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Acid induced unfolding of anthrax protective antigen

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Abstract

Acidic pH plays an important role in the membrane insertion of protective antigen (PA) of anthrax toxin leading to the translocation of the catalytic moieties. The structural transitions occurring in PA as a consequence of change in pH were investigated by fluorescence and circular dichroism measurements. Our studies revealed the presence of two intermediates on-pathway of acid induced unfolding; one at pH 2.0 and other at pH 4–5. Intrinsic fluorescence measurements of these intermediates showed a red shift in the wavelength of emission maximum with a concomitant decrease in fluorescence intensity, indicative of the exposure of tryptophan residues to the bulk solvent. Furthermore, no significant change was seen in the secondary structure of PA at a pH of 2.0, as indicated by far UV–CD spectra. The low pH intermediate of PA was characterized using the hydrophobic dye, 8-anilino-1-naphthalenesulfonate, and was found to have properties similar to those of a molten globule state.

Keywords: Anthrax protective antigen; Acid; Unfolding; Intermediate; Molten globule; Structural transitions

The charge–charge interactions and salt bridges that are present on protein surfaces and interfaces play an important role in the protein structure and function [1–3]. In this regard, acid induced unfolding of proteins offers an easy way to understand the role of these electrostatic interactions. Acid induced denaturation occurs due to the intramolecular charge repulsion, which leads to the destabilization of native conformation of protein [4,5]. In general, acid denaturation has been shown to result in denatured states that are less unfolded than those obtained with high concentrations of GdmCl or urea [6,7]. Thus analysis of the protein structural stability in different pH allows the identification and characterization of specific electrostatic interactions that make significant contributions to determine the free energy difference between the folded and unfolded state [8]. It also helps in understanding the kinetic or thermodynamic intermediates in the folding pathway of a protein [8].

Protective antigen (PA) is the central receptor binding moiety of anthrax toxin that mediates the translocation of catalytic moieties, lethal factor (LF) and edema factor (EF), into the cytosol of mammalian cells [9]. The first step in the intoxication process of anthrax toxin involves binding of PA (83 kDa) to anthrax toxin receptor (ATR) present on the cell surface [10,11]. Subsequently, PA is activated by cell surface proteases, which induce conformational changes in the protein that allow its efficient interaction with LF/EF [12,13]. The complex is then internalized by clathrin-dependent endosomal uptake [14]. This is accompanied by membrane insertion through domain II of PA followed by the translocation of LF/EF into the cytosol [15–17]. The whole process is triggered by the acidic pH inside the endosomes [18].

The pH plays an important role in the PA mediated translocation of LF and EF. Pre-treatment of macrophages with agents, which elevate endosomal pH, protects them from the lethal effect of toxin [19]. Internalization of LF is experimentally prevented by incubation at 4 °C, but acidifying the medium to pH 5 or less can overcome this inhibition, indicating that passage through acidic environment is required for anthrax toxin

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to exert its effect [19,20]. PA has been shown to get inserted into phospholipid bilayers and cell membranes to form ion-permeable channels in a pH-dependent fashion [18,21,22]. Petosa et al. [23] proposed that acidification induces conformational changes in a loop (residues 302–325) present in domain II of PA, allowing a membrane-spanning hairpin to unfold and insert into the endosomal membrane. In spite of being a critical step, no study has been done so far to characterize the acid induced unfolding of PA. Thus, present study was designed to investigate the structural changes occurring in PA as a consequence of change in pH by fluorescence and circular dichroism measurements. We propose that there exist at least two intermediates in the acid induced unfolding of PA and one intermediate behaves like a classical molten globule. To the best of our knowledge this is the first study to understand the unfolding mechanism of this protein.

Materials and methods

Materials. Bacterial culture media were purchased from Difco Laboratories, USA. All other chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).

Purification of PA. PA was purified from culture supernatant of *Bacillus anthracis* BH441 as described earlier [12]. In brief, culture was grown overnight, centrifuged and supernatant was concentrated using a 30 kDa cutoff filter in an Amicon concentrator (Millipore, USA). PA was further purified by anion exchange Q–Sepharose column interfaced with a Pharmacia FPLC system using a linear gradient of 0–500 mM NaCl in 20 mM, pH 8.0, Tris–HCl buffer.

The protein concentration was determined by Bradford reagent using BSA as standard.

Effect of pH. PA (0.65 μM) was incubated with 20 mM buffer of different pH ranging 2–9 for 1 h at room temperature before taking the measurements. Buffer alone was used as controls.

Intrinsic fluorescence studies. Fluorescence emission spectra were recorded on a Jobin Yvon FluoroMax-3 luminescence spectrometer using a 10 mm path length quartz cell at 25 °C. The protein samples (0.65 μM) were excited at 290 nm and the emission spectra were recorded from 300 to 400 nm. The excitation and emission band passes were kept as 10 nm. The baseline correction was done with buffer alone prior to every run.

Circular dichroism spectral studies. CD measurements were carried out on a JASCO J-715 spectropolarimeter at 25 °C. A path length of 10 mm was used and the spectra were averaged over six scans at a scan speed of 200 nm/min. The protein concentration was kept as 0.65 μM. The values obtained were corrected for baseline with buffer alone.

ANS binding studies. 8-Anilino-1-naphthalenesulfonate (ANS) binding studies were monitored by spectrofluorescence measurements conducted on Jobin Yvon FluoroMax-3 spectrofluorometer. A quartz cell of 10 mm path length was used for all experiments keeping the slit width for excitation and emission wavelengths as 10 nm. The excitation wavelength was 365 nm and the emission spectrum was recorded from 400 to 600 nm. The protein concentration was kept as 0.65 μM.

Results

The conformational changes occurring in PA at different pH values were probed by intrinsic fluorescence studies. Fig. 1A shows that a decrease in pH from 8.0 to 2.0 leads to a red shift in the wavelength of fluorescence emission from 335 to 343 nm, suggesting an exposure of tryptophan residues to the bulk solvent. Furthermore, there was a concomitant fall in fluorescence intensity with decrease in pH and a clear biphasic transition can be seen with a highly populated intermediate at pH of 4–5 (Fig. 1B).

To further characterize the nature of the intermediate we analyzed the exposure of hydrophobic regions of PA

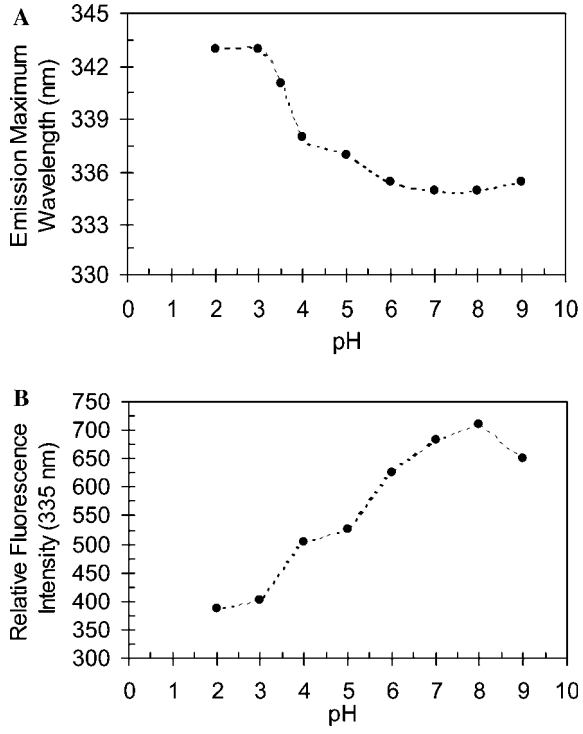

Fig. 1. Effect of pH on intrinsic fluorescence of PA. Fluorescence emission spectra of PA in different pH were recorded as described under "Materials and methods." Each spectrum was averaged over four scans. The emission maxima (λ_{max}) (A) and relative fluorescence intensity at 335 nm (B) of PA are presented here with increasing pH.

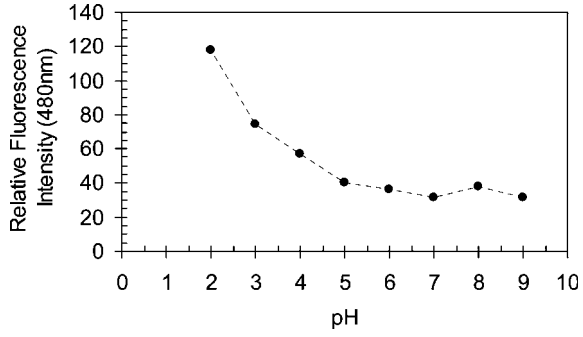

Fig. 2. 8-Anilino-1-naphthalenesulfonate (ANS) binding studies. ANS was added in samples prior fluorescence measurements. The samples were excited at 365 nm and emission spectra were recorded from 400 to 600 nm. The relative fluorescence intensity at 480 nm is shown here as a function of pH.

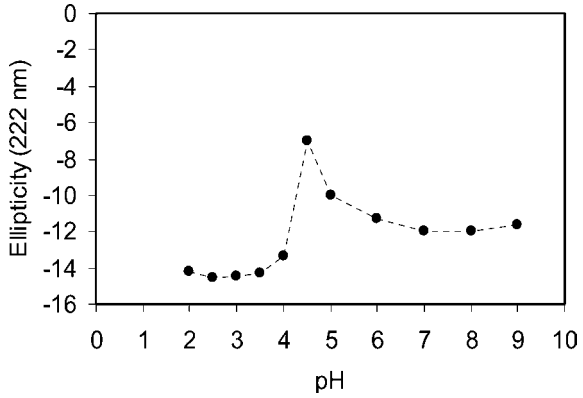

Fig. 3. Effect of pH on ellipticity of PA. UV–CD spectra of PA in different pH were recorded as described under "Materials and methods." A quartz cell of 10 mm path length was used and the spectra were averaged over 6 scans. Ellipticity of PA at 222 nm is shown here as a function of increasing pH.

using ANS [24]. The fluorescence emission of ANS is known to increase when the dye binds to hydrophobic regions of a protein. Fig. 2 illustrates the relative fluorescence intensity at 480 nm with increasing pH. It was observed that there was an increase in the fluorescence emission intensity of ANS with a decrease in pH with a maximum intensity at pH 2.0.

The secondary structure of PA at different pH values was analyzed by far UV–CD. Fig. 3 shows a change in ellipticity at 222 nm as a function of pH. It can be clearly seen that there is insignificant change in the protein conformation between the pH values of 2–4. But at a pH of 4.5 there was marked change in the relative ellipticity, suggesting a decrease in the α-helical content of the proteins. Furthermore, there was a gradual decrease in the relative ellipticity at 222 nm with increase in pH.

Discussion

It is generally accepted that protein denaturation is a highly cooperative process for small globular proteins and may be approximated by a two state model with no significant intermediates existing during the transition from native state to unfolded state [25]. However, one or more intermediates have been shown in the unfolding process for many proteins investigated in recent years. An earlier study to elucidate the effect of acid on the denaturation of some 20 monomeric proteins indicated that these proteins fall in distinct categories displaying certain acid unfolding transitions that depend on the type of protein and the buffer conditions [26].

Since PA experiences a pH environment ranging from neutral in extracellular space to acidic in endosomal compartments, the structure and stability of this protein was examined as a function of pH. Circular dichroism (CD) and intrinsic fluorescence of PA were monitored as a function of pH to provide information on changes in structure or stability.

The spectral analysis of PA at different pH values revealed the presence of an unfolded intermediate at a pH of 4–5 and another intermediate at a pH of 2.0. Intrinsic fluorescence spectra of the pH 2.0 intermediate showed a red shift of 8 nm indicating the exposure of hydrophobic residues which was also confirmed by an increase in the ANS fluorescence intensity. On contrary, the far UV–CD spectrum revealed that the secondary structure of pH 2.0 intermediate is similar to the native state of the protein. These results suggest that this unfolding intermediate has properties very similar to those of the molten globule [27,28].

Furthermore, around a pH of 4–5 there is a decrease in the α-helical content of the protein and only a 3 nm red shift in the fluorescence emission spectrum, suggesting the presence of another unfolding intermediate in this pH range. It is important to note that the pH inside the endosomes is also in this range and the formation of such an intermediate may have some role in membrane insertion [28]. Similar studies on the PA oligomer are underway and may shed more light into the possible role of this intermediate in membrane insertion.

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