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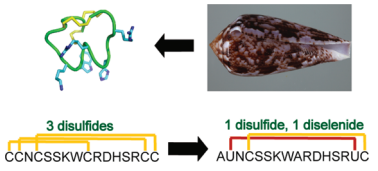
Disulfide-Depleted Selenoconopeptides: Simplified Oxidative Folding of Cysteine-Rich Peptides

Tiffany S. Han,[†] Min-Min Zhang,[†] Konkallu Hanumae Gowd,[†] Aleksandra Walewska,[†,‡] Doju Yoshikami,[†] Baldomero M. Olivera,[†] and Grzegorz Bulaj*[,§]

[†]Department of Biology, University of Utah, Salt Lake City, Utah, 84112, [‡]Faculty of Chemistry, University of Gdansk, 80-952 Gdansk, Poland, and [§]Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah, 84108

ABSTRACT Despite the therapeutic promise of disulfide-rich, peptidic natural products, their discovery and structure/function studies have been hampered by inefficient oxidative folding methods for their synthesis. Here we report that converting the three disulfide-bridged μ-conopeptide KIIIA into a disulfide-depleted selenoconopeptide (by removal of a noncritical disulfide bridge and substitution of another disulfide bridge with a diselenide bridge) dramatically simplified its oxidative folding while preserving the peptide's ability to block voltage-gated sodium channels. The simplicity of synthesizing disulfide-depleted selenopeptide analogues containing a single disulfide bridge allowed rapid positional scanning at Lys7 of μ-KIIIA, resulting in the identification of K7L as a mutation that improved the peptide's selectivity in blocking a neuronal ($Na_v1.2$) over a muscle ($Na_v1.4$) subtype of sodium channel. The disulfide-depleted selenopeptide strategy offers regioselective folding compatible with high-throughput chemical synthesis and on-resin oxidation methods, and thus shows great promise to accelerate the use of disulfide-rich peptides as research tools and drugs.

KEYWORDS Conotoxins, diselenide bridges, selenocysteines, oxidative folding, disulfide-rich peptides



Peptidic, cysteine-rich natural products, such as neurotoxins from spiders, scorpions and predatory marine snails comprise millions of unique, disulfide-rich peptides.[1−4] The diversity of these peptides is reflected in the vast repertoire of their targeting specificities (e.g., the different molecular isoforms of ion channels and neurotransmitter receptors).[5] These neurotoxins hold great promise to provide novel drug treatments for various diseases; in fact, one has already been approved by the FDA for patients experiencing neuropathic pain.[6−9] However, due to synthetic challenges, only a small fraction of these promising peptide-based neurotoxins has been characterized to date. The discovery of novel peptidic toxins has been dominated by the traditional approach of venom fractionation/screening, followed by microsequencing of the isolated bioactive peptide. Recent advances in venomics and DNA sequencing have significantly accelerated the pace of acquiring new peptide sequences.[2,10−14] The rapid progress in molecular biology has outpaced the capacity for chemically synthesizing disulfide-rich peptides encoded in cDNA and genomic sequences.[3]

A critical step in chemical synthesis of disulfide-rich peptides is their oxidative folding.[15,16] A key challenge that significantly hampered discovery and structure/function studies of these peptides is the time-consuming, multistep procedures required for regioselective formation of the correct disulfide bridges. There have been considerable efforts to improve oxidative folding methods that are broadly applicable to cysteine-rich peptide families that differ in disulfide-bridging scaffolds. One recent advancement in the oxidative folding methods is the use of polymer-supported oxidation.[17−19] Simplifying oxidative folding by replacing disulfide bridges with isosteric diselenide bridges[20−27] is another attractive strategy, but it has yet to be widely used by peptide chemists. The advantage of the selenopeptide technology is the redox-favored formation of the diselenide bridge compared to presence of disulfide bridges, ensuring the regioselective formation of cross-links. An early work of Moroder and co-workers showed that replacing a disulfide bridge with a diselenide bridge did not affect the bioactivity of endothelin.[28] The benign consequences of the disulfide-to-diselenide substitution on the bioactivity were subsequently confirmed with α-, ω- and μ-conotoxins,[23,25−27] suggesting that the selenopeptide technology might be applicable to a wider range of bioactive, disulfide-rich peptides.

Central to the strategy described here is the growing body of evidence that many disulfide-rich peptides do not need all

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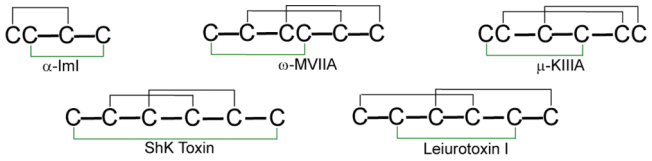

Figure 1. Selected disulfide scaffolds found among neurotoxins for which one of the native disulfide bridges has been shown to be noncritical for bioactivity.

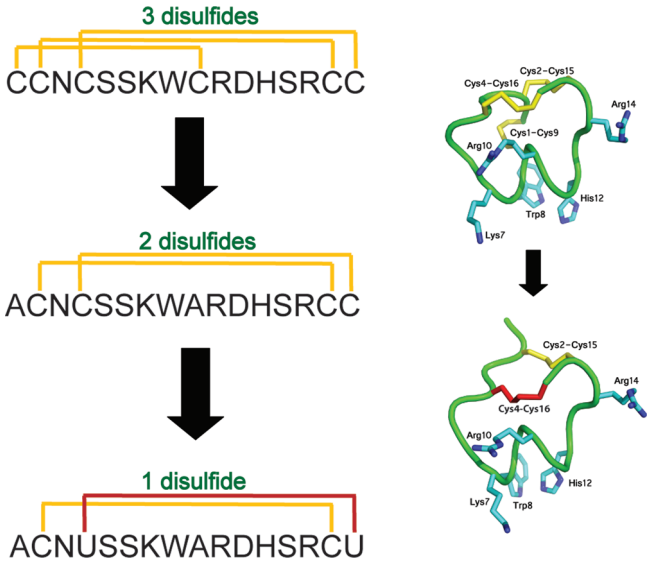

Figure 2. The disulfide-depleted selenopeptide strategy to simplify oxidative folding. The removal of a noncritical disulfide bridge in μ-KIIIA yielded μ-KIIIA[C1A,C9A].[31,33] Next, one of two critical disulfide bridges was replaced by a redox-favored diselenide bridge, allowing a conversion of the three disulfide-bridged conotoxin KIIIA into disulfide-depleted selenopeptide ddKIIIA analogues. Model structures of μ-KIIIA and the disulfide-depleted KIIIA[C1A,C9A] analogue were prepared using the NMR-based coordinates.[33] (Note that the second disulfide bridge in KIIIA-[C1A,C9A] was colored red.) The key amino acid residues shown here, including Lys7, were identified using the Ala-walk strategy.[38] All of the structures were visulized with the use of PyMOL program (Delano, W. L. *The PyMOL Molecular Graphics System* (2002) on World Wide Web http://www.pymol.org).

their native disulfide bridges to retain biological activity. Illustrated in Figure 1 are several peptides in which the deletion of a disulfide bridge did not appreciably affect their structure or function (ShK, scorpion toxins HsTX1[29] or leiurotoxin,[30] examples of *Conus* peptides are reviewed in ref 31). For example, removal of a disulfide in ω-conotoxin MVIIA, which contains a so-called inhibitory cystine knot (ICK) motif, did not abolish its affinity for calcium channels.[32] Likewise, two of three disulfide-depleted analogues of conotoxin KIIIA retained their ability to block the neuronal sodium channels subtype $Na_V1.2$.[31,33] Interestingly, naturally occurring disulfide-depleted peptide analogues, including conkunitzin-S1 and other conkunitzins derived from *C. striatus, C. magus, C. consors* and *C. catus*, were reported.[34−36] Thus, one strategy to simplify the oxidative folding of disulfide-rich peptides is the removal of a noncritical disulfide bridge. Reducing the number of Cys residues from 6 to 4 results in a reduction in the number of possible oxidation isomers from 15 to 3.

In this work, we report a proof-of-concept study in which the selenopeptide technology was applied to disulfide-depleted (dd) analogues (Figure 2). μ-Conopeptide KIIIA, which has 16 AA residues and three disulfide bridges, is a sodium channel blocker with demonstrated analgesic activity in an animal model of pain.[31,33,37,38] To fully exploit the promising pharmacological and therapeutic properties of μ-conopeptides, such as KIIIA, optimization studies that require fast and efficient folding methods are essential (chemical synthesis was indeed a major bottleneck in prior structure−function studies of KIIIA).[38] We previously showed that the disulfide-depleted analogue of μ-conopeptide KIIIA, KIIIA[C1A,C9A], retained structural and functional properties of the wild-type KIIIA.[31,33] By creating the disulfide-depleted selenoconopeptide KIIIA analogues (dd-sec-KIIIA), we were able to reduce the number of disulfide bridges from three to one, while maintaining the bioactivity of this peptide.

To simplify oxidative folding of μ-conotoxin KIIIA, we designed two dd-sec-KIIIA analogues with a diselenide bridge replacing either of the two remaining native disulfide bridges: KIIIA[C1A,C2U,C9A,C15U] and KIIIA[C1A,C4U,C9A, C16U] (designated ddKIIIA-1 and ddKIIIA-2, respectively). The KIIIA selenoconopeptide analogues were chemically synthesized on solid support using the standard Fmoc chemistry and the selenocysteine/cysteine protection groups are illustrated in Figure 3a. The cleavage from the resin and the reduction of the crude peptide was followed by HPLC purification using the protocol described previously.[25] The presence of DTNP in the cleavage mixture is critical for the removal of the *p*-methoxybenzyl groups.[25,39,40] Based on our previous experience with selenoconopeptides,[25,27] the immediate treatment of the crude peptide with DTT results in the linear form that contains the diselenide bridge. This finding was further confirmed using dd-KIIIA-1 as a model peptide (Figure S1 in the Supporting Information). Oxidative folding of the analogues containing a preformed diselenide bridge was promoted by a mixture of 1 mM GSSG and 1 mM GSH. Such oxidizing conditions favor formation of the native disulfide bridges in μ-conotoxins.[41] The HPLC elution profiles of the analogues before and after oxidation are shown in Figure 3b. In each case, the oxidation reaction yielded a single peak, as expected from yielding a single folding species containing only one disulfide bridge. The chemical identity of the oxidized species was confirmed by mass spectrometry (Table S1 in the Supporting Information). It is worth mentioning here that the process described above from the cleavage step to the final purification of the oxidized species can be further optimized by performing all steps as a one-pot procedure, for example using Clear-Ox as an oxidizing agent,[17−19] while HPLC purification steps can be replaced by solid-phase extraction. Oxidizing conditions other than the glutathione-based redox buffer used in this study may also be employed to promote a disulfide bond formation in disulfide-depleted selenopeptides.[26,42]

Both KIIIA selenopeptide analogues retained comparable activity in blocking $Na_V1.2$, a neuronal subtype of sodium

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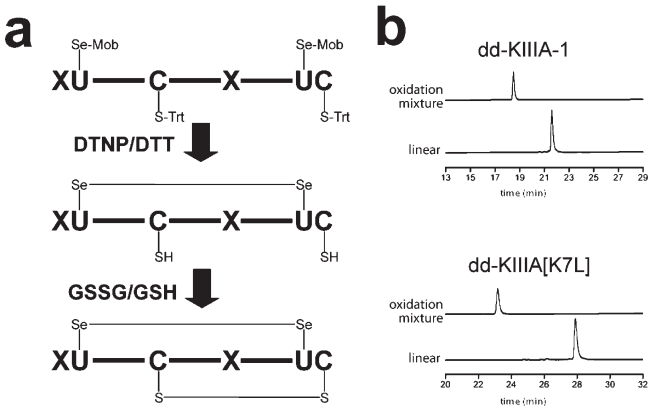

Figure 3. Chemical synthesis and oxidative folding of the disulfide-depleted selenopeptide analogues of KIIIA. (a) An overview of the chemical synthesis strategy for ddKIIIA analogues. The selenopeptide analogue is removed from a resin by the treatment with an enriched reagent K (DTNP removes Mob-protecting groups from Sec residues, whereas subsequent treatment with DTT removes the resulting 5-thionitropyridyl derivatives by thiolysis). The redox-favored diselenide bridge is formed at low pH, and its presence was confirmed by mass spectrometry and alkylation analyses (Figure S1 in the Supporting Information). The formation of the remaining single disulfide bridge is promoted by oxidants, such as glutathione. (b) HPLC elution profiles of the oxidative folding of two ddKIIIA analogues. The HPLC-purified linear form contains preexisting diselenide bridge, imposing only one possible oxidation product, as confirmed by HPLC analyses of the oxidation mixtures.

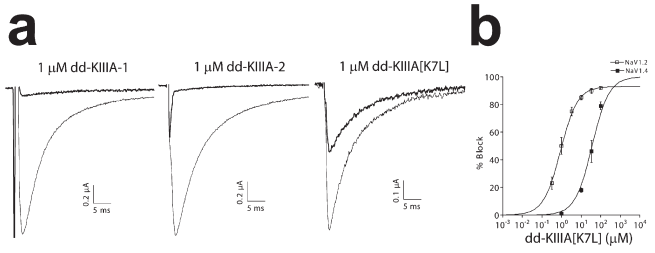

Figure 4. Bioactivity of dd KIIIA analogues in blocking the $Na_V1.2$ subtype of sodium channels expressed in oocytes. (a) Sodium currents in the absence (gray traces) and presence (black traces) of the selenoconopeptide analogues. (b) Dose response curves of the block of $Na_V1.2$ and $Na_V1.4$ by ddKIIIA[K7L], an analogue with sharpened selectivity toward $Na_v1.2$.

channel, as shown in Figure 4a. Interestingly, both ddKIIIA analogues exhibited comparable relative activities in blocking $Na_V1.2$ and $Na_V1.4$, a skeletal muscle subtype (data not shown). Based on calculated K_d values, there was 20-fold (for ddKIIIA-1) and 18-fold (for ddKIIIA-2) preference for blocking $Na_v1.2$ relative to $Na_V1.4$. These results are consistent with previous structure/function studies[31,33,38] in which the wild type KIIIA and KIIIA[C1A,C9A] were shown to exhibit 17-fold and 30-fold preference, respectively, for blocking $Na_V1.2$ over $Na_V1.4$.

Next, we tested the feasibility of the new chemical strategy for SAR studies by carrying out a limited positional scanning of Lys7, which was previously identified as a selectivity "hot-spot" for better discrimination between $Na_V1.2$ and $Na_V1.4$.[38] The K7A mutation in KIIIA did not affect the ability of the analogue to block $Na_V1.2$, but it decreased the block of $Na_V1.4$.[38] We designed and synthesized several KIIIA[C1A, C2U,C9A,C15U] analogues (ddKIIIA[K7X]) varying in Lys7 substitutions, namely, with Ala, Phe, Ser, Thr, Asp, Gly, Leu, Val or Dap (diaminopropioic acid). The ddKIIIA[K7X] analogues were folded using the identical protocol as used for ddKIIIA-1 and ddKIIIA-2, in each case yielding a single oxidation product, confirmed by mass spectrometry to contain a diselenide and a disulfide bridge. The HPLC profiles of the one-step oxidation of ddKIIIA[K7L] are shown in Figure 3b. The electrophysiological characterization of the analogues resulted in the identification of the analogue ddKIIIA[K7L] which exhibited over 70-fold preference for blocking $Na_V1.2$ over $Na_V1.4$ (Figure 4b). The detailed pharmacological characterization of the resulting analogues suggested uncoupling of efficacy and affinity of KIIIA inhibition of subtypes of sodium channels, and will be published elswhere (Zhang et al., manuscript submitted). The results of this rapid positional-scanning study emphasizes the advantage of disulfide-depleted selenopeptide technology for accelerated SAR studies on disulfide-rich peptides. We acknowledge here that further studies are needed to scrutinize the structural and functional consequences of the disulfide-to-diselenide replacements.

Our findings indicate that the use of selenocysteine chemistry can significantly facilitate the discovery of novel disulfide-rich peptides. In the postgenomic era, high volume DNA sequencing and rapid cloning methods can provide access to millions of new peptide sequences, and alignments of DNA-derived sequences of cysteine-rich peptides facilitate analysis of evolutionary conserved patterns of cysteine residues. Furthermore, an accumulated knowledge from the biochemical studies has already improved the prediction accuracy of the disulfide connectivities in cysteine-rich peptides. Thus, proper design and synthesis of disulfide-depleted selenopeptides based on newly discovered sequences will provide a rapid synthetic advantage. For some disulfide scaffolds and sequences, the disulfide-depleted selenopeptide technology may produce unsatisfactory results; however, our tests (Gowd et al., manuscript in preparation) of disulfide-depleted selenoconopeptide analogues of ω-GVIA, which has an inhibitory cysteine knot (ICK) motif, confirmed previous findings with ω-conotoxin MVIIA that a removal of a disulfide did not significantly affect bioactivity. The potential advantage of working with disulfide-depleted analogues of cysteine-rich peptides might spur additional structure/function studies to identify which disulfide bridges are not critical for different disulfide scaffolds across various peptide groups spanning mollusks or spider or scorpion neurotoxins, plant-derived peptides such as cyclotides or proteinase inhibitors, amphibian-based antimicrobial peptides, and endogenous disulfide-rich peptides such as hepcidin, to name few examples.

Noteworthy, the disulfide-depleted selenopeptide strategy described here is compatible with two recent advances in the oxidative folding technology, namely, the integrated oxidative folding that includes the NMR-based peptide mapping[25,27,43,44] and the selenium-directed on-resin oxidation.[26] In the integrated oxidative folding, the use of diselenide and selectively $^{15}N/^{13}C$-labeled disulfide bridges improves the oxidative

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folding yields while allowing concurrent rapid disulfide mapping. Thus, combining the disulfide-depleted selenopeptide technology with the integrated oxidative folding should ease SAR studies on bioactive peptides containing four disulfide bridges, as illustrated in Figure S2 in the Supporting Information. Furthermore, synthesis of disulfide-depleted selenopeptide analogues on a solid support containing safety-catch linkers (SCAL)[26,45] should facilitate efficient high-throughput synthesis of cysteine/selenocysteine-rich peptides via the selenium-directed on-resin oxidation, as recently described by the Alewood group.[26] Taken together, combining all above listed technological advances in the oxidative folding extends the applications of the disulfide-deficient selenopeptides to study peptides containing three and perhaps even four disulfide bridges.

These results provide proof-of-principle for developing a high-throughput chemical synthesis and folding strategy for disulfide-rich peptides. The bottleneck in their accelerated discovery and characterization will be shifted to functional screening, in particular against voltage- and ligand-gated ion channels and receptors, which many neurotoxins target. The minimalist methodology described above showed greatly accelerated SAR studies for lead optimization, making the development of new biotherapeutics more facile.[3] Overall, this approach contributes significantly toward discovery and structure/function characterization of disulfide-rich peptides.

SUPPORTING INFORMATION AVAILABLE Experimental procedures, HPLC elution profiles and mass spectrometry analyses of deprotected, reduced and alkylated disulfide-depleted selenoconopeptide dd-KIII-1 (Figure S1), scheme for applying the disulfide-depleted selenopeptide approach to four-disulfide-containing peptides (Figure S2) and a summary of mass spectrometry and HPLC retention times of all synthesized disulfide-depleted selenoconopeptide analogues (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author: *Corresponding author. Phone: (801) 581-4629, Fax: (801) 581-7087. E-mail: bulaj@pharm.utah.edu.

Author Contributions: T.S.H., M.M.Z., A.W., K.H.G., B.M.O., D.Y., and G.B. designed this research project. T.S.H., M.M.Z., K.H.G., and A.W. performed experiments and analyzed data. T.S.H., K.H.G., B.M.O., D.Y., and G.B. wrote the manuscript.

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ABBREVIATIONS dd, disulfide-depleted; DTT, dithiothreitol; ddKIIIA, disulfide-depleted selenoconopeptide KIIIA analogue; Fmoc, Fmoc (*N*-(9-fluorenyl)methoxycarbonyl); GSH, reduced glutathione; GSSG, oxidized glutathione; KIIIA, μ-conotoxin KIIIA; Na_V, voltage-gated sodium channel.

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