Conformational fluctuations in anthrax protective antigen: a possible role of calcium in the folding pathway of the protein

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Abstract Protective antigen (PA) is the central receptor binding component of anthrax toxin, which translocates catalytic components of the toxin into the cytosol of mammalian cells. Ever since the crystal structure of PA was solved, there have been speculations regarding the possible role of calcium ions present in domain I of the protein. We have carried out a systematic study to elucidate the effect of calcium removal on the structural stability of PA using various optical spectroscopic techniques, limited proteolysis and mutational analysis. Urea denaturation studies clearly suggest that the unfolding pathway of the protein follows a non-two state transition with apo-PA being an intermediate species, whereas the folding pathway shows that calcium ions may be critical for the initial protein assembly.

Key words: Anthrax toxin; Protective antigen; Calcium; Proteolysis; Denaturation; Folding pathway

1. Introduction

The native conformation of a protein is maintained by non-covalent interactions and even a small or a localized structural change can affect the global protein stability by switching it to random coil state [1–4]. In this regard, metal binding proteins serve as good model systems for studying structure–stability relationships as the unfolded states of both the ion-bound and the ion-free (apo) forms are the same in principle [5]. Among the metalloproteins, the calcium binding proteins form a major subclass and perform variegated biological functions [6–8]. In many cases, it has been shown that conformational changes induced by calcium binding are functionally important, enhance structural stability and decrease the structural perturbations relative to the ion-free form [9–11].

Protective antigen (PA), the central component of the anthrax toxin, is a tetra-domain protein that delivers catalytic moieties, viz. edema factor (EF) and lethal factor (LF), into the cytosol of mammalian cells [12]. During the intoxication process of anthrax toxin, PA is cleaved by cell surface proteases like furin and in vitro by trypsin at the ^{164}RKKR^{167} site present in domain I, to generate a 63 kDa C-terminal fragment (PA_{63}) which actively participates in the subsequent steps [13,14]. The cleavage has been shown to be very specific and deletion of this site abolishes the LF binding activity of PA [14]. X-ray diffraction data of PA revealed the presence of a pair of adjacent calcium ions coordinated to a variant of the EF hand motif in domain I [15]. A recent report showed that these ions are tightly bound to PA as indicated by their slow exchange rate with labeled Ca^{2+} ions [16]. Furthermore, the exchange rate was found to be enhanced in the case of PA_{63} heptamer but there was a reduction in this rate on binding of LF to the heptamer, reflecting ligand (LF)-dependent stabilization of domain I [16]. But surprisingly, detailed analysis of structural transitions and biochemical properties of protein in the Ca^{2+} ion-bound and unbound state is not available in the literature. In the present paper, the role of calcium ions in providing structural stability to PA was investigated by proteolysis, spectroscopic analyses, denaturation studies and mutational analyses. We show that apo-PA appears to be an intermediate in the unfolding pathway of the protein, while binding of calcium and folding of PA may occur as a concerted mechanism.

2. Materials and methods

2.1. Materials

Bacterial culture media were purchased from Difco Laboratories, USA. The enzymes and chemicals for DNA manipulations were obtained from New England BioLabs, USA. All other chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA). Urea was recrystallized with ethanol before the experiments.

2.2. Purification of PA and preparation of apo-PA

PA was purified from culture supernatant of *Bacillus anthracis* BH441 as described previously [13]. For preparation of apo-PA, PA (2.0 mg/ml) was taken in 10 mM HEPES, pH 7.2, 100 mM NaCl (buffer A), incubated with 3 M urea and 10 mM EGTA for 3 h at 28°C and then dialyzed extensively against buffer A at 4°C. Buffer A used for studies was treated with Chelex-100 resin to remove any free calcium. The removal of calcium from PA was confirmed by analyzing the samples on atomic absorption spectrophotometer (GBC Scientific Equipment, model GBC 932AA).

The protein concentration was determined by the method of Bradford with bovine serum albumin as a standard.

2.3. Fluorescence studies

The samples (0.65 μM) were excited at 290 nm and fluorescence emission spectra were recorded from 300 to 400 nm on a spectrofluorometer (Jobin Yvon FluoroMax-3) in a 10 mm path length quartz

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cell at 25°C. Excitation and emission band passes were kept at 10 nm. The baseline was corrected with buffer alone prior to every run.

2.4. Circular dichroism (CD) studies

CD measurements were carried out on a Jasco J-715 spectropolarimeter at 25°C. The protein concentration was kept at 0.65 and 18 μM for far- and near-UV CD measurements respectively and spectra were corrected for baseline with buffer alone.

2.5. Urea denaturation studies

PA and apo-PA (0.65 μM) were incubated with different concentrations of urea for 1 h at 25°C before taking the measurements. Each concentration of urea was taken as appropriate controls.

2.6. Data analysis

A three state model of unfolding was used for biphasic urea denaturation curve. The equation describing a three state denaturation transition [17] is:

$$F_{\text{app}} = \frac{\exp[(-\Delta G_{\text{IU}}^{\text{H}_2\text{O}} + m_{\text{IU}}c)/RT] \times [Z + \exp[(-\Delta G_{\text{NI}}^{\text{H}_2\text{O}} + m_{\text{NI}}c)/RT]]}{1 + \exp[(-\Delta G_{\text{IU}}^{\text{H}_2\text{O}} + m_{\text{IU}}c)/RT] \times [1 + \exp[(-\Delta G_{\text{NI}}^{\text{H}_2\text{O}} + m_{\text{NI}}c)/RT]]} \quad (1)$$

where F_{app} is the apparent fraction of folded protein, Z normalizes the optical properties of the intermediate to those of the native and unfolded protein forms and $\Delta G_{\text{NI}}^{\text{H}_2\text{O}}$ and $\Delta G_{\text{IU}}^{\text{H}_2\text{O}}$ are the free energy changes in the absence of denaturant for the native (N) to intermediate (I) and intermediate to unfolded (U) transitions, respectively, m_{NI} and m_{IU} are cooperativity parameters of the native to intermediate and intermediate to unfolded transitions, respectively, and c is the denaturant concentration. F_{app} values were calculated using the equation:

$$F_{\text{app}} = (y_i - y_{\text{U}})/(y_{\text{N}} - y_{\text{U}})$$

where y_i is the observed value of the spectroscopic parameter at denaturant concentration i and y_{U} and y_{N} are the values of the unfolded and native forms of the protein, respectively. Non-linear least squares fitting of Eq. 1 to urea denaturation curve was performed using the program SPSS 11.0.

2.7. Mutagenesis of PA gene and their expression

Mutations in the PA gene were introduced using appropriate mutagenic overlapping primers in the previously described plasmids pYS5 or pMS1 [18,19]. PA mutants were expressed either in a non-toxigenic *B. anthracis* BH441 strain or in *Escherichia coli* BL21 cells.

PA and mutated PA genes were subcloned in an expression plasmid containing 6×His tag and T7 promoter. Coupled in vitro transcription and translation was carried out in RTS-500 instrument using *E. coli* circular template kit (Roche Diagnostics, Germany). The reaction mixture was prepared according to the manufacturer's instructions and 15 μg plasmid DNA was used for a reaction of ~20 h. At the end, aliquots were stored at −20°C until further use.

2.8. Cell culture and cytotoxicity assay

The J774A.1 macrophage cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. Proteins were assayed for toxicity in the macrophage lysis assay as described previously [20].

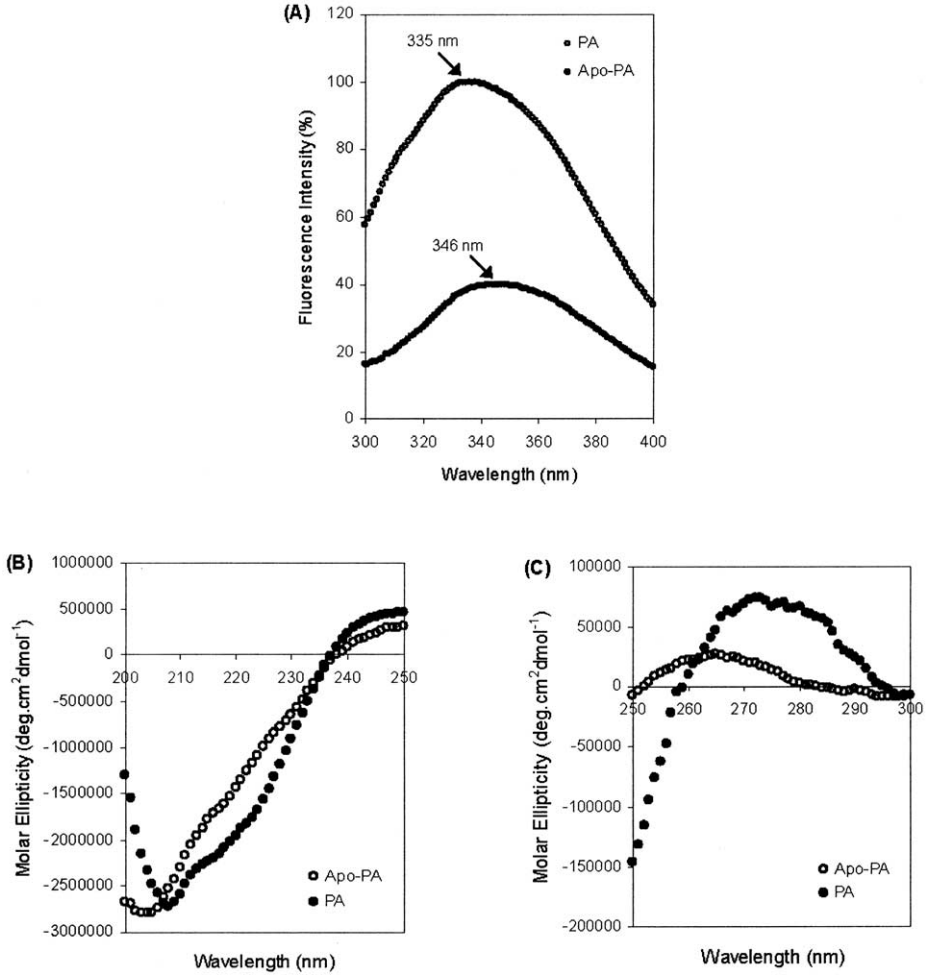

Fig. 1. A: Fluorescence emission spectra. Emission spectra of PA and apo-PA were recorded as described in Section 2. Arrow indicates emission maxima of samples. Each spectrum is an average of four scans. B,C: CD spectra. Far-UV CD (B) and near-UV CD (C) spectra of PA and apo-PA were recorded as described in Section 2. A path length of 10 mm was used and the spectra were averaged over six scans.

3. Results and discussion

3.1. Spectral properties

Comparison of the spectroscopic properties of PA and apo-PA revealed that the removal of Ca^{2+} ions from PA has a profound effect on its molecular conformation. PA had a tryptophan fluorescence with an emission λ_{max} of 335 nm, suggesting that the tryptophan moieties are buried in the hydrophobic core of PA. In comparison, apo-PA had only 40% of the fluorescence intensity of PA with an 11 nm red shift in emission maximum indicating the exposure of tryptophan residues to the bulk solvent (Fig. 1A) [21].

The far-UV CD spectrum of PA has two minima at 208 and 220 nm with the 208 nm band having greater intensity (Fig. 1B) indicative of a classical α+β protein [22]. In contrast to this, apo-PA showed a minimum at 205 nm suggesting an increased randomness in the structure. Comparison of near-UV CD spectra revealed a substantial loss in the tertiary structure of PA on removal of calcium (Fig. 1C) [23]. Furthermore, it was observed that the structure of the protein was restored when apo-PA was supplemented with external Ca^{2+}, whereas Mg^{2+}, which is highly abundant in vivo, restored it to only 60–70% as monitored by spectroscopic methods (data not shown). These results suggest that the presence of calcium ions in PA is of utmost importance for maintaining its overall conformation.

3.2. Urea denaturation studies

To elucidate the thermodynamic stability, we carried out equilibrium unfolding studies on PA and apo-PA using urea as the denaturant.

Far-UV CD studies on urea-induced unfolding of PA were carried out to investigate the changes in its secondary structure with increasing concentration of urea. Up to a urea concentration of 1.0 M, there was a slight increase in the ellipticity at 222 nm of both apo- and native PA (Fig. 2A) probably due to slight compactness in the structure as reported earlier for many other proteins [24–26]. But the ellipticity at 222 nm was completely lost as the urea concentration reached 6 M. The C_m value (concentration of urea at which 50% population is unfolded) for unfolding of PA was ~3.6 M.

The alteration in microenvironment of tryptophan residues on removal of calcium from PA was monitored by studying changes in tryptophan fluorescence intensity and emission maximum (λ_{max}) as a function of denaturant concentration. Between 0 and 2 M urea, there was a substantial fall in the fluorescence intensity of PA (data not shown) and an increase in the λ_{max} emission from 335 nm to 347 nm (Fig. 2B). On further increasing the denaturant concentration, native protein completely unfolded at 6 M urea with a shift in emission λ_{max} to 356 nm. Furthermore, PA shows a typical biphasic curve suggesting the presence of an intermediate around 2 M urea concentration (Fig. 2B). This fact was reconfirmed when the denaturation curves of PA as monitored by tryptophan fluorescence and CD were found to be non-coincidental (Fig. 2C) further suggesting a 'non-two state transition' for PA as reported earlier for other proteins [27–29].

The denaturation midpoint of the first phase occurs near 1.3 M urea, while the second phase has a midpoint near 4.45 M urea (Fig. 2B). The denaturation curve was fitted to a three state model as described in Section 2. On the basis of this fit (Fig. 2D), the following values (±S.E.M.) were obtained for:

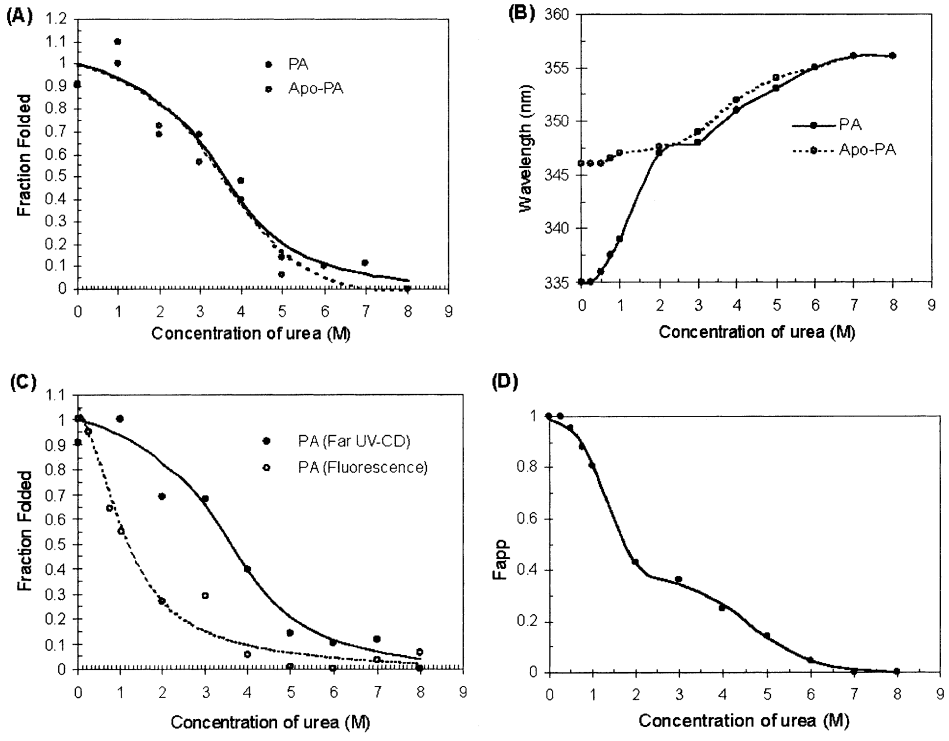

Fig. 2. Urea denaturation profile. Samples were incubated with increasing concentration of urea for 1 h prior to the spectral studies. Far-UV CD (ellipticity at 222 nm) (A) and λ_{max} emission (B) of PA and apo-PA are presented with increasing concentrations of urea. Superimposition of urea-induced changes in fluorescence and far-UV CD of PA is shown in C. D shows the apparent fraction of unfolded protein (F_{app}) with varying concentrations of denaturant after performing non-linear least squares fitting of Eq. 1 to the urea denaturation curve using the program SPSS 11.0. For panels A and C, the value at 0 M urea concentration is represented as 1, i.e. folded state.

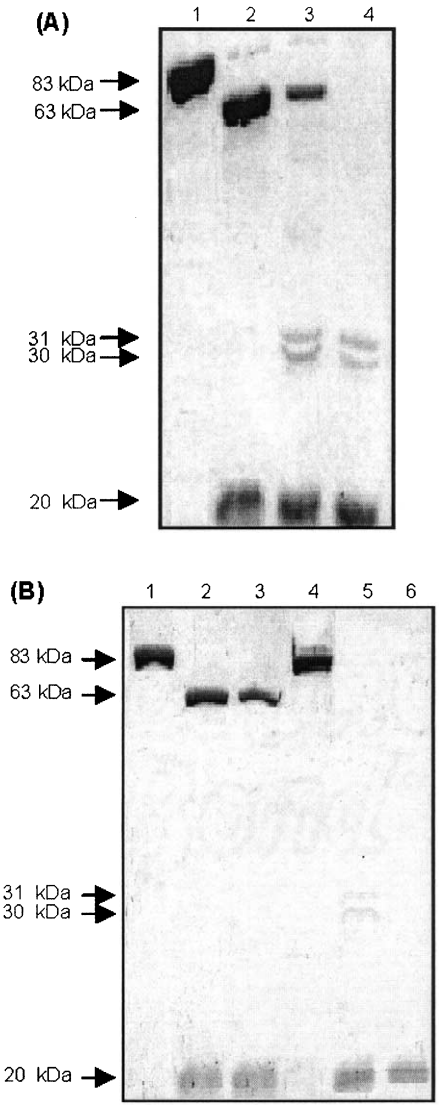

Fig. 3. Trypsin susceptibility analysis. PA, apo-PA and PA pre-incubated with 2 M urea (1 mg/ml) were treated with trypsin and analyzed on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie brilliant blue R. A: Lane 1: PA; lane 2: PA treated with trypsin for 20 min; lanes 3 and 4: PA preincubated with 1 and 2 M urea respectively followed by trypsin treatment for 20 min. B: Lane 1: PA; lanes 2 and 3: PA treated with trypsin for 20 and 45 min respectively; lane 4: apo-PA; lanes 5 and 6: apo-PA treated with trypsin for 20 and 45 min respectively.

$\Delta G^{H_2O}_{NI}$, -2.32 ± 0.219 kcal/mol; m_{NI}, 1.819 ± 0.211 kcal/mol^2; $\Delta G^{H_2O}_{IU}$, -4.05 ± 0.730 kcal/mol; m_{IU}, 0.877 ± 0.156 kcal/mol^2, and Z, 0.371 ± 0.025. The values of calculated free energy changes suggest that the intermediate state is energetically more similar to the native state than the unfolded state.

Closer inspection of fluorescence and far-UV CD spectra of 2 M urea-treated PA revealed distinct similarities with apo-PA (Fig. 2A,B), indicating that apo-PA might be an intermediate in the unfolding pathway of PA. The experimental evidence comes from the fact that electrophoretic analysis of tryptic digest of 2 M urea-treated PA behaved in a similar fashion as apo-PA with the loss of a 63 kDa band and appearance of 30 kDa and 31 kDa bands along with 20 kDa (Fig. 3A,B). A similar observation for apo-PA has been reported recently by Gao-Sheridan et al. [16]. These two new polypeptides (30 and 31 kDa) were also susceptible to proteolysis and degraded on prolonged incubation of apo-PA with trypsin over a period of 45 min while PA_{63} was stable within this period (Fig. 3B). To rule out the possibility that increased proteolysis is a consequence of urea used during preparation of apo-PA, PA treated with urea and dialyzed against buffer A was used as a control which behaved in a similar fashion as PA (data not shown).

3.3. Mutational analysis

The crystal structure of PA reveals the presence of a pair of octahedral complexes composed of eight amino acid residues and a water molecule on domain I chelating two calcium ions [15]. In order to understand the folding behavior of calcium-depleted PA we generated single, double and triple mutants by replacing the amino acids ligating calcium ion with alanine (Table 1). The PA genes carrying the mutation were cloned and expressed in a bacillus-based vector (plasmid pYS5) and an *E. coli*-based vector (plasmid pMS1) as described previously [18,19]. The expressed mutant proteins were found to be highly unstable and degraded during the purification process. To circumvent this, attempts were made to express the mutant proteins by coupling in vitro transcription and translation in a cell-free Rapid Translation System (RTS 500). The RTS system was initially standardized for native PA and the yield of purified PA was 30–40 μg from a 1 ml reaction mixture. The mutant proteins were also expressed under similar conditions and expression was found to be comparable to native PA as shown by Western blot of the reaction mixture (Fig. 4A). Strangely, even in the presence of a cocktail of protease inhibitors extensive degradation of mutant proteins hampered our attempts to purify them. This implies the role of the Ca^{2+} binding region of domain I in protein assembly and initial folding.

3.4. Cytotoxicity assay

The biological activities of apo-PA and RTS-expressed mutant PA proteins in combination with LF (1.5 μg/ml) were determined in a cytotoxicity assay using J774A.1 macrophages as described previously [20]. The cytotoxicity measurements could not be done for apo-PA, as the cell culture medium

Table 1
Description of PA mutant produced in RTS and their cytotoxicity on J774A.1 cells

Mutant	Cytotoxicity (LC_{50}) (ng/ml)
PA	~450
D177A	>1220
D179A	>1250
D181A	>1170
I183A	>1200
E188A	>1220
D235A	>1150
S222A and K225A	>1210
D179A, D181A and E188A	>1260

The residues involved in calcium binding were replaced with alanine (individually or in combination) and produced in a cell-free system. For cytotoxicity experiments, cells were incubated with increasing amounts (volume of reaction mixture) in combination with LF (1.5 μg/ml). After 4 h, 0.5 mg/ml MTT was added to the cells and incubated for 45 min. LC_{50} is the amount required to kill 50% of cells. Each value represents the average of three different experiments done in triplicate.

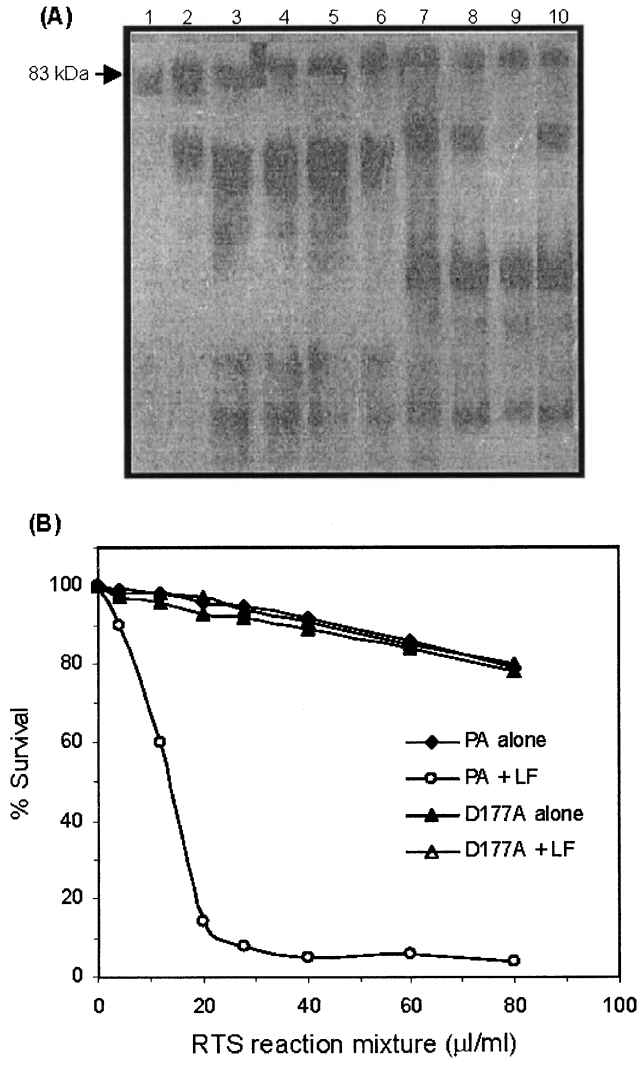

Fig. 4. A: Immunoblot analysis. Samples were separated on 10% SDS–PAGE, transferred onto nitrocellulose paper, and probed with anti-PA polyclonal antibody. The blot was developed using the enhanced chemiluminescence kit (Amersham Biosciences). Lane 1: PA; lanes 2–10: 10 µl of RTS reaction mixture of PA, single mutants D177A, D179A, D181A, I183A, E188A, D235A, double mutant [S222A and K225A] and triple mutant [D179A, D181A and E188A] (summarized in Table 1) respectively. B: Cytotoxicity assay. J774A.1 cells were cultured in 96 well plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum prior to 24 h of experiment. Cells were incubated with LF (1.5 µg/ml) and varying amounts (4, 12, 20, 28, 40, 60 and 80 µl/ml of cell culture medium) of PA and D177A (mutant PA as described in Table 1) for 4 h at 37°C. At the end of the experiment, toxicity was determined with the MTT assay. Results of a representative mutant protein D177A are plotted here while results with other mutants are given in Table 1. Each value represents the mean of three different experiments done in triplicate.

was rich in calcium and would readily convert apo-PA to PA. Calcium-depleted media could not be used as the presence of extracellular calcium has been shown to be essential for the activity of lethal toxin [30]. The cytotoxicity assay on mutant proteins was carried out with crude RTS mixture as attempts to purify them were not successful as mentioned above. Densitometry and Western blot analysis indicated that ~30–40% of the crude protein extract was the intact 83 kDa form (data not shown). Crude RTS mixture containing native PA showed 50% killing of J774A.1 cells at 15 µl/ml (~450 ng PA) in combination with LF. However, mutant proteins were found to be non-toxic at measurable concentrations (Fig. 4B). Crude mixture above 80 µl/ml of cell culture medium started showing toxicity even in the absence of LF; therefore, it was not possible to test the potency of mutant proteins beyond this concentration. The lack of toxicity of PA mutants can be attributed either to its unavailability due to rapid degradation or to defects in its ability to perform various steps of cytotoxicity. Experiments to probe the exact nature of the defect such as receptor binding or cleavage were not possible due to the unavailability of purified proteins. These results imply that the amino acids ligating calcium ions play a major role in providing structural stability to PA and loss in the cytotoxicity of mutant proteins may be due to their inability to bind calcium.

3.5. Conclusion

Our study shows that the removal of calcium ions from PA leads to global conformational changes and affects protein stability as revealed by the optical spectroscopic properties and limited proteolysis profiles of PA and apo-PA. Mutational studies suggested the critical role of the Ca^{2+} binding region of domain I in protein assembly and initial folding indicating that the protein folding and binding of calcium ions may follow a concerted mechanism or may have unstable intermediates. In contrast, apo-PA was relatively stable as compared to mutant proteins, which may be due to intra- and intermolecular interactions that come into play once the protein is in the folded state [31]. Furthermore, urea denaturation studies revealed that apo-PA is an intermediate species in the unfolding pathway of PA and energetically it is more similar to the native folded state of PA than the unfolded state. Thus, the presence of calcium ions in PA is quite imperative for initial folding of PA to its proper conformation, which is partially maintained even on stripping off the calcium ions.

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