Investigation of the unfolding pathway of *Bacillus thuringiensis* Cyt2Aa2 toxin reveals an unfolding intermediate

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**Abstract**

Cyt2Aa2 is a cytolytic toxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. Its active form has a lethal activity against specific mosquito larvae. We characterized an unfolding pathway of Cyt2Aa2 using a guanidinium hydrochloride denaturation. The results revealed three-state transition with a detectable intermediate in a condition with 3–4 M of CuHCl. The conformational free energies for native and intermediate state unfolding were 5.82 ± 0.47 and 16.85 ± 1.47 kcal/mol, respectively. Kinetic analysis suggested that the activation energy of both transitions was around 23–25 kcal/mol, with a rate-limiting step in the second transition. These results have established an energy profile of the Cyt2Aa2 toxin in various conformations involved in the unfolding/refolding pathway. Further characterization of the intermediate state by dye-binding assay, intrinsic fluorescence, and circular dichroism spectroscopy demonstrated characteristics of a molten globule state. This revealed intermediate could play an active role in the structural folding and biological activity of the toxin.

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1. Introduction

*Bacillus thuringiensis* is a spore-forming, Gram-positive soil bacterium which produces parasporal proteins during sporulation (Nickerson et al., 1975). The produced endotoxins can be solubilized in alkaline pH, and become insecticidal after proteolysis by insect gut proteases (Murphy et al., 1976; Bulla et al., 1977; Andrews et al., 1985; Armstrong et al., 1985). The binding of an active toxin on the brush border membrane of a susceptible insect could result in the formation of ion channels or pores, leading to osmotic imbalance, cell swelling and osmotic lysis (Hofte and Whiteley, 1989; Schnepf et al., 1998).

The cytolytic toxin Cyt2Aa2 is produced by *B. thuringiensis* subsp. *darmstadiensis* (Promdonkoy et al., 2003). This toxin is synthesized as a 29-kDa protoxin and then proteolytically processed into a 25-kDa active form. Its toxicity is found against Stegomyia and *Culex* sp. mosquito larvae (Galjart et al., 1987). The X-ray structure of Cyt2 toxin contains a single domain of α/β architecture comprising six α-helices and seven β-sheets (Li et al., 1996). Cyt toxin can bind and form pores in a synthetic lipid membrane without the requirement of a receptor (Thomas and Ellar, 1983). The precise mechanism of action for Cyt toxin is still unclear, and may be based on either pore-forming (Promdonkoy and Ellar, 2000, 2003) or detergent-like model (Butko, 2003). To study the details of membrane interaction, stable conformational states of the toxin should be identified and characterized. The present study aims to analyze the conformational states of Cyt2Aa2 toxin using a chemically induced unfolding experiment. The identified conformational states and calculated transitional free energy between each state in the unfolding pathway could help reveal an energy map of the toxin. In addition, the stable intermediate state can also be characterized further to provide a clue to its possible involvement in the structural folding and biological function of Cyt2Aa2 toxin.

2. Materials and methods

2.1. Protein expression and purification

Cyt2Aa2 protein was expressed at 37°C in *Escherichia coli* strain JM 109 (Promdonkoy et al., 2003) in the presence of 0.1 mM IPTG. The culture media was LB broth containing 100-μg/ml ampicillin. The cell culture was disrupted using a French pressure cell. The harvested inclusion protein was solubilized in 50 mM carbonate buffer (pH 10.0). The soluble toxin was then chromatographically purified using a Superdex-200 HR10/30 size-exclusion column (Amersham). Protein concentration was determined based...
on Bradford dye-binding assay and far-UV absorption (Waddell, 1956).

2.2. Circular dichroism spectroscopy

CD spectra were obtained by a Jasco J-715 spectropolarimeter, purged with oxygen-free nitrogen (Jasco, Japan). The instrument was calibrated daily with 1.0 mg/ml (+)-10-camphorsulphonic acid (CSA), yielding an intensity ratio between 192 and 290 nm greater than 2.0. A series of 0.4–0.6 mg/ml was loaded into a cylindrical quartz cuvette of 0.5-cm path length. Scanning rate was set at a rate of 20 nm/min, with 1.0-s response time, 50-millidegree sensitivity and four accumulations. All spectra were subtracted by baseline spectra of buffers containing an appropriate concentration of GuHCl.

2.3. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence spectra of Cyt2Aa2 toxin in various concentrations of GuHCl were recorded for subtraction. Intensity changes at a particular wavelength (465 nm) versus GuHCl concentrations were documented. The transitional midpoint $C_{50\%}$ and unfolding free energy of the protein in the absence of denaturant $\Delta G_w$ were obtained by curve fitting (Ibarra-Molero and Sanchez-Ruiz, 1996).

2.4. Steady-state unfolding

A series of GuHCl stock from 0 to 6.0 M was freshly prepared and used to unfold the protein at 4°C. The purified toxin was incubated overnight in various concentrations of GuHCl, and then monitored for conformational state by fluorescence spectroscopy. An accurate concentration of GuHCl in each individual condition was confirmed for conformational state by fluorescence spectroscopy. An accurate concentration of GuHCl was calibrated daily with 1.0 mg/ml (+)-10-camphorsulphonic acid (CSA), and analyzed in a rectangular quartz cuvette of 0.5-cm path length. Scanning rate was set at a rate of 20 nm/min, with 1.0-s response time, 50-millidegree sensitivity and four accumulations. All spectra were subtracted by baseline spectra of buffers containing an appropriate concentration of GuHCl.

2.5. Kinetic unfolding

The toxin (20–40 µg/ml) was mixed with various concentrations of GuHCl. The fluorescence decay spectra was recorded at 340 nm over a time course from 2000 to 5000 s, using an excitation wavelength of 280 nm. The bandwidths of excitation and emission were 5 nm. The fluorescence decay spectra were subtracted by baseline spectra obtained in the first 50 s. Each curve was then fitted to the first order single exponential equation (using the SigmaPlot 6.0 software suite):

$$I_t = I_0 + I_{\text{obs}} \exp(-k_{\text{obs}} t)$$

where $I_t$ is the signal intensity at a given time, $I_0$ is the signal intensity at the plateau, $I_{\text{obs}}$ is the initial intensity. $\Delta I$ is the difference of $I_t$ and $I_0$, $k_{\text{obs}}$ is the kinetic rate constant (which is denaturant dependent), and $t$ is time. The ln $k_{\text{obs}}$ was plotted against the GuHCl concentration and fitted with the linear equation

$$\ln k_{\text{obs}} = m[C\text{GuHCl}] + \ln k_w$$

where $\ln k_w$ is the natural log of the kinetic rate constant in water, $m$ is the slope, and $[C\text{GuHCl}]$ is the concentration of GuHCl. The $k_w$ value was used for the activation energy calculation

$$k_w = \frac{\ln \tau}{h} \exp(-\frac{\Delta G_w}{RT})$$

where $\ln \tau$ is Boltzmann’s constant $\times 1.3807 \times 10^{-23}$ J/K, $h$ is Planck’s constant $\times 6.6261 \times 10^{-34}$ m$^2$ kg/s, $T$ is absolute temperature (K), R is the gas constant $\times 8.314$ J/mol K and $E_{ac,w}$ is the activation energy.

2.6. ANS binding assay

1-Anilino-8-naphthalene-sulfonate (ANS) was applied to determine the conformational state of an unfolding intermediate. Cyt2Aa2 protoxin (30 µg/ml) was incubated in various concentrations of GuHCl for 16–18 h. ANS was then added to a final concentration of 100 µM, mixed and incubated for 5 min in the dark. The samples were scanned for emission spectra from 420 to 600 nm at an excitation wavelength of 350 nm. Slit width for excitation and emission spectra was 5 nm. The spectra of blank solution (without protein) were recorded for subtraction. Intensity changes at a particular wavelength (465 nm) versus GuHCl concentrations were documented.

3. Results and discussion

3.1. Steady-state unfolding and transitional free energy analysis

We employed intrinsic fluorescence spectroscopy to monitor for conformational states of Cyt2Aa2 in various GuHCl concentrations. The toxin in an initial condition of carbonate buffer gave a fluorescence emission spectrum with $\lambda_{\max}$ around 330 nm. When the denaturant was gradually increased in the unfolding condition, the spectra progressively changed, with a reduction of emission

![Fig. 1. Intrinsic fluorescence spectra of Cyt2Aa2 toxin in various concentrations of guanidinium hydrochloride. Purified toxin of 20–40 µg/ml was incubated overnight in 0.0–6.4 M GuHCl. The emission spectra were obtained from 300 to 500 nm, with an excitation at 280 nm.](image-url)
The concentration at a half unfolding ([GuHCl]50%) and transitional of GuHCl, respectively. Based on the three-state model equation, I and U can be obtained at approximately 0–2, 3–4 and 6–7 M conformations of the toxin. The steady-state conformations for N, (N), intermediate (I) and unfolded states (U). This suggests that conformational states could be assumed to represent the native corresponding to a three-state transitional model. These three revealed the resulting curve demonstrated a well-defined feature corresponding to a three-state transitional model. These three revealed conformational states could be assumed to represent the native (N), intermediate (I) and unfolded states (U). This suggests that GuHCl could bind and help stabilize intermediate and unfolded conformations of the toxin. The steady-state conformations for N, I and U can be obtained at approximately 0–2, 3–4 and 6–7 M of GuHCl, respectively. Based on the three-state model equation, a curve fitting was performed which yielded values for denaturant concentration at a half unfolding ([GuHCl]50%) and transitional slope (m). These data were then used to determine the conformational free energy of protein in a denaturant-free condition (ΔG_{ac}).

A number of independent repeats, we could report a conformational free energy of the native state at 5.82 ± 0.47 kcal/mol, while the free energy of the intermediate against the fully unfolded state was 16.85 ± 1.47 kcal/mol. The reverse process of these conformational changes was also analyzed by a refolding experiment. Interestingly, the derived refolding curve and free energy values were found to be very similar to those obtained from the unfolding study. These results confirmed that the two investigated pathways are simply a reversal process of the same route and existing conformations. When considering the completed transition, starting from native to unfolded state, the summation of conformational free energy found for Cyt2Aa2 toxin was 22.67 ± 1.94 kcal/mol. The reverse process of these conformational changes was also analyzed by a refolding experiment. The combined data from steady-state and kinetic analyses can provide necessary information for the construction of a conformational energy map of the unfolding toxin. Conformational free energy (ΔG_{ac,w}) of the three conformational states together with the activation energy (E_{ac,w}) of both transitions were mapped along the pathway progression, as shown in Fig. 4. This energy map displays an unfolding pathway starting from a lower-energy native state, and proceeding to higher-energy intermediate and unfolded states, respectively. The transition between each conformational state involves thermodynamic free energy around 5 and 16 kcal/mol, and activation energy around 23–24 kcal/mol. This energy map for the Cyt toxin family was experimentally established for the first time in this study. It could provide relative energy characteristics for the study of protein structure and stability, and could be used as a reference for structural engineering of the mutant toxins.
Several reports on the protein folding pathway (Goldberg et al., 1990; Pitsyn et al., 1990; Sugawara et al., 1991) involve a molten globule state formation. Moreover, the molten globule states for diphtheria toxin (Chenal et al., 2002), anthrax protective antigen (Gupta et al., 2003) and colicins (Zakharov and Cramer, 1997) had been shown to be responsible for their functions in protein–lipid membrane interactions. For B. thuringiensis toxin, a molten globule has been proposed for Cyt1A toxin in the presence of liposome vesicles, using differential scanning calorimetry and CD spectroscopy (Butko et al., 1997). The toxin binds and releases the dye from lipid membrane vesicles at low pH (Butko et al., 1996, 1997). It has been proposed that the molten globule structure binds to the lipid membrane independent from the net charge of the membrane. The importance of a molten globule for biological functions could also be inferred for Cyt2Aa2. Our data directly suggest a presence of molten globule in its unfolding and refolding pathway. When the native and intermediate states of the toxin are related in terms of mechanism of action, it is clear that the native conformation is required for the production of toxin, providing a stable form of protease resistance. However when the toxin undergoes a proteolytic activation and conformational change, a formation of molten globule could then be required for an active role in toxin and membrane interaction. Future investigation of the functional role and interacting mechanism of the intermediate revealed in this work could help provide a basic understanding of the toxin structure as well as a better mechanism model to be used for the application of Cyt2A toxin.

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Fig. 4. The energetic map of Cyt2Aa2 unfolding/refolding, showing the relative energy levels for native (N), intermediate (I) and unfolded (U) states. The activation energy was also labeled for each conformational transition.

Fig. 5. Circular dichroism spectra of Cyt2Aa2 toxin in the native, intermediate and unfolded states. Purified toxin of 0.3 mg/ml was applied, and the spectra expressed in [θ]max(deg cm² dmol⁻¹).


