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Ribosomal Protein P2 from apicomplexan parasite *Toxoplasma gondii* is intrinsically a molten globule

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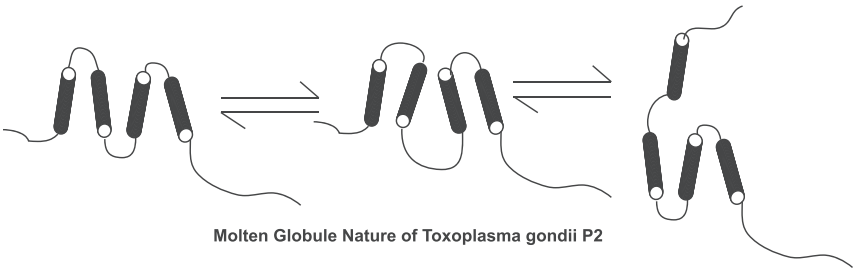
HIGHLIGHTS

- *Toxoplasma gondii* P2 is a monomer at pH 2.0 and oligomerises at higher pH values.
- TgP2 consists of only helices and unstructured regions.
- TgP2 is intrinsically a molten globule.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii is an apicomplexan parasite, which causes toxoplasmosis. Toxoplasma P2 (TgP2) is a ribosomal protein and exists as supramolecular assembly with other proteins in the ribosome. It is also shown that TgP2 is involved in some extra ribosomal functions. However, till date the protein has evaded structural characterization by any of the known techniques. In this background, we report here a systematic study using a variety of biophysical techniques and NMR, under different conditions of pH and temperature, and deduce that TgP2 consists of only helices and unstructured regions, is a monomer at low pH but forms multimers at higher pH, and has intrinsically a molten globule structure. The C-terminal half is flexible and the helices are concentrated in the N-terminal half of the chain. The dynamism inherent to the molten globule structure may have functional implications for its extra-ribosomal functions. which is contrast to that of human P2.

1. Introduction

Apicomplexa are a large group of eukaryotic micro-organisms which possess a unique organelle known as apicoplast and an apical complex structure. These are unicellular, spore forming parasites of animals which penetrates the host cells [1]. *Toxoplasma gondii* is an apicomplexan parasite which causes toxoplasmosis. This parasite affects all warm blooded animals. In *T. gondii* three infectious stages were observed; tachyzoites which are rapidly dividing and slowly growing bradyzoites and sporozoites [2]. Tachyzoites enters into the host cells by phagocytosis [2].

T. gondii P2 is a ribosomal stalk protein and exists as supramolecular complex with P0–$(P1–P2)_2$. These proteins are observed to be partially disordered as observed in Plasmodium and human [3,4]. Sequence

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comparison of P2 from *T. gondii* (Tg) and *Plasmodium falciparum* (Pf) shows ~70% similarity, which would suggest similar behaviors. In fact, PfP2 and TgP2 have been observed to be structurally quite similar [5]. *Plasmodium falciparum* P2 (PfP2) and *T. gondii* P2 (TgP2) do show oligomerization signatures. However, the PfP2 oligomerization was observed to be SDS-resistant, whereas TgP2 oligomerization was found to be not SDS-resistant. During nuclear division of the parasite, PfP2 translocates to RBC surface as a tetramer [6] while this behavior is not observed for TgP2. On the other hand, TgP2 was detected on the free tachyzoite surface [5]. These observations suggest certain important functions of apicomplexan ribosomal P2 proteins in host cell invasion [5]. Till date, to the best of our knowledge, there is no report on the structural characteristics of this protein. In this background we report here by CD, fluorescence, DSC and NMR investigations that TgP2 is intrinsically a molten globule, a characteristic which is in contrast to that of HuP2 whose structure has been reported [7]; HuP2 forms a stable dimer at physiological conditions. This molten globule nature of TgP2 could have functional implications because of the conformational adjustability in such structures.

2. Materials and methods

2.1. Protein expression and purification

TgP2 expression was tried with different vectors, but the protein expression was found to be best with PET vector. However, the PET vector itself codes for a 30 residue affinity tag including six histidines. This sequence has one thrombin site after 15 residues of tag. We worked with both the full length protein as well as the one derived after thrombin cleavage. Histidine-tagged P2 was overexpressed in *Escherichia coli* BL21 (DE3) cells after inducing with 500 μM isopropyl-β-D-thiogalactopyranoside at 37 °C for 4–5 h. The protein was affinity purified on Ni-NTA beads (Sigma-Aldrich) and eluted with Tris buffer (pH 7.5; 20 mM) containing NaCl (150 mM), imidazole (250 mM) and DTT (5 mM). Thrombin digestion was done which cleaves the tag and then was incubated with benzamidine sepharose to remove thrombin from the solution. MALDI spectra of these two proteins and sample purity are shown in supplementary Fig. S1. For NMR experiments, isotope-enriched (^{15}N or $^{15}N/^{13}C$) TgP2 was prepared using M9 media containing $^{15}NH_4Cl$ and ^{13}C glucose as the sole sources of nitrogen and carbon, respectively. The purified protein was concentrated to ~500 μM concentration.

2.2. Gel filtration of recombinant TgP2

AKTA device from GE Healthcare was used for gel filtration which was coupled to UV spectrometer. The gel filtration profile of recombinant TgP2 at pH 2 and 7.4 was determined using Superdex-75 column along with the markers (BSA and Lysozyme) run on the same column. The parameters used for gel filtration were: pressure 0.5 MPa, column bed volume 120 ml, and flow rate: 0.5 ml/min. All runs were done under reduced condition (5 mM DTT). This profile was then compared with standards run on the same column.

2.3. Circular dichroism (CD) spectroscopy

Circular Dichroism (CD) experiments were performed on a JASCO-810 spectropolarimeter for observing alterations in the secondary and tertiary structures of the protein. The protein concentrations used were 5 μM and 50 μM for far-UV (190–260 nm) and near-UV CD (260–360 nm) experiments, respectively. CD spectra were obtained using a 0.2 cm and 1 cm quartz cell in the far and near-UV regions respectively. The spectropolarimeter was purged with nitrogen gas prior to the experiments. All the CD measurements were corrected by subtracting the buffer spectra and were taken as an average of three

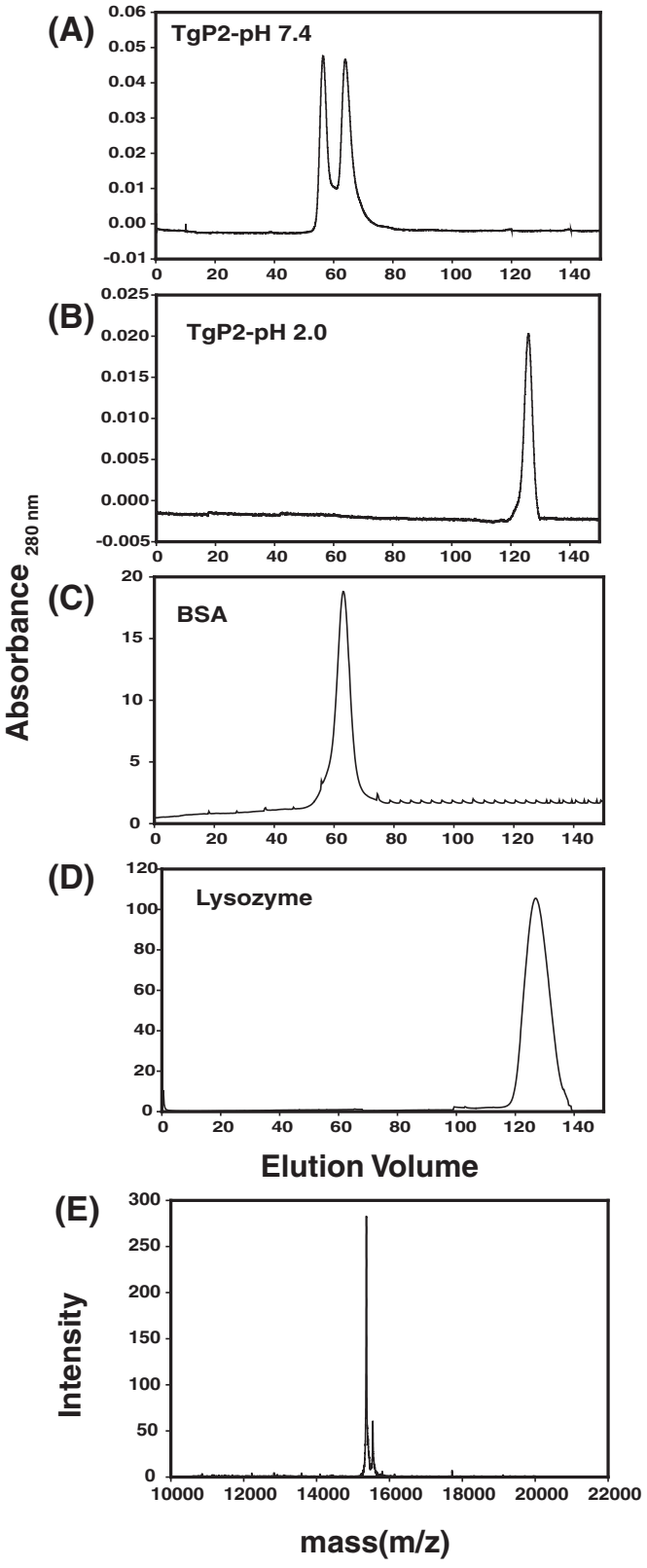

Fig. 1. Gel filtration profiles: (A) recombinant TgP2 at pH 7.4 (loading: 1 ml of 4 mg/ml), (B) recombinant TgP2 at pH 2.0 (loading: 1 ml of 4 mg/ml) (C) BSA protein marker (loading:1 ml of 1 mg/ml) run on the same column; (D) Lysozyme protein marker (loading:1 ml of 1 mg/ml) run on the same column; (E) MALDI spectrum of unlabelled TgP2 at pH 2.0.

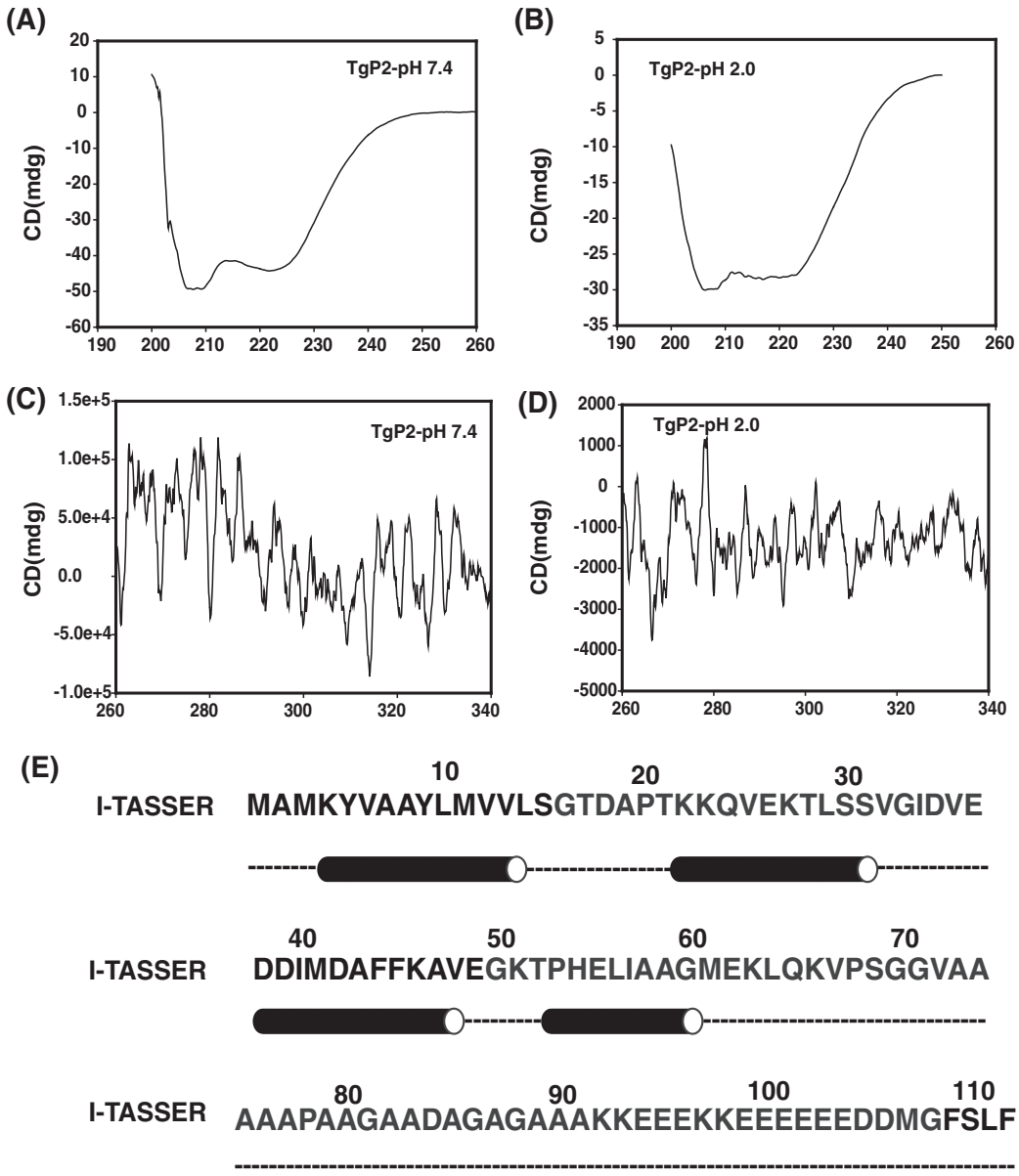

Fig. 2. CD spectra and secondary structure predictions: (A) far-UV CD spectrum of 50×10^{-3} mM TgP2 at pH 7.4; (B) far-UV CD spectrum of 50×10^{-3} mM TgP2 at pH 2.0; (C) near-UV CD spectrum of 50×10^{-3} mM TgP2 at pH 7.4; (D) and near-UV CD spectrum of 50×10^{-3} mM TgP2 at pH 2.0. (E) Summary of structure prediction details of PfP2 using I-TASSER. Cylinders show α-helical regions, and lines show random coils.

accumulations at a scan rate of 50 nm min^{-1}, and a response time of 1 s. The molar ellipticity $[\theta]$ was calculated from the observed ellipticity θ as

$$[\theta] = 100 \cdot \left(\frac{\theta}{c \cdot l}\right) \quad (1)$$

where c is the concentration of the protein in M (mol dm^{-3}) and l is the path length of the cell in centimeters.

2.4. Mass spectrometry of TgP2 using MALDI-TOF

TgP2 protein sample was subjected to mass spectrometry using MALDI from Bruker Corporation (model number 201344). The protein was dissolved in phosphate buffer, pH 7.4 and glycine buffer pH 2.0, and reconstituted using 50% acetonitile and 0.1% TFA (500 μl of 100% acetonitrile 499 μl of distilled water + 1 μl of TFA). The reconstituted protein was loaded on a MALDI plate using saturated solution of α-cyanohydroxyl cinnamic acid (Bruker 201344) (20 mg/ml) in 50% acetonitrile and 0.1% TFA.

2.5. Fluorescence spectroscopy

The fluorescence spectroscopic measurements for the binding of 1-anilino-naphthalene-8-sulfonate (ANS) with TgP2 were carried out on a Cary Eclipse fluorescence spectrophotometer, Agilent Technology with a 3 mL quartz cell of 1 cm path length. For ANS binding experiments, the ANS concentration was kept at 200 μM which was determined by using an extinction coefficient of ANS as E_{350} = 5000 M^{-1} cm^{-1} [8]. The concentration of TgP2 was 20 μM in all the experiments. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 365 nm to selectively excite the ANS molecules, and the emission spectra were monitored in the wavelength range of 370–650 nm. The background emission spectra of the ANS solutions in buffer were subtracted from those of protein with ANS solutions.

2.6. Differential scanning calorimetry

The thermal denaturation experiments were carried out on Nano DSC (TA-instruments). The experiments were performed at pH 2.0 and pH 7.4. For all DSC experiments, the TgP2 concentration was kept at 0.142 mM and the scan rate used was 1 °C min^{-1}. Before loading into the calorimetric vessels all the solutions were thoroughly degassed using Barnstead (Thermolyne) degassing unit from Nuova. The excess heat capacity versus temperature plots were analyzed by Nanoanalyser software supplied by TA-Instruments. The reversibility of the thermal transitions was checked by heating the samples to just above the transition temperature, cooling immediately and then reheating at the same scan rate.

2.7. NMR spectroscopy

All the NMR experiments were recorded at 298 K on a Bruker 800 MHz spectrometer equipped with a triple resonance cryo-probe with an actively shielded Z-gradient. Series of two-dimensional $^{1}H–^{15}N$ HSQC were recorded at different pH (2.0, 3.0. 5.6, 7.4) and temperatures, 15, 25 and 35 °C. Further, series of two-dimensional and three-dimensional experiments were carried out on native TgP2 at pH 2. Backbone H^{N} and ^{15}N resonance assignments TgP2 at pH 2.0 were obtained using 3D HNN and HN(C)N triple resonance experiments [9,10]. 3D HNCA, HN(CO)CA, CBCANH, and CBCA(CO)NH [11,12] provided additional checks and facilitated the assignment. The ^{1}H chemical shift was referenced to HDO while the ^{13}C and ^{15}N chemical shifts were indirectly referenced to DSS [13].

3. Results and discussion

3.1. TgP2 oligomerizes at physiological pH but is a monomer at pH 2

We have recorded gel filtration profile of recombinant TgP2 at 400 μM and 100 μM concentrations at pH, 7.4 and 2.0. The results were identical in both cases. Fig. 1A shows the chromatograms obtained with protein concentration of 400 μM at pH 7.4. For comparison the profile obtained for BSA marker (66 kDa) is also shown in Fig. 1C. From this it follows that TgP2 oligomerizes in solution. When the individual fractions were collected and rerun on the column, the same elution profiles were observed that the two oligomeric species are in equilibrium in solution.

Fig. 1B shows the FPLC chromatogram of TgP2 at pH 2. It can be clearly seen that the molecule is a monomer with a molecular weight of 15.5 kDa. For comparison the profile obtained for Lysozyme marker (14.3 kDa) is also shown in Fig. 1D. This fraction of TgP2 was subjected to MALDI for an accurate estimate of molecular size, and this is shown in Fig. 1E.

3.2. Structural features of TgP2

3.2.1. Secondary and tertiary structural characteristics

Fig. 2 shows the CD spectra of TgP2 at pH 7.4 and 2.0. In either case we see that the far-UV CD spectrum (Fig. 2A and C) exhibits two negative bands at 210 nm and 222 nm which are indicative of helical structures. It is interesting to note from the ellipticities at the two pH values that the helical content is nearly the same in both the situations. Noting that at pH 2, the chain is a monomer, and at pH 7.4 it is a multimer, it follows that oligomerization does not change the helical content in the structure. Quantitative analysis of the data indicates that the helical content is between 35–40%. The near-UV CD spectra of TgP2 at pH 7.4 and 2.0 (Fig. 2B and D) showed practically no ellipticity in the wavelength range of 260 nm–340 nm, indicating absence of tertiary structure in the protein. These are very characteristic features of molten globules. That means the monomer is a molten globule and this property is preserved even on oligomerization.

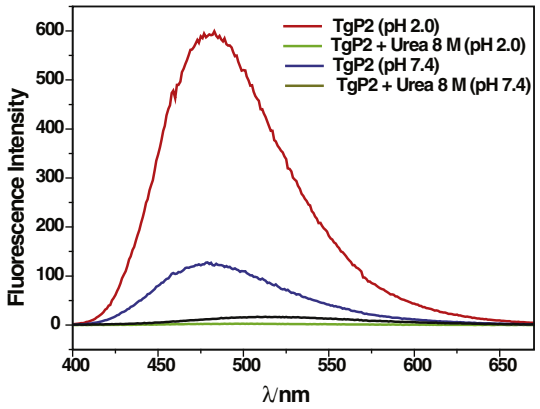

Fig. 3. Binding of 200×10^{-3} mM ANS to 20×10^{-3} mM TgP2 at pH 2.0 and 7.4 in absence and presence of 8 M urea.

3.2.2. Secondary structure predictions

We predicted the secondary structure elements in the protein using the prediction algorithm, I-TASSER [14] and the results of this are shown in Fig. 2E. It appears that the sequence consists of only helices (roughly 35%) and the rest is unstructured. The calculated helical content from prediction algorithm I-TASSER matches very closely to that of estimated from the CD spectra (35%). This suggests that the helices are concentrated in the first half of the sequence and the entire C-terminal half is unstructured.

3.3. Fluorescence spectroscopy

The binding of hydrophobic fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), to TgP2 at pH 2.0, and 7.4 were studied. It is well reported in literature that ANS has much stronger affinity to the protein "molten globule" state, with a pronounced secondary structure, but without a tightly packed tertiary structure as compared with its affinity to the completely folded and coil-like proteins [15]. Fig. 3 represents the binding of ANS with the native and 8 M urea induced denatured state of TgP2. It is clear from this figure that the binding of ANS is much more efficient with TgP2 at pH 2.0 compared to that at pH 7.4.

These results suggest that predominantly monomeric TgP2 at pH 2.0 has exposed hydrophobic patches, thereby offers more hydrophobic surface area for binding of ANS molecules. On the other hand TgP2 at pH 7.4, due to multimerization, offers reduced hydrophobic surface area for binding to ANS molecules. This is reflected in the reduction of

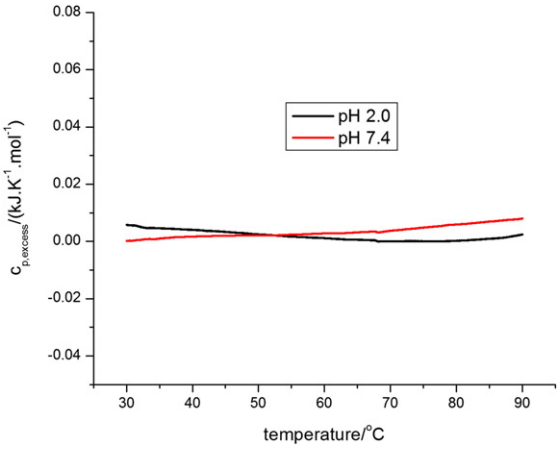

Fig. 4. DSC profiles of the thermal denaturation of 0.142 μM TgP2 at pH 2.0 and 7.4 at a scan rate of 1 °C min^{-1}.

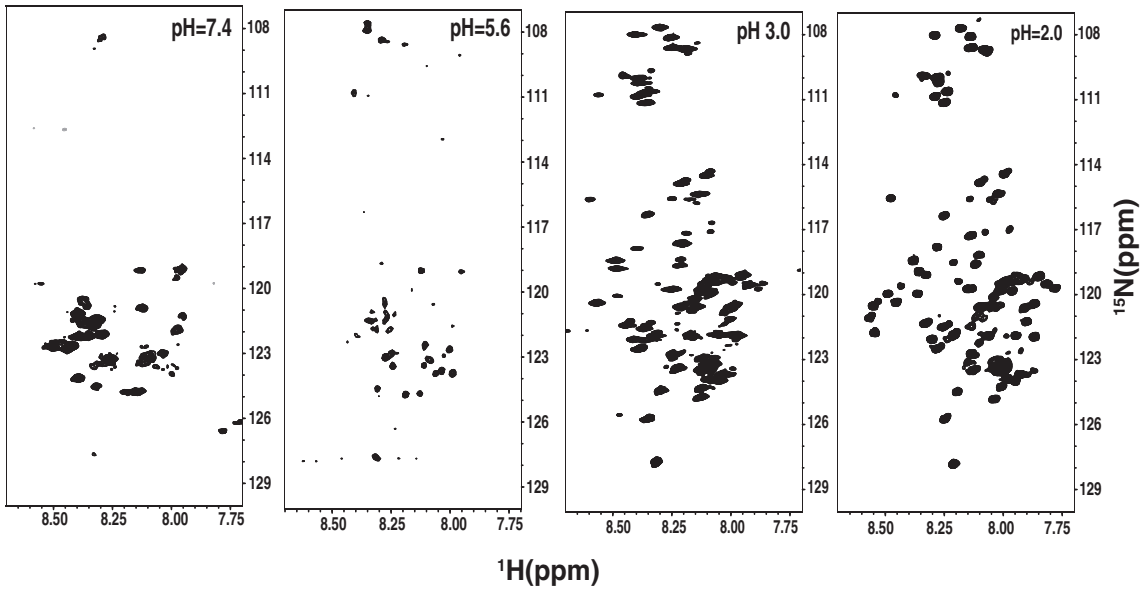

Fig. 5. 2D ^{1}H–^{15}N HSQC spectra at different pH values: TgP2 spectra at 25 °C at different pH values (2.0, 3.0, 5.6 and 7.4). The spectra were recorded on a Bruker 800 MHz NMR spectrometer.

ANS fluorescence emission intensity at pH 7.4 as shown in Fig. 3, although the molten globule like structure of monomeric TgP2 is still retained in the multimer. Fig. 3 also shows that ANS does not bind to 8 M urea induced denatured state of TgP2.

3.4. Differential scanning calorimetry

In order to examine the cooperativity of unfolding of the molten globule state of TgP2, we have done DSC measurements of the protein at pH 2.0 and pH 7.4. At pH 2.0 TgP2 is in monomeric state and pH 7.4, in multimeric state. Fig. 4 shows the excess heat capacity curves of TgP2 at pH 2 and 7.4 as a function of temperature. As seen in this Figure, no cooperative thermal transition of the protein at both the pH was observed. This suggests that the molten globule state of TgP2 does not exhibit the enthalpy change of unfolding in a cooperative manner upon heating. These results are consistent with the observation of Yatuni et al. [16] that the molten globule state of protein does not undergo cooperative transition.

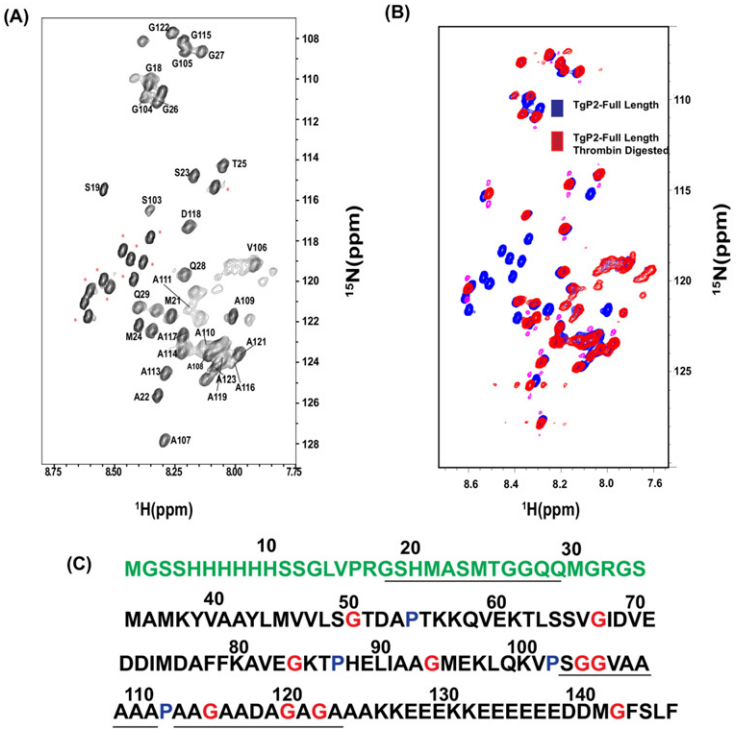

Fig. 6. NMR assignments in TgP2. (A) 2D ^{1}H, ^{15}N HSQC spectrum of TgP2 in glycine buffer at pH 2.0 and 25 °C. Residue specific assignment for each peak is marked on the spectrum. * identifies peaks coming from vector residues; (B) overlay of HSQC spectra before and after thrombin digestion; (C) assigned residues are underlined on the sequence. Green residues belong to the vector.

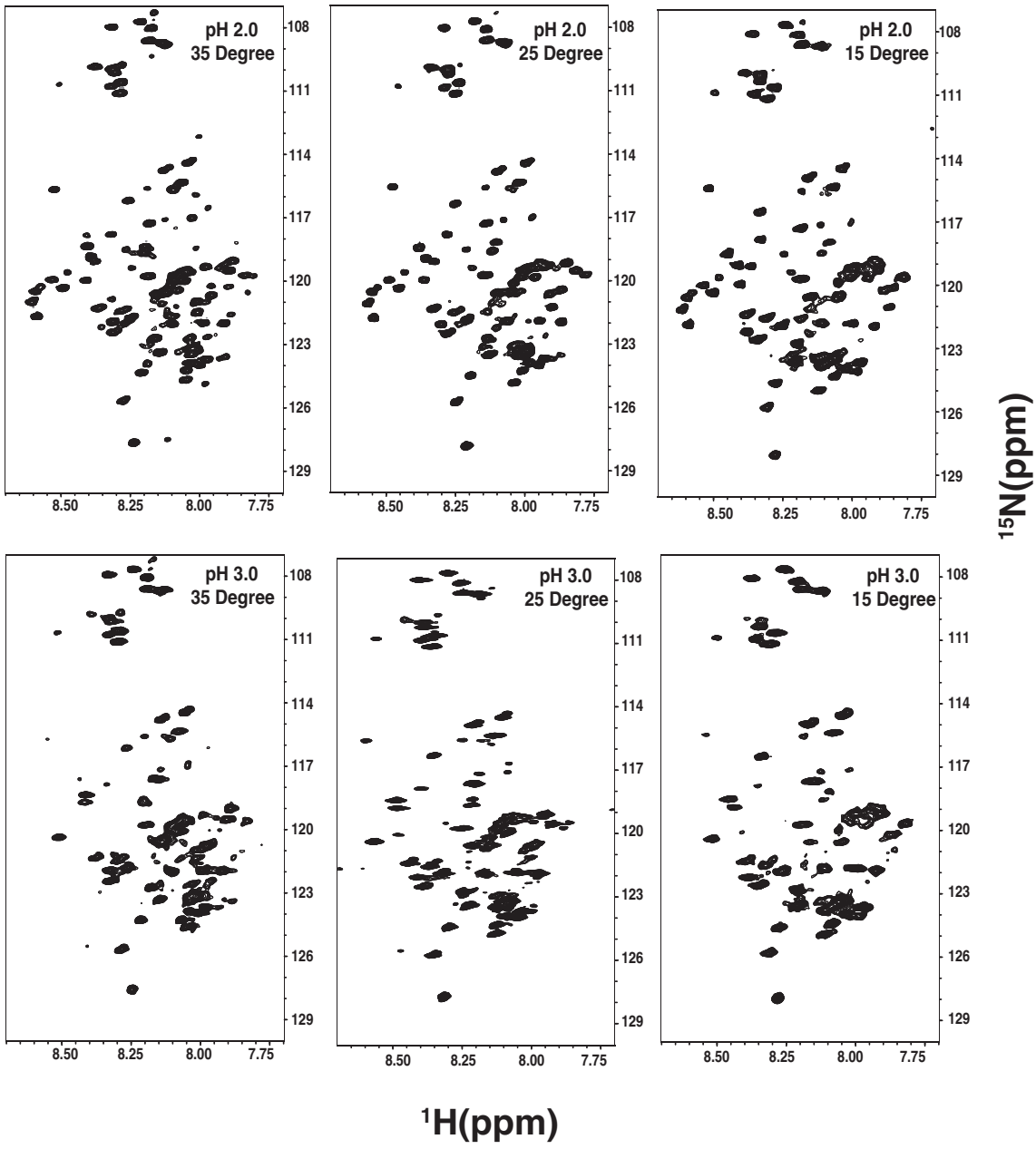

Fig. 7. 2D ^{1}H–^{15}N HSQC spectra at different temperature values: 2D ^{1}H–^{15}N HSQC spectra of TgP2 at pH 2.0 and 3.0 at different temperatures (15 °C, 25 °C, 35 °C). The spectra were recorded on a Bruker 800 MHz NMR spectrometer.

3.5. NMR analysis of TgP2 characteristics

Next, we investigated the structural characteristics of the TgP2 protein by NMR by monitoring the changes that occur in the spectra when pH and temperature are systematically changed. Fig. 5 shows ^{1}H–^{15}N HSQC spectra of TgP2 at pH values of 2.0, 3.0, 5.6 and 7.4. The spectra change quite dramatically as the pH is increased from 2.0 to 7.4, implying that significant changes occur in the structure and dynamics of the protein.

At pH 2.0, the peaks are relatively sharper, indicating a rather stable segment, but the peak count is much smaller than is expected for a monomeric protein; we expect 141 peaks (33 from the vector and 108 from the TgP2 sequence) whereas we observe only ~70 peaks. We could assign about ~40 peaks by multidimensional NMR experiments (Fig. 6A). When the HSQC spectra were recorded with another construct wherein the first 15 residues were deleted, the spectra showed 12 peaks less, and the overall appearance of the spectrum did not change (Fig. 6B), implying that the extra residues coming from the vector do not influence the structural characteristics of the protein to any significant extent. From the assigned peak list 12 peaks belong to the vector. Thus it appears that among the observed peaks at least 24 peaks belong to the vector. We deduce therefore that the HSQC spectrum reflects a maximum of about 46 peaks actually belonging to TgP2, of which 23 peaks could be assigned; the remaining peaks are very weak, did not show connectivities in the multidimensional NMR spectra and thus could not be assigned. We observe that the assigned peaks belong to the C-terminal which has been predicted to be disordered and thus flexible. This indicates that peaks from the remaining residues (belonging to the N-terminal half of the protein) in the monomer are not visible possibly due to extreme line broadening which could be due to the unstable tertiary structure in the protein causing significant domain motions at intermediate time scale. This is also the region where helices are predicted to be present along the chain. Thus, these observations are in conformity with the conclusion of molten globule nature of TgP2

monomer at pH 2.0. When the pH is increased to 3.0, there is a substantial line broadening and the peak count reduces. At pH 5.6 the peaks appear sharper but the count is substantially reduced, and at pH 7.4 the lines are again very broad and the peak count is also very less. These variations must be attributed to variations in the structural stabilities of the individual monomers in the multimers, which seem to retain the molten globule character, on one hand, and to monomer–multimer or multimer–multimer exchange, on the other.

Fig. 7 shows the changes that occur in the spectra when the temperature of the solution is varied from 15 °C to 35 °C, at two pH values, 2.0 and 3.0, under which conditions the molecule would mostly be a monomer. Here again we see substantial changes in the spectral features and this must imply changes in the inter-conversion rates between the multiple conformations populating the molten globule state of the monomer. This data is again completely consistent with the conclusion that the monomer is a molten globule.

4. Concluding remarks

We have described in this paper, the structural characteristics of TgP2 protein determined using a variety of biophysical techniques and NMR spectroscopy. It turns out that the protein is intrinsically a molten globule which has a tendency to self-associate at physiological conditions. This is in sharp contrast to the behavior of human P2, also a ribosomal protein, which exhibits a stable dimeric structure under similar conditions. The dynamic inter conversion between multiple conformations inherent to the molten globule structure may have implications to the extra ribosomal functions TgP2 is shown to be involved in, since that may facilitate interactions with different kinds of partners.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2015.03.008.

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