

Our preliminary characterization was performed by measuring protein solubility in 50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.8. Interestingly, a numbers of mutant toxins were found to be insoluble in the native buffer condition. These mutants include D259N, Y267F, T285V, K487A and R627A. Since the R203Q template protein and original wild type are well soluble in the alkali carbonate buffer, a drastic change of solubility in these mutant toxins can be used as a strong indication for an incorrect folding of the native toxin structure. An explanation based on the selection of these mutated residues should be addressed in terms of loss in specific stabilizing forces or bonding. According to a x-ray structure of the active Cry4B, the residues D259, Y267, T285, K487 and R627 are the conserved residue lie within the five conserved blocks of toxin molecule, providing either hydrogen bond or salt bridge for structural stabilization. An important observation is that these stabilizing bonds and interaction were found between these mutated residues and residues located closely in space but far away in the linear sequence. Mutations that eliminate these stabilizing forces have a significant effect to the ability of protein to adopt the correct native conformation.

3.4 Proteolytic Processing of Soluble Toxins

The 130-kDa soluble template protein (R203Q) and L175V, I189L, R251A, H466Y, D470N, E629Q mutant toxins, were then subjected to proteolytic processing by trypsin to produce the 65-kDa active toxins. Upon an analysis by SDS-PAGE, it was found that the L175V, I189L, R251A, H466Y, and D470N mutants yielded the 65-kDa products similar to their R203Q template. While the E629Q mutant toxin gave no detectable product of a 65-kDa protein. Even though the E627Q mutant was found to be soluble in an alkali carbonate buffer like other, a complete digestion by trypsin has suggested that the soluble conformation of this mutant is somehow different from the native toxin. Therefore, the stabilizing interaction provided by this E627 residue is critical for the folding of native structure.

3.5. Chromatographic Purification of Mutants

The 65-kDa soluble R203Q template protein and its mutants, L175V, I189LR251A H466Y, D470N, were purified using size-exclusion column. All samples showed chromatographic profiles containing the 65-kDa product peak at retention volume approximately 13-14 ml. These purified fractions were confirmed in SDS-PAGE as a single band.

4. Characterization of Protein Unfolding for Mutants

Our basis for site-directed mutagenesis study is focused on a removal of hypothesized stabilizing forces provided by a numbers of residues within the conserved sequence blocks. These selected residues were chosen based on the examination on the three dimensional structure of Cry4B. We have started by an investigation of the hydrophobic interaction located on the conserved block I of domain I.

4.1. Roles of the Hydrophobic Interaction in conserved Block I

According to the structural feature of domain I which is a helical bundle among seven helices, we were interested in the highly conserved helix-5 located in the center of domain I. This central helix is surrounded by the other helices and it represents the conserved block I among the sequences of Cry toxins. The residues in this conserved block are mostly an amino acid with hydrophobic side chain. These hydrophobic residues should provide a suitable environment for structural packing in the core of domain interior. From other reports and our experiences in mutating a residue in this conserve block, these mutations usually lead to the expression of the insoluble protein product. These results has already confirmed the critical role of residues in this conserved block I on the formation of correct native folding. Here in this work we choose to make a rather conserved mutation from L175 to V and I189 to L. Our conservative changes among L, V and I has led to the expressed mutant proteins with comparable solubility to their template. The advantage of getting these mutant proteins in a soluble form is that they can be subjected to further analysis in protein unfolding experiment.

From basic characterization of the biochemical properties, the constructed L175V and I189L mutants were found to have a comparable expression level, solubility and proteolytic processing pattern to that of the R203Q template. This is a good indication that the mutants can fold into the native-like conformation. However, an unfolding experiment has revealed different unfolding curves between these two mutants (Figure 28). We found that the unfolding curve of L175V is shifted to the lower concentration of denaturant while I189L give an identical curve to the template protein. After model fitting analysis their transitional mid point and unfolding free energy were list in Table 2.

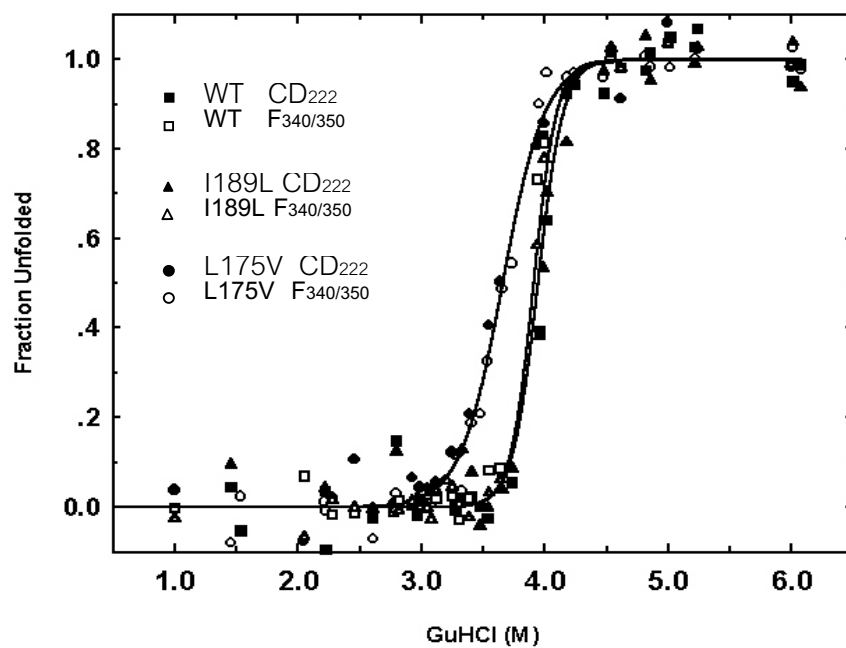


Figure 28: Unfolding curves of the block I mutants showing LO175V, I189L and their R203Q template.

Toxins	$[\text{GuHCl}]^{50\%}$ (M)	m (cal/mol·M)	$\Delta G^{\circ}_{U,W}$ (kcal/mol)	$\Delta\Delta G^{\circ}_{U,W}$ (kcal/mol)
R203Q	3.92	5890	23.09	0.00
L175V	3.66	3445	12.61	-10.48
I189L	3.94	5774	22.75	-0.34

Table 2: Steady state parameters derived from unfolding experiment of L175V, I189L and T203Q

According to the calculated unfolding free energy, the native structure of L175V mutant is destabilized by 10.48 kcal/mol. This destabilization is very significant when compared to the unfolding free energy of R203Q template at 23.09 kcal/mol. Since our analysis for stabilizing interactions in the three-dimensional structure of domain I showed only the closed packing of hydrophobic cluster along the central helix-5, the destabilizing effect could be due to an interference on the hydrophobic core packing of this mutant.

However, the characterized unfolding free energy of I189L mutant remains unchanged. We have analyzed the mutational effect using a contact sphere around I189L and L175V. The model revealed that a substitution of L175 with V has generated a large cavity while a substitution of I189 with L has no significant change within the closed packing. This observation is also found in several cases of protein folding study. Here we have confirmed the significant role of specific hydrophobic residues of the conserved block I on folding and stability of the molecular structure of the native toxin.

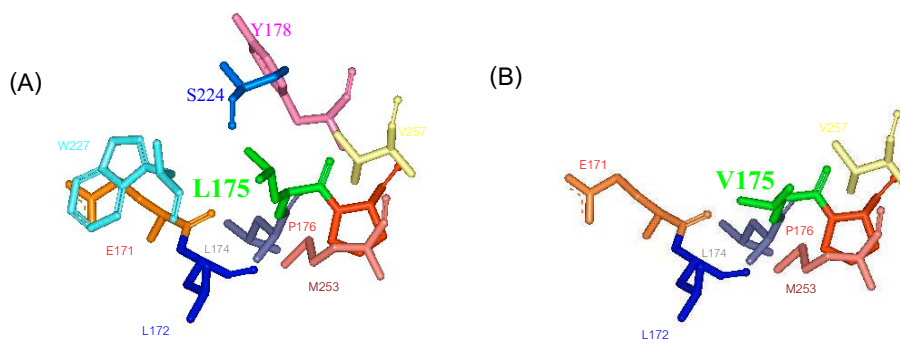


Figure 29: Molecular contact within a sphere of 5 Å radius around the L175 (A) and V175 (B)

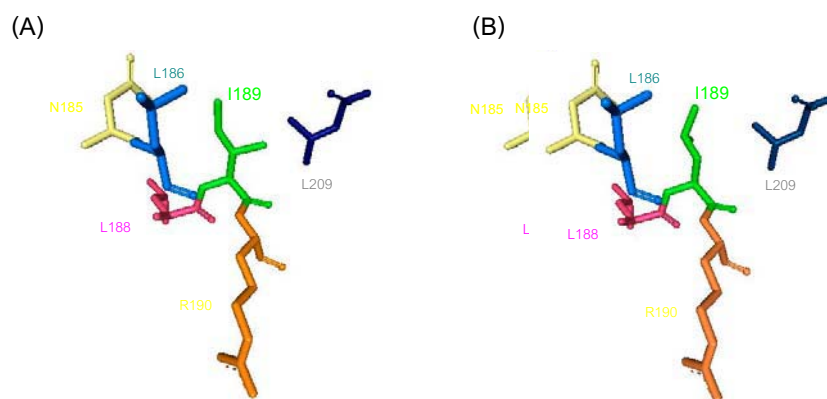


Figure 30: Molecular contact within a sphere of 5 Å radius around the I189 (A) and L189 (B)

We have continued the characterization of these two mutants, L175V and I189L by an analysis of unfolding kinetics. Both mutants have demonstrated an exponential decay of the fluorescence intensity similar to their R203Q template (Figure 31-32).

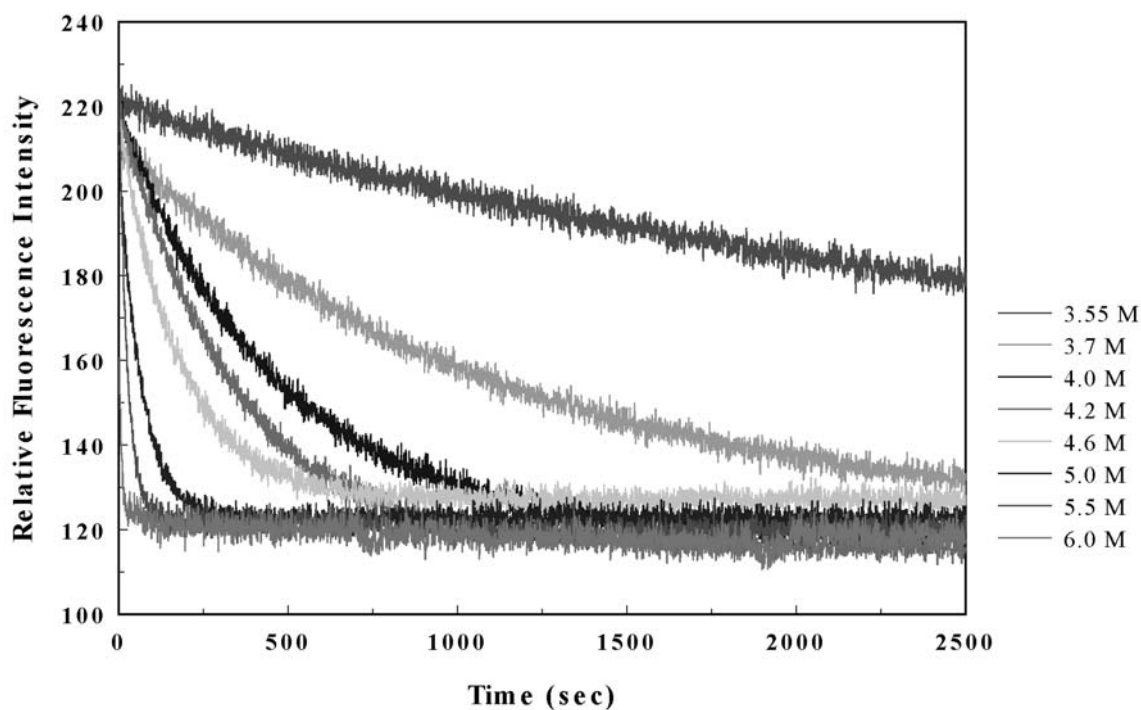


Figure 31: Analysis of unfolding kinetics of L175V mutant

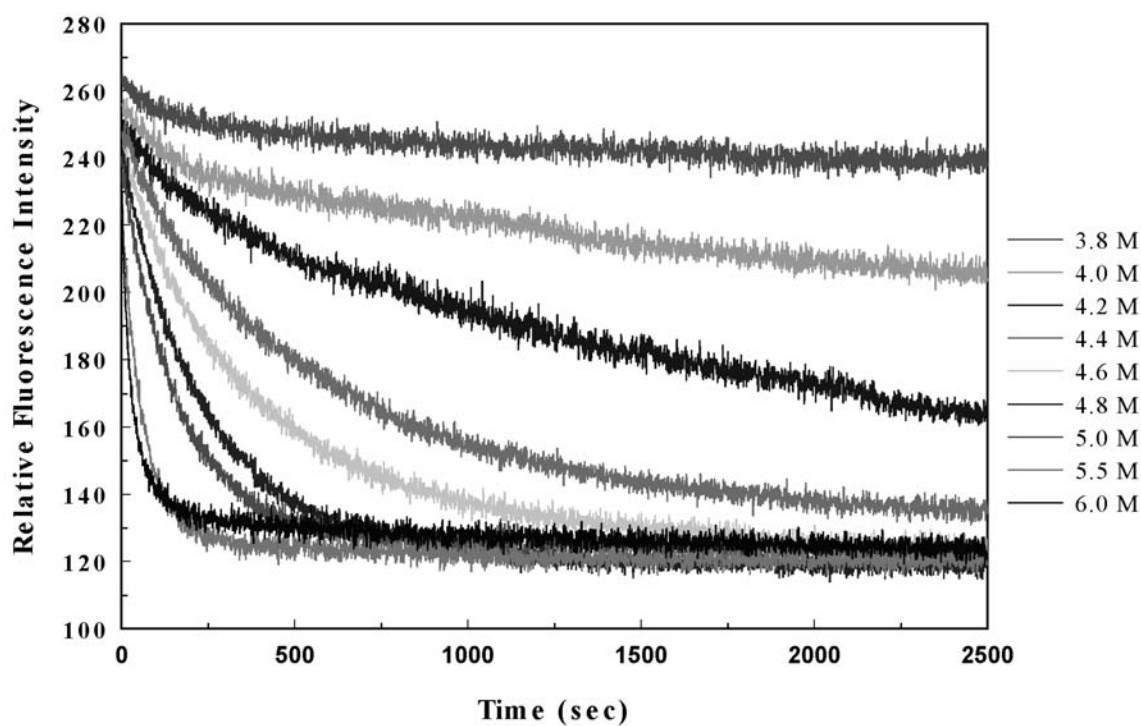


Figure 32: Analysis of unfolding kinetics of I189L mutant

Our calculation for the unfolding rate constant (Figure 33) and activation energy has revealed a similar value for both mutants (Table 3). It means that the introduced mutation on both positions has no effect on the kinetics or mechanism of the protein during an unfolding process. A combined data of unfolding free energy and activation energy was then used to sketch an energy profile of unfolding for each mutant (Figure 34). This energy profile can graphically demonstrate the mutational effect of L175V and I189L. It shows that the native state of L175V is destabilized by 10.4 kcal/mol relative to I189L and the R203Q template. The map also shows that the activation energy used to overcome the transitional barrier of the two mutants remain unchanged.

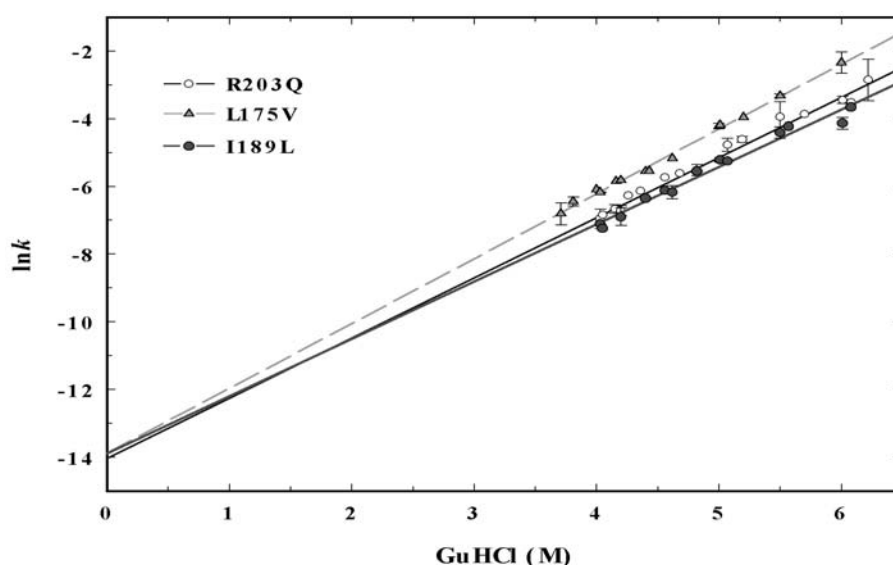


Figure 33: Determination of the unfolding rate constant of L175V and I189L

Toxins	$\ln k_{U,W}^0 \pm \text{SEM}^{/1}$	$k_{U,W}^0 \pm \text{SEM}$ ($\times 10^{-7} \text{ s}^{-1}$) ^{/2}	$Ea_{U,W}^0 \pm \text{SEM}$ (kcal/mol) ^{/3}	$\Delta Ea_{U,W}^0 \pm \text{SEM}$ (kcal/mol) ^{/5}
R203Q	-13.94 ± 0.09	8.90 ± 0.85	25.71 ± 0.06	
L175V	-14.03 ± 0.36	9.06 ± 3.01	25.76 ± 0.21	$+0.06 \pm 0.18$
I189L	-13.80 ± 0.30	10.99 ± 2.81	25.62 ± 0.17	-0.08 ± 0.23

Table3: Kinetics parameters from the unfolding of L175V and I189L in comparison with the R203Q template

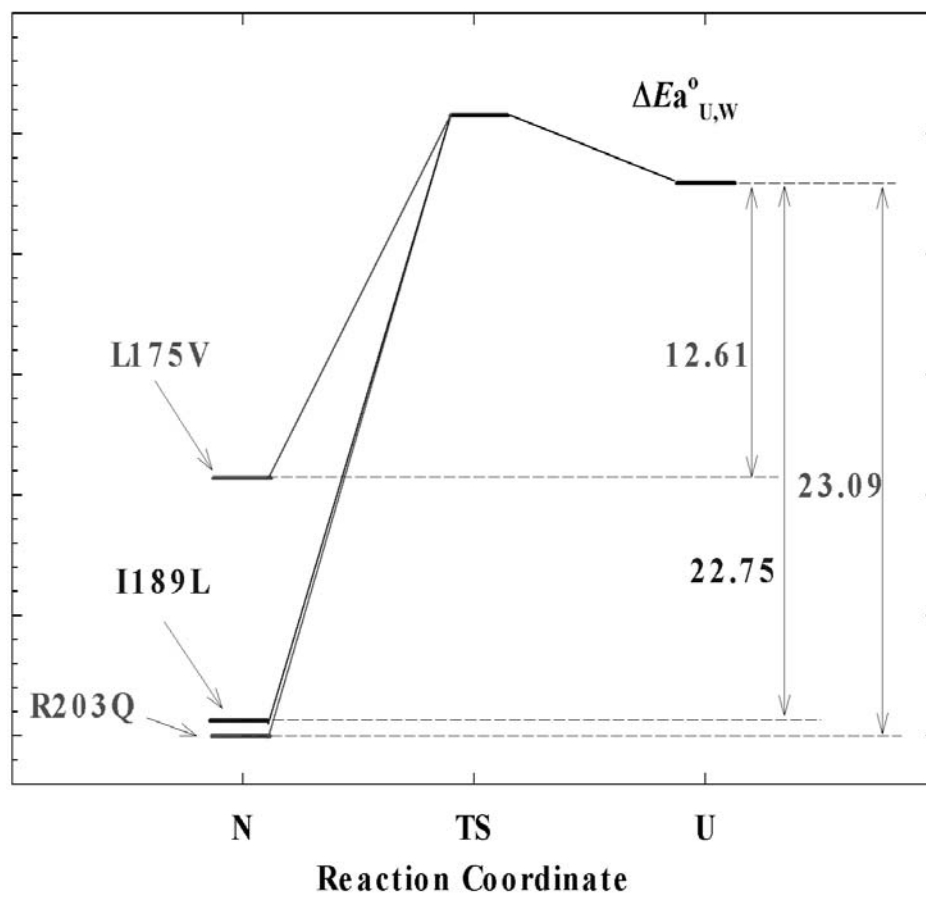


Figure 34: Energy map of protein unfolding constructed from a combined data from steady state and kinetics experiment

4.2. Roles of Hydrogen Bonding

A number of mutant toxins were constructed to investigate the role of identified hydrogen bonding provided by the residues of conserved sequence blocks. These mutants are R190, D191 (Block 1), Y267F, T285V, H466Y (Block 3), D470N, K487A, R627A and E629Q (Block 5). They were successfully constructed by PCR-based site-directed mutagenesis using R203Q as a template.

Biochemical characterization of these mutants showed that only H466Y, D470N and E629Q are soluble in the alkali carbonate buffer pH.9. The other expressed protein products of R190A, D191N, Y267F, T285V, K487A and R627A were found to be insoluble in the same buffer condition. Further processing of the 130-kDa soluble protoxins by trypsin gave an expected active toxin molecule of 65 kDa for H466Y and D470N but gave no stable product for E629Q mutant. This is suggesting that although E629Q was obtained as the soluble product but the mutant can not adopt a correct folding leading to a complete digestion by proteases.

In summary we have successfully obtained 9 mutant proteins from the template. Two of them (H466Y and D470N) is suggested to be in the native conformation based on solubility and the processing pattern. The other seven mutants (R190A, D191N, Y267F, T285V, K487A, R627A and E629Q) are likely to have lost their native folding. An analysis of the x-ray structure of wild type toxin had indicated stabilizing hydrogen bonds between those mutated residues with other residues nearby. The criterion used for defining these hydrogen bonds is an existing of the hydrogen donor (D) and acceptor (A) with in the distance ranging around 2.5-3.3 angstrom apart. The Angle between D-H-A is also set below 90 degree.

The critical role of hydrogen bond revealed in these mutants can be discusses as a crucial stabilization required for the polypeptide chain to adopt the correct folding of the native toxin. These identified hydrogen bonds are listed in Table 4 and demonstrated in Figure 34-41. One would see that a majority of the eliminated hydrogen bond has a critical role for the protein to adopt the native folding, while a few of them do not show any effect in biochemical property. If we analyzed the position of amino acids that interact with our mutated residues, we can classify them into two groups. One is an amino acid located far away in sequence but come closer to the mutated residue in space. This kind of interaction provides stabilization for the tertiary structure of protein. The other group is a neighboring amino acid located nearby in the sequence and in space. Our result suggest that an elimination of the interaction contributing in tertiary structure stabilization will obstruct the

protein to adopt the correct folding as we saw in R190A, D191N, Y267F, T285V, K487A, R627A and E629Q.

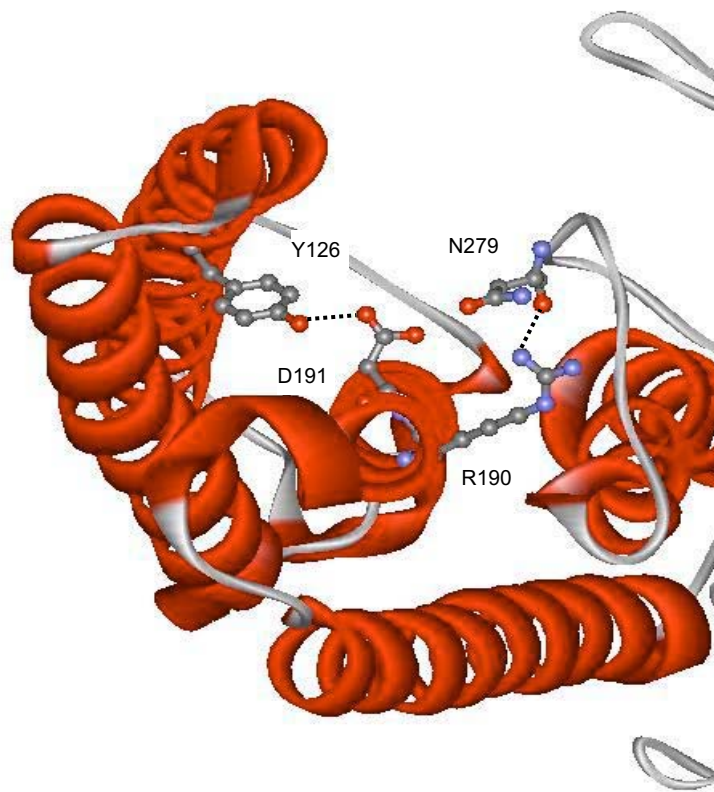


Figure 34: X-ray structure of the Cry4B toxin showing hydrogen bonding of R190 and D191

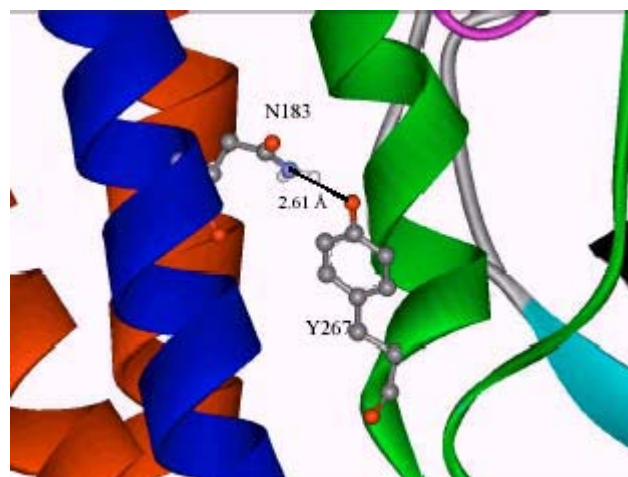


Figure 35: X-ray structure of the Cry4B toxin showing hydrogen bonding of Y269

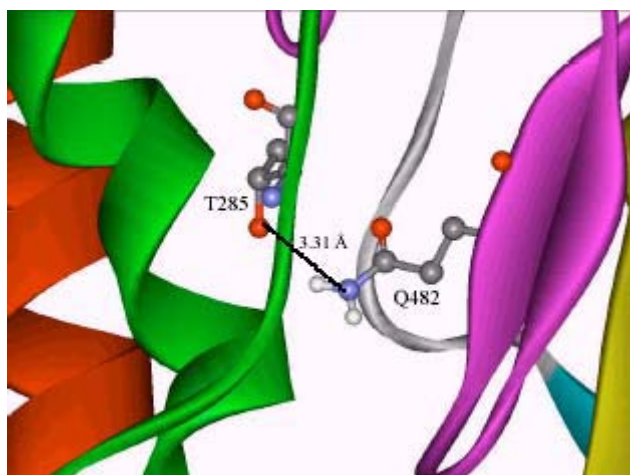


Figure 36: X-ray structure of the Cry4B toxin showing hydrogen bonding of T285

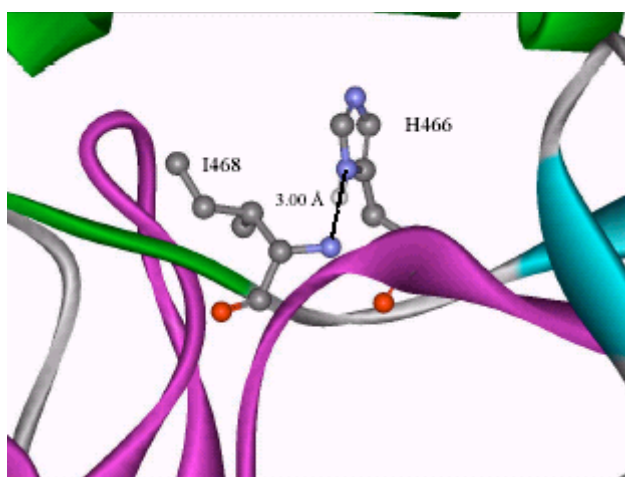


Figure 37: X-ray structure of the Cry4B toxin showing hydrogen bonding of H466

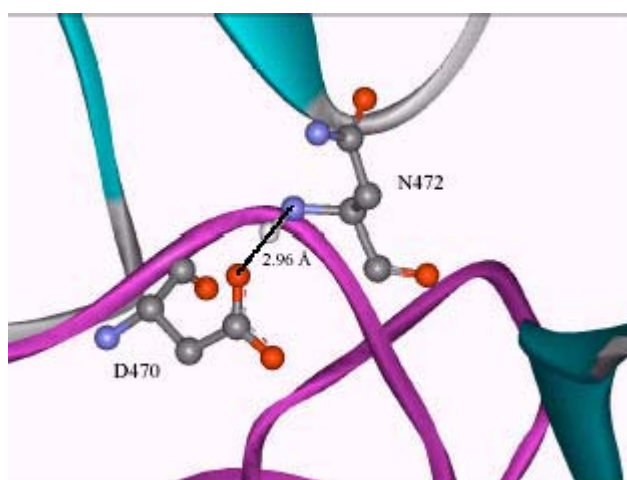


Figure 38: X-ray structure of the Cry4B toxin showing hydrogen bonding of D470

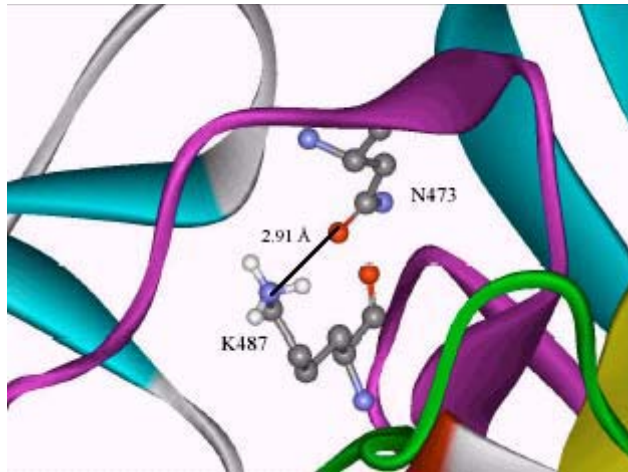


Figure 39: X-ray structure of the Cry4B toxin showing hydrogen bonding of K187



Figure 40: X-ray structure of the Cry4B toxin showing hydrogen bonding of R627

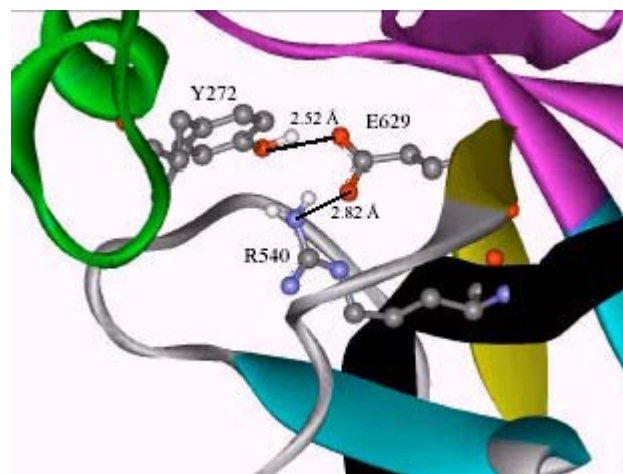


Figure 41: X-ray structure of the Cry4B toxin showing hydrogen bonding of E629

Block	Mutation	Type of interaction	Interaction removed	Bond distance Å	Correct folding
1	R190A	H-bond	R190 - N279	2.89	No
1	D191A	H-bond	D191 - Y126	2.79	No
3	Y267F	H-bond	Y267 - N183	2.61	No
3	T285V	H-bond	T285 - Q482	3.31	No
3	H466Y	H-bond	H466 - I468	3.00	Yes
3	D470N	H-bond	D470 - N472	2.98	Yes
3	K487A	H-bond	K487 - N473	2.91	No
5	R627A	H-bond	R627 - Y272	3.12	No
5	E629Q	H-bond	E629 - Y272	2.52	No
		H-bond	E629 - R627	2.73	

Table 4: A list of mutants with hydrogen bond eliminated from each conserved block

For the soluble H466Y and D470N mutants which is thought to be in the correct native folding, further analysis by an unfolding experiment was then employed to assess for their molecular stability compared to the template. The results showed that their unfolding curves and the calculated unfolding free energy are very much closed to their template toxin (Table 5). This is a confirmation that mutation at the position H466Y and D470N does not have any affect on both structural folding and molecular stability of the toxin.

	$[\text{GuHCl}]^{50\%}$ (M) $\pm$ SD	m (kcal.mol ⁻¹ .M ⁻¹) $\pm$ SD	$\Delta G^{\circ}_{U, W}$ (kcal.mol ⁻¹) $\pm$ SD	$\Delta\Delta G^{\circ}_{U, W}$ (kcal.mol ⁻¹)
R203Q (template)	3.81 $\pm$ 0.00	6.22 $\pm$ 0.04	23.72 $\pm$ 0.17	0.00
H466Y	3.80 $\pm$ 0.02	5.62 $\pm$ 0.33	21.39 $\pm$ 1.36	-2.33
D470N	3.74 $\pm$ 0.04	6.25 $\pm$ 0.24	23.38 $\pm$ 1.15	-0.34

Table 5: Steady state parameters and unfolding free energy of H466Y and D470N mutants

4.3. Role of Electrostatic interaction

We have designed and constructed more mutants to investigate the role of selected salt bridge provided by residues of the conserved sequence blocks. These mutants are R251A and D259N (Block 2). These mutants were successfully constructed by PCR and characterized for their biochemical property. We have found that the two mutants can be expressed at a comparable level to that of the R203Q template. However the expressed D259N is found insoluble in the alkali carbonate buffer. The drastic change of toxin solubility has suggested that the expressed D259N mutant could not adopt the native conformation (Table 6).

However the expressed R251A was obtained as a soluble protein and can be processed to the 65-kDa active form. A possible explanation is based on the different nature between both salt bridges as identified from the x-ray structure of toxin. We found that the first electrostatic interaction or salt-bridge formed between R251 and neighboring D248 residue can help stabilizes the local folding of protein secondary structure (Figure 42). But the other interaction between D259 and R289 help stabilize protein tertiary structure (Figure 43). It is likely that an elimination of the interaction that stabilizes tertiary structure has led to a failure in adopting the native folding.

Block	Mutation	Type of interaction	Interaction removed	Bond distance A	Correct folding
2	R251A	Salt bridge	R251 - D248	2.89	Yes
2	D259N	Salt bridge	D259 - R289	2.78	No

Table 6: A list of mutants with salt bridges eliminated

We have continued to analyze the soluble mutant toxin, R251A by an unfolding experiment. After a construction of unfolding curve and fitting the data to the two-state transition model, it was found that R251A give an identical unfolding curve and very similar unfolding free energy to the R203Q template (Table 7). This result means that a perturbation on the electrostatic interaction between R251 - D248 has no effect on structural folding and molecular stability of the toxin.

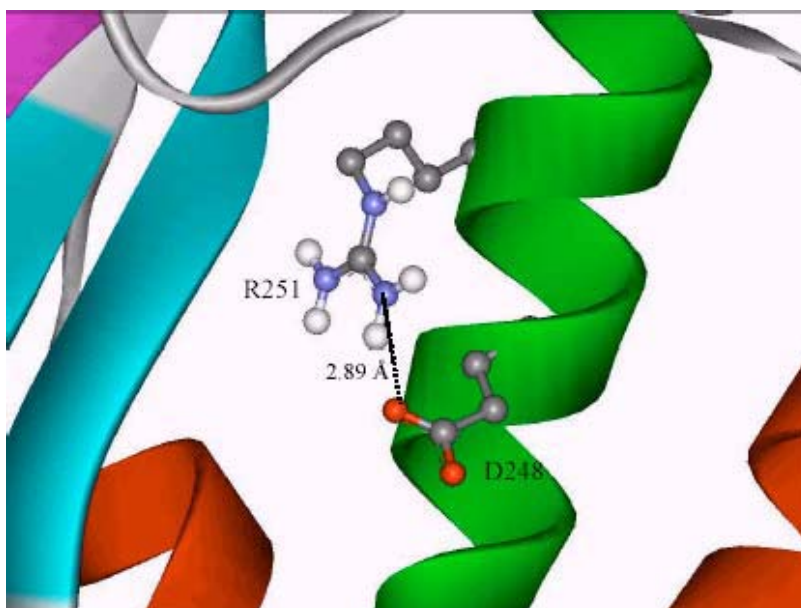


Figure 42: X-ray structure of the Cry4B toxin showing an electrostatic interaction formed between R251 and D248

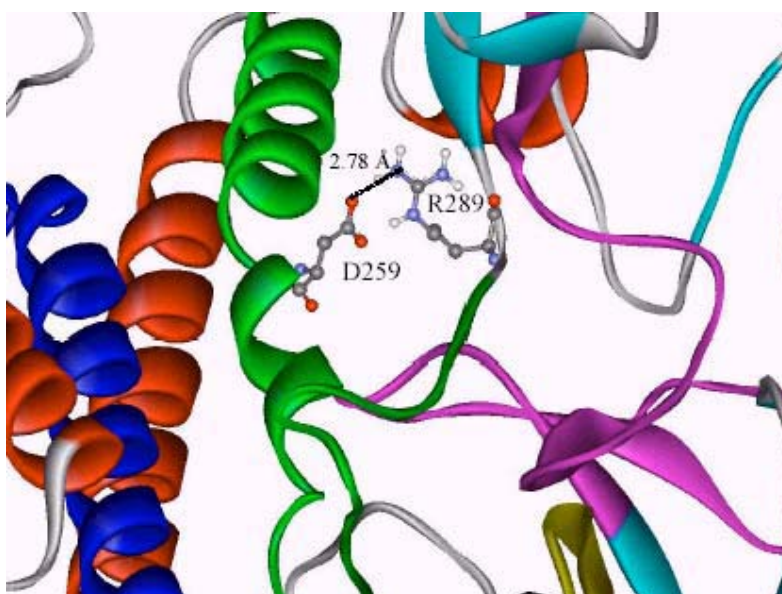


Figure 43: X-ray structure of the Cry4B toxin showing an electrostatic interaction formed between D259 and R289

	$[\text{GuHCl}]^{50\%}$ (M) $\pm$ SD	m (kcal.mol ⁻² .M ⁻¹) $\pm$ SD	$\Delta G^{u,w}$ (kcal.mol ⁻²) $\pm$ SD	$\Delta\Delta G^{u,w}$ (kcal.mol ⁻¹)
R203Q (template)	3.81 $\pm$ 0.00	6.22 $\pm$ 0.04	23.72 $\pm$ 0.17	0.00
R251A	3.80 $\pm$ 0.03	6.58 $\pm$ 0.00	24.98 $\pm$ 0.18	+1.26

Table 7: Steady state parameters and unfolding free energy of R251A

5. Characterization of Larvicidal Activity

According to all the generated mutants, they were characterized for the basic biochemical property in terms of expression level, solubility, proteolytic processing pattern. Here we also examined the biological activity of these mutant toxins. After feeding the 2-day old *A. aegypti* larvae with the cells expressing for the toxin, the mortality rate of larvae was then reported. We found that the mutants adopting the native-like structure (R203Q, L175V, I189L, R251A, H466Y and D470N) showed their toxicity similar to the wild type around 80-90%. On the contrary, those mutants which are insoluble or sensitive to a complete digestion by trypsin showed a marked drop of their activity against the larvae. These results confirm that the biological functionality is closely related to the correct structural folding of the toxins.

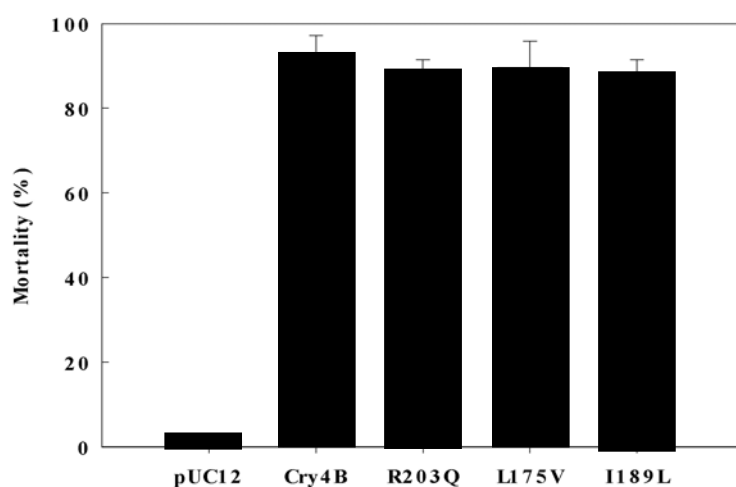


Figure 44: Larvicidal activity of wild type, R203Q template and mutants

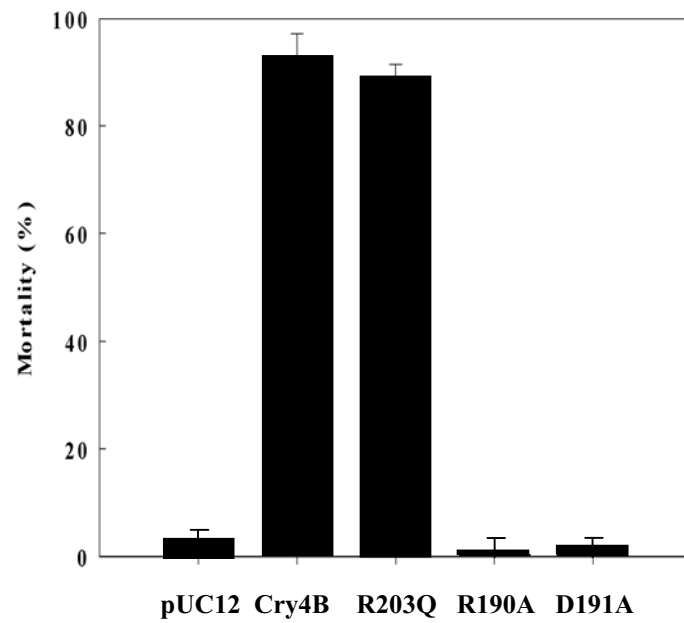


Figure 45: Larvicidal activity of wild type, R203Q template and mutants

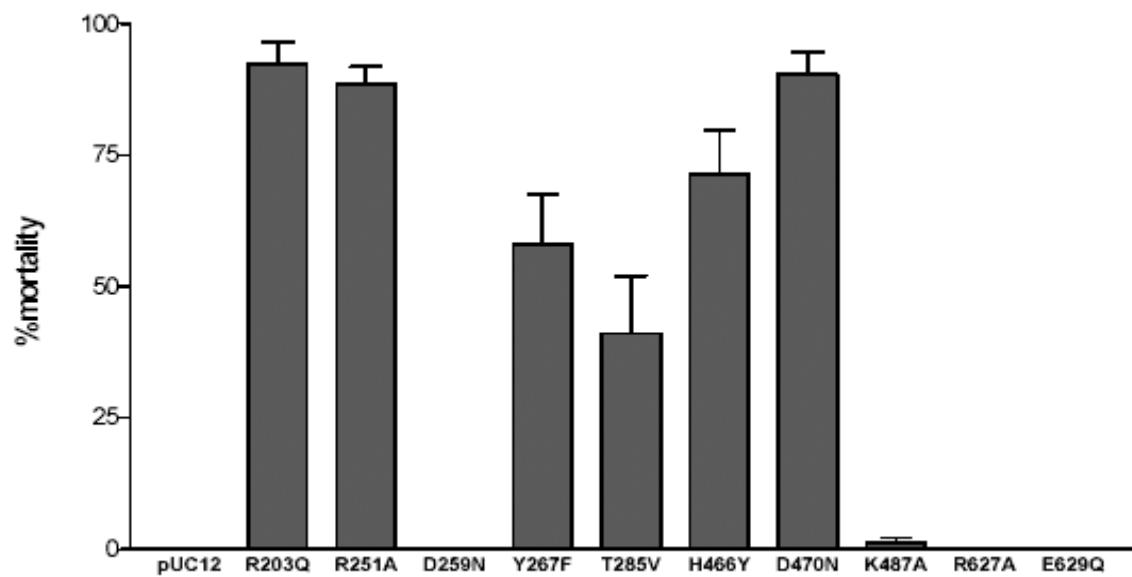


Figure 46: Larvicidal activity of R203Q template and mutants

Concluding Remarks

1. Characterization of protein unfolding for wild type Cry4B has revealed an unfolding free energy of 17.86 kcal/mol and activation energy of 25.77 kcal/mol, respectively.
2. A single chain R203Q mutant has demonstrated an enhanced stability with an unfolding free energy of 23.09 kcal/mol and activation energy of 25.71 kcal/mol.
3. The mutant plasmids, pL175V, pI189L, pR190A, pD191A, pR251A, pD259N, pY267F, pT285V, pH466Y, pD470N, pK487A, pR627A and pE629Q, were successfully constructed by PCR-based site directed mutagenesis using pR203Q as a template, and their nucleotide sequences were confirmed by automated DNA sequencing.
4. The expression levels of these mutant toxins are comparable to that of R203Q template.
5. Based toxin solubility and proteolytic processing pattern, L175V, I189L, R251A, H466Y and D470N mutant toxins were expressed in the native-like conformation while R190A, YD259N, Y267F, T285V, K487A and R627A could not adopt the correct folding due to losing of hydrogen bond and electrostatic interaction in the conserved blocks.
6. The chemical unfolding curves and unfolding free energy of the purified I189L, R251A, H466Y and D470N were found similar to that of R203Q template. However a perturbation of the hydrophobic packing inside the domain interior of L175V has led to a significant destabilization of molecular structure by 10.49 kcal/mol
7. Stabilizing hydrogen bond and electrostatic interaction that helps maintain protein tertiary structure is more important than those interactions within a secondary structure element.
8. Specific stabilizing interaction formed between the residues located on the five conserved blocks is highly critical for the correct folding of Cry4B.

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Outputs from the Project

1. Publications in an international journal

1.1 Krittanai, C., Bourchookarn, A., Pathaichindachote, W. and Panyim, S. (2003)

Mutation of the hydrophobic residues on helix $\alpha 5$ of the *Bacillus thuringiensis* Cry4B affects structural stability. ***Protein & Pept. Letters*** 4: 361-368.

1.2 Krittanai, C., Sangcharoen, A. and Taepanunt, W. Stabilizing interaction provided by the five conserved blocks of Cry4B toxin are critical for the correct folding. ***In Preparation.***

2. Presentations in an international symposium

2.1 Krittanai, C., Pathaichindachote, W., Bourchookarn, A., Angsuthanasombat, C. and Panyim, S. Investigation of Denaturation Curves for Cry 4B Delta-Endotoxin The 46th Annual Meeting of Biophysical Society, San Francisco, USA., 23-27 February 2002.

2.2 Ounjai, P., Boonserm, P., Krittanai, C., Katzenmeier, G., Panyim, S. and Angsuthanasombat, C. Mutagenic Analysis of a Highly Conserved Arginine in an Alternating Arginine Region within Domain III of the *Bacillus thuringiensis* Cry4B Toxin The 46th Annual Meeting of Biophysical Society, San Francisco, USA., 23-27 February 2002.

2.3 Krittanai, C., Bourchookarn, A. and Sangcharoen, A. Investigation of the *Bacillus thuringiensis* Cry4B Protein Unfolding by Circular Dichroism and Fluorescence Spectroscopy The Inaugural Austral-Asian Biospectroscopy Conference. Suranaree University of Technology, Thailand, February 3-7, 2003.

2.4 Krittanai, C., Bourchookarn, A., Sangcharoen, A. and Panyim, S. Mutation of the Hydrophobic Residues in the Interior of the *Bacillus thuringiensis* Cry4B Domain I Affects Structural Folding and Stability The 5th European Symposium of the Protein Society, Florence, Italy, March 29 – April 2, 2003

3. Presentation in national symposium

3.1 Characterization of Protein Unfolding for *Bacillus thuringiensis* Cry4B Toxin by Stopped-flow Circular Dichroism Spectroscopy

In Protein Network Symposium on Protein Structure and Molecular Enzymology

August 29-30, 2002, Faculty of Science, Mahidol University

3.2 Elimination of an Internal Cleavage Improved Structural Stability of the *Bacillus thuringiensis* Cry4B Toxin

In Protein Network Symposium on Protein Structure and Molecular Enzymology

August 29-30, 2002, Faculty of Science, Mahidol University

3.3 Characterization of Protein Unfolding for *Bacillus thuringiensis* Cry4B Toxin by Stopped-flow Circular Dichroism Spectroscopy

In Protein Network Symposium on Protein Structure and Molecular Enzymology

August 29-30, 2002, Faculty of Science, Mahidol University

3.4 Basic Structure of the insecticidal protein from *Bacillus thuringiensis*

In "Bacillus thuringiensis Symposium: From Basic to Applied Research"

May 22-23, 2003, Institute of Molecular Biology and Genetics, Mahidol University

3.5 Investigation of Molecular Stabilization and Charge Interaction in the Conserved Regions of *Bacillus thuringiensis* Cry4B Toxin

In The 28th Science and Technology of Thailand Congress

October 24-26, 2002, Queen Sirikit Convention Center

3.6 Site-Directed Mutagenesis and Stability Characterization of Mosquitocidal Cry4B Toxin:

A Contribution of Hydrophobic Residues on Helix $\alpha 5$ Regions

In The 28th Science and Technology of Thailand Congress

October 24-26, 2002, Queen Sirikit Convention Center

4. Graduate student training / MSc. thesis

Miss Wanwarang Pathaichindachote

Mr Apichai Bourchookarn

Miss Anchaneer Sangcharoen

MUTATION OF THE HYDROPHOBIC RESIDUE ON HELIX $\alpha 5$ OF THE *BACILLUS THURINGIENSIS* CRY4B AFFECTS STRUCTURAL STABILITY.

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Abstract: Cry4B toxin is a mosquito-larvicidal protein from the *Bacillus thuringiensis* subsp. *israelensis*. We have investigated the role of two conserved hydrophobic residues of Cry4B in structural stabilization. Substitutions of the leucine-175 and isoleucine-189 on helix $\alpha 5$ with valine and leucine did not affect the expression level, solubility and proteolytic processing. Steady state analysis of an unfolding experiment as monitored by circular dichroism and fluorescence spectroscopy demonstrated a typical two-state transition. The determined unfolding free energy for the L175V mutant revealed a structural destabilization of 10.49 kcal/mol relative to the wild type. However unfolding kinetic analysis gave identical activation energy for wild type and both mutants. Our findings suggested that a perturbation on the close packing of the hydrophobic side chains in protein interior could lead to a significant destabilization of the native conformation.

Keywords: *Bacillus thuringiensis*, protein folding, hydrophobic packing, free energy, mutagenesis, circular dichroism, intrinsic fluorescence.

INTRODUCTION

Since a general globular protein folds in a way that keeps the hydrophobic side chains inside the interior and avoids contact with physiological water, hydrophobic interaction has been proposed to be one of the major driving forces for protein folding [1, 2]. Recently an optimal van der Waals close packing of the side-chain has been shown to also play a significant role in stabilization of the molecular structure [3, 4]. Several site-directed mutagenesis studies on the buried non-polar amino acid have reported an effect on structural folding and stability [5-8]. These mutational effects can be experimentally determined as a change of free energy in an unfolding/refolding process. A number of small or single-domain proteins such as ribonuclease [9], myoglobin [10], chymotrypsin inhibitor [11], staphylococcal nuclease [12], lysozyme [13] and barnase [14] have been energetically studied. However, a study on a larger protein like Cry4B is

still lacking. Even though the unfolding study of the proteins in the same family such as Cry1Ab, Cry1Ac and Cry3Aa [15-17] have been published. These works were somewhat qualitative and not directly involved with energetic effect of mutation.

The Cry4B toxin from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is a protein with specific toxicity to *Aedes* mosquito larvae. The 130-kDa protoxin form is generally converted into a 65-kDa functional molecule by proteolytic processing inside the midgut of larvae. X-ray structure of Cry4B showed a similar fold to other Cry toxins like Cry1A and Cry3A [18-20]. Domain I, a seven-helical bundle, has been proposed to function as a membrane pore-forming domain [21]. Domain II and III were suggested to be involved in the membrane binding [22, 23]. Several works on site-directed mutagenesis have reported significant changes of the biochemical properties of the mutants, especially when the mutation was introduced on the central helix $\alpha 5$ [24-26]. These effects include a complete loss of solubility, mosquito-larvicidal activity, and increased sensitivity for proteolytic digestion. These abnormalities were proposed as a result of incorrect folding of the toxin structure or molecular destabilization upon perturbation on the critical amino acid residues.

This work aims to characterize the mutational effect of the hydrophobic residues, L175 and I189, on the central helix $\alpha 5$ of domain I. These two residues were selected based on their conservation among the toxin family and they are well buried inside a hydrophobic core of the domain. The L175V and I189L mutants were constructed and their conformational states in the unfolding experiment were monitored by circular dichroism and fluorescence spectroscopy. The unfolding free energy and activation energy was experimentally determined and compared to the template toxin. The differences in energetic parameters can then reveal an energetic role of specific mutation on structural folding and molecular stability.

MATERIALS AND METHODS

Site-directed mutagenesis: PCR based site-directed mutagenesis was performed on the pMU388/R203Q plasmid containing *cry4B* gene with previous point mutation for R203Q mutant. The reaction employed a pair of forward and reverse synthetic oligonucleotide primers (Genset, Singapore) under a high fidelity *Pfu* DNA polymerase (Promega, USA). Parental DNA template was removed by *DpnI* digestion. *E. coli* strain JM109 was transformed with mutant plasmids and screened by restriction analysis. DNA sequences were confirmed by analysis on an ABI-PRISM 377 automated sequencer (Perkin Elmer, USA).

Protein expression and purification: Expression was performed with 0.1 mM IPTG induction in LB broth containing a 100- μ g/ml ampicillin at 37 °C for 12-16 hours. After cell disruption by French Press Cell, an inclusion product was solubilized in 50 mM carbonate buffer pH 10.0 and processed with TCPK-treated trypsin. Purified protein was obtained from Superdex-200 HR10/30 size-exclusion column (Amersham). Protein concentration was determined based on a far-UV absorption on using a Cary300 Bio UV-Visible spectrophotometer (Varian, Australia). Conc. (in mg/ml) = $0.144 \times (A_{215} - A_{225})$ [27].

Steady state unfolding: The purified protein was incubated in a series of GuHCl concentrations from 0-6.0 M overnight. The conformational state was monitored at 25°C by circular dichroism and

fluorescence spectroscopy. Accurate concentration of GuHCl stock was determined from a refractive index measurement as described by Nozaki [28].

Unfolding kinetics: Fluorescence spectra were obtained in a rapid mixing between protein in carbonate buffer pH 10.0 and various proportions of GuHCl. The experiment was performed with a final protein concentration of 10 µg/ml. All solutions and sample were degassed well before mixing.

Circular dichroism measurement: CD spectra were obtained from a Jasco J-715 spectropolarimeter (Jasco, Japan). Instrument was calibrated with 1.0 mg/ml of (+)-10-camphorsulphonic acid (CSA) and purged with nitrogen. Samples (0.4-0.6 mg/ml protein) were analyzed at 25°C in a cuvette with 0.02-cm path length. Scanning was set from 190 to 260 nm with 20-nm/min scanning rate, 1.0-second response time, and 50-millidegree sensitivity for at least 4 accumulations. All sample spectra were subtracted with baseline.

Intrinsic fluorescence measurement: Intrinsic emission fluorescence spectra were recorded from 300 to 500 nm on a Perkin Elmer LS-50B luminescence spectrometer. Excitation was set at 280 nm. Samples containing 20-40 µg/ml of protein were analyzed in a cuvette with 0.5-cm path length. Scanning rate was controlled at 50 nm/min. At least three repetitive scans were recorded and averaged.

Data Analysis: The unfolding curves were derived from changes of CD intensity at 222 nm and the fluorescence emission ratio 340/350 nm ($F_{340/350}$). Apparent fraction of unfolding (f_{app}) was calculated from an equation:

$$f_{app} = \frac{I_{obs} - (\alpha_N + \beta_N [C])}{(\alpha_U + \beta_U [C]) - (\alpha_N + \beta_N [C])}$$

Where I_{obs} is an observed spectral intensity, α_N and α_U are Y-intercept for native and unfolded state. β_N and β_U are slopes at low and high GuHCl concentration. $[C]$ is the GuHCl concentration. Transitional midpoint, $[C]^{50\%}$ and unfolding free energy of protein in an absence of denaturant, $\Delta G_{(H_2O)}^0 = m[C]^{50\%}$ at 25 °C were determined by fitting with the model equation [29]:

$$f_{app} = \frac{(\alpha_N + \beta_N [C]) + (\alpha_U + \beta_U [C]) \exp \{ m ([C] - [C]^{50\%}) / RT \}}{1 + \exp \{ m ([C] - [C]^{50\%}) / RT \}}$$

For kinetic data, the exponential decay were fitted to the first order kinetic using the equation:

$$I_t = I_\infty + \Delta I \exp^{-kt}$$

Where I_t and I_∞ is an observed intensity and intensity at the infinite. ΔI is the maximum intensity change. Rate constant from the fitting was then used to calculate for the free energy of activation (E_a).

$$K = (k_B T / h) \exp^{(-E_a / RT)}$$

When k_B and h is the Boltzmann's and Plank's constants. T and R is an absolute temperature and molar gas constant. The reported free energy of activation in an absence of denaturant $E_a^0_{(H_2O)}$ was obtained from a linear plot of E_a and GuHCl concentration.

RESULTS AND DISCUSSION

Construction and biochemical characterization of mutants

There are a number of works that showed an anomaly from site-directed mutagenesis studies of several Cry toxins, especially when a substitution was made on the amino acid residues located on the

central helix region of domain I [24-26]. Those mutants were found to be highly susceptible to the proteolytic digestion, with complete loss of toxin solubility and mosquito-larvicidal activity. According to the recently elucidated three-dimensional structure of the 65-kDa active toxin, the two investigated hydrophobic residues L-175 and I-189 in this work are located on the central helix $\alpha 5$ [18]. Any substitutions made at these two positions are expected to bring a directed perturbation into the domain interior. Interestingly, our constructed mutants, L175V and I189L have demonstrated native-like properties. They had a similar expression level, solubility in alkali buffer, and resistance to the proteolytic digestion (data not shown). It is appeared that the perturbation introduced by our side chain substitutions was too gentle to disrupt the general folding of the toxin. However this gave us an excellent opportunity to prepare these samples in a solution and then characterize them further for structural stability.

Steady-state unfolding analysis

Steady state unfolding has displayed a conformational change from the native to unfolded state upon an increasing concentration of GuHCl. Circular dichroism spectra representing the protein in native state were comprised of two negative bands around 208 and 222 nm and one intense positive band around 192 nm (**Figure 1a**). A significant loss of secondary structure was observed when the protein was incubated with an increasing concentration of denaturant. This unfolded state was represented by the CD spectra containing a single negative band with decreased intensity. The unfolding curves derived from the maximal change of CD intensity at 222 nm demonstrated a sigmoidal shape. The experiment was also performed in parallel by using intrinsic emission fluorescence. The transition from native to unfolded state was observed as an intensity change and a shift of λ_{max} from 340 to 350 nm (**Figure 1b**). It was found that the unfolding

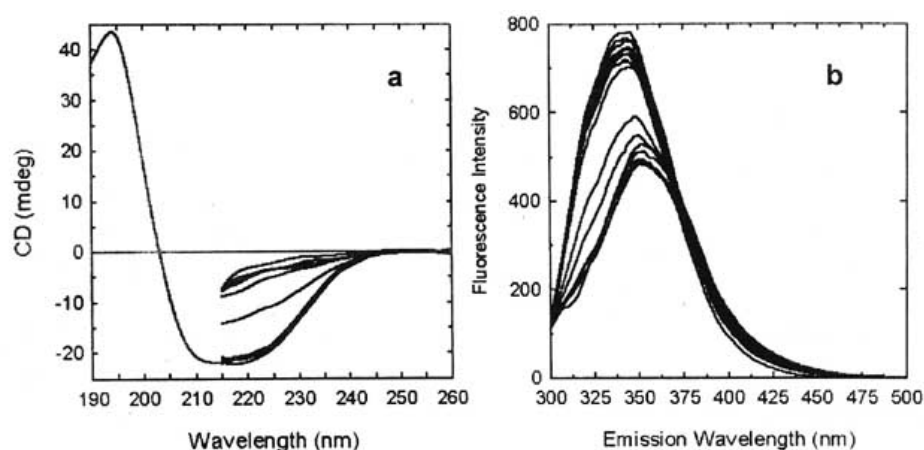


Figure 1. Conformational changes of toxins from native to unfolded states as monitored by (a) circular dichroism and (b) fluorescence spectroscopy.

curves derived from both CD and fluorescence spectra were identical. The obtained sigmoidal curves for both wild type and mutants are basically assumed as an indication for the two-state transition (**Figure 2**). These transitions were rapid and there was apparently no sign of stable intermediate detected. Since an analysis for unfolding free energy by extrapolation of the calculated free energy to the denaturant-free condition is sometimes not reliable [29], our free energy determination was performed based on the fitting model. The calculated unfolding free energies in an absence of denaturant ($\Delta G^0_{H_2O}$) for wild type, I189L and L175V were 23.10, 22.78 and 12.61 kcal/mol respectively. In general these free energies represent an energy gap between the native and unfolded state of proteins. Based on the baseline value obtained from the wild type, the mutation of isoleucine to leucine at position 189 seems to have no effect on structural stability of the protein. On the other hand, the mutation of leucine to valine at position 175 has a significant effect on the free energy. It was obvious that the perturbation introduced by this mutation can narrow down the energy gap and destabilize the native state up to 10.49 kcal/mol. Since the mutation from leucine to valine should not change any charge interaction, side-chain polarity or hydrogen bond, the explanation for the destabilization is likely to be an effect from the perturbation of van der Waals close packing of the hydrophobic side-chains. This suggesting justification is in good agreement with several studies in which protein stability were modulated by an improper closed-packing of the hydrophobic side-chains, especially when the perturbation was introduced inside structural interior [3-5].

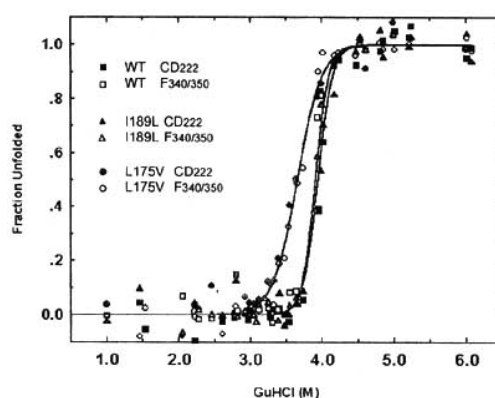


Figure 2. Unfolding curves of Cry4B and its mutants. The unfolding free energy determined from these curves were 23.10, 22.78 and 12.61 kcal/mol for wild type, I189L and L175V respectively.

Unfolding kinetics analysis

Apart from the steady state analysis in which the only initial and final states are taken into account, we have extended our investigation to determine whether the introduced perturbation affects the unfolding transition. In the kinetic experiment, fluorescence intensity corresponding to the native state at 340 nm was found to decrease rapidly upon a mixing of purified protein with GuHCl. An exponential decay

trace can be interpreted as an indication for a fast transition from native to unfolded state (**Figure 3a**). Rate constants from the best fit to the first order kinetics revealed a linear dependence between their logarithms and denaturant concentration (**Figure 3b**). The calculated activation energy in an absence of denaturant for wild type, L175V and I189L were 25.88, 25.75 and 25.54 kcal/mol respectively. Since these obtained values were virtually the same, we can assume that the mutations in both mutants do not affect the energy barrier or the mechanism of the unfolding transition.

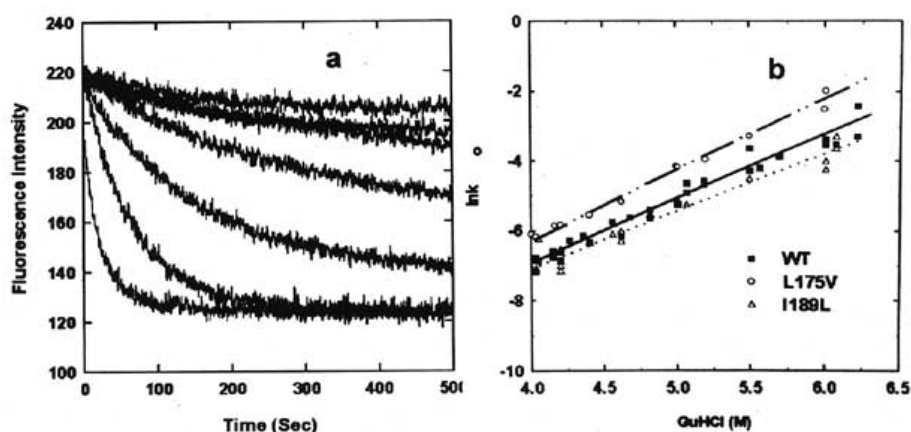


Figure 3. (a) Exponential decay of the fluorescence intensity at 340 nm after the protein was mixed with various GuHCl concentrations. (b) The linear plot of $\ln k$ against GuHCl concentrations. An activation energy (E_a) derived from these plots were 25.88, 25.75 and 25.54 kcal/mol for wild type, L175V and I189L respectively.

Reaction coordinates and mutational effect

To maintain structural and functional significance, most proteins are stabilized in the native state with conformational energy around 5-20 kcal/mol lower than the unfolded state [30]. The conformational free energy of the template protein in this work (ca. 23 kcal/mol) was comparable to those reported values. The positive sign of the calculated free energy also indicates that the transition from folded to unfolded state is spontaneous. The graphical view of unfolding can be illustrated as a path from the native state at lower energy to the unfolded state at higher energy. The determined free energy and activation energy obtained from our steady state and kinetic analysis were combined to describe the unfolding coordinates of Cry4B (**Figure 4**). This reaction coordinate has provided us a quantitative means to reveal a mutational effect of specific residue on structural folding and stability of the toxin. In this work the mutational effect on unfolding free energy of L175V were much larger than I189L mutant (ca. 10.49 kcal/mol). An analysis on rotamer optimization in both mutants suggested that the destabilization effect in L175V is probably due to a marked loss of van der Waals contact while it remains unchanged in I189L mutant (data not shown). This similar interior cavity has also been reported to affect structural stabilization in other proteins including

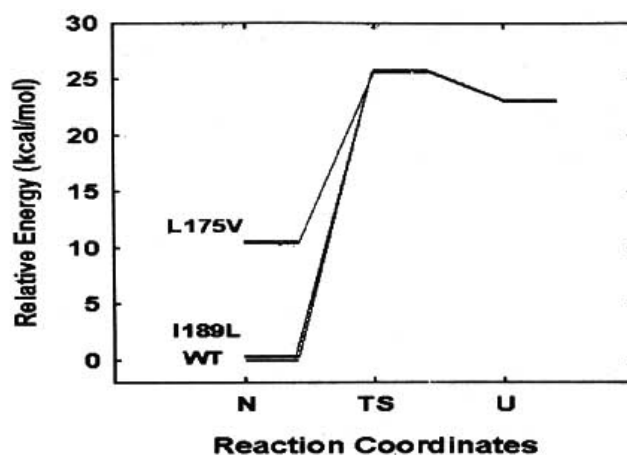


Figure 4. A graphical view of reaction coordinate for an unfolding of wild type and mutant Cry4B constructed from the steady state free energy and kinetic activation energy.

staphylococcal nuclease [3]. However activation energy for the unfolding of these two mutants were maintained to the same value of their template (ca.25.54-25.88 kcal/mol). It was apparent that the introduced mutation has a significant effect only on the stabilization of folded state without participating in the unfolding mechanism of the toxin.

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