Protocol

Journal of PeptideScience

Received: 8 April 2010 Revised: 29 June 2010 Accepted: 30 June 2010 Published online in Wiley Online Library: 2 September 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1283

Optimization of oxidative folding methods for cysteine-rich peptides: a study of conotoxins containing three disulfide bridges

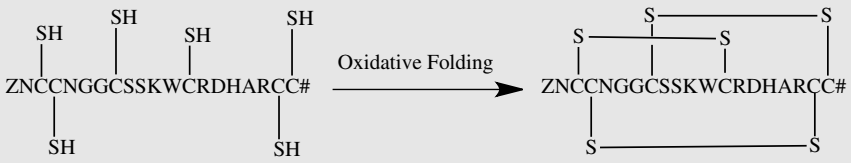
Andrew M. Steiner and Grzegorz Bulaj*

The oxidative folding of small, cysteine-rich peptides to selectively achieve the native disulfide bond connectivities is critical for discovery and structure-function studies of many bioactive peptides. As the propensity to acquire the native conformation greatly depends on the peptide sequence, numerous empirical oxidation methods are employed. The context-dependent optimization of these methods has thus far precluded a generalized oxidative folding protocol, in particular for peptides containing more than two disulfides. Herein, we compare the efficacy of optimized solution-phase and polymer-supported oxidation methods using three disulfide-bridged conotoxins, namely μ-SIIIA, μ-KIIIA and ω-GVIA. The use of diselenide bridges as proxies for disulfide bridges is also evaluated. We propose the ClearOx-assisted oxidation of selenopeptides as a fairly generalized oxidative folding protocol. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: oxidative folding; disulfide; ClearOx; glutathione; diselenide; conotoxin; protocol; selenocysteine; cysteine-rich peptides

Generalized Reaction Scheme



Z denotes pyroglutamate, and # denotes amidation at the C-terminus.

General Optimized Procedure(s)

1. Glutathione : Tenfold concentrated solutions were used to prepare a folding reaction with 1 mM oxidized glutathione, 1 mM reduced glutathione, 0.1 M Tris, pH 7.5, 0.1 mM EDTA, 20 μM μ-Conotoxin SIIIA. The reaction was allowed to proceed for 16 h and was quenched by acidification with formic acid to a final concentration of 8%. *2. Dimethyl Sulfoxide* : To a dilute solution of dimethyl sulfoxide (11.63%) were added 10-fold concentrated solutions to a final concentration of 0.1 M Tris, pH 7.5, 0.1 mM EDTA and 20 μM μ-Conotoxin SIIIA, making the final DMSO concentration 10%. The reaction proceeded for 16 h and was quenched by acidification with formic acid to a final concentration of 8%. *3. ClearOx* : To 12 molar equivalents of swelled and washed resin was added a solution of 3 mM μ-Conotoxin SIIIA in 50% acetonitrile, 50% 0.1 M MOPS, pH 7. The reaction proceeded for 1 h, and was quenched by 100-fold dilution with 0.1% TFA in water.

Scope and Comments

Disulfide bonds conformationally restrict a peptide, which is crucial to their biological activity. However, achieving specificity in the topology of disulfide formation during chemical synthesis is difficult, in particular for peptides containing more than two disulfide bridges (for two disulfide-bridged peptides, the orthogonal side chain protection of pairs of cysteines and regioselective folding is the method of choice [1]). The challenge in chemical synthesis of disulfide-rich peptides is reflected in their oxidative folding yields, ranging from prohibitively low to

* *Correspondence to: Grzegorz Bulaj, Department of Medicinal Chemistry, College of Pharmacy, University of Utah, 421 Wakara Way, Suite 360, Salt Lake City, UT 84108, USA. E-mail: bulaj@pharm.utah.edu*

Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84108, USA

Abbreviations used: *DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTNP, 2,2′-dithiobis(5-nitro-pyridine); GSSG, oxidized glutathione; GSH, reduced glutathione; MOPS, 3-morpholinopropane-1-sulfonic acid; Tris, tris(hydroxymethyl)aminomethane.*

quantitative [2]. Coincidently, a majority of cysteine-rich bioactive peptides contain three or more disulfide bridges, including plant-derived protease inhibitors, defensins from both vertebrates and invertebrates and neurotoxins from spiders, scorpions and mollusks. Despite the fact that these peptides comprise a pool of millions of bioactive compounds, many of which have already become research tools and even therapeutics, their discovery and structure–function studies have progressed at a relatively slow pace. Due to their small size, chemical synthesis has been the method of choice over recombinant expression, but efficient oxidative folding methods remain the bottleneck in exploring the full potential of cysteine-rich peptides [3–5].

Herein, we compare oxidation methodologies in the context of conotoxins, a large class of cysteine-rich peptides that includes one of the only venom-derived, FDA-approved biopharmaceuticals to date [6–9]. These peptides are very short (10–40 residues), usually with 2–4 disulfide bonds, and exhibit a wide range of folding yields (Table 1). *Conus* peptides are an excellent model system for the study of disulfide bond formation because they are natural product peptides that are sufficiently complex and diverse to address the myriad variables of oxidative folding.

There are several methods used to oxidize cysteine-rich peptides to their folded products; these methods fall into three broad categories: solution-phase, polymer-bound oxidants and on-resin oxidation. To form the disulfide, deprotonation of the thiol is required to make the reactive nucleophilic thiolate [20,21]; consequently, the pH must be buffered (near neutral) to control the thiol/thiolate equilibrium (see Table S1, Supporting Information, for pH dependence). The protonation/deprotonation of cysteine residues is sequence dependent [22], and this property could be used to modulate reactivity of individual cysteines. The other variables in the folding reaction, such as peptide concentration, temperature and folding time should be optimized. Further optimization of peptide folding can be accomplished using folding additives. These factors can often be predicted based on the sequence of the peptide. Salt can suppress electrostatic interactions [23], and organic solvents that are miscible with water, detergents or 1-ethyl-3-methylimidazolium acetate can significantly increase the folding efficiency of hydrophobic peptides [13,24,25].

Solution-phase oxidation is the most commonly employed method to form intramolecular disulfide bonds in peptides [13,23,24,26,27]. Solution-phase oxidation requires very dilute peptide (20–50 μM), in order to ensure that the disulfide bonds form intramolecularly. At higher peptide concentrations, the effective concentration of the intramolecular thiols is surpassed by the concentration of intermolecular thiols, leading to oligomers and resulting in lower folding yields [1]. As solution-phase oxidation has been thoroughly explored, there are numerous possible oxidants (glutathione, selenoglutathione, cysteine, cystamine, DMSO, (±)-trans-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC) [28] and molecular oxygen are common examples), with context-dependent variations in the folding efficiency. Although selenoglutathione can oxidize peptides at mildly acidic pH (~5), to our knowledge, it has not been used to fold peptides with three or more disulfides [29]. Glutathione-based redox buffers are common, as they are believed to better reflect oxidation pathways *in vivo* [30,31]. The folding efficiency and extent of oxidation are also influenced by the ratio of GSSG to GSH (Figure 2). Dilute dimethyl sulfoxide (final concentration 5–10%) is another common oxidant. The product ratios from glutathione and DMSO methods are often very similar, although dimethyl sulfoxide oxidation can give a slightly more random distribution of folding isomers (Figure 3), and often requires longer folding times (Table S4). Molecular oxygen is also used to oxidize thiols to disulfides. Drawing oxygen from either air or oxygen atmosphere, this method relies on low levels of oxygen dissolved in solution; consequently, these methods are relatively slow (usually requiring several days) [1].

Folding at high dilution presents problems with both scaled-up and higher throughput folding; these problems can often be overcome using polymer-bound oxidation. With this type of oxidation, the oxidant is bound to a solid support at low loading levels, exploiting the pseudodilution effect to fold peptides at significantly higher concentrations; nonetheless, intramolecular disulfide bonding remains preferred. The low loading levels ensure that each molecule of the peptide is isolated, and intramolecular disulfide bonds are favored. With respect to polymer-bound folding methods, we evaluated the use of ClearOx resin. Solution-phase oxidants to fold peptides isolated on a hydrocarbon-based resin has been previously explored [14]; however, the (relative) folding yields did not justify further consideration of these methods (Table 1). On more complicated or difficult folding substrates, agarose-bound folding media that include folding chaperones and oxidant have also been employed [15].

With peptides containing one or two disulfides, oxidative folding before cleavage from the resin has been reported [5,32,33]. Although on-resin folding methods are quite effective for folding one or two disulfide bonds, successful folding of three or more disulfide bonds with resin-bound peptide has not been reported to our knowledge.

μ-Conotoxin SIIIA (shown in generalized reaction scheme) has three disulfide bridges and has been the subject of several folding studies [10,26,27]. Noteworthy, μ-SIIIA is a potent blocker of neuronal subtypes of sodium channels and exhibits analgesic activity in animal models of pain [34]. In addition, SIIIA has a known three-dimensional structure [35]; it is used here as the model peptide to compare various oxidation methods. The wider applicability of these folding methods is demonstrated with additional *Conus* peptides, μ-KIIIA and ω-GVIA, and these results are presented in the Supporting Information.

To describe the efficiency of the folding methods, we employed two terms: 'relative folding yield' (accumulation of the native species, relative to all folding species, determined from HPLC separations of the quenched folding mixtures) and 'apparent folding yield' (recovery of the native species, relative to the amount of linear form used in the folding reaction). As illustrated in Figure 1, relative folding yield is taken to mean the fraction of the integrated HPLC peak corresponding to the natively folded species, relative to the total integrated HPLC area corresponding to all folding species in a given HPLC chromatogram. This is the canonical measurement of refolding efficiency on the analytical scale. Apparent folding yield is the fraction of correctly folded peptide to peptide in the reaction, accomplished by comparison of the HPLC peak corresponding to the natively folded species in the reaction with the integral of the same peak of a known quantity of very pure (≥95% by analytical HPLC) reference folded peptide. In selenocysteine-containing peptides, the non-selenocysteine form was used for the calculation of apparent folding yield. Interestingly, these are quite distinct measurements of folding efficiency (Figure 1).

Both relative folding yield and apparent folding yield are useful measurements of folding efficiency; their difference lies in what is used as the theoretical value for 100% conversion. The canonical measurement (relative folding yield) takes this value as

Journal of PeptideScience

Table 1. Comparing oxidative folding yield for various cysteine-rich peptides

Peptide	Origin	Folding method	Yield (%)	Ref.
μ-SIIIA	*C. striatus*	Solution	18[a] ± 5[a]	10
μ-GIIIA	*C. geographus*	Solution	49.3 ± 3[a]	11
ω-MVIIC	*C. magus*	Solution[b]	8[a] ± 1[a]	11
ω-MVIIC	*C. magus*	ClearOx[b]	13[a] ± 2[a]	11
δ-PVIA	*C. purpurascens*	Solution[b]	2.1 ± 0.5[a]	12
δ-PVIA	*C. purpurascens*	Solution[c]	6.4 ± 0.8[a]	12
α-ImI	*C. imperialis*	Solution[d]	73 ± 4	13
α-GI	*C. geographus*	Solution	68	14
α-GI	*C. geographus*	Hydrocarbon resin	34	14
α-GI	*C. geographus*	ClearOx	80–88	14
μ-PIIIA	*C. purpurascens*	Solution	20 ± 1[a]	14
μ-PIIIA	*C. purpurascens*	Hydrocarbon resin	15 ± 1[a]	14
μ-PIIIA	*C. purpurascens*	ClearOx	32.5 ± 2.5[a]	14
Cn5	Scorpion	Resin-bound Media[e]	87	15
α-AahI	Scorpion	Solution	0.3%-2%	16
β-CssII	Scorpion	Solution	34.1	17
AAI	*A. hypocondriacus*	Solution	>95	18
Urotensin II	Human[f]	ClearOx	54	19

[a] These values are approximated from the graph provided in the publication.
[b] This is thermally optimized, but without folding additives, and folding additives improve folding yield.
[c] Tween-40 was used as a folding additive.
[d] Methanol was used as a folding additive.
[e] A special preparation of a ternary matrix (miniGroEL/DsbA/PPI/Agarose) was used to fold the peptide.
[f] Peptide is present in humans, but was originally discovered in teleost fish.

the integral of the entire folding area, hence assuming that all oxidation products are represented in the HPLC chromatogram (e.g. there is no aggregation). Conversely, apparent folding yield uses a separate HPLC chromatogram of the same peptide that has been folded, purified and quantified (e.g. by spectrophotometry), hence comparing with an amount of the folded species that would represent 100% conversion. Although neither is equivalent to the gold standard of a mass-to-mass calculation of yield, we use apparent folding yield throughout in order to meaningfully compare oxidation methods with different propensities for aggregation. Also of note, the proposed intermediate for the polymer-supported oxidation method that we employ is resin-bound, and hence is not present in the HPLC separation. Canonical folding yield data is available in the Supporting Information.

Experimental Procedures

Reduced peptides

Peptides were synthesized on a single-channel automated peptide synthesizer using Rink Amide resin and standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. All cysteine residues were trityl protected. Selenocysteine residues were 4-methoxybenzyl protected. Peptides without selenocysteine residues were cleaved from resin with Reagent K (TFA : phenol : water : thioanisole : 1,2-ethanedithiol; 33 : 2 : 2 : 2 : 1 by volume). Selenocysteine-containing peptides were cleaved from resin with enriched Reagent K[†] (TFA : phenol : water : thioanisole : DTNP; 178 : 14 : 5 : 1.6 : 1 by volume). DTNP is included in order to remove the *p*-methoxybenzyl protecting group from selenocysteine, which produces an adduct of selenocysteine with 2-thio-5-nitro-pyridine [36,37]. Cleaved peptides were filtered and precipitated with methyl *tert*-butyl ether. Selenocysteine-containing peptides were then treated with 50 mM DTT (in 0.1 M Tris, pH 7.5 with 0.1 mM EDTA) for 2 h, in order to remove the 2-thio-5-nitro-pyridine adduct. The diselenide-containing peptide is recovered following HPLC purification [38]. Peptides without selenocysteine were also purified by HPLC on a Waters 600 chromatograph with a dual-wavelength absorbance detector using Vydac C_{18} semipreparative and preparative columns. All SIIIA HPLCs (linear and folded) were run with a linear gradient from 4.5 to 31.5% acetonitrile, with 0.9% change per minute, maintaining 0.1% TFA throughout. Masses of the linear/diselenide peptides were validated by electrospray ionization mass spectrometry.

Solution-phase oxidation

Glutathione-based oxidation

Oxidative folding with glutathione-based redox buffers was accomplished with varying ratios of GSSG and GSH. Typical reaction conditions were 0.1 M Tris, 0.1 mM EDTA, 0.5–2 mM GSSG, 0–2 mM GSH and 20 µM μ-SIIIA (final concentration), pH 7.5. The pH dependence was investigated using MOPS in lieu of Tris/EDTA as the buffer (Supporting Information). The buffered glutathione redox system was first established, and the peptide was added as the final component. Reactions were set up in triplicate on an analytical scale (14 nmol of peptide), and were allowed to proceed for 16 h. The temperature dependence of solution-phase folding was evaluated at 4, 22 and 37 °C using a mixture of 1 mM GSSG, 1 mM GSH. In order to manipulate product ratios in a solution-phase oxidation reaction, folding temperature and time are commonly optimized in parallel. The effect of pH was tested using three buffer systems. Folding at acidic pH was assayed with 0.1 M sodium acetate/acetic acid buffer (pH 4); mid-range pH's were considered

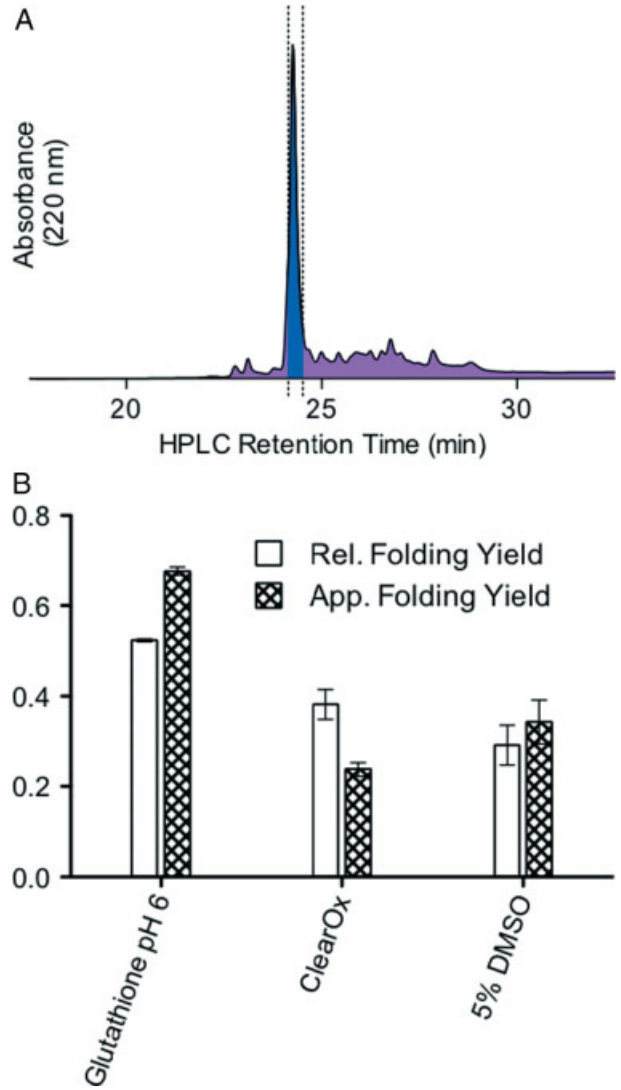


Figure 1. Methods for determining folding yields used in this study. (A) Sample analytical HPLC separation of a quenched SIIIA folding reaction. Relative folding yield is calculated by taking the peak area of the natively folded peak (blue), and dividing by the total area (sum of blue and purple). To calculate apparent folding yield, the peak area of a folded and purified sample (with the same total amount of peptide as the folding reaction) is used as a reference, representing 100% yield. The apparent folding yield is then the area of the peak in the folding reaction (blue) divided by the average area of reference peaks (mean of three runs). (B) A comparison between relative folding yield and apparent folding yield of selected conditions for oxidation of SIIIA.

with 0.1 M MOPS (pH 6–8); 0.1 M sodium carbonate/bicarbonate buffer (pH 10) was used to assess folding under basic conditions. Quenching by acidification was accomplished by the addition of formic acid to a final concentration of 8% (final pH ≈1.78).

Dimethyl sulfoxide mediated oxidation

Folding reactions contained 0.1 M Tris, pH 7.5, 0.1 mM EDTA, 5–10% dimethyl sulfoxide and 20 μM μ-SIIIA (final concentrations). Because the reaction begins when the peptide contacts DMSO, the buffered, dilute DMSO was prepared, and the peptide was added as the final component. Reactions were set up in triplicate on an analytical scale (14 nmol of peptide). After 16 h, formic acid was added to a final concentration of 8% to quench the folding

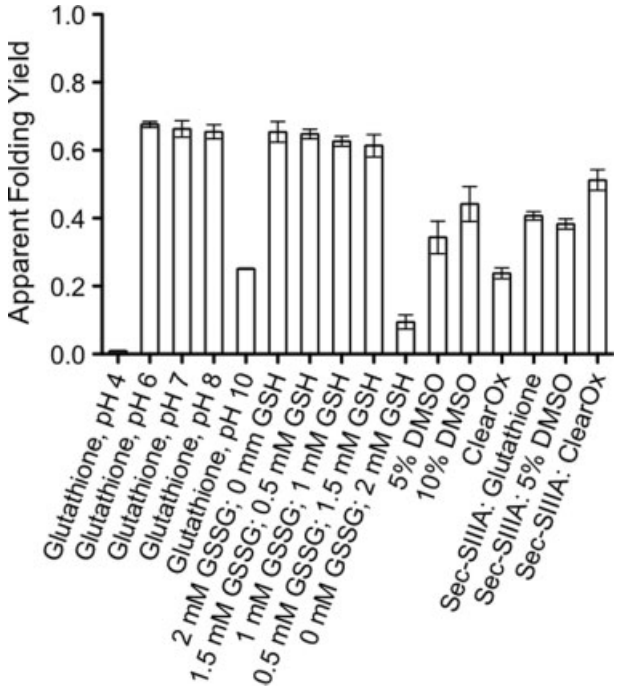


Figure 2. Apparent folding yield of SIIIA oxidation under various oxidation conditions. Error bars show standard deviation from three analytical HPLC separations. Unless otherwise stated, 'Glutathione' indicates 1 mM oxidized (GSSG) and 1 mM reduced (GSH) glutathione. All reactions with glutathione or DMSO proceeded for 16 h. ClearOx folding reactions were carried out with 12 equivalents of ClearOx resin and proceeded for 1 h. HPLC separations were monitored by measuring UV absorbance at 220 nm.

reaction (final pH ≈1.83). A final concentration of 10% dimethyl sulfoxide was found to be most effective to fold μ-SIIIA.

A summary of various folding conditions investigated and the resulting apparent folding yields is shown in Figure 2, with representative HPLC chromatograms of the folding reactions shown in Figure 3.

Polymer-supported oxidation

ClearOx is a commercially available resin that has been preloaded with Ellman's reagent (DTNB), immobilizing the peptide by reaction of the peptide thiolate with the on-resin disulfide, producing a peptide-resin disulfide intermediate [19]. Because ClearOx is preloaded, the low loading conditions are preestablished to favor intramolecular disulfide bonding at millimolar peptide concentrations (indicated by the apparent folding yield, shown in Figure 2).

ClearOx-based folding

Twelve molar equivalents of resin per disulfide (1 equiv. = 2.768 μg resin/nmol peptide/disulfide to be oxidized) were swelled in dichloromethane for 30 min. For folding 30 nmoles of SIIIA, 2.93 mg of ClearOx was employed. 20–30 mg of ClearOx was prepared in a single vessel, using 300–700 μl of each of the swelling and washing solvents. To begin washing, excess dichloromethane was pipetted off, and replaced with dimethylformamide. The same process was repeated with centrifugation to wash the resin with methanol, then 50% acetonitrile in water and finally 50% acetonitrile, 0.05 M MOPS, pH 7. To ensure that the resin remains swelled, the solution is never entirely removed, enforcing the low loading conditions that allow efficient folding while the solution is

Journal of PeptideScience

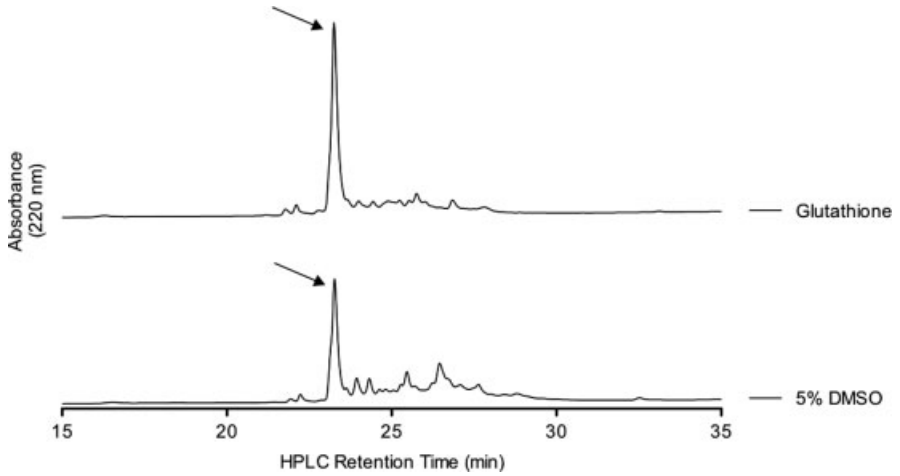


Figure 3. Representative HPLC traces of glutathione- and DMSO-assisted folding of SIIIA. Both folding reactions were carried out at pH 7.5 for 16 h at room temperature. Also, both HPLC separations were done with the same gradient, from 4.5 to 31.5% acetonitrile, with 0.9% change per minute, and maintaining 0.1% TFA throughout.

changed from dichloromethane to one that is more amenable to oxidative folding of peptides. The swelled and washed resin was then divided into separate vessels for each folding reaction. 50% acetonitrile, 0.05 M MOPS, pH 7.0 was used to redissolve μ-SIIIA to 3 mM. To minimize folding and aggregation due to air-mediated oxidation, the peptides were resuspended as shortly before use as possible. Following resin preparation, all excess wash solution was pipetted off and replaced with solution containing 3 mM μ-SIIIA. Reactions were done in triplicate on an analytical (30 nmol) scale. Folding reactions proceeded for 1 h in 50% acetonitrile, 50% 0.1 M MOPS, pH 7.0. Reactions were quenched by acidification, accomplished by 100-fold dilution with 0.1% TFA in water (final pH $\approx$2.17). Air oxidation and disulfide shuffling are possible unless the reaction is acidified; for preparative work, this can also be done by the addition of formic acid in order to maintain a small reaction volume. The peptide solution was then removed from the polymer-bound oxidant following centrifugation.

The amount of cosolvent present during ClearOx-based oxidative folding may affect both the relative folding yield and apparent folding yield; although this effect tends to vary based on the oxidation substrate, 50% acetonitrile is sufficiently generalizable to be a starting point for subsequent optimization. Optimization remains necessary, although the variables to be optimized are rather limited to the amounts of cosolvent and ClearOx resin in the folding reaction (see Supporting Information on folding of ω-conotoxin GVIA). Noteworthy, further optimization of ClearOx-assisted folding may involve changing to more acidic pH, as described previously [4,19].

Folding efficiency with ClearOx was found to be optimal at room temperature, although the folding time varies substantially among peptides (Tables S3–S5, Supporting Information). Due to generally shorter folding times and higher peptide concentrations (which provide for preferable acidification conditions), ClearOx has significant potential for higher throughput peptide folding.

Selenocysteine

Selenocysteine residues have recently been shown to simplify oxidative folding and facilitate disulfide mapping in peptides [5,10,38–42]. The replacement of both cysteines of a single disulfide bridge with selenocysteines allows one bridge to be formed directly after deprotection and treatment with DTT [38]. The strong reduction potential of selenium prevails over encoded sequence information [40], and consequently, the preformed bridge reduces the number of possible disulfide isomers. The preformed bridge serves as both a combinatorial constraint with respect to the number of possible folding isomers as well as an entropic constraint in the oxidative folding process, both of which benefit the folding efficiency. The combinatorial effect remains constant; however, the entropic effect depends on the location of the diselenide bridge [10,38]. The use of diselenide bridges has been shown to complement solution-phase oxidative folding [10,38,39]. As replacement of the first disulfide of μ-SIIIA with a diselenide gave the largest increase in relative folding yield [10], this substrate, namely [C3U;C13U] SIIIA (Sec-SIIIA) was used in the folding experiments.

Folding of selenocysteine-containing peptides

In solution, selenocysteine-containing peptides were folded under the same conditions as the same peptide without the diselenide. Briefly, folding reactions contained 0.1 M Tris, pH 7.5, 0.1 mM EDTA, 20 μM peptide, and either 1 mM each of GSSG and GSH or 5–10% DMSO. With ClearOx, the resin preparation was identical; however, the amount of resin used was decreased in order to maintain 12 equivalents of resin per disulfide bond to be closed in the folding reaction. Apparent folding yield calculations of selenocysteine-containing peptides were accomplished by normalizing to a known quantity of the folded form of the same peptide without the diselenide proxy. Figure 4 shows the effect of the diselenide, with analytical HPLC traces of parallel-foldings of SIIIA with Sec-SIIIA, both at 3 mM peptide concentration using ClearOx resin as the oxidant. The effect on folding efficiency is summarized in Figures 2 and S1, Supporting Information. When combined with a similar observation that the ClearOx-assisted oxidation of a selenopeptide analog of GVIA (Table S5 and Figure S5) also produced relatively high folding yields, these results suggest that the above strategy could be considered as a more generalized folding protocol.

Summary and Limitations

Optimized solution-phase folding often produces the highest reaction yields; however, there are intrinsic costs to oxidative

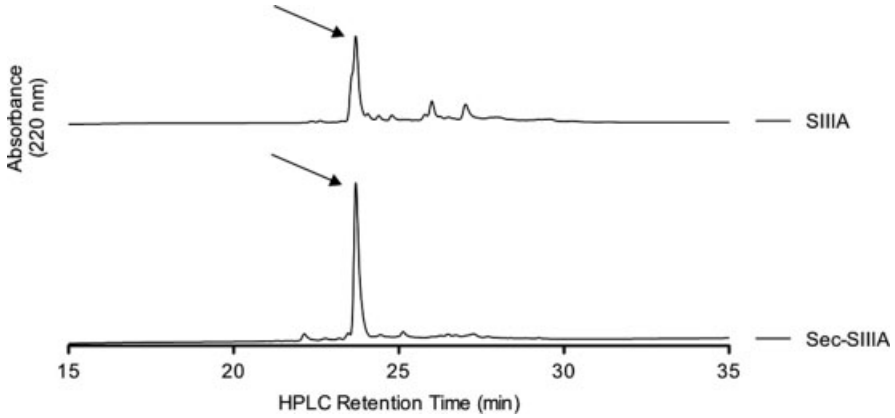


Figure 4. Analytical HPLC traces of SIIIA (top) and Sec-SIIIA (bottom) foldings with ClearOx. Arrows indicate the native species. Both reactions were carried out at 3 mM peptide using 12 equiv. of ClearOx in 50% acetonitrile in 0.1 M MOPS, pH 7.0 for 1 h, and were quenched by 100-fold dilution with 0.1% TFA.

folding in the solution phase. The requirement for high dilution conditions presents problems for large-scale and high-throughput applications. However, with relatively minor costs, these problems may largely be overcome using resin-bound oxidants, such as ClearOx. For example, to oxidize 1 g of μ-SIIIA using solution phase methods would require a reaction volume of 22.7 l. However, to oxidize 1 g of μ-SIIIA using ClearOx would require a reaction volume of only 151 ml. The optimization of folding time with ClearOx is significantly more reliable when separate reactions for each timepoint are carried out because swelled ClearOx resin is not readily pipetted.

Selenocysteine proxies for disulfides can significantly increase the relative folding yield, and simplify the HPLC folding pattern, allowing for easier purification. Although the apparent folding yield for some peptides is not necessarily significantly increased with a diselenide proxy (Figures 2 and S1), the benefit to apparent folding yield for other selenopeptides can be quite substantial (Figure S5 and Table S5). We suggest that ClearOx-assisted oxidation of selenopeptides (Figure 2 and S5) should be considered for higher throughput folding of cysteine-rich peptides.

Given the unprecedented molecular diversity of cysteine-rich peptides with respect to both disulfide scaffolds and primary amino acid sequences, a word of caution about the generality of the recommended folding methods should be made. The selection of oxidation method and extent of optimization of the reaction conditions will depend on the peptides' structure, quantities required for subsequent studies, as well as on the purpose of studying the peptides. When smaller quantities ($<$1–2 mg) of a peptide are needed, it is recommended to start with optimization of the solution-phase folding methods. For folding larger quantities of a peptide, the resin-bound oxidants should be considered. For folding difficult peptides, or for higher throughput oxidative folding of multiple peptides or combinatorial libraries, the use of selenopeptide analogs and resin-bound oxidants is suggested.

Acknowledgements

This work was supported by N.I.H. Program Project Grant GM 48677 (to G.B.), and the Willard Eccles Fellowship Fund Predoctoral Fellowship (to A.M.S.). We thank Drs. Robert Shackmann and Scott Endicott from the DNA/Peptide Synthesis Core Facility at the University of Utah for their help in peptide synthesis. We also thank Peptides International, Inc. for kindly providing us with ClearOx™ resin (ClearOx™ is a trademark of Peptides International, Inc.).

Supporting information

Supporting information may be found in the online version of this article.

References

1 Annis I, Hargittai B, Barany G. Disulfide bond formation in peptides. *Methods Enzymol.* 1997; **289**: 198–221. DOI: 10.1016/S0076-6879(97)89049-0.
2 Bulaj G, Walewska A. Oxidative folding of single-stranded disulfide-rich peptides. In *Oxidative Folding of Peptides and Proteins*, Buchner J, Moroder L (Eds). RSC Biomolecular Sciences: Cambridge, UK 2009; 279–286. DOI: 10.1039/9781847559265.
3 Bulaj G, Olivera BM. Folding of conotoxins: formation of the native disulfide bridges during chemical synthesis and biosynthesis of Conus peptides. *Antioxid. Redox Signal.* 2008; **10**(1): 141–155. DOI: 10.1089/ars.2007.1856.
4 Zhang J, Diamond S, Arvedson T, Sasu BJ, Miranda LP. Oxidative folding of hepcidin at acidic pH. *Biopolymers* 2010; **94**(2): 257–264. DOI: 10.1002/bip.21383.
5 Muttenthaler M, Nevin ST, Grishin AA, Ngo ST, Choy PT, Daly NL, Hu SH, Armishaw CJ, Wang CI, Lewis RJ, Martin JL, Noakes PG, Craik DJ, Adams DJ, Alewood PF. Solving the α-conotoxin folding problem: efficient selenium-directed on-resin generation of more potent and stable nicotinic acetylcholine receptor antagonists. *J. Am. Chem. Soc.* 2010; **132**(10): 3514–3522. DOI: 10.1021/ja910602h.
6 Armishaw CJ, Alewood PF. Conotoxins as research tools and drug leads. *Curr. Protein Pept. Sci.* 2005; **6**(3): 221–240. DOI: 10.2174/1389203054065437.
7 Han TS, Teichert RW, Olivera BM, Bulaj G. Conus venoms - a rich source of peptide-based therapeutics. *Curr. Pharm. Des.* 2008; **14**(24): 2462–2479. DOI: 10.2174/138161208785777469.
8 Livett BG, Gayler KR, Khalil Z. Drugs from the sea: conopeptides as potential therapeutics. *Curr. Med. Chem.* 2004; **11**(13): 1715–1723. DOI: 10.2174/0929867043364928.
9 Halai R, Craik DJ. Conotoxins: natural product drug leads. *Nat. Prod. Rep.* 2009; **26**(4): 526–536. DOI: 10.1039/b819311h.
10 Walewska A, Zhang MM, Skalicky JJ, Yoshikami D, Olivera BM, Bulaj G. Integrated oxidative folding of cysteine/selenocysteine containing peptides: improving chemical synthesis of conotoxins. *Angew. Chem. Int. Ed. Engl.* 2009; **48**(12): 2221–2224. DOI: 10.1002/anie.200806085.
11 Lopez-Vera E, Walewska A, Skalicky JJ, Olivera BM, Bulaj G. Role of hydroxyprolines in the *in vitro* oxidative folding and biological activity of conotoxins. *Biochemistry* 2008; **47**(6): 1741–1751. DOI: 10.1021/bi701934m.

12 Buczek P, Buczek O, Bulaj G. Total chemical synthesis and oxidative folding of δ-conotoxin PVIA containing an N-terminal propeptide. *Biopolymers* 2005; **80**(1): 50–57. DOI: 10.1002/bip.20211.
13 Nielsen JS, Buczek P, Bulaj G. Cosolvent-assisted oxidative folding of a bicyclic α-conotoxin ImI. *J. Pept. Sci.* 2004; **10**(5): 249–256. DOI: 10.1002/psc.531.
14 Green BR, Bulaj G. Oxidative folding of conotoxins in immobilized systems. *Protein Pept. Lett.* 2006; **13**(1): 67–70. DOI: 10.2174/092986606774502162.
15 Altamirano MM, García C, Possani LD, Fersht AR. Oxidative refolding chromatography: folding of the scorpion toxin Cn5. *Nat. Biotechnol.* 1999; **17**(2): 187–191. DOI: 10.1038/6192.
16 M'Barek S, Fajloun Z, Cestèle S, Devaux C, Mansuelle P, Mosbah A, Jouirou B, Mantegazza M, Van Rietschoten J, El Ayeb M, Rochat H, Sabatier JM, Sampieri F. First chemical synthesis of a scorpion α-toxin affecting sodium channels: the Aah I toxin of *Androctonus australis hector*. *J. Pept. Sci.* 2004; **10**(11): 666–677. DOI: 10.1002/psc.582.
17 Estrada G, Garcia BI, Schiavon E, Ortiz E, Cestele S, Wanke E, Possani LD, Corzo G. Four disulfide-bridged scorpion beta neurotoxin CssII: heterologous expression and proper folding *in vitro*. *Biochim. Biophys. Acta* 2007; **1770**(8): 1161–1168. DOI: 10.1016/j.bbagen.2007.04.006.
18 Čemažar M, Zahariev S, Lopez JJ, Carugo O, Jones JA, Hore PJ, Pongor S. Oxidative folding intermediates with nonnative disulfide bridges between adjacent cysteine residues. *Proc. Natl. Acad. Sci. U.S.A.* 2003; **100**(10): 5754–5759. DOI: 10.1073/pnas.2225470100.
19 Darlak K, Wiegandt Long D, Czerwinski A, Darlak M, Valenzuela F, Spatola AF, Barany G. Facile preparation of disulfide-bridged peptides using the polymer-supported oxidant CLEAR-OX™. *J. Pept. Res.* 2004; **63**(3): 303–312. DOI: 10.1111/j.1399-3011.2004.00153.x.
20 Bach RD, Dmitrenko O, Thorpe C. Mechanism of thiolate-disulfide interchange reactions in biochemistry. *J. Org. Chem.* 2008; **73**(1): 12–21. DOI: 10.1021/jo702051f.
21 Snow J, Finley J, Friedman M. Oxidation of sulfhydryl groups to disulfides by sulfoxides. *Biochem. Biophys. Res. Comm.* 1975; **64**(1): 441–447. DOI: 10.1016/0006-291X(75)90272-7.
22 Bulaj G, Kortemme T, Goldenberg DP. Ionization-reactivity relationships for cysteine thiols in polypeptides. *Biochemistry* 1998; **37**(25): 8965–8972. DOI: 10.1021/bi973101r.
23 Kubo S, Chino N, Kimura T, Sakakibara S. Oxidative folding of ω-conotoxin MVIIC: effects of temperature and salt. *Biopolymers* 1996; **38**(6): 733–744. DOI: 10.1002/(SICI)1097-0282(199606)38:6<733::AID-BIP5>3.0.CO;2-S.
24 DeLa Cruz R, Whitby FG, Buczek O, Bulaj G. Detergent-assisted oxidative folding of δ-conotoxins. *J. Pept. Res.* 2003; **61**(4): 202–212. DOI: 10.1034/j.1399-3011.2003.t01-1-00048.x.
25 Miloslavina AA, Leipold E, Kijas M, Stark A, Heinemann SH, Imhof D. A room temperature ionic liquid as convenient solvent for the oxidative folding of conopeptides. *J. Pept. Sci.* 2009; **15**(2): 72–77. DOI: 10.1002/psc.1106.
26 Wang CZ, Zhang H, Jiang H, Lu W, Zhao ZQ, Chi CW. A novel conotoxin from Conus striatus, μ-SIIIA, selectively blocking rat tetrodotoxin-resistant sodium channels. *Toxicon* 2006; **47**(1): 122–132. DOI: 10.1016/j.toxicon.2005.10.008.
27 Bulaj G, West PJ, Garrett JE, Watkins M, Marsh M, Zhang MM, Norton RS, Smith BJ, Yoshikami D, Olivera BM. Novel conotoxins from *Conus striatus* and *Conus kinoshitai* selectively block TTX-resistant sodium channels. *Biochemistry* 2005; **44**(19): 7259–7265. DOI: 10.1021/bi0473408.
28 Woycechowsky KJ, Wittrup KD, Raines RT. A small-molecule catalyst of protein folding in vitro and in vivo. *Chem. Biol.* 1999; **6**(12): 871–879. DOI: 10.1016/S1074-5521(00)80006-X.
29 Beld J, Woycechowsky K, Hilvert D. Catalysis of oxidative protein folding by small-molecule diselenides. *Biochemistry* 2008; **47**(27): 6985–6987. DOI: 10.1021/bi8008906.
30 Saxena VP, Wetlaufer DB. Formation of three-dimensional structure in proteins. I. Rapid nonenzymic reactivation of reduced lysozyme. *Biochemistry* 1970; **9**(25): 5015–5023. DOI: 10.1021/bi00827a028.
31 Hwang C, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 1992; **257**(5076): 1496–1502. DOI: 10.1126/science.1523409.
32 Galande AK, Weissleder R, Tung CH. An effective method of on-resin disulfide bond formation in peptides. *J. Comb. Chem.* 2005; **7**(2): 174–177. DOI: 10.1021/cc049839r.
33 Galanis AS, Albericio F, Grøtli M. Enhanced microwave-assisted method for on-bead disulfide bond formation: synthesis of α-conotoxin MII. *Biopolymers* 2009; **92**(1): 23–34. DOI: 10.1002/bip.21116.
34 Green BR, Catlin P, Zhang MM, Fiedler B, Bayudan W, Morrison A, Norton RS, Smith BJ, Yoshikami D, Olivera BM, Bulaj G. Conotoxins containing nonnatural backbone spacers: cladistic-based design, chemical synthesis, and improved analgesic activity. *Chem. Biol.* 2007; **14**(4): 399–407. DOI: 10.1016/j.chembiol.2007.02.009.
35 Yao S, Zhang MM, Yoshikami D, Azam L, Olivera BM, Bulaj G, Norton RS. Structure, dynamics, and selectivity of the sodium channel blocker mu-conotoxin SIIIA. *Biochemistry* 2008; **47**(41): 10940–10949. DOI: 10.1021/bi801010u.
36 Harris KM, Flemer S, Hondal RJ. Studies on deprotection of cysteine and selenocysteine side-chain protecting groups. *J. Pept. Sci.* 2007; **13**(2): 81–93. DOI: 10.1002/psc.795.
37 Schroll AL, Hondal RJ. Further development of new deprotection chemistry for cysteine and selenocysteine side chain protecting groups. *Adv. Exp. Med. Biol.* 2009; **611**: 135–136.
38 Gowd K, Yarotskyy V, Elmslie K, Skalicky J, Olivera B, Bulaj G. Site-Specific effects of diselenide bridges on the oxidative folding of a cystine knot peptide, ω-selenoconotoxin GVIA. *Biochemistry* 2010; **49**(12): 2741–2752. DOI: 10.1021/bi902137c.
39 Armishaw CJ, Daly NL, Nevin ST, Adams DJ, Craik DJ, Alewood PF. α-selenoconotoxins, a new class of potent α_7 neuronal nicotinic receptor antagonists. *J. Biol. Chem.* 2006; **281**(20): 14136–14143. DOI: 10.1074/jbc.M512419200.
40 Fiori S, Pegoraro S, Rudolph-Böhner S, Cramer J, Moroder L. Synthesis and conformational analysis of apamin analogues with natural and non-natural cystine/selenocystine connectivities. *Biopolymers* 2000; **53**(7): 550–564. DOI: 10.1002/(SICI)1097-0282(200006)53:7<550::AID-BIP3>3.0.CO;2-O.
41 Han T, Zhang M, Gowd K, Walewska A, Yoshikami D, Olivera B, Bulaj G. Disulfide-Depleted Selenoconopeptides: Simplified Oxidative Folding of Cysteine-Rich Peptides. *ACS Med. Chem. Lett.* 2010; **1**(4): 140–144. DOI: 10.1021/ml900017q.
42 Mobli M, de Araújo AD, Lambert LK, Pierens GK, Windley MJ, Nicholson GM, Alewood PF, King GF. Direct visualization of disulfide bonds through diselenide proxies using ^{77}Se NMR spectroscopy. *Angew. Chem. Int. Ed. Engl.* 2009; **48**(49): 9312–9314. DOI: 10.1002/anie.200905206.

