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Dissecting a Role of Evolutionary-conserved but Non-critical Disulfide Bridges in Cysteine-Rich Peptides Using ω -Conotoxin GVIA and its Selenocysteine Analogs

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Abstract

Conotoxins comprise a large group of peptidic neurotoxins that employ diverse disulfide-rich scaffolds. Each scaffold is determined by an evolutionarily conserved pattern of cysteine residues. Although many structure-activity relationship studies confirm the functional and structural importance of disulfide crosslinks, there is growing evidence that not all disulfide bridges are critical in maintaining activities of conotoxins. To answer the fundamental biological question of what the role of non-critical disulfide bridges is, we investigated function and folding of disulfide-depleted analogs of ω -conotoxin GVIA (GVIA) that belongs to an inhibitory cystine knot (ICK) motif family and blocks N-type calcium channels. Removal of a non-critical Cys1–Cys16 disulfide bridge in GVIA or its selenopeptide analog had, as predicted, rather minimal effects on the inhibitory activity on calcium channels, as well as on in vivo activity following intracranial administration. However, the disulfide-depleted GVIA exhibited significantly lower folding yields for forming the remaining two native disulfide bridges. The disulfide-depleted selenoconotoxin GVIA analog also folded with significantly lower yields, suggesting that the functionally non-critical disulfide pair plays an important cooperative role in forming the native disulfide scaffold. Taken together, our results suggest that distinct disulfide bridges may be evolutionary preserved by the oxidative folding or/and stabilization of the bioactive conformation of a disulfide-rich scaffold.

Keywords

disulfide bridges; conotoxins; structure-function; oxidative folding; calcium channels

INTRODUCTION

Nature employs disulfides in the construction of stable structural motifs in peptides that are secreted by the cell. Peptides containing multiple disulfide bonds are better protected from stress and unfolding conditions found in extracellular environments.^{1–6} One such class of

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disulfide- rich natural peptides having well defined structures are conotoxins, neuroactive modulators derived from marine cone snails.^{7–10} Conotoxins are short peptides of varying length from 6–50 residues and containing 1–5 disulfide bonds which provide characteristic scaffolds to the peptides and stabilize the biologically active conformation.¹⁰ Although multiple disulfide bonds generally contribute to the stability of native conotoxin fold, the removal of one of the native disulfide bond was found to not affect their structure or function in conkunitzin-S1,¹¹ μ -conotoxin KIIIA,¹² ω -conotoxin MVIIA,¹³ α -conotoxin ImI,¹⁴ and α -conotoxin GI.¹⁵ These findings indicate that disulfides are differentially associated with structure and function of disulfide rich conopeptides (a disulfide can be critical for structure or function or both), which could be dissected by studying the corresponding disulfide-depleted peptides. The best example of one such study is ω -conotoxin MVIIA, an N-type Ca^{2+} channel blocker isolated from the marine snail *C. magus* having the inhibitory cystine knot (ICK) motif.^{13, 16} The fundamental question of why nature conserves functionally non-critical disulfide bridges still remains unanswered.

Inhibitory cystine knot (ICK) motif is a common structural motif found in peptides of diverse origin including toxins from plants, antimicrobial peptides from insects, and in venoms of scorpion, spider and cone snail.^{17–21} They share a unique knotted topology consisting of three disulfide bridges, with one disulfide crossing a loop formed by the two other disulfides and inter-connecting peptide backbone. The ICK motif containing conotoxins with the cysteine pattern C---C---CC---C---C are widely characterized from *Conus* venom; ω -conotoxin (inhibitor of Ca^{2+} channel),²² κ -conotoxin (modulator of K^{+} channel),²³ δ -conotoxin (delay inactivation of Na^{+} channel),²⁴ and μO -conotoxin (inhibitor of Na^{+} channel activation).^{25–26} ω -Conotoxins are the best characterized ICK motif containing conopeptides (Figure 1a), including ω -conotoxin MVIIA which is an FDA-approved drug for the treatment of intractable pain (ziconotide, Prialt).^{27–30} The role of disulfide bonds in structure-activity relationships of ω -conotoxins were investigated by Sabo and coworkers,³¹ Norton and coworkers³² and the Goldenberg group.¹³ As summarized in Table 1, the first disulfide bond has a minimal contribution to the activity, whereas removal of either the second or third disulfide bond drastically affected the activity of peptide.

ω -Conotoxin GVIA (Figure 1b) is a standard pharmacological tool for inhibiting synaptic transmission.^{29–30} Previous studies by the Sabo group and the Norton group showed that the disulfide bond between Cys1 and Cys16 in GVIA had minimal contribution for the activity.^{31–32} Based on these findings, we selected GVIA to ask a more general fundamental question: what is the role of a functionally non-critical disulfide bridge? Keeping in mind that the functional role was assessed based on *in vitro* assays, one possibility was that the disulfide bridge might be important for *in vivo* activity (when injected into an animal). We also hypothesized a potential role of a functionally non-critical disulfide in oxidative folding that affects efficient biosynthesis of a disulfide-rich scaffold, rather than in stabilizing the “end-point” bioactive conformation. To address the above questions, we re-assessed the role of the functionally non-critical disulfide in folding and activity of ω -conotoxin GVIA using disulfide depleted analogs, namely dd-GVIA and dd-Sec-GVIA (Figure 2). Replacing disulfide bridges with diselenide bridges was previously shown to be an effective strategy to simplify the oxidative folding of cysteine-rich peptides, without affecting their native conformation or biological activity.^{33–43} Our findings indicate that the first disulfide bridge (Cys1–Cys16) in ω -conotoxin GVIA is important for directing the correct formation of the other two native disulfides. The results are discussed in the evolutionary context of conserving the functionally non-critical disulfides in cysteine-rich natural peptides.

MATERIALS AND METHODS

Peptide synthesis

Peptides were synthesized using solid phase peptide synthesis methodology, with Fmoc chemistry and activated OPfp esters of protected amino acids.

Natively folded dd-GVIA—Side chains of one pair of natively connected cysteines were protected with trityl (Trt) group, and the other pair of natively connected cysteines were protected with the acetamidomethyl group. Peptide was cleaved from the resin and side chains were deprotected using reagent K [TFA/thianisole/phenol/water/ethanedithiol (82.5:5:5:5:2.5)], precipitated with MTBE and washed several times with MTBE. The peptide was purified using C₁₈ RP-HPLC over linear gradient of 10–40 % buffer B (90% acetonitrile containing 0.1% TFA) for 40 min. The purified peptide contains two acetamidomethyl-protected cysteines and two free thiols. Disulfide bond formation between the free thiols was achieved by incubating 20 μ M peptide in a folding buffer containing 100 mM Tris-HCl (pH 7.5), 1 mM GSSG and 2 mM GSH. The purified single disulfide containing peptide was treated with 10 mM I₂ (at acidic pH) to simultaneously deprotect acetamidomethyl groups and form a disulfide bond between corresponding cysteines. The natively folded peptide was purified by RP-HPLC and characterized using mass spectrometry {[M+H]⁺_{oxi} = 2974.2 Da (calculated), 2974.3 Da (observed)}.

Natively folded dd-Sec-GVIA—The side chains of selenocysteines were protected with a p-methoxybenzyl (Mob) group and cysteines were protected with trityl (Trt) groups. Peptides were removed from resin and side chains were deprotected using enriched reagent K [trifluoroacetic acid (TFA)/thianisole/phenol/water (90:2.5:7.5:5) and 1.3 equiv of DTNP (2,2'-dithiobis(5-nitropyridine))] and precipitated using MTBE. Crude peptide was treated with 50 mM DTT in 100 mM Tris-HCl (pH 7.5) and subsequently purified using preparative RP-HPLC with a C₁₈ column over a linear gradient of 10–40 % buffer B (90% acetonitrile containing 0.1% TFA) for 40 min. Observed mass of the peptide was 2 Da less than the predicted mass, confirming the presence of a preformed diselenide in the linear peptide {[M+H]⁺_{red} = 3074.2 Da (calculated), 3072.4 Da (observed)}. This was further supported by an alkylation reaction using iodoacetamide; the resulting species was shown by mass spectrometry to contain a diselenide bridge.^{38–39} The remaining free thiols were oxidized using 10 mM I₂ and subsequently purified by RP-HPLC and characterized using mass spectrometry {[M+H]⁺_{oxi} = 3070.2 Da (calculated), 3070.3 Da (observed)}.

Oxidative Folding

Free thiol containing linear peptide dd-GVIA was obtained by incubating natively folded dd-GVIA in 10 mM DTT containing 10 mM Tris-HCl (pH 7.5) at 37°C, purified by RP-HPLC and characterized using mass spectrometry. Note that the linear peptide dd-Sec-GVIA contains a preformed diselenide bridge. The folding reaction (or) stability assay was initiated by resuspending 5 nmol of linear peptide or natively folded peptide into 200 μ L of folding buffer containing 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione (GSSG), and 2 mM reduced glutathione (GSH). The reaction was quenched after 2 hours by acidification with formic acid (10% final concentration). Samples were analyzed using C₁₈ analytical HPLC over a linear gradient of 10–40% buffer B (90% acetonitrile containing 0.1% TFA) in 40 min. Accumulation of natively folded peptide at the steady state was calculated by integrating the HPLC chromatogram.

Proteolytic digestion and mass spectrometry

The disulfide/selenosulfide connectivity in non-natively folded dd-GVIA and dd-Sec-GVIA were studied by coupling tryptic digestion with mass spectrometry. 5 nmol of non-natively

folded dd-GVIA (or) dd-Sec-GVIA were dissolved in 100 mM Tris-HCl (pH 7.5), 5 μ l of trypsin (25ng/ μ l) was added and reaction mixture was incubated at 37°C for 4–8 hr. The resulting peptide fragments were separated using analytical RP-HPLC over a linear gradient of 10–80% buffer B (90% acetonitrile containing 0.1% TFA) in 60 min, and fractions were subsequently analyzed using mass spectrometry. The MALDI-Mass Spectra were acquired at the Mass Spectrometry and Proteomic Core facility of the University of Utah.

Measurement of N-Type Calcium Currents (CaV2.2)

HEK cell Transfection—HEK293 cells were maintained in DMEM/Glutamax® medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA), at 37°C in 5% CO₂ incubator. The cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cDNA plasmids were: 1.93 μ g α_{1B} -CFP, 1.55 μ g $\alpha_2\delta$, and 0.97 μ g β_{2a} subunits. N-channel expressing cells were visualized by CFP, which was attached to the N-terminus of α_{1B} (α_{1B} -CFP; from Dr. Blaise Peterson, Penn State College of Medicine). Transfected cells were split into 35 mm dishes that also served as the recording chamber.

N-current Measurement and Analysis—HEK cells were voltage-clamped as previously described^{44–45} in an external solution containing (in mM): 145 N-methyl-D-glucamine (NMG)•Cl, 10 NMG•HEPES, 5 BaCl₂. The internal (pipette) solution contained (in mM): 104 NMG•Cl, 14 creatine•PO₄, 6 MgCl₂, 10 NMG•HEPES, 10 NMG₂•EGTA, 5 Tris•ATP, and 0.3 Tris•GTP. The osmolarity of the external solution was 300 mOsm, while that of the internal solution was 280 mOsm. Both the internal and external solutions were adjusted to pH 7.4 using NMG base. Leak current was subtracted online using a -P/4 protocol for ionic currents. All recordings were carried out at room temperature and the holding potential was -120 mV. Ionic currents were digitized at 50 kHz after analog filtering at 5 – 10 kHz. Solutions were applied using a gravity-fed perfusion system with an exchange time of 1–2 sec. All toxin concentrations were 1 μ M. Data were analyzed using IgorPro (WaveMetrics, Lake Oswego, OR) as previously described.⁴⁷

Behavioral Assay—The natively folded GVIA, Sec-GVIA, dd-GVIA and dd-Sec-GVIA and the misfolded dd-GVIA and dd-Sec-GVIA were injected intracranially to 21-to-23-day-old Swiss Webster mice using a syringe with a 29-gauge needle. 1 nmol of each of the peptides was dissolved separately in 15 μ l of saline and subsequently injected in mice; an equal volume of normal saline was used as a control. After injection, mice were placed in a cage for observation. All of the native peptides exhibited the shaking syndrome, which is characterized by a persistent body tremor, and this behavior was maintained for a long time in GVIA and Sec-GVIA, as compared to dd-GVIA and dd-Sec-GVIA. Mice injected with misfolded dd-GVIA and dd-Sec-GVIA did not exhibit the shaking syndrome; rather, the injected mice were quite passive, occasionally shaking their bodies.

RESULTS

Design of dd-GVIA and dd-Sec-GVIA

To investigate the role of the functionally non-critical disulfide in the folding and activity of ω -conotoxin GVIA, dd-GVIA and dd-Sec-GVIA were designed and synthesized. Table 1 summarizes the effect of removal of a disulfide bond on the activity of ω -conotoxins. It is apparent from Table 1 that removal of the first disulfide bridge in ω -conotoxins resulted in decreased activity by 15–70 fold. The loss of activity observed by removal of any other native disulfides is 200–5000 fold. Hence, the removal of first disulfide bridge in ω -conotoxin GVIA results in peptide that retains the ability to modulate N-type calcium currents. It is worth mentioning that the previous studies on the role of removing the first

disulfide in ω -conotoxin GVIA are not conclusive because the disulfide connectivity of the peptides used in these studies were unknown³¹ or misfolded.^{32, 46}

The removal of the first disulfide bridge in ω -conotoxin GVIA resulted in two-disulfide containing dd-GVIA. The removal of first disulfide bridge in ω -conotoxin GVIA reduces the possible number of disulfide isomers from 15 to 3. Figure 2 shows a schematic representation of the design of disulfide-depleted-seleno- ω -conotoxin GVIA (dd-Sec-GVIA). The design of dd-Sec-GVIA involves incorporation of selenocysteine into dd-GVIA. Systematic replacement of native disulfide bridges with a diselenide bridge in ω -conotoxin GVIA has resulted in increased yield of natively folded peptide in all Sec-GVIA analogues. The most prominent effect was observed when the second native disulfide bridge was replaced with a diselenide bridge (net yield enhancement of natively folded peptide is 19%).³⁸ Thus, removal of the functionally non-critical first disulfide bridge and incorporation of a diselenide bridge in the second native disulfide position in ω -conotoxin GVIA resulted in biologically active dd-Sec-GVIA. The incorporation of a diselenide bridge in dd-GVIA has further simplified the folding problem and eliminated the need for disulfide mapping in the folded peptide (dd-Sec-GVIA needs to form only one disulfide bond to achieve the natively folded state).

Synthesis of natively folded *dd*-GVIA and *dd*-Sec-GVIA

The natively folded dd-GVIA was accessed using an orthogonal protection scheme; one pair of natively connected cysteines were protected with the trityl (Trt) group (Cys8 and Cys19) and the other pair of natively connected cysteines were protected with the acetamidomethyl (Acm) group (Cys15 and Cys26). Figure 3a shows the regioselective folding of dd-GVIA. Upon cleavage from the resin, trifluoroacetic acid (TFA)-labile trityl groups yielded free thiols, and TFA-resistant acetamidomethyl groups retain protected thiols, as described in methods. The disulfide bond formation between free thiols was achieved using an oxidized/reduced glutathione buffer, resulting in formation of one native disulfide bridge. The deprotection of the Acm group and simultaneous disulfide bond formation between corresponding thiols was achieved using molecular iodine, yielding natively folded dd-GVIA. The identity of natively folded dd-GVIA was confirmed by mass spectrometric studies following the two successive folding steps, each of which resulted in a single major peak. Synthesis of natively folded dd-Sec-GVIA was achieved as described in Experimental section, Sec residues were incorporated into the sequence using p-methoxybenzyl-protected selenocysteine and side chain deprotection was achieved using DTNP. Figure 3b shows the regioselective folding of dd-Sec-GVIA. The crude peptide obtained following cleavage from the resin contains two DTNP-selenocysteine adducts, which were subsequently removed by thiolysis using 50 mM DTT. The spontaneous oxidation of the selenols to a diselenide yields a linear dd-Sec-GVIA containing a preformed diselenide bridge, which was actually detected.^{37–41} Observed mass of linear dd-Sec-GVIA analogue was 2 Da less than the predicted mass and had two alkylation sites (iodoacetamide was used as alkylating agents), confirming the presence of a preformed diselenide bridge. The formation of a disulfide bond between free thiols was achieved using molecular iodine which leads to rapid and irreversible disulfide bond formation yielding natively folded dd-Sec-GVIA. Similar to dd-GVIA, the elution profiles of the folding pathway of dd-Sec-GVIA yielded a single major peak corresponding to folded peptides. Mass spectrometric studies confirmed the identity of natively folded dd-Sec-GVIA. It is evident from Figure 3 that the regioselective folding of dd-GVIA consists of two folding steps and that of dd-Sec-GVIA consists of only one folding step, demonstrating that the incorporation of selenocysteine simplifies the oxidative folding.

Oxidative folding studies of *dd*-GVIA and *dd*-Sec-GVIA

Incubation of the native forms of disulfide-rich peptides in redox buffers, such as a mixture of oxidized/reduced glutathione, can be used as a measure of the stability of the corresponding disulfide isomer at the given redox potential.³⁶ The glutathione mediated folding of disulfide rich peptides involves thiol-disulfide exchange reactions and a steady-state folding mixture reflects the thermodynamic stability of the resulting disulfide isomers of cysteine-rich peptides. To investigate the stability of natively folded GVIA analogs, natively folded *dd*-GVIA and *dd*-Sec-GVIA were incubated in redox-buffer containing oxidized (1 mM GSSG) and reduced (2mM GSH) glutathione; these experimental conditions were shown previously to promote efficient folding of GVIA.^{47–48}

Figure 4a shows elution profiles of steady-state folding mixtures of *dd*-GVIA, *dd*-Sec-GVIA and the corresponding peptides GVIA and Sec-GVIA. Similar elution profiles were obtained by incubating corresponding linear peptides in the folding buffer under identical experimental conditions. The elution profile of *dd*-GVIA at the steady state, in the presence of the glutathione mixture retains about 15 % of natively folded peptide and that of GVIA retains about 60 % of natively folded peptide. The major peak accumulated at the steady state in *dd*-GVIA corresponds to the non-natively folded species (observed mass is 2974 Da). The tryptic digest of non-natively folded *dd*-GVIA yielded fragments of mass 1745 Da and 1247/1265Da, which could be ascribed to peptide fragment AKSOGSSCSOTSYNCAR and SCNOYTKRCY* (Note: O denotes hydroxyproline, * denotes an amidated C-terminus and cysteines are disulfide bonded), respectively. The disulfide connectivity in non-natively folded *dd*-GVIA is Cys8–Cys15 and Cys19–Cys26. These results were further supported by the folding studies of [C1S,C16S]GVIA by Flinn. *et.al.*,³² where the major folded fraction corresponds to the Cys8–Cys15 and Cys19–Cys26 connectivity.

The elution profile of *dd*-Sec-GVIA at the steady state, in the presence of the glutathione mixture, retains about 45 % of natively folded peptide and that of Sec-GVIA retains about 78 % of natively folded peptide. The major satellite peak accumulated at the steady state in *dd*-Sec-GVIA has the mass 3070 Da, indicating the formation of non-natively folded and seleno-sulfide containing *dd*-Sec-GVIA. The satellite peak was attributed to the isomer Sec8-Cys15 and Sec19-Cys26, since its retention time and shift in retention time (as compared to that of natively folded peptide) was similar to non-natively folded *dd*-GVIA having the Cys8–Cys15 and Cys19–Cys26 connectivity. To further confirm the presence of seleno-sulfide bonds in the satellite peak, the peptide was subjected to tryptic digestion and the resulting fragments were analyzed using mass spectrometry. Incubation of the satellite peak of *dd*-Sec-GVIA with trypsin yielded fragments of mass 1793 Da & 1295/1313 Da, which corresponds to the fragment AKSOGSSUSOTSYNCAR and SUNOYTKRCY* (Note: cysteine and selenocysteine are bonded), further confirming presence of Sec8-Cys15 and Sec19-Cys26 connectivity in non-natively folded *dd*-Sec-GVIA. Note also that the natively folded and non-natively folded *dd*-Sec-GVIA were sensitive to dissolved oxygen species, and tended to redistribute among all three the possible disulfide/selenosulfide isomers of *dd*-Sec-GVIA over time.

Figure 4b shows accumulation of the natively folded and biologically active conformer of GVIA, *dd*-GVIA, Sec-GVIA and *dd*-Sec-GVIA at steady state in the presence of a reduced/oxidized glutathione mixture. It is evident from Figure 4b that incorporation of selenocystine at native disulfide connectivity in GVIA (Sec-GVIA and *dd*-GVIA (*dd*-Sec-GVIA)) has a profound influence on improving the yield of natively folded peptide. The net increase in yield of natively folded peptide by incorporation of selenocystine in GVIA is 20 %, and that of *dd*-GVIA is 29%. The greater redox stability and more rapid formation of diselenide bonds, as compared to disulfide bonds, significantly improves folding yields, and preferential formation of diselenides over selenosulfides further simplifies disulfide mapping

in the folded peptide. Impairment in folding pattern of GVIA and Sec-GVIA upon removing the functionally non-critical disulfide confirms the role that the Cys1–Cys16 disulfide bond plays in the folding of ω -conotoxin GVIA.

Biological activities of dd-GVIA and dd-Sec-GVIA

We previously demonstrated that GVIA and Sec-GVIA produced an equivalent block of N-type calcium currents current following a 16 minute application.³⁸ The disulfide deficient peptides, dd-GVIA and dd-Sec-GVIA, also block N-type calcium currents expressed in HEK293 cells, but with a slower time course (Figure 5a). The blocking time course for each peptide was well described by a single exponential function to yield the time constant, τ . The τ values for dd-GVIA and dd-Sec-GVIA were significantly different from that of GVIA and Sec-GVIA (Figure 5b). As we had previously suggested,³⁸ the substitution of Cys8 and Cys19 by selenocysteine had no significant effect on the kinetics of N-current block since τ of GVIA and Sec-GVIA were not statistically different. The selenocysteine change also did not impact the block by the disulfide deficient peptide, since the block τ was statistically equivalent between dd-GVIA and dd-Sec-GVIA. We conclude that the removal of the first disulfide pair does not prevent block of N-type channels, but does slow that block by roughly 50%.

To investigate the role of removal of the functionally non-critical disulfide in the *in vivo* activity of ω -conotoxin GVIA, dd-GVIA, Sec-GVIA, dd-Sec-GVIA and the non-natively folded dd-GVIA and dd-Sec-GVIA were injected intracranially into mice. GVIA and Sec-GVIA were previously shown to induce shaking syndrome in mice upon intracranial injection, which is characterized by a persistent body tremor, and this behavior was maintained for a long time in a dose-dependent manner.^{22, 38} dd-GVIA and dd-Sec-GVIA also exhibit shaking syndrome in mice, confirming that the removal of the functionally non-critical disulfide did not affect the biological activity of ω -conotoxin GVIA. However, the shaking syndrome induced by dd-GVIA and dd-Sec-GVIA in mice was not as long-lasting as either GVIA or Sec-GVIA. The non-natively folded dd-GVIA and dd-Sec-GVIA did not exhibit shaking syndrome; rather, the injected mice were passive, occasionally shaking their bodies. The distinct behavioral symptoms elicited by natively folded and non-natively folded disulfide depleted peptide analogues demonstrate that the topology of disulfide connectivity is critical to the biological activity. Similar behavioral symptoms were elicited by natively folded GVIA and dd-GVIA and their corresponding selenocysteine analogues, confirming that the removal of the disulfide between Cys1 and Cys16 did not substantially affect the biologically active conformation of ω -conotoxin GVIA.

DISCUSSION

While cone snails have a very efficient mechanism to incapacitate their prey, they are not as mobile as other species, and therefore rely on high-efficiency hunting mechanisms. Consequently, the implications of a failed envenomation event are dramatically larger for snails than they are for other venomous animals that are more able to pursue their prey as it attempts to escape. Therefore it is likely that the evolutionary pressures on cone snails are stronger than those on other venomous animals. For instance, if an injection of venom takes two minutes to paralyze the prey, the cone snail would remain hungry, where a snake or scorpion would likely not. This is probably the explanation for the atypically high evolutionary rate of venom peptides in *Conus*.⁴⁹ However, as discussed below, conotoxins offer a unique opportunity to study evolutionary and molecular mechanisms by which genetic information is translated into folding and activity of disulfide-rich polypeptides.

As illustrated in Figure 6a, the high rate of evolution of *Conus* venom toxins is in striking contrast with discrete conservation of various portions of a toxin precursor gene.⁴⁹ From a

mechanistic point of view, even more puzzling is the fact that the cysteine scaffold remains highly conserved at the level of Cys codon usage (TGC or TGT), while adjacent codons belong to hypervariable sequences of intercysteine loops.^{50–52} Despite this extreme conservation of the cysteine scaffold, this does not imply a conservation of pharmacological properties.¹⁰ For example, Figure 6b shows two common disulfide scaffolds found in conotoxins blocking sodium channels,^{53, 54} but one scaffold (i) also blocks calcium channels and potassium channels (ω - and κ -conotoxins, respectively), as well as delaying the inactivation of sodium channels (δ -conotoxins), whereas the other scaffold (ii) is also capable of functioning as a noncompetitive inhibitor of nicotinic acetylcholine receptors.⁵⁵ Noteworthy, despite an evolutionary conservation of Cys positions and codons, both scaffolds provide examples of conotoxins containing functionally non-critical disulfide bridges.

However, despite the highly conserved disulfide scaffolds in *Conus*, there are several instances of evolutionary divergence of disulfide scaffolds in these marine snails. While it often is difficult to reliably determine which is the “ancestral” fold, the conservation of cysteine codons and pro-peptide region strongly suggest evolutionary linkage between the α -conotoxins (bearing two disulfide bridges) and the α A-conotoxins (bearing three disulfide bridges).⁵⁰ Additionally, the examples of Kunitz domains in conotoxins indicate that the evolution of disulfide bridges does not bear an inherent directionality (gain/loss of bridges). The Kunitz domain is a very ancient fold, being present in both plants⁵⁶ and animals,⁵⁷ with bovine pancreatic trypsin inhibitor (BPTI) being the prototypical example. From this ancient fold/cysteine scaffold, cone snails have developed a class of conotoxins, referred to as conkunitzins. The conkunitzin family has sequences that show a deletion of a disulfide bridge (illustrated in Figure 6c) that is highly conserved in non-*Conus* Kunitz domains,⁵⁸ as well as in other conkunitzins that contain two Kunitz domains in tandem, with addition of a fourth disulfide bridge on the N-terminal Kunitz scaffold.⁵⁹

One outstanding question that remains is that the mechanism by which the cysteine scaffold is so highly conserved remains unknown. The extent of conservation decreases from the signal peptide to the mature toxin region (Figure 6a). However, the placement of cysteine, and even the cysteine codons within the mature toxin region are much more highly conserved than the remainder of the toxin region. While it has been proposed that this is due to a polymerase that preferentially preserves cysteine codons,⁶⁰ a DNA polymerase capable of recognizing frame shifts has yet to be discovered. Consequently, it is possible that the cysteine scaffold is preserved by another mechanism, likely relying on evolutionary pressures. A given disulfide bond may be either critical to oxidative folding or to biological activity (or both), or may even be expendable. Thus, the extent to which the sequence encodes folding information to direct the folding to the native disulfide connectivity is critical to the ability to add and/or remove disulfide bridges to an existing scaffold. Herein we explore the ability to delete a native disulfide bridge from ω -conotoxin GVIA, and consider the evolutionary pressures present to preserve a given disulfide scaffold.

The potential to dissect the roles of individual disulfide bridges in a short, disulfide-rich peptide allows for a much more thorough analysis of the evolutionary pressures present to maintain each bridge. In Figure 4, it is evident that the first disulfide bridge is critical to the efficient folding of GVIA, while Figure 5 shows that the same disulfide has a minimal effect on the biological activity of GVIA. Other work on deleting the second and third disulfide bridges demonstrated significant loss of the activity.³² Using ω -conotoxin GVIA as a model for inhibitory cystine knot (ICK) peptides, it is evident that each of the disulfides serves a critical purpose in generating the final, biologically active compound. Consequently, the evolutionary pressure to efficiently produce active peptide has caused the retention of the disulfide bridges in almost all ICK peptides, with the only potential exception being U1-

liotoxin-Lw1a, from the scorpion *Liocheles waigiensis*,⁶¹ which may also be a precursor which never acquired a third disulfide bridge.

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ABBREVIATIONS

U or Sec	selenocysteine
GVIA	ω -conotoxin GVIA
Sec-GVIA	ω -conotoxin GVIA containing diselenide between Sec8–Sec19
dd-GVIA	disulfide depleted ω -conotoxin GVIA
dd-Sec-GVIA	disulfide depleted ω -conotoxin GVIA containing diselenide between Sec8–Sec19
GSH	reduced glutathione
GSSG	oxidized glutathione
ICK	Inhibitory Cystine Knot
Ca_v2.2	N-type calcium channel
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
TFA	Trifluoroacetic acid
MTBE	Methyl <i>tert</i> -butyl ether

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(a)

Species	Name	Sequence Alignment
<i>C. geographus</i>	GVIA	---CKSPGSSCSPTSYN---CCR-SCNPYTKRCYX---
<i>C. tulipa</i>	TVIA	---CLSPGSSCSPTSYN---CCR-SCNPYSRK---
<i>C. radiates</i>	RVIA	---CKPPGSPCRVSSYN---CCS-SCSKSYNKKCG---
<i>C. geographus</i>	GVIIA	---CKSPGTPCSRGMRD---CCT-SCLLYSNKCRRY---
<i>C. magus</i>	MVIIA	---CKGKGAKCSRLMYD---CCTGSCRS--GKCX---
<i>C. consors</i>	CnVIIA	---CKGKGAPCTRLMYD---CCHGSCSSSKGRCX---
<i>C. catus</i>	CVID	---CKSKGAKCSKLMYD---CCSGSCSGTVGRCX---
<i>C. magus</i>	MVIIC	---CKGKGAPCRKTMVD---CCSGSCGR-RGKCX---
<i>C. magus</i>	MVIID	---CQGRGASCRKTMYN---CCSGSCNR--GRCX---
<i>C. catus</i>	CVIA	---CKSTGASCRRTSYD---CCTGSC-R-SGRCX---
<i>C. striatus</i>	SVIB	---CKLKGQSCRKTSYD---CCSGSCGR-SGKCX---
<i>C. textile</i>	TxVII	---CKQADEPCDVFSLD---CCTGICLG---VCMW---
<i>C. striatus</i>	SVIA	---CRSSGSPCGVTS-I---CCG-RCYR--GKCTX---
<i>C. pennaceus</i>	PnVIA	--GCLEVDYFCGIPFANNGLCCSGNCVF---VCTPQ---
<i>C. pennaceus</i>	PnVIB	DDDCEPPGNFCGM-IKIGPPCCSGWCFF---ACA---

(b)

Figure 1.

ω -Conotoxins derived from the marine cone snails. (a) Multiple sequence alignment of diverse ω -conotoxins. Absolutely conserved residues were shaded and they include Cys residues. Observed modes of disulfide connectivity in ω -conotoxins were also highlighted. Sequences were retrieved from conoserver and alignments were achieved using ClustalW software. (b) The orientation in three dimensional space of the conserved Cys residues of ω -conotoxins, depicted using ω -conotoxin GVIA as an example. The structure of GVIA (PDBID: 2CCO) was modeled using PyMOL software.

Figure 2.

Design of disulfide depleted seleno- ω -conotoxin GVIA was accomplished using redox favored selenocysteine and removing the functionally non-critical disulfide. Systematic disulfide-to-diselenide scanning in ω -conotoxin GVIA suggested that the second disulfide bridge is ideal for replacement with selenocysteines.³⁸ Structure/function studies on the role of disulfide bridges in ω -conotoxin GVIA suggested that removal of first disulfide bridge had relatively small effects on the functional properties of ω -conotoxin GVIA.^{31–32} Combination of disulfide deletion and incorporation of a diselenide in ω -conotoxin GVIA resulted in dd-Sec-GVIA, which requires the formation of only one disulfide, thus removing the need for disulfide mapping of the folded peptides.

Figure 3.

Comparison of regioselective folding of dd-GVIA and dd-Sec-GVIA. (a) Two step oxidative folding of dd-GVIA. The first disulfide bond formation was mediated by redox buffer (GSH/GSSG) and second disulfide bond formation was achieved using iodine. (b) One step oxidative folding of dd-Sec-GVIA. Crude peptide was treated with dithiothreitol (DTT) to remove the DTNP adduct, which is followed by spontaneous diselenide bond formation. The diselenide directed folding requires only one folding step (disulfide bond formation using iodine) to achieve the natively folded peptide. [Note: Asterisk (*) indicates the desired product].

Figure 4.

The role of the biologically non-critical disulfide in the oxidative folding of ω -conotoxin GVIA and Sec-GVIA. (a) RP-HPLC elution profiles of steady state folding mixtures of GVIA, Sec-GVIA and the corresponding disulfide depleted analogues. Incubation of linear (or) natively folded peptides in the folding mixture containing GSH/GSSG (2:1) at steady-state yielded an identical elution profile (Note: The asterisk (*) indicates the biologically active and natively folded peptides). (b) Steady state accumulation of biologically active and natively folded peptide during oxidative folding (at room temperature). Error bars represent the standard error of mean, derived from four independent experiments.

Figure 5.

The blocking effect of GVIA, Sec-GVIA and their corresponding disulfide depleted analogues on the N-type calcium channel. (a) The blocking time courses from three N-type calcium channel-expressing HEK cells exposed to 1 μ M of either GVIA (closed circles), dd-GVIA (open squares) or dd-Sec-GVIA (closed squares). The smooth gray lines are single exponential fits that begin with the toxin onset. (b) Peptide blocking time constant (block τ) measured from N-type calcium channels expressed in HEK293 cells. The bar graph shows mean $\pm$ SD for data from 4–7 cells (indicated within each bar). The lower case letters indicate significant differences ($p < 0.05$, ANOVA with Tukey HSD posthoc analysis).

(a)

(b)

(c)

Figure 6.

Biochemical synthesis of cysteine rich conotoxins and disulfide scaffolds found among conotoxins. (a) The precursor prepropeptide with highlighted conserved and variable regions. (b) Two disulfide scaffolds found in conotoxins. (i) shows the disulfide scaffold for ω , κ , δ and μ O pharmacological families, and (ii) shows the disulfide scaffold for the μ and ψ pharmacological families. The scaffold shown in (i) can act as a calcium channel blocker, potassium channel blocker, delay of sodium channel inactivation, or a sodium channel blocker. Conversely, the scaffold shown in (ii) can act as a sodium channel blocker or a noncompetitive inhibitor of nicotinic acetylcholine receptors. (c) Konkunitizin scaffold with a naturally-deleted disulfide bridge marked by a dashed line. Conserved Cys-Cys motifs in Kunitz domain were replaced by Gly-Gln motif in konkunitizin.

Table 1Summary of the role of disulfide bonds on the activity of ω -conotoxins.

Peptide	Disulfide connectivity	Biological activity	References
GVIA	1–16; 8–19; 15–26	63 ^a	(31)
[C1A,C16A] GVIA	ND	“	“
[C8A,C19A] GVIA	ND	933 ^a	“
[C15A,C26A] GVIA	ND	NA ^a	“
		NA ^a	
GVIA	1–16; 8–19; 15–26	10 nM ^b	(32)
[C1S,C16S] GVIA	8–15; 19–26	> 1 μ M ^b	“
[C8S,C19S] GVIA	ND	> 1 μ M ^b	“
[C15S,C26S] GVIA	1–16; 8–19	> 10 μ M ^b	“
ω -MVIIA-Gly	1–16; 8–20; 15–25	2.5 nM ^c	(13)
[8–20, 15–25] _{1,16 Ala}	8–20; 15–25	170 nM ^c	“
[1–16, 15–25] _{8,20 Ala}	1–16; 15–25	517 nM ^c	“
[1–16, 8–20] _{15,25 Ala}	1–16; 8–20	13000 nM ^c	“
RCM ω -MVIIA-Gly	Linear Peptide	37000 nM ^c	“

(Where ND: Not Determined; NA: Not Active (LD₅₀>6000 μ g/kg);^aBiologically activity of the peptide was determined by their LD₅₀ (μ g/kg) in the gold fish assay;^bActivity of the peptides was accessed using the twitch responses of the rat vas deferens to sympathetic nerve stimulation;^cFunctions of the peptides was established by competitive binding assay using ¹²⁵I-labeled ω -GVIA on chick brain synaptosomes)