Journal of Biotechnology 141 (2009) 137–141

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Anchanee Sangcharoen[a], Weerachon Tepanant[a], Somruathai Kidsanguan[a], Boonhiang Promdonkoy[b], Chartchai Krittanai[a,*]

[a] *Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Putthamonthon 4 Road, Salaya, Nakhonpathom 73170, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, 113 Phahonyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand*

ARTICLE INFO

*Article history:*
Received 1 November 2008
Received in revised form 4 March 2009
Accepted 16 March 2009

*Keywords:*
Cyt2Aa2
*Bacillus thuringiensis*
Protein unfolding
Molten globule
Intrinsic fluorescence
Circular dichroism

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 GuHCl. The conformational free energies for native and intermediate state unfolding were $5.82 \pm 0.47$ and $16.85 \pm 1.47$ 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.



## 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

* Corresponding author. Tel.: +662 800 3624x1410; fax: +662 441 9906.
*E-mail address:* stckt@mahidol.ac.th (C. Krittanai).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.03.012

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 sample of 0.4–0.6 mg/ml was loaded into a cylindrical quartz cuvette of 0.02-cm path length (Hellma, USA) and analyzed from 190 to 260 nm. Scanning 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

Emission spectra were monitored from 300 to 500 nm on Jasco FP-6300 and Perkin Elmer LS-50B spectrofluorometers, based on an excitation of intrinsic fluorescence from aromatic side chains at 280 nm. Samples containing 20–40 μg/ml of protein were analyzed in a rectangular quartz cuvette of 0.5-cm path length. Scanning rate was 50 nm/min. At least three repetitive scans were obtained and averaged.

### 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 by a refractive index, as described by Nozaki (1972). An unfolding curve of the toxin was constructed from a fluorescence intensity ratio between 330 and 350 nm ($F_{330/350}$). The observed spectral intensity ($I_{obs}$) was fitted by the three-state equation:

$$I_{obs} = \frac{I_N + I_I\,(\exp\,(m_{NI}[C]\text{-}m_{NI}[C]^{NI\ 50\%})/RT) + I_U\{(\exp\,(m_{NI}[C]\text{-}m_{NI}[C]^{NI\ 50\%})/RT) \times (\exp\,(m_{IU}[C]\text{-}m_{IU}[C]^{IU\ 50\%})/RT)\}}{1+(\exp\,(m_{NI}[C]\text{-}m_{NI}[C]^{NI\ 50\%})/RT) + \{(\exp\,(m_{NI}[C]\text{-}m_{NI}[C]^{NI\ 50\%})/RT) \times (\exp\,(m_{IU}[C]\text{-}m_{IU}[C]^{IU\ 50\%})/RT)\}}$$

$I_N = \alpha_N + \beta_N[C]$, $I_I = \alpha_I + \beta_I[C]$, and $I_U = \alpha_U + \beta_U[C]$

where $I_N$, $I_I$ and $I_U$ are the intensity for N, I and U states; $\alpha$ and $\beta$ are $Y$-intercepts and slopes of these states; and [C] is the GuHCl concentration. The transitional midpoint $[C]^{50\%}$ and unfolding free energy of the protein in the absence of denaturant $\Delta G^{\circ}_{w} = m[C]^{50\%}$ at 25 °C were obtained by curve fitting (Ibarra-Molero and Sanchez-Ruiz, 1996).

### 2.5. Kinetic unfolding

The toxin (20–40 μg/ml) was mixed with various concentrations of GuHCl. The fluorescence spectra decay 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):

$$I_t = I_\alpha + \Delta I \exp^{(-k_{obs})t}$$

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

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

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

$$k_w = \left(\frac{k_B T}{h}\right) \exp^{(-E_{ac,w})/RT}$$

where $k_B$ is Boltzmann's constant ($1.3807 \times 10^{-23}$ J/K), $h$ is Planck's constant ($6.6261 \times 10^{-34}$ m$^2$ kg/s), $T$ is absolute temperature (K), $R$ is the gas constant (1.987 cal/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

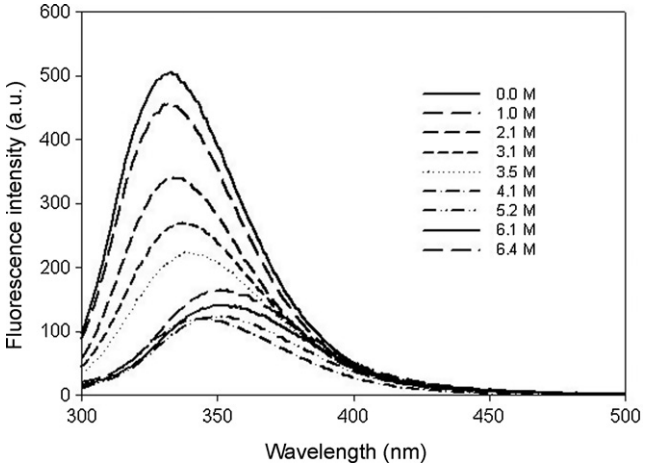


**Fig. 1.** Intrinsic fluorescence spectra of Cyt2Aa2 toxin in various concentrations of guanidinium hydrochloride. Purified toxin of 20–40 μg/ml was incubated overnight in 0.0–6.4 M GuHCl. The emission spectra were obtained from 300 to 500 nm, with an excitation at 280 nm.