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Dynamics of Saxitoxin Binding to Saxiphilin C-lobe Reveals Conformational Change

Penny Lewis[a], **Ingrid Fritsch**[a], **Robert E. Gawley**[a,*], **Ralph Henry**[b], **Alicia Kight**[b], **Jackson O. Lay Jr.**[c], **Rohana Liyanage**[c], and **Jeanne McLachlin**[b]

a *Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701*

b *Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701*

c *Statewide Mass Spectrometry Facility,University of Arkansas, Fayetteville, AR 72701*

Abstract

Thermodynamic parameters (ΔG, ΔH, ΔS, ΔC_p) have been determined to evaluate the dynamics of binding of saxitoxin to the c-lobe of saxiphilin. We have developed an improved method to rapidly express and purify recombinant saxiphilin c-lobe, and fully characterized it by mass spectrometry for the first time. Surface plasmon resonance was used to characterize the interaction between saxitoxin and immobilized c-lobe. At 298K, c-lobe binds saxitoxin with K_D = 1.2 nM, $\Delta H° = -11.7 \pm 0.8$ kcal/mol, and $\Delta S° = 1.17 \pm 0.07$ cal/mol·K. Analysis of ΔC_p of toxin association at several temperatures suggests that hydrophobic forces contribute to the binding event. Additionally, changes in 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence upon binding to c-lobe in the presence and absence of saxitoxin support a conformational change in c-lobe upon saxitoxin binding.

Keywords

Saxitoxin; saxiphilin; PST; paralytic shellfish toxins; ligand binding; ΔC_p; ANS

INTRODUCTION

Saxitoxin is a bisguanidinium alkaloid that is the parent structure of the paralytic shellfish toxins, or PSTs, and is the only marine natural product that has been declared a chemical weapon (Hall et al. 1990; Llewellyn 2006). PSTs are biosynthesized by several species of dinoflagellates in marine waters and by five genera of freshwater cyanobacteria, making them ubiquitous in a variety of environments (Llewellyn 2006; Oshima et al. 1990). These poisons are neurotoxins that act by blocking ion currents in most, but not all, isoforms of voltage-gated sodium channels (Bricelj et al. 2005; Kao 1981). Extreme cases of paralytic shellfish poisoning result in respiratory paralysis (Acres and Gray 1978). Saxitoxin is the most toxic of the PSTs, and as such, has been the most studied.

Saxiphilin is a serum protein that was originally isolated from bullfrogs, but has also been found in other organisms such as amphibians, fish, arthropods, and reptiles (Llewellyn et al. 1997). Saxiphilin is related to the transferrin class of proteins; although it does not bind iron, it binds saxitoxin very strongly. Bullfrog saxiphilin binds saxitoxin from pH 5.5 – 9, with

* Corresponding author. Tel. +1 479 575 6933; fax +1 479 575 5178. Email address: bgawley@uark.edu.

K_D ~0.2 nM at 0 °C and neutral pH (Llewellyn et al. 1997; Llewellyn and Moczydlowski 1994; Mahar et al. 1991). Saxiphilin sequesters the toxin in the blood stream, precluding its binding to sodium channels. Perhaps, saxiphilin evolved in these species as a survival mechanism in environments where saxitoxin and other PSTs are present, although it may have another, unknown, role in ectothermic animals in which it is found (Llewellyn 2006). Saxiphilin binds other PSTs with varying affinity, but the affinity does not appear to correlate strongly with toxicity relative to saxitoxin.(Llewellyn 2006)

Morabito et al. found that the saxitoxin binding site is located in the saxiphilin carboxy-terminal domain (hereinafter referred to as "c-lobe") (Morabito et al. 1995). Successful expression of saxiphilin has been accomplished by Morabito *et al.* in insect cells transfected with a baculovirus expression vector containing the saxiphilin coding sequence. Krishnan *et al.* expressed the 361 amino acid c-lobe using the same approach and showed that carboxy-terminal Flag and hexahistidine (His_6) tags, which aided in immunodetection and affinity purification, do not hinder c-lobe binding to saxitoxin in solution (Krishnan et al. 2001).

As part of a program to develop a device for the detection of paralytic shellfish toxins using a displacement assay, we required quantities of saxiphilin c-lobe. We therefore developed an improved method for the production of recombinant c-lobe using lipid-mediated transfection. To characterize the protein, we employed unequivocal mass spectral characterization of the entire sequence of the recombinant c-lobe. We then used surface plasmon resonance spectroscopy to study the dynamics of binding of saxitoxin to c-lobe that had been immobilized on a surface. We find that c-lobe that has been immobilized through an exposed lysine, via a biotin-streptavidin construct, maintains high affinity for saxitoxin and can be used to study the dynamics of toxin binding at several temperatures. We note that the free energy of binding of immobilized c-lobe is similar to that found with native saxiphilin in free solution. More importantly, changes in heat capacity (ΔC_p), fluorescence enhancements, and wavelength shifts of ANS reveal a previously unnoticed conformational change in c-lobe upon binding of saxitoxin.

Materials and Methods

Preparation of polyclonal antibody to saxiphilin c-lobe

Rabbit polyclonal antibody to c-lobe with His_6 tag was prepared by Cocalico Biologicals against purified c-lobe inclusion bodies expressed by *Escherichia coli.* The c-lobe coding sequence was codon optimized for expression in *E. coli* and synthesized by GeneArt Inc. (Toronto, Ontario, Canada) with the addition of an NcoI restriction site at the 5′ end and a HindIII site at the 3′ end for ligation into appropriately restricted pET-22b (Novagen, EMD Biosciences). The sequence was confirmed by GeneArt, and is included as Supplementary Material. All cloned sequences were verified by DNA sequencing (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR). The ligated plasmid was transformed into *E. coli* strain BL21 Star (Invitrogen) and expression was induced with isopropyl-β-D-thiogalactoside to a final concentration of 1 mM at an optical density of 0.66 (OD_{600}). After overnight expression at 21 °C, c-lobe present as insoluble inclusion bodies was isolated as described by Cline *et al.* (Cline et al. 1993) and further purified by 12.5% SDS-PAGE followed by electroelution of Coomassie blue-stained c-lobe excised from the gel.

Saxiphilin c-lobe expression and purification in insect cells

High Five™ cells (Invitrogen) were cultured as adherent cells at 27 °C in serum-free Express Five™ insect cell media (Gibco) supplemented with 20 mM L-glutamine and penicillin-streptomycin solution (10 units/mL penicillin, 0.01 mg/mL streptomycin). DNA coding for c-lobe, with the coding sequence for signal peptide at the 3′ end, was synthesized by GenScript

Corporation (Piscataway, NJ) using codon optimization for high level expression in insect cells. The sequence was verified by GenScript Corporation, and is included as Supplementary Material. A coding sequence was added to the 3′ end of the c-lobe DNA resulting in addition of Flag and His_6 tags at the c-terminus of the recombinant protein (DYKDDDDKIVGGHHHHHH; underlined residues serve as a spacer between the two tags). A SacI restriction site at the 5′ end and an XbaI restriction site at the 3′ end were included to facilitate ligation into appropriately restricted pIZT/V5-His plasmid (Invitrogen). All cloned sequences were verified by DNA sequencing (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR). *E. coli* transformed with the ligated plasmid were then used to prepare DNA suitable for transfection into insect cells. High Five™ cells were transfected using Cellfectin™ (Invitrogen) in unsupplemented Express Five™ media according to the manufacturer's instructions. The transfection mixture was then replaced with supplemented Express Five™ media (5×10^5 cells/mL).

After 30 h of recovery, cells were selected for plasmid uptake and expression by including 100 μg/mL Zeocin™ (Invitrogen) in the supplemented culture medium. After 1 week of selection, the stably transformed cells were plated at a density of ~1.5×10^6 cells/mL into supplemented media containing 100 μg/mL Zeocin™ and grown for 12–15 days (without feeding or passaging the cells). The expressed c-lobe was secreted into the growth media and recovered after cells were pelleted by centrifugation for 10 min at 1000 g. The supernatant was filtered through a 0.2 μm disposable bottle-top filter membrane (Nalgene) and buffer exchanged into a solution of 20 mM sodium phosphate, 150 mM NaCl, pH 7 (buffer A) using hollow fiber filtration with a 10 kDa molecular weight cut-off cartridge (UFP-10-E-4MA, Amersham) and a Watson-Marlow 323 peristaltic pump. C-lobe was bound to His-Select HF Nickel Affinity resin (Sigma) using the C-terminal His_6 tag and washed with buffer containing 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8. Weakly bound proteins were removed by washing the column with 26 mM imidazole (6.7% elution buffer; 93.3% bind/wash buffer) prior to elution with 250 mM imidazole (100% elution buffer: 250 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8). Eluted c-lobe was desalted by passage over a HiPrep 26/10 column (Amersham) equilibrated with buffer A. The purified protein was stored at −80°C after being concentrated to ~0.1 mg/mL with a VivaSpin™ 10 kDa molecular weight cutoff filter (VivaScience). C-lobe concentration and purity were estimated by Coomassie Blue staining using bovine serum albumin as a standard. No sample was subjected to repeated freeze-thawing.

Saxiphilin c-lobe characterization

To confirm the identity of c-lobe and processing of the N-terminal signal sequence, purified c-lobe was blotted to polyvinylidine difluoride membrane and used for N-terminal sequencing by Edman degradation (Protein Sequencing Core, University of Arkansas). As additional confirmation of identity, and to confirm the integrity of purified c-lobe, the average molecular weight of purified c-lobe was estimated (± 100 Da) by MALDI-TOF (Reflex III, Bruker Daltonics, Billerica, MA) MS using a 1 M solution (90% methanol, 0.1% formic acid) of 2,5-dihydroxybenzoic acid as matrix. Purified c-lobe was also subjected to MALDI-TOF peptide mass fingerprinting to confirm the amino acid sequence and confirm presence of the His_6-tag. Trypsin digestion was performed using both standard in-solution and "in-gel" digestion protocols with reduction by dithiothreitol and alkylation using iodoacetamide. The protease was sequencing-grade modified trypsin (V511A, Promega, Madison, WI). For peptide fingerprinting, both 2,5-dihydroxybenzoic acid (DHB) and α-cyano hydroxycinnamic acid (HCCA, saturated solution in 30% acetonitrile, 0.1% formic acid) were used as matrix compounds in separate experiments. Peaks in the enzymatic "peptide mass fingerprint" experiments were first manually analyzed in silico for tryptic fragments predicted by the PeptideMass tool available on the ExPASy proteomics server (http://www.expasy.ch/)

(Gasteiger et al. 2005; Wilkins et al. 1997). Because this step corresponds to fitting the ions to a known sequence, a second approach which treats the protein as an unknown and explores the likelihood of a coincidental close match between experimental and predicted ions was also used. A monoisotopic peak list was compiled by manual removal of isotopes, and the mass list submitted to MASCOT (Matrix Science Ltd, www.matrixscience.com) for comparison of the 25 most abundant monoisotopic tryptic fragments with enzymatic fragments predicted for all proteins in the Swiss Protein 49.0 (Swiss Prot) database. Database search parameters were 1 missed cleavage, 0.2 Dalton mass error, and carbamiodomethyl alkylation.

Surface plasmon resonance studies

SPR kinetic and thermodynamic studies were performed on Biacore S51 and T100 instruments (Biacore AB, Uppsala, Sweden) at the University of Utah, Center for Biomolecular Interaction Analysis. C-lobe was biotinylated by reaction with succinimidyl-6-[biotinamido]-6-hexanamidohexanoate (NHS-LC-LC-biotin) (Pierce) in a 1:1 molar ratio for 1 h at room temperature in 20 mM sodium phosphate, 150 mM NaCl, pH 7 (buffer A); final volume = 50 μL. The reaction was quenched by desalting c-lobe into buffer A using SMART System (Pharmacia) equipped with a Pharmacia Fast-Desalt column. Biotinylated c-lobe was captured on the streptavidin-coated surface of a Series S Sensor Chip SA (Biacore) by applying desalted, biotinylated c-lobe at 90 μL/min for 20 min until the binding was saturated. Saxitoxin certified standard (NRC-CNRC Institute for Marine Biosciences) was diluted with buffer A from 65 μM in 0.003 M HCl to 250, 200, 150, 100, 50 nM for kinetic studies, and to 250, 50, 10 nM for thermodynamic studies. Buffer A containing STX flowed over the chip beginning at time 0 s (association phase); buffer A without STX flowed over the chip (dissociation phase) beginning at time 60 s until t > 500 s. The flow rate over the sensor chip was 90–100 μL/min. Scrubber2 software (Biologic Software Pty, Australia) was used to fit data and calculate kinetic values to a standard 1:1 interaction model including a term for mass transport (Myszka et al. 1998).

Fluorescence

8-anilino-1-naphthalenesulfonic acid (ANS, 160 μM) and c-lobe (10 μM) in buffer A were placed in a microcuvette (100 μL). Fluorescence spectra were collected by exciting at 390 nm (slit width 10 nm) and monitoring emission from 400–600 nm (slit width 15 nm). Dried saxitoxin was dissolved in the ANS/c-lobe solution at a concentration of 10 μM and fluorescence spectra were recorded again.

Results and Discussion

Saxiphilin c-lobe expression and purification

Baculovirus expression vectors have been used to successfully produce both saxiphilin (Morabito et al. 1995) and saxiphilin c-lobe (Krishnan et al. 2001) in insect cells. However, the rigors associated with this technology led us to explore alternatives for c-lobe production. Lipid-mediated transfection of High Five™ insect cells with pIZT/V5-His/c-lobe expression vector combined with selection proved to be an effective method to secure a stable population of insect cells expressing c-lobe at high levels (Figure 1a). Compared to expression of c-lobe in High Five™ cells infected with a recombinant baculovirus vector, which yields ~0.6 mg/L (Krishnan et al. 2001), we were able to produce ~1.3 mg/L of purified c-lobe in a stable insect cell population following selection of expressing cells in Zeocin (Figure 1b). An alternative purification approach was also investigated. The high level of purity obtained is a significant improvement over previous reports, in which c-lobe was only partially purified using nickel-affinity chromatography (Krishnan et al. 2001). C-lobe was purified by fast protein liquid chromatography (FPLC) in a single affinity step using the C-terminal His_6 tag to bind a His-Select HF Nickel Affinity resin (see Experimental). Based on staining with Coomassie Blue,

the eluted c-lobe protein was purified to near homogeneity (>95%) (Figure 1b). The increased purity resulted from using 26 mM imidazole (6.7% elution buffer) to elute weakly bound protein prior to elution of c-lobe with buffer containing 250 mM imidazole. These modifications provide a ready source of His_6-tagged c-lobe and eliminate the steps required to generate and test a recombinant virus expressing saxiphilin c-lobe.

Characterization of recombinant saxiphilin c-lobe

The c-lobe produced above was characterized in a variety of ways. Presence of the c-lobe C-terminal His_6 tag was supported by binding of c-lobe to nickel affinity resin, and by immunodetection with antibodies specific for c-lobe and for the His_6 tag at the C-terminus (italics, Figure 2a). The 9 *N*-terminal amino acids identified by Edman sequencing (underlined, Figure 2a) showed that the signal sequence had been cleaved from c-lobe secreted into the media, indicating that c-lobe was properly processed.

The c-lobe sequence, including the His_6 tag, was unequivocally confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of tryptic digest fragments. Mass spectral peptide mapping experiments done using both in-gel and solution phase digestion all gave the same identification (P31266 SAX RANCA) (Morabito and Moczydlowski 1994), which was the only statistically significant hit obtained from a search of the entire database. SAX RANCA scored 92 on a probability-based Mowse Score where a score of 67 corresponds to 95% confidence; all other hits scored below 67. Based on the peptide map and a database search, the identity of c-lobe could be established unequivocally with detection of amino acids 484–844 corresponding to the carboxy-terminal domain (c-lobe) of unprocessed precursor saxiphilin determined from Rana catesbiana mRNA (Morabito and Moczydlowski 1994). Tryptic fragment masses predicted by PeptideMass from the expected sequence (Figure 2a) confirmed that the entire sequence of c-lobe was present including the His_6 tag (Figure 2b–c). Additionally, the average molecular weight of intact purified c-lobe measured by MALDI-TOF MS was within instrument error of the expected mass calculated from the His_6-tagged c-lobe sequence (MW = 41,854 ± 100 Da; Figure 2a and d).

Kinetics of saxitoxin binding to saxiphilin c-lobe

Previous studies used radioassay (^{3}[H]-saxitoxin) to determine the equilibrium constant for dissociation of the saxitoxin:c-lobe complex (Llewellyn and Doyle 2001; Morabito et al. 1995). Surface plasmon resonance (SPR) employs an optical detection method for real-time monitoring of protein-protein and protein-small molecule interactions and provides a highly sensitive method to examine the kinetic and thermodynamic characteristics of protein-small molecule interactions (Huber et al. 2004; Katsamba et al. 2001). Using SPR, neither analyte nor ligand must be labeled for detection of the binding event. However, attachment to the SPR sensor surface may result in conformational restrictions that could change the binding characteristics of proteins. For these studies, we chose to modify c-lobe by covalent attachment of biotin via a linker chain, making it possible to anchor c-lobe to covalently immobilized streptavidin. The biotin linker targets lysine amino groups exposed on the c-lobe exterior. We selected a tether length (Lys-NHCO$(CH_2)_5$NHCO$(CH_2)_5$NH-biotin) that hopefully would allow movement of c-lobe independent of the surface. This immobilization strategy has the possible disadvantage of tethering c-lobe in more than one orientation, since there may be more than one site of attachment to an exposed lysine. An advantage is that the high affinity of biotin for streptavidin ensures that c-lobe will remain on the surface even for the length of time required to conduct multiple SPR studies.

Association and dissociation rate constants were determined for different concentrations of saxitoxin with biotin-conjugated c-lobe from SPR sensorgrams at temperatures ranging from 11 °C to 37 °C (Figure 3). Association and dissociation rate constants were calculated from

their respective regions of each sensorgram, with the change from association to dissociation made at 60 s by change of buffer (Figure 3). No response was observed between STX and a streptavidin reference surface (data not shown). The sensorgram data was globally fit to curves calculated from a 1:1 complex formation model. Kinetic data from the sensorgrams shown in Figure 3 and listed in Table 1 were used to calculate equilibrium dissociation constants at each temperature (Table 1). The dissociation constants range from 0.9 to 5.0 nM, in excellent agreement with the value of 0.9 nM, measured at 0 °C by radio-ligand assay with unbound c-lobe in solution (Llewellyn and Doyle 2001;Morabito et al. 1995). Importantly, these data demonstrate that the binding characteristics of c-lobe are not altered by reaction with lysine residues on the surface of the protein, or by its immobilization on a surface. Using this construct, previous experimental hurdles associated with measuring thermodynamic properties of the c-lobe:saxitoxin interactions using radiolabeled toxin can be overcome using SPR.

Dynamics of saxitoxin binding to saxiphilin c-lobe

Determination of thermodynamic properties of the saxitoxin:c-lobe interaction were made by analysis of the kinetic data derived from SPR in Table 1. Data reported by Morabito, et. al (Morabito et al. 1995), is included for comparison. Both the association and dissociation rates obtained from SPR measurements for the saxitoxin:c-lobe complex are accelerated with increasing temperature.

The free energy of binding at the 5 temperatures ranges from −11.8 to −12.0 kcal/mol (Table 2). Assuming a generous experimental error of ± 25% in K_D (the observed standard deviation in K_D corresponds to ±0.4%) propagation of error gives approximately ± 0.11 cal/mol in ΔG °. A van't Hoff plot (Figure 4a) of the equilibrium association constants ($K_A = 1/K_D$) reveal that ΔH° = −11.7 ± 0.8 kcal/mol, and calculation from the values of ΔG° and ΔH° gives ΔS° = 1.17 ± 0.07 cal/mol·K (at 298 K). To our knowledge, this is the first thermodynamic analysis of the dynamics of saxitoxin binding to recombinant c-lobe. Llewellyn *et al.* reported that saxitoxin binding to native saxiphilin from bullfrog plasma in the temperature range 0 – 25 °C had values of ΔH° = −8.3 kcal/mol and ΔS° = 13.8 cal/mol·K (Llewellyn and Moczydlowski 1994); at 25 °C these values correspond to ΔG° = −12.4 kcal/mol. These data reveal that the free energy changes (ΔG°) of saxitoxin binding to recombinant c-lobe and to native saxiphilin at 25 °C are approximately equal, but the enthalpic contribution is lower and the entropic contribution is higher in the native protein.

Heat capacity change

For both protein folding and protein binding, the change in heat capacity (ΔC_p) between the unfolded/unbound and folded/bound states as a function of temperature is informative about the driving forces of the folding or interaction. Specifically, positive ΔC_p values for complex dissociation are indicative of burial of a polar region of the protein during binding (Gallagher and Sharp 1998), while large negative ΔC_p values for complex dissociation are traditionally interpreted as burial of a hydrophobic region of the protein during binding (Privalov and Gill 1988; Stites 1997; Sturtevant 1977). Using the Gibbs-Helmholtz equation, ΔC_p was determined from the thermodynamic parameters ΔG and ΔH as a function of temperature (Pace and Laurents 1989):

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)]$$

where T_m is the midpoint temperature (402 K, at which $K_D = 1$) determined from a plot (Figure 4b) of ΔG versus temperature, ΔH_m is the enthalpy at T_m (5.5 kcal/mol), and ΔG values were calculated using the Gibbs equation from K_D values obtained at temperatures 11– 37 °C. At each temperature, the value of ΔC_p is large and negative (Table 2), indicating that hydrophobic forces are involved in the saxitoxin binding event.

Saxitoxin is doubly charged at pH 7, which was used for the binding experiments. The pK_a of the C8 (five-membered ring) guanidinium ion is 8.24, and that of the C2 (six-membered ring) guanidinium ion is 11.3 (Rogers and Rapoport 1980). At pH 7, both guanidiniums are protonated and saxitoxin is present as a dication. Additionally, while the 3-dimensional structure of c-lobe (and therefore the structure of the toxin binding site) is unknown, Llewellyn and Moczydlowski suggest that the saxitoxin binding site of c-lobe contains an aspartic acid and a glutamic acid (Llewellyn and Moczydlowski 1994). Both residues are negatively charged at neutral pH, so the initial saxitoxin:c-lobe interaction is electrostatic.

However, the negative $\Delta H°$, the positive $\Delta S°$, and the negative ΔC_p, suggest binding that is stabilized by, or accompanied by, hydrophobic effects. We conclude that the most likely source of the observed hydrophobic effect is a conformational change in c-lobe following the initial electrostatic binding to saxitoxin. This hypothesis is supported by the following fluorescence experiments.

Conformation changes monitored by ANS fluorescence

Thermodynamic data and values of ΔC_p suggest that the c-lobe:saxitoxin binding event is stabilized by hydrophobic interactions. These hydrophobic interactions may either be due to interaction between saxitoxin and the c-lobe binding cavity—which seems unlikely due to the reasons discussed above—or due to conformational change of c-lobe. To test for conformational change in c-lobe upon binding of saxitoxin, fluorescence studies using 8-anilino-1-naphthalene sulfonic acid (ANS) were performed. ANS is a weak fluorophore that exhibits increased fluorescence and a blue shift when associated with hydrophobic surfaces of proteins (Melo et al. 2001; Uversky et al. 1998). It can therefore be used as a probe to monitor protein tertiary structure (such as hydrophobic packing) in solution (Golynskiy et al. 2005; Twigg et al. 2001).

As shown in Figure 5, in the presence of c-lobe, ANS fluorescence shifts 19 nm to shorter wavelength and increases in intensity by 130% (F_{max} from 335 to 772), indicating interaction of ANS with a hydrophobic region either on the protein surface or in the saxitoxin-binding cavity. When saxitoxin is introduced to the ANS/c-lobe solution, fluorescence intensity further increases (F_{max} = 952) and a blue shift in emission maximum is still observed. Were ANS interacting with c-lobe in the saxitoxin-binding cavity, introduction of saxitoxin would displace ANS from the binding cavity, resulting in a fluorescence spectrum similar to ANS alone (Figure 5b). However, this is not observed. A second possibility is that ANS interacts with a hydrophobic surface on the c-lobe external to the saxitoxin-binding cavity (Figure 5c); this scenario is consistent with the increase in ANS fluorescence upon introduction of saxitoxin. The increase in ANS fluorescence in the presence of saxitoxin (compare dashed line with solid line in Figure 5a) indicates that a conformational change occurs in c-lobe upon binding of saxitoxin.

Conclusion

Saxitoxin is a bisguanidinium dication with more heteroatoms than carbons that interacts electrostatically with negatively charged aspartic acid and glutamic acid residues in c-lobe (Llewellyn and Moczydlowski 1994). Thermodynamic parameters determined by SPR indicate that the saxitoxin:c-lobe interaction is stabilized by hydrophobic forces, consistent with a conformational change in c-lobe upon binding of toxin that was revealed by changes in ANS fluorescence in the presence of c-lobe with and without saxitoxin. We suggest that initial electrostatic binding of the toxin prompts a change in c-lobe conformation that sequesters the toxin.

As part of this study, we developed an improved procedure for the expression and purification of recombinant saxiphilin c-lobe. Mass spectral characterization of the entire primary sequence was also accomplished through mass spectral analysis of trypsin digests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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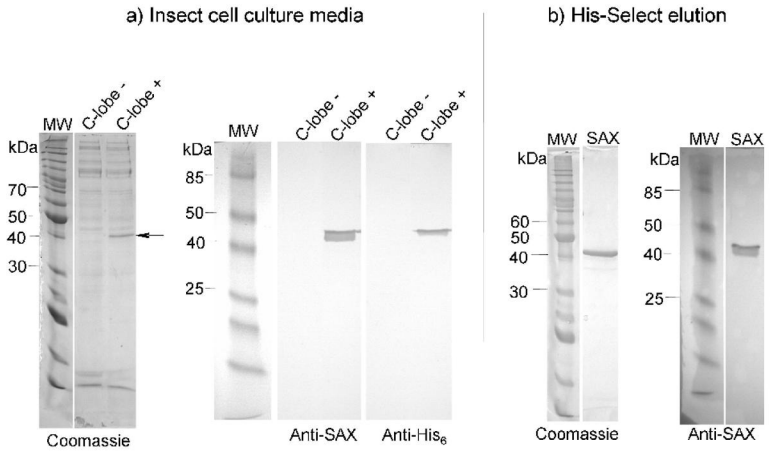

Figure 1. Secreted saxiphilin c-lobe, present in stainable quantities in the media, is purified to homogeneity

Saxiphilin c-lobe that has been secreted into the media is present in stainable quantities after 6 days of expression (a, Coomassie, indicated by arrow). High Five™ insect cells were transformed with plasmid lacking (a, −C-lobe) or containing (a, +C-lobe) the coding sequence for His_6-tagged SAX c-lobe. Transformed cells were grown for 6 days. An equal volume of media from each population was examined by electrophoresis using 12.5% SDS-PAGE and stained (Coomassie) or blotted and probed with antibody against saxiphilin c-lobe or c-terminal His_6. (b) c-lobe purified by nickel affinity chromatography as described in Materials and Methods was analyzed by electrophoresis using 12.5% SDS-PAGE and either stained (b, Coomassie) or blotted and probed with antibody to c-lobe (b, Anti-SAX).

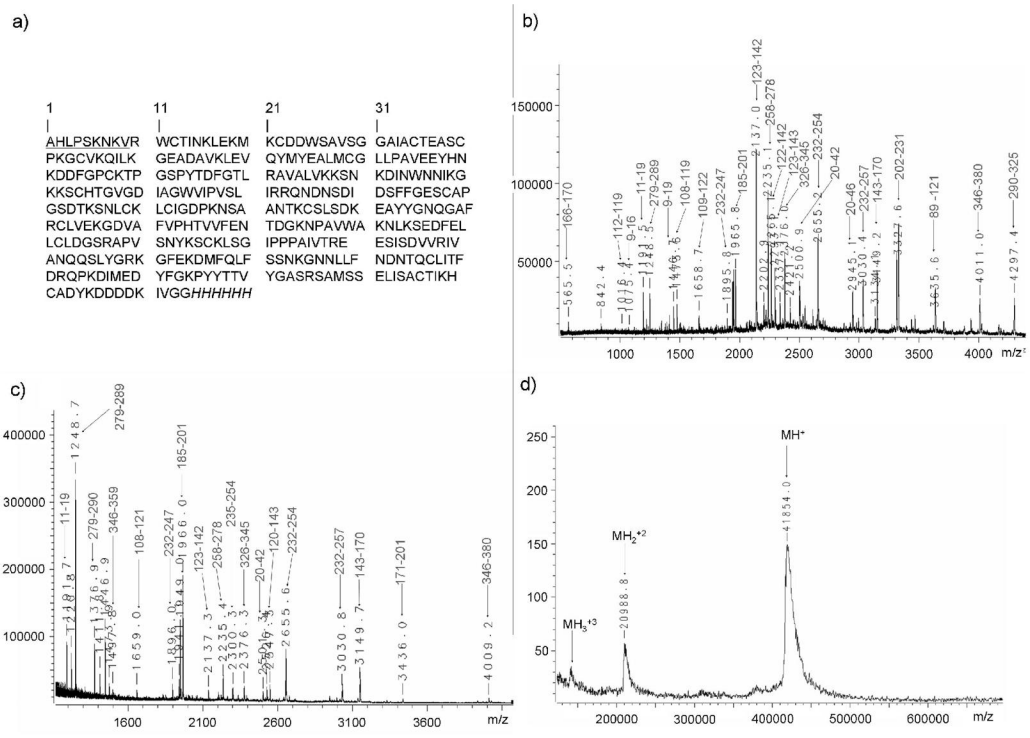

Figure 2. Saxiphilin c-lobe characterization using MALDI-TOF MS

Expected c-lobe sequence. Underlined residues confirmed by Edman degradation; italicized residues are the signal tag (a). MALDI-TOF mass spectrum of intact (purified) c-lobe (MH+) obtained using 2,5-dihydroxybenzoic acid (DHB) matrix (b). MALDI-TOF peptide mass fingerprints obtained using DHB and α-cyano-4-hydroxycinnamic acid (HCCA) (c and d, respectively), with assignment of peaks corresponding to expected tryptic fragments.

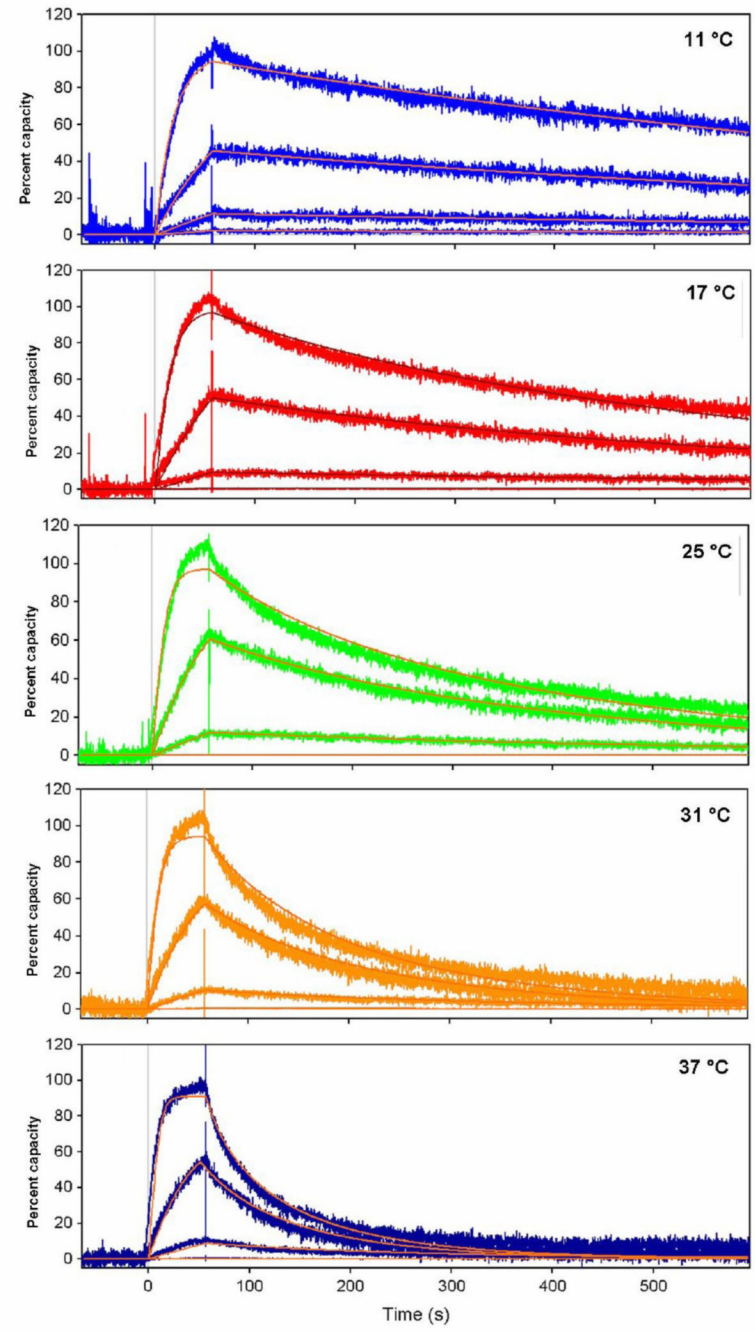

Figure 3. SPR analysis of saxitoxin:c-lobe interaction shows saxitoxin concentration-dependent response at multiple temperatures

Sensorgrams collected at 11, 17, 25, 31, and 37 °C. The concentrations of toxin were 250, 50, and 10 nM, and are illustrated in that order from top to bottom at each temperature. Each toxin sample was flowed over biotinylated c-lobe captured on an immobilized-streptavidin chip. 20 mM sodium phosphate, 150 mM NaCl, pH 7 (buffer A) containing saxitoxin was flowed over the surface beginning at time 0 s and was switched to buffer A lacking toxin only at 60 s. N = 5 for each data set. Smooth curves (which are sometimes obscured by experimental traces) represent the best fit of the binding responses to a 1:1 interaction model.

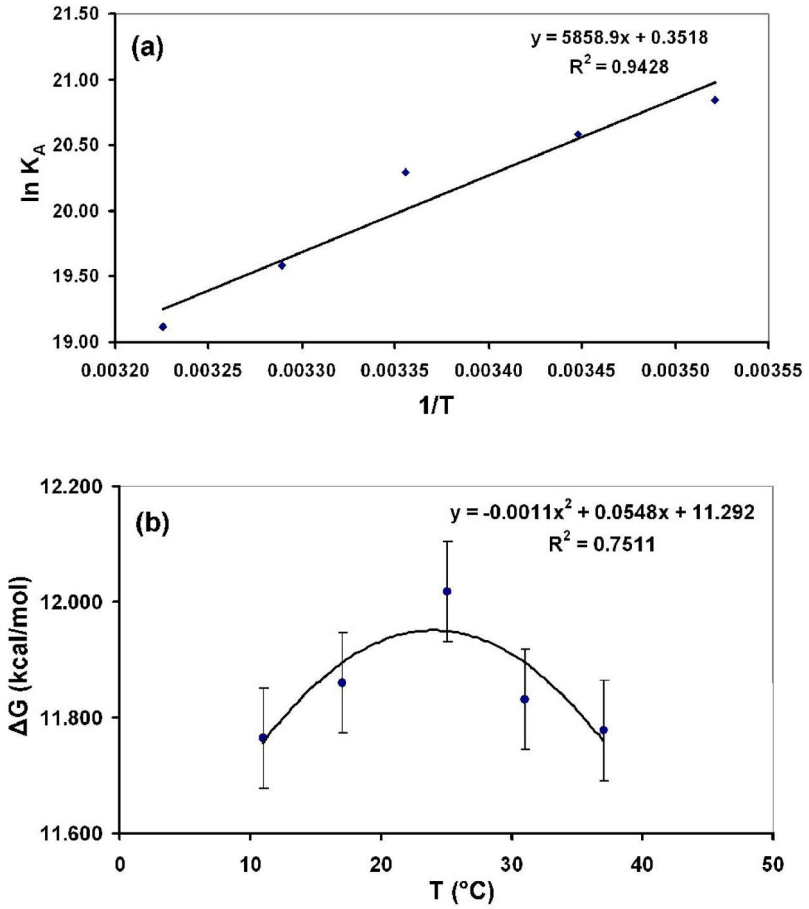

Figure 4.

(a) van't Hoff analysis of saxitoxin:c-lobe interaction. Thermodynamic values were determined from a plot of equilibrium association constants calculated from kinetic data for each temperature using the van't Hoff equation: $\ln K_D = -(\Delta H/R)(1/T) + (\Delta S/R)$ in which the term $\Delta H/R$ corresponds to the slope of the van't Hoff plot. Values of ΔG were calculated from dissociation constants at each temperature using the equation: $\Delta G = -RT \ln K_D$ in which R and T have their usual meanings. The value of ΔS at 273 K was then calculated from ΔH and ΔG using the relationship $\Delta G = \Delta H - T\,\Delta S$. Error bars based on one standard deviation lie within the markers on the plot. **(b) Plot of ΔG of saxitoxin:c-lobe dissociation *vs* temperature.** Error bars are set at ±0.1 kcal/mol. The midpoint temperature (402 K), where $K_D = 1$, was determined from the least squares fit of the data.

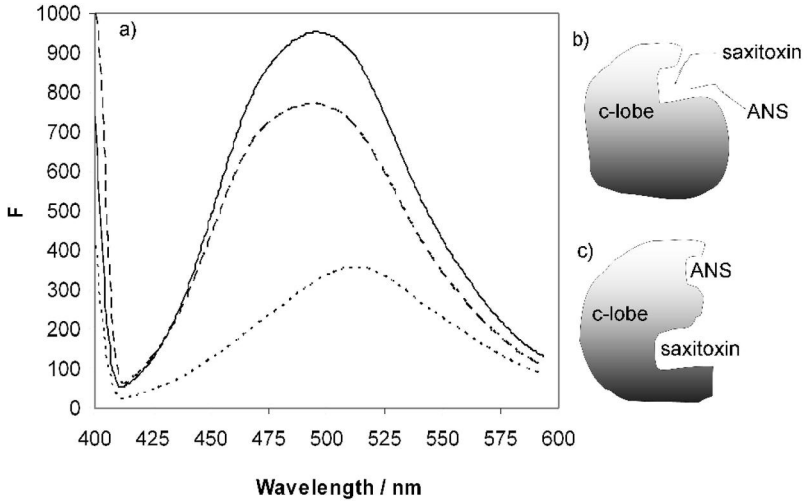

Figure 5. Fluorescence intensity of ANS increases and shifts wavelength in the presence of c-lobe and c-lobe with saxitoxin

(a) Fluorescence intensity of 160μM ANS (dotted line) increases from 335 to 772 (130%), with concomitant shift of λ_{max} from 513 nm to 494 nm, in the presence of 10 μM c-lobe (dashed line). When 10 μM STX is also present (solid line) a further increase in fluorescence is observed (F_{max} = 952). Control experiments under identical conditions but without c-lobe showed no change in ANS fluorescence; also, no fluorescence was observed from either c-lobe or saxitoxin. Data are an average of seven scans. λ_{ex} = 390 nm; Buffer = 20 mM Na_2HPO_4, 150 mM NaCl, pH 7. **(b)** ANS associated with c-lobe in the saxitoxin binding site would be displaced by the natural ligand, saxitoxin, resulting in an ANS fluorescence spectrum like that of ANS in the absence of c-lobe. This effect is not observed. **(c)** ANS associated with c-lobe in a hydrophobic patch exterior to the STX binding site remains associated with c-lobe when saxitoxin is introduced, consistent with data shown in Figure 5a.

Table 1

Rates and equilibrium dissociation constants of saxitoxin:c-lobe at multiple temperatures.[b]

Temp. (°C)	$k_a \times 10^6$ (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
0[a]	*1.7 ± 0.1*	*0.00070 ± 0.00002*	*0.9 ± 0.1*
11	1.1 ± 0.002	0.00098 ± 0.000003	0.9 ±0.003
17	1.8 ± 0.01	0.0021 ± 0.0001	1.2 ± 0.004
25	2.7 ± 0.009	0.0041 ± 0.0001	1.2 ± 0.005
31	2.7 ± 0.01	0.0084 ± 0.00004	3.1 ± 0.01
37	5.5 ± 0.07	0.027 ± 0.0003	5.0 ± 0.02

[a] Data from Morabito, et al. (Morabito et al. 1995).

[b] Standard errors represent statistical errors from the fitting of single experiments.

Table 2

Change in heat capacity (ΔC_p) for dissociation of saxitoxin:c-lobe, calculated from ΔG at multiple temperatures using T_m = 402 K and ΔH_m = 5.5 kcal/mol.

Temp. (°C)	ΔG (kcal/mol)	ΔC_p (cal/mol·K)
11	11.8	−530
17	11.9	−600
25	12.0	−730
31	11.8	−820
37	11.8	−920