Integrated Oxidative Folding

DOI: 10.1002/anie.200806085

# Integrated Oxidative Folding of Cysteine/Selenocysteine Containing Peptides: Improving Chemical Synthesis of Conotoxins**

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Bioactive disulfide-rich peptides, such as neurotoxins from spiders, scorpions, and cone snails, plant cyclotides, antibacterial peptides, and protease inhibitors, form an incredibly diverse group of natural products estimated to consist of millions of distinct sequences. Many of these peptides are promising drug leads as analgesics, antihypertensive, antiarrhythmic, antitumor, antiviral, or antibiotic therapeutics.[1–3] However, an efficient oxidative folding and determination of the resulting disulfide connectivities are the most common bottlenecks in their chemical syntheses that slow the progress of drug discovery and development.[4] To address these two challenges simultaneously, we developed an integrated approach that combines the use of diselenide and selectively ($^{15}N/^{13}C$)-labeled disulfide bridges. We synthesized conotoxin analogues, in which the selenocysteines significantly improved folding yields and the labeled cysteines allowed the correctly folded species to be rapidly identified by NMR spectroscopy.

Numerous strategies have been developed to improve the oxidative folding of disulfide-rich peptides.[5,6] A replacement of disulfide bridges by more redox-stable diselenide crosslinks has been employed for peptides containing either one or two disulfide bridges.[7–12] Substitution of a pair of cysteines with selenocysteines should guide the formation of disulfide bridges between the remaining cysteines,[8] because the more stable diselenide forms first (the redox potential of a diselenide bridge ($E_o = -381$ mV) is significantly lower than that of a disulfide bridge ($E_o = -180$ mV)[13]) providing a topological constraint for the formation of the remaining disulfides and reducing the number of possible disulfide connectivities.[14] Once a disulfide-rich peptide is synthesized and oxidized, the disulfide bridges must be determined. To overcome multiple challenges of traditional disulfide mapping, we recently developed an NMR-spectroscopy-based strategy to rapidly determine disulfides using differential isotope labeling of pairs of Cys, followed by detecting NOESY crosspeaks between cross-disulfide $H^{\alpha}/H^{\beta2}/H^{\beta3}$ protons.[15] Our search for improved oxidation strategies led us to the idea of combining selenocysteines and $^{15}N/^{13}C$-labeled cysteines, which we successfully applied to μ-conotoxins.

The concept of "integrated oxidative folding" is illustrated in Figure 1a. For three-disulfide-bridged peptides, the analogues containing diselenide and the isotope-labeled disulfide bonds should: 1) fold more efficiently, since the pre-existing diselenide bridge reduces the number of possible disulfide crosslinks and directs formation of the remaining disulfides, and 2) provide unambiguous evidence of the correct pairing of all three crosslinks; the cross-disulfide NOESY crosspeaks from the selectively labeled pair of the cysteine residues and the thermodynamically preferred diselenide bridge would suffice to make such a claim. To obtain proof-of-concept, we selected the three disulfide-bridged peptide, μ-conotoxin

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[**] This work was supported in part by the NIH grants: R21 NS055845 (G.B.) and Program Project GM 48677 (B.M.O., G.B., D.Y.). We thank Drs. Robert Schackmann and Scott Endicott from the DNA/Peptide Synthesis Core for their help with chemical synthesis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200806085.

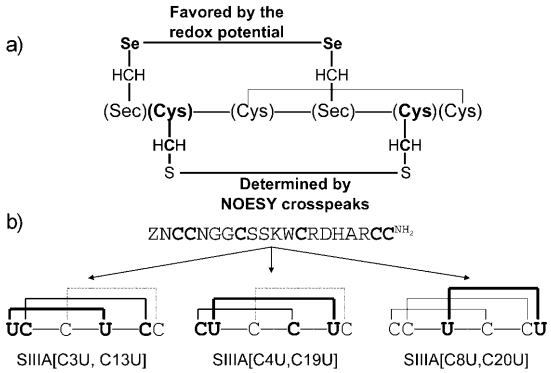


***Figure 1.*** Integrated oxidative folding. a) μ-Conotoxin scaffold containing one diselenide bridge, one isotope-labeled disulfide bridge, and one normal disulfide bridge. The formation of the diselenide bridge is thermodynamically preferred and improves the oxidative folding, while the formation of the labeled disulfide bridge is readily confirmed by detecting cross-disulfide NOEs. Sec = selenocysteine, **Cys** = labeled cysteines, Cys = unlabeled cysteines. b) Structures of μ-SIIIA (top) and μ-selenoconotoxin analogues of SIIIA studied in this work (bottom, in which **U** = selenocysteine, **C** = labeled cysteine, C = cysteine).

SIIIA, for which folding, structure, and function are well studied.[16–19] Figure 1b shows the structure of SIIIA and three analogues in which one pair of the cysteine residues forming a native disulfide bridge was replaced by a pair of selenocysteine residues. The analogues were synthesized using the Fmoc-based chemistry (see Experimental Section). The cysteine thiols were protected with the trityl groups, whereas selenocysteine residues were protected with 4-methoxybenzyl (Mob) groups. Both protecting groups were removed during the cleavage of the peptides from the resin. The Mob group came off easily with 2-2′-dithiobis-(5-nitropyridine) (DTNP). A mechanism underlying the removal of the Mob group and the formation of the diselenide bridge was recently studied.[20,21] A critical step in an efficient recovery of the reduced conotoxins containing a diselenide bridge appeared to be the reduction of the crude (post-cleavage) peptide with dithiothreitol. Mass spectrometry analysis confirmed the existence of the preformed diselenide bridge in the otherwise reduced SIIIA analogues (an alkylation of free Cys thiols with 4-vinylpyridine yielded SIIIA[C3U,C13U] = 2726.9, SIIIA-[C4U,C19U] = 2726.7, SIIIA[C8U,C20U] = 2727.0).

The diselenide-containing peptides were subjected to oxidative folding in the mixture of the oxidized glutathione (1 mM GSSG) and reduced (10 mM GSH) glutathione (Figure 2). The identity of each folded analogue ($[MH^+]_{calcd} = 2302.7$) was confirmed by MALDI-TOF mass spectrometry: SIIIA[C3U,C13U] $[MH^+]_{exp} = 2302.5$, SIIIA-[C4U,C19U] $[MH^+]_{exp} = 2302.6$, SIIIA[C8U,C20U] $[MH^+]_{exp} = 2302.4$. The highest steady-state accumulation of the native form was found for SIIIA[C3U,C13U] and the lowest for SIIIA. The number of the folding intermediates was much lower for all three diselenide-containing analogues compared to SIIIA, and only few minor folding species were detected to contain mixed disulfides with glutathione, as determined by mass spectrometry. Noteworthy, further improvements of the folding yields might be achieved by optimizing concentrations of GSSG and GSH. All SIIIA analogues blocked $Na_v1.2$ subtype of voltage-gated sodium channels (Supporting Information; Table S1 and Figure S1), the $K_d$ values were: $47 \pm 16$ nM for SIIIA, $46 \pm 38$ nM for SIIIA[C3U,C13U], $67 \pm 18$ nM SIIIA[C4U,C19U], and $37 \pm 6$ SIIIA[C8U,C20U] (mean $\pm$ SD, $N \geq 3$).

The position-specific introduction of the $^{15}N/^{13}C$ labeled Cys residues in μ-selenoconotoxin SIIIA analogues, (Figure 1b), allowed us to rapidly determine the disulfide connectivities. The $^{15}N/^{13}C$-labeled cysteines in the two μ-selenoconotoxin analogues, SIIIA[C3U,C13U, $^{15}N/^{13}C$ enriched C4 and C19] and SIIIA[$^{15}N/^{13}C$ enriched C3 and C13,C4U,C19U], were identified in 2D [$^{13}C$,$^{1}H$] HSQC NMR spectroscopy experiments. The methine and methylene resonances were assigned in both analogues using the reported chemical shifts for μ-SIIIA (Figure 3).[19] Following resonance assignment, 2D $^{13}C$-F2-edited NOESY was recorded to identify cross-disulfide NOEs consistent with a disulfide bond. These are boxed in Figure 3 panels (b) and (d). Thus, we were able to deduce the proper connectivity of the crosslinks in the μ-selenoconotoxin analogues by: 1) preforming the diselenide bridge, and 2) detecting cross-disulfide NOEs for the disulfide bond with $^{13}C/^{15}N$ enrichment.

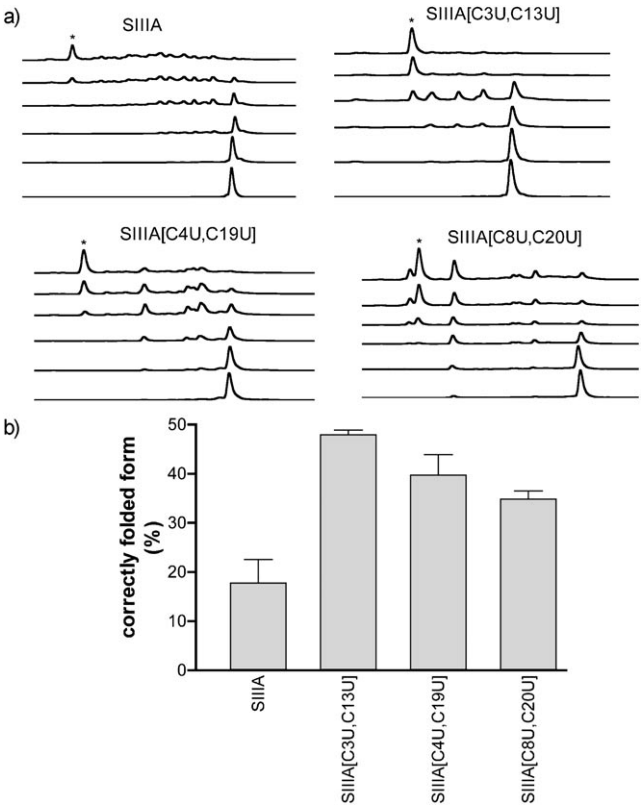


***Figure 2.*** Oxidative folding of μ-selenoconotoxin SIIIA analogues. a) HPLC elution profiles of μ-SIIIA and μ-selenoconotoxin analogues of μ-SIIIA folded with a mixture of 1 mM oxidized and 10 mM reduced glutathione. The oxidation mixtures were quenched by acidification after 1, 10, 30, 60, and 120 min and analyzed by reversed-phase $C_{18}$ analytical HPLC. Asterisk indicates the native form of the peptide; labeled peaks were collected and analyzed by mass spectroscopy. b) Correctly folded yields at the steady-state of μ-SIIIA and its μ-selenoconotoxin analogues. Error bars represent standard errors ($N = 3$).

To examine applications of μ-selenoconotoxins for peptide engineering, we designed two non-natural selenoconotoxin SIIIA analogues (Figure 4): in the first analogue, AHX-Sec-SIIIA, two adjacent Ser residues were replaced by 6-aminohexanoic acid, whereas the second analogue, DOTA-Sec-SIIIA, contained Lys-DOTA at the N-terminus. The oxidative folding of both analogues resulted in an accumulation of a single major species (Figure 4c). Both analogues retained the ability to block $Na_V1.2$ sodium currents, and the DOTA-Sec-SIIIA was fluorescent when it chelated terbium ($Tb^{+3}$) (Supporting Information, Figure S2). To investigate whether other μ-selenoconotoxins also exhibit improved folding properties, we introduced a pair of selenocysteines into poorly folding μ-conotoxin SmIIIA.[22] The folding yield the SmIIIA[C3U,C15U] (41%) was significantly improved compared to the wild-type peptide (12%), whereas the replacement of disulfide by diselenide bridges did not markedly compromise bioactivity (Supporting Information, Figure S3).

Herein, we described a novel approach to the oxidative folding of disulfide-rich peptides that combines the use of

  

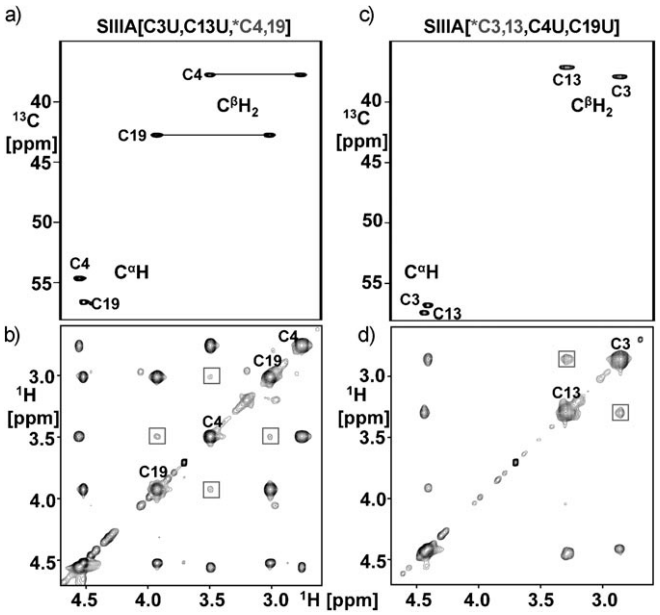


***Figure 3.*** NMR-spectroscopy-based determination of disulfides in two μ-selenoconotoxin SIIIA analogues. NMR spectra at 15 °C for 1 mM SIIIA[C3U,C13U,$^{15}$N/$^{13}$C enriched C4 and C19] prepared in 40 mM NaPi (pH 6.2), 50 mM NaCl, 90% $H_2O$, and 10% $D_2O$ are shown in panels (a) and (b), and SIIIA[$^{15}$N/$^{13}$C enriched C3 and C13,C4U,C19U] in identical solution conditions are shown in panels (c) and (d). Panels (a) and (c) show the 2D [$^{13}$C,$^{1}$H] constant time HSQC spectra and panels (b) and (d) the 2D $^{13}$C-F2-edited [$^{1}$H,$^{1}$H] NOESY spectra. The proton dimensions (abscissa) are aligned for panel pairs (a)/(b) and (c)/(d). Non-degenerate C4 and C19 $C^{\beta}H_2$ signals are connected with a line (shown in panel (a)), the degenerate C3 and C13 $C^{\beta}H_2$ signals are shown in panel (c). NOE crosspeaks confirming the C4–C19 and C3–C19 disulfides are boxed. Intraresidue NOEs can be easily traced in the Figure. A few natural abundance signals are present in the NOESYs but posed no problems with interpretation.

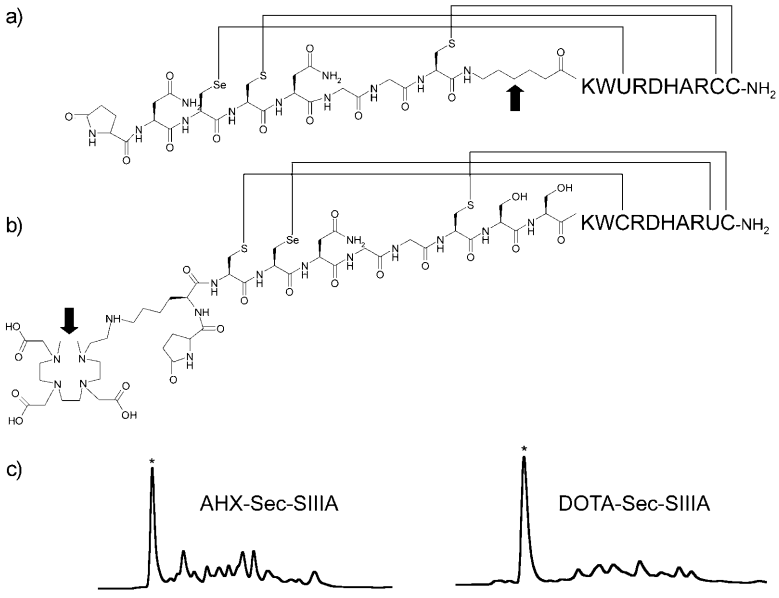


***Figure 4.*** Structures of AHX-Sec-SIIIA (a) and DOTA-Sec-SIIIA (b). The arrow points to the backbone spacer, 6-aminohexanoic acid, or the DOTA moiety. c) HPLC elution profiles of the folding reactions. Asterisk indicates the folding species that was functionally characterized.

diselenide and selectively ($^{15}$N/$^{13}$C)-labeled disulfide bridges: introduction of selenocysteines significantly improves folding yields while the labeled cysteines allow the correctly folded species to be rapidly identified by NMR spectroscopy. Integrated oxidative folding opens new opportunities in chemical syntheses of peptides containing even four disulfide bridges (Supporting Information, Figure S4). Since this technology is compatible with higher throughput chemical syntheses, it will also accelerate drug discovery and development. For larger polypeptides, this strategy may be exploited in conjunction with recombinant methods or native chemical ligation.[23,24] Furthermore, integrated oxidative folding is useful in studying folding mechanisms, since it offers a means of dissecting a role of individual (native and non-native) disulfide bridges in the folding pathways. Taken together, the integrated oxidative folding approach is likely to have an impact on both the basic and applied research of disulfide-rich peptides.

## *Experimental Section*

Peptides were synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, as described elsewhere.[16,22] Fmoc-protected selenocysteine with the selenium-p-methoxybenzyl protection was purchased from ChemImpex International, (Wood Dale, IL). Fmoc-protected $^{15}$N/$^{13}$C cysteine residue (U-13C3, 97–99%; 15N, 97–99%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Cysteine residues were protected with S-trityl groups. Peptides were cleaved from the resin for 4 h with the enriched reagent K (trifluoroacetic acid (TFA)/thioanisole/phenol/water (90:2.5:7.5: 5, v/v/v/v) and 1.3 equivalents DTNP [2,2′-dithiobis(5-nitropyridine)]).[20] The selenoconotoxins were washed with methyl *tert*-butyl ether and treated for 2 h with DTT (threo-1,4-dimercapto-2,3-butanediol; 50 mM), Tris (tris(hydroxymethyl)aminomethane; 0.1M), EDTA (ethylenediaminetetraacetic acid; 1 mM), pH 7.5 at room temperature, then quenched with 8% formic acid. The peptides were purified using a C18 HPLC and gradient from 5 to 30% of 0.1% (v/v) TFA in 90% acetonitrile in 25 min. Oxidative folding was performed in Tris-HCl (0.1M), EDTA (1 mM; pH 7.5), GSSG (1 mM), GSH (10 mM) at room temperature. SIIIA[C3U,C13U]DOTA (220 nmol) was dissolved in water (220 μL), then ammonium acetate (40 μL; pH 6.1) and terbium (III) chloride hexahydrate (22 μL; 0.01M) was added. The mixture was shaken at 50 °C overnight, pH value was adjusted to 9 with NaOH (10 mM). The solution was shaken for 30 min at 0 °C, centrifuged at 7000 RPM for 5 min, filtered through a 0.22 μm filter, then washed with water and vacuum-dried.

Two-dimensional [$^{13}$C,$^{1}$H] HSQC and 2D [$^{1}$H,$^{1}$H] NOESY were recorded[25] at 15 °C on a Varian Inova 600 NMR spectrometer with a cryogenic $^{1}$H/$^{13}$C/$^{15}$N probe. Data were processed with FELIX2004 and analyzed using SPARKY (T. D. Goddard and D. G. Kneller, UCSF). Chemical shifts are published.[19]

Electrophysiological assays were carried out as described in Zhang et al.[26]

Received: December 13, 2008
Published online: February 10, 2009

**Keywords:** conotoxin · cysteine · NMR spectroscopy · oxidative folding · selenium

  

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