state, resulting in overall greater stability of the molten globule state than the folded conformations.

This investigation identified a number of forms of *A. niger* RNase under different conditions. The presence of such multiple partially folded intermediates that differ in the amount of secondary structure, globularity, stability, and compactness has also been reported for other proteins (Tcherkasskaya and Ptitsyn 1999; Uversky et al. 1999). These partially folded intermediates may be important in the correct folding of the enzyme. It is being speculated that these partially folded intermediates probably consist of ensembles of substrates with a common core of native-like secondary structure which are responsible for their stability. Consequently, it is likely that the intermediates may represent the equilibrium counterparts of transient kinetic intermediates (Uversky et al. 1998). The overall unfolding scheme of *A. niger* RNase is presented in Fig. 7.

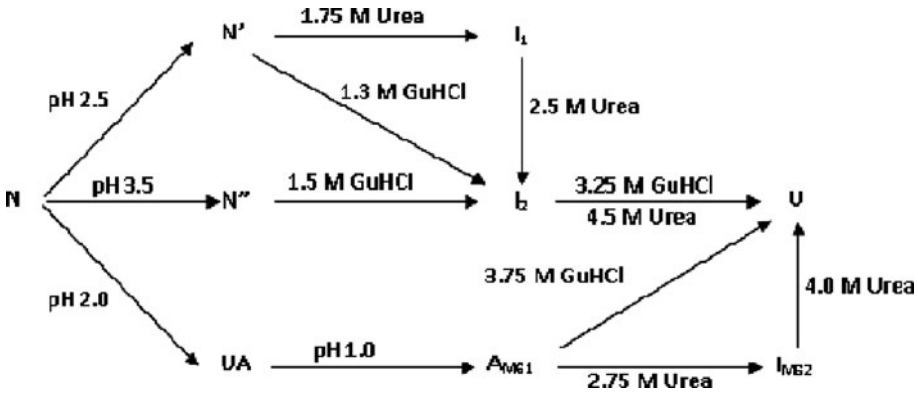

Fig. 7 Unfolding of *A. niger* RNase

The unfolding of *A. niger* RNase occurred through two non native states at pH 3.5 and 2.5 which are more susceptible to denaturation than the native state at pH 7.0 and may provide important insights into the molecular organization of *A. niger* RNase and its folding behavior. Unfolding of *A. niger* RNase by various denaturants was also monitored at pH 1.0 (molten globule state). GuHCl unfolding at pH 3.5 and 2.5 reveals the presence of intermediate states with similar specific conformational properties which can thus be assigned as the same intermediate state which populates under different conditions. Urea-induced unfolding at pH 2.5 resulted in the accumulation of two intermediate states, one with high secondary structure and less tertiary structure and the other with comparatively less secondary structure and no tertiary structure; these states are designated the more and less ordered intermediates, respectively, in the unfolding pathway. The latter intermediate shares some properties with the intermediate identified in GuHCl denaturation of *A. niger* RNase at pH 3.5 and 2.5. Accumulation of intermediates in the GuHCl and urea-induced unfolding pathway is observed for number of proteins of two-domain or multidomain nature (Baldwin and Rose 1999; Fersht 1999). Thus, it is likely that *A. niger* RNase has two domains which behave differently under different denaturing conditions leading to the formation of a number of intermediate states. Further temperature and urea-induced unfolding of *A. niger* RNase in the molten globule state at pH 1.0 are three-state-equilibrium in nature, suggesting the unfolding of two structural parts or domains in steps. The urea denaturation curves for cardiac troponin I (Morjana and Tal 1998) are in three-state equilibrium, which indicates the presence of a stable folding intermediate which might be related to the two domain architecture of troponin I.

Conclusion

In the study reported here it was established that *A. niger* RNase unfolds through an intermediate state and the protein unfolds sequentially. *A. niger* RNase followed a two-state unfolding mechanism during urea, GuHCl, and thermal denaturation. The pH dependence of ΔG^{H2O} suggests that electrostatic interactions among the charged groups make a significant contribution to the conformational stability of the enzyme. Similar unfolding behavior is observed for other RNase Rs (Deshpande et al. 2003). However, the nature of the intermediates and the conditions under which they occur vary with each protein. To a first approximation, some generalization of the folding behavior of RNases of the T2 family can be drawn. This class of proteins unfolds through intermediates, and the two domains in the molecular structure unfold sequentially. However, the properties of the intermediates and the conditions under which they are populated may be different, depending on the stability and structural integrity of the protein molecule.

Acknowledgments We are especially thankful to Dr Radheshyam Maurya (Department of Animal Science, University of Hyderabad, Hyderabad, India) for providing laboratory facilities. GRK wants to thank the University Grants Commission, Government of India, for financial support, and the School of Biochemical Engineering, Institute of Technology, BHU, Varanasi, India, for providing laboratory and technical support. Financial assistance to AS and MK in the form of a research fellowship from Council of Scientific and Industrial Research, Government of India, is gratefully acknowledged.

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