Journal of Peptide Science
J. Peptide Sci. **10**: 249–256 (2004)
Published online 12 November 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.531

JOURNAL OF PEPTIDE SCIENCE

Cosolvent-Assisted Oxidative Folding of a Bicyclic α-Conotoxin ImI

JACOB S. NIELSEN, PAWEL BUCZEK and GRZEGORZ BULAJ*

Cognetix, Inc., 421 Wakara Way, Suite 201, Salt Lake City, Utah 84108, USA

Received 20 February 2003
Accepted 26 June 2003

Abstract: α-Conotoxin ImI is a 12-amino acid peptide, found in the venom of the marine snail *Conus imperialis*. This conotoxin is a selective antagonist of α7 nicotinic acetylcholine receptors. To produce biologically active α-ImI, disulfide bonds must be formed between Cys2–Cys8 and Cys3–Cys12. Oxidative folding of bicyclic conotoxins, such as α-ImI, has been traditionally achieved using two-step oxidation protocols with orthogonal protection on two native pairs of cysteines. In this work, two alternative oxidation protocols were explored: (1) the recently described one-pot oxidation of t-butyl/4-methylbenzyl protected Cys pairs and (2) direct oxidative folding. In contrast to the first method, the latter one resulted in high yields of correctly folded α-ImI. The addition of organic cosolvents, such as methanol, ethanol or isopropanol into the folding mixture significantly increased the accumulation of the native peptide. This effect was also observed for another conotoxin, α-PnIA. It is suggested that cosolvent-assisted direct oxidation might be of general use for other bicyclic α-conotoxins, but efficiency should be assessed on a case-by-case basis.

Keywords: conotoxin; cosolvent-assisted folding; oxidative folding; disulfide bond formation

INTRODUCTION

α-Conotoxins comprise a large group of bicyclic peptide toxins targeting nicotinic receptors [1,2]. These peptides contain four cysteine residues, arranged in the characteristic pattern: CC—C—C (Table 1). The three dimensional structure of bioactive α-conotoxins is stabilized by two overlapping native disulfide bonds [3,4]. Because of their small size, typically ranging from 10 to 15 amino acids, α-conotoxins are amenable to solid-phase peptide synthesis. Relatively low yields of cyclization preclude the efficient production of the synthetic bioactive peptides. Since the first regioselective synthesis of α-GI [4], multiple synthetic strategies have been tested to produce α-conotoxins [5–8]. The Barany group explored two-step regioselective strategies for producing α-SI using different orthogonal protection schemes. The one-step strategy with immobilized Ellman's reagent and on-resin cyclization were also tested. Recently, a novel one-pot oxidation method was described for α-conotoxin SI [9]. This regioselective strategy involved simultaneous cleavage and oxidation of the first bridge, followed by subsequent deprotection/oxidation of the second bridge at elevated temperature.

α-ImI is one of the best characterized members of the α-conotoxin family [10–20]. This peptide is a selective antagonist of α7 nicotinic acetylcholine receptors (nAChRs) with an affinity in

Abbreviations: ACN, acetonitrile; Acm, acetamidomethyl; DMSO dimethyl sulfoxide; 4-MeBz, 4-methylbenzyl; MTBE methyl *tert*-butyl ether; t-Bu, *tert*-butyl; TFA, trifluoroacetic acid; nAChRs, nicotinic acetylcholine receptors; Tris, tris-[hydroxymethyl] aminomethane; Trt, trityl

* Correspondence to: Dr Grzegorz Bulaj, Cognetix, Inc., 421 Wakara Way, Suite 201, Salt Lake City, Utah 84108, USA;
e-mail: gbulaj@cognetix.com
Contract/grant sponsor: National Institute of Health; Contract/grant number: AG18662-01.

Table 1 Structure of α-Conotoxins Targeting Nicotinic Acetylcholine Receptors (nAChRs)

Conotoxin	Source	Sequence	Target nAChR
α-ImI	*C. imperialis*	G**CC**SDPR**C**AWR**C**[a]	neuronal α7
α-GI	*C. geographus*	E**CC**NPA**C**GRHYS**C**[a]	skeletal muscle
α-SI	*C. striatus*	I**CC**NPA**C**GPKYS**C**[a]	skeletal muscle

[a] *C*-terminal α-carboxyl group is amidated.

the submicromolar range. α-ImI competes with α-bungarotoxin for the α7 receptor. Interestingly, the α-bungarotoxin-sensitive α7 nAChRs have been recently shown to be a target of the β-amyloid peptide, the binding of which causes neurotoxicity in Alzheimer's patients [21,22]. Since the α7 nAChRs may be therapeutic targets for neuroprotection against amyloid-induced neurotoxicity, there is a need to develop highly selective and potent ligands for these receptors. α-ImI-based analogs may be a good source of such ligands. Recently, a toxin similar to α-ImI, α-ImII, was discovered from the cone snail *Conus imperialis* [23]. Despite the sequence similarity between the two conotoxins, α-ImII targets a different binding site on the α7 nAChRs. This example illustrates the potential of developing ImI-based selective ligands for the α7 receptors.

To produce synthetic α-ImI, a one-pot regioselective oxidation was tested, as described by Cuthbertson and Indrevoll [9] and a direct oxidative folding, where all four cysteines were allowed to form disulfide bridges simultaneously. Direct oxidative folding of α-ImI was found to be a very effective strategy. In contrast, the one-pot regioselective method did not yield detectable amounts of the native α-ImI, even though this method was effective in producing other α-conotoxins such as α-GI (our work) or α-SI [9]. The direct oxidative folding may be an efficient alternative strategy for producing native bicyclic conotoxins.

MATERIALS AND METHODS

Solid-phase Peptide Synthesis

Peptides were synthesized on an Advanced Chemtech 357 FBS peptide synthesizer using amide MBHA Rink resin and standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. Side chain protection of non-Cys residues was in the form of t-Bu (Ser, Asp), Pmc-6-sulfonyl (Arg) and t-Boc (Trp). Protection of Cys residues for the one-pot/two step method was as follows: Cys2 and Cys8 were protected as Cys(t-Bu), while Cys3 and Cys12 were protected as Cys(4-MeBz). Protection of Cys residues for the two-step folding was as follows: Cys2 and Cys8 were protected as Cys(Trt), while Cys3 and Cys12 were protected as Cys(Acm). For the direct oxidation protocol, all four Cys residues were trityl protected. The peptides were removed from a solid support by 3 h treatment with reagent K (TFA/water/ethanedithiol/phenol/thioanisole; 90/5/2.5/7.5/5 by volume). The cleaved peptides were filtered, precipitated with MTBE, pre-cooled at $-20\,^{\circ}C$, and washed several times with cold MTBE. The linear peptides were purified by reversed-phase HPLC using a preparative C18 Vydac column (catalogue# 218TP1022, 10 mm $\times$ 250 mm) in a linear gradient from the initial 70% solvent A and 30% solvent B to the final 50% solvent A and 50% solvent B in 40 min, where solvent A = H_2O, 0.1% TFA, solvent B = 90% ACN in H_2O/0.1% TFA. The flow rate was 15 ml/min. The separations were monitored by UV absorbance at 220 nm.

One-pot Oxidation of t-Butyl/4-Methylbenzyl Protected Cysteines

Cys-protected peptide (4 mg) was treated with 8 ml of a mixture of TFA/DMSO/anisole; 97.9/2/0.1 by volume. The mixture was stirred at room temperature for 40 min. An additional 160 µl of the TFA/DMSO/anisole mixture was added, and the flask was then placed in an oil bath and heated at $70\,^{\circ}C$ for 3 h. TFA was removed by N_2 stream and the peptide was precipitated by addition of MTBE. The precipitate was analysed by analytical reversed-phase HPLC using a C18 Vydac column (catalogue 218TP54, 4.6 mm $\times$ 250 mm) in a linear gradient from the initial 90% solvent A and 10% solvent B to the final 40% solvent A and 60% solvent B in 25 min. The flow rate was 1 ml/min and the elution was monitored by UV absorbance at 220 nm.

Two-step Oxidation of S-Trityl/S-Acm Protected Cysteines

To produce α-ImI with the native Cys connectivity (Cys2-Cys8 and Cys3-Cys12), 7.3 mg of the linear α-ImI (Cys3 and Cys12 with Acm protection) was dissolved in 25 ml of 10% ACN in H_2O. The peptide solution was added slowly to 25 ml solution of a solution of 10 mM $K_3[Fe(CN)_6]$, 0.1 M Tris base, pH adjusted to 7.6 with acetic acid. The solution was mixed for 1.5 h at room temperature. The peptide was removed from the folding mixture by solid-phase extraction using Strata C18-E cartridges (50 μm) from Phenomenex. The monocyclic peptide was treated with 1 mM iodine in 50% ACN in H_2O to remove the Acm protecting groups from the second pair of cysteines and concurrently oxidize the second disulfide bridge. The identity of the final product was confirmed by MALDI-TOF analysis (observed MH^+ (monoisotopic) 1351.6, calculated 1351.5). Other peptides used as reference standards ('misfolded' α-ImI with the Cys connectivity Cys2-Cys12 and Cys3-Cys8, α-GI, α-PnIA) were also folded in the same manner as the native α-ImI and their identities were confirmed by MALDI-TOF analysis.

Direct Oxidative Folding

Folding reactions were carried out in buffered solutions (0.1 M Tris-HCl, 1 mM EDTA, pH 8.7) containing an appropriate concentration of the linear peptide and a mixture of 2 mM reduced and 1 mM oxidized glutathione. The analytical folding reactions were initiated by adding 20 μl of concentrated linear peptide (200–500 μM in H_2O, 0.01% TFA) into 0.2 ml of folding mixture. The final peptide concentration ranged from 20 to 200 μM, but 20 μM was used for screening various folding conditions. After an appropriate time, aliquots were withdrawn and the reaction was quenched by acidification with 20 μl of formic acid. For samples with a high concentration of organic cosolvents (>40% vol/vol), the quenched folding reactions were dried in a speed-vac and then resuspended in 0.1% TFA in H_2O. The reaction mixtures were analysed by analytical reversed-phase HPLC separations using a Vydac C18 column (4.6 mm × 250 mm). The following separation conditions were used for α-ImI: a linear gradient from the initial 90% solvent A and 10% solvent B to the final 65% solvent A and 35% solvent B in 10 min. For α-GI, a linear gradient was from the initial 90% solvent A and 10% solvent B to the final 70% solvent A and 30% solvent B in 20 min. For α-PnIA, a linear gradient was from the initial 85% solvent A and 15% solvent B to the final 60% solvent A and 40% solvent B in 15 min. In each case, the flow rate was 1 ml/min and the HPLC eluent was monitored at 220 nm. The folding yields were determined as a ratio of the steady-state accumulation of the native form, relative to all folding species, calculated from the integrated peak areas.

RESULTS

To produce a bicyclic α-ImI, two oxidative folding strategies were tested: (1) the one-pot oxidation of t-butyl/4-methylbenzyl protected Cys pairs and (2) direct oxidative folding. For each strategy, linear peptides were synthesized on solid support using standard Fmoc protocols. In order to monitor folding reactions by HPLC, two reference isoforms were prepared: (1) α-ImI with the native Cys connectivity Cys2–Cys8 and Cys3–Cys12 [2–8, 3–12] and (2) the misfolded form with the Cys connectivity Cys2–Cys12 and Cys3–Cys8 [2–12, 3–8]. These two isoforms were produced using a standard two-step oxidation protocol with Trt/Acm protected Cys pairs. The first pair of Cys was oxidized with $K_3[Fe(CN)_6]$ and the second pair was oxidized with iodine. The third possible isoform was unlikely to accumulate in significant quantities, since it contained an unfavorable vicinal disulfide bond between Cys2 and Cys3 [24].

Oxidation of α-ImI Containing t-Butyl/4-Methylbenzyl Protected Cysteines

First, the folding protocol as described by Cuthbertson and Indrevoll [9] was tested. Our procedure included purification of the linear form by reversed-phase HPLC prior to the first oxidation step (Figure 1A). The identity of the purified peptide was confirmed by MALDI-TOF mass spectrometry. After a 40 min incubation in the TFA/DMSO/anisole mixture at room temperature, the reaction mixture was heated for 3 h at 70 °C. The final oxidation products were precipitated with cold MTBE and analysed by C18 reversed-phase HPLC, as shown in Figure 1B. Since none of the chromatographic peaks corresponded to the properly folded α-ImI, the effects of various oxidation times on the final distribution of folding products were examined. The first oxidation step ranged from 10 to 60 min, followed by a standard 3 h oxidation at a higher temperature

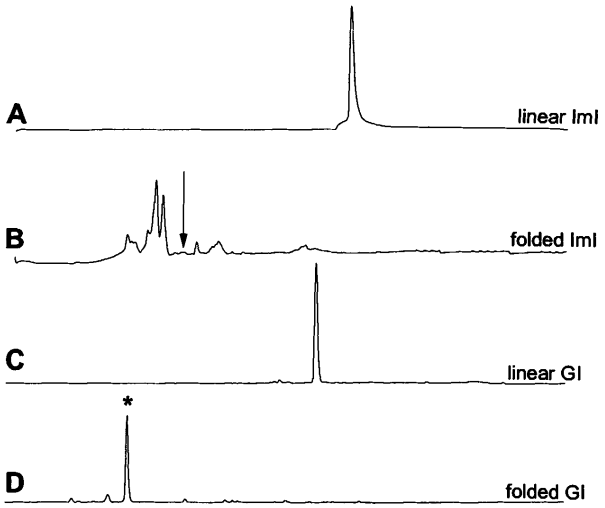

Figure 1 HPLC analysis of the one-pot regioselective folding of α-ImI and α-GI. **A**. The linear form of α-ImI used for the one-pot folding. **B**. Final folding products of α-ImI, arrow indicates a retention time corresponding to the native α-ImI. **C**. The linear form of α-GI used for the one-pot reaction. **D**. Folding reaction products, star indicates the correctly folded α-GI.

(70 °C). Various durations for the second oxidation step ranging from 1 to 6 h (with 40 min for the first oxidation) were tested. However, none of the combinations resulted in a substantial accumulation of the properly folded α-ImI. To verify an effectiveness of the one-pot oxidation method, α-GI was synthesized and oxidized with a final yield of 36% using the identical protocol as that for α-ImI and α-SI [9] (40 min at room temperature, 4 h at 70 °C). As shown in Figure 1C and D, a major oxidation product appeared to correspond to the properly folded α-GI.

Direct Oxidative Folding

The second strategy involved a direct oxidation of α-ImI, where all S-Trt protection groups were removed during a cleavage of the peptide. Folding reactions were carried out in the presence of reduced and oxidized glutathione in the buffered solutions, pH 8.7. The reactions were quenched by acidification and separated by analytical C18 reversed-phase HPLC. As illustrated in Figure 2A, oxidative folding of the fully unprotected α-ImI resulted in the equal accumulation of two major products (each accounted for approximately 40% of the total products). The HPLC coelution experiments identified these products as: (1) correctly folded

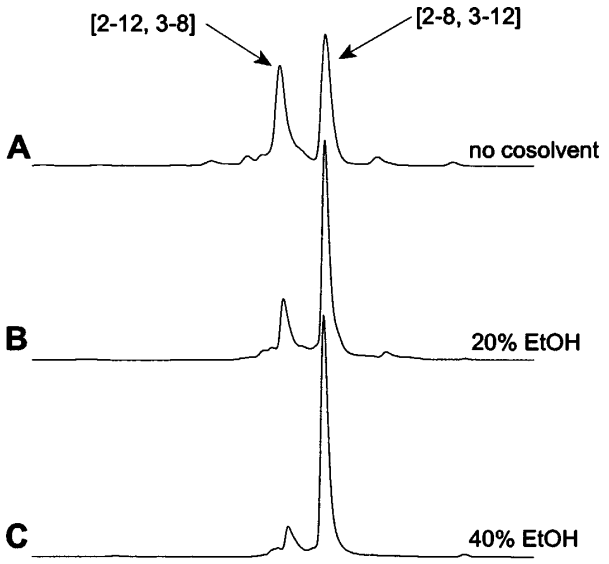

Figure 2 Direct oxidative folding of α-ImI in the presence of 1 mM oxidized and 2 mM reduced glutathione, pH 8.7 and 25 °C. The reaction was quenched after 2 h by acidification and the folding mixture was separated on an analytical C18 HPLC column. **A**. No cosolvents in the folding mixture. **B** and **C**. In the presence of 20% and 40% ethanol, respectively.

species [2–8, 3–12] and (2) the misfolded form [2–12, 3–8].

To explore factors that could increase accumulation of the correctly folded form, a limited folding screen was used. This empirical-based screen included testing several folding conditions such as high ionic strength, low temperature, or folding additives, which previously were shown to improve folding yields for other conotoxins [25–27]. Similar 'random' folding screens were successfully applied to improve folding of other polypeptides [28,29]. Figure 3 summarizes the effects of these factors on the accumulation of the native form. Neither lower temperature, higher ionic strength, nor the presence of a chaotropic agent changed the overall distribution of folding products. Only 40% glycerol or 20% ethanol significantly shifted the equilibrium toward the native α-ImI (Figure 2B). The effects of various organic cosolvents and their concentrations on the accumulation of the native α-ImI was further examined, as illustrated in Figure 4. The addition of 20% or 60% methanol changed the folding equilibrium in a concentration-dependent manner ($p = 0.0003$), but no significant differences were observed for ethanol- or isopropanol-assisted folding at concentrations above 20%. The cosolvent-assisted folding was not significantly affected by changes of the

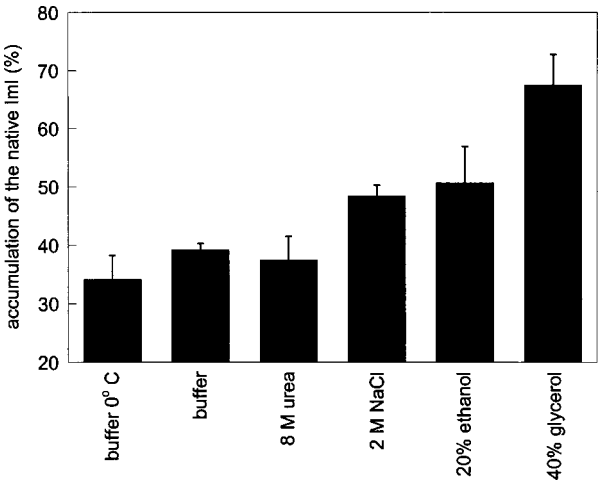

Figure 3 Effects of various folding conditions on the accumulation of the native α-ImI. The bars represent the folding yields, expressed as the accumulation of the native α-ImI, relative to the other folding species. The folding reactions were carried out in the presence of 1 mM oxidized and 2 mM reduced glutathione, pH 8.7 and reached equilibrium after 2 h. The reactions were quenched by acidification and concentrations of folding species were determined by HPLC. The average values (bars) and standard errors (error bars) were calculated from three experiments ($p < 0.05$ for 2 M NaCl and 20% ethanol, $p < 0.001$ for 40% glycerol).

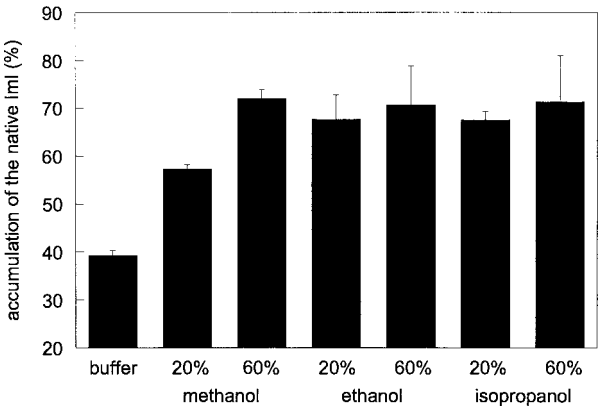

Figure 4 Effects of organic cosolvent on the accumulation of the native α-ImI. The bars represent the folding yields determined by HPLC analysis, as shown in Figure 3. Statistically significant differences were only found between 20% and 60% methanol ($p < 0.0005$). The average values (bars) and standard errors (error bars) were calculated from three experiments.

temperature within the range from 0° to 25 °C, as illustrated in Table 2. Only at the lowest (−20 °C) and the highest (+45 °C) temperatures, was accumulation of the native form slightly reduced. For practical reasons, 20% ethanol was used as a folding additive, since no significant dilutions were required for purification of the folded peptide using reversed-phase HPLC methods.

Table 2 Effect of Temperature on the Cosolvent-assisted Folding of α-ImI. The Values Represent the Accumulation of the Native α-ImI, Relative to the Other Folding Species. The Average Values and Standard Errors were Calculated from Three Experiments

	Temperature (°C)			
Cosolvent	−20	0	25	45
Buffer	n.d.	34 ± 4	39 ± 1	32 ± 1
60% MeOH	67 ± 3	73 ± 4	72 ± 2	50 ± 2
60% EtOH	60 ± 5	71 ± 8	71 ± 8	62 ± 7
60% IsopOH	51 ± 3	64 ± 7	71 ± 10	47 ± 1

To assess the applicability of cosolvent-assisted folding, the direct oxidation of two other α-conotoxins, namely α-GI and α-PnIA [30], was studied in the presence of 20% ethanol. α-PnIA (GCCSLPPCAANNPDYC-NH_2) shares some conformational similarity with α-ImI [10,11], but PnIA is more hydrophobic. Both α-PnIA and α-GI were oxidized with a mixture of 2 mM reduced and 1 mM oxidized glutathione in 0.1 M Tris-HCl, pH 8.7, 0.1 mM EDTA in the presence or absence of 20% ethanol. Figure 5 summarizes HPLC analysis of the folding reactions after 4 h. For α-GI, relatively high folding yields were observed in the absence of cosolvent (Figure 5A) and were not further improved by addition of 20% ethanol (Figure 5B). However, the accumulation of the correctly folded α-PnIA increased from approximately 30% to 65% when the cosolvent was present in the folding mixture (Figure 5C and D). An increase of ethanol concentration from 20% to 40%, further improved folding of α-PnIA, resulting in almost 75% yields (Figure 5E).

DISCUSSION

The production of bicyclic peptides may be achieved by several alternative synthetic schemes: (1) regioselective, two-step oxidation of orthogonally protected Cys pairs, either in solution or on-resin; (2) one-pot regioselective oxidation with orthogonal protection

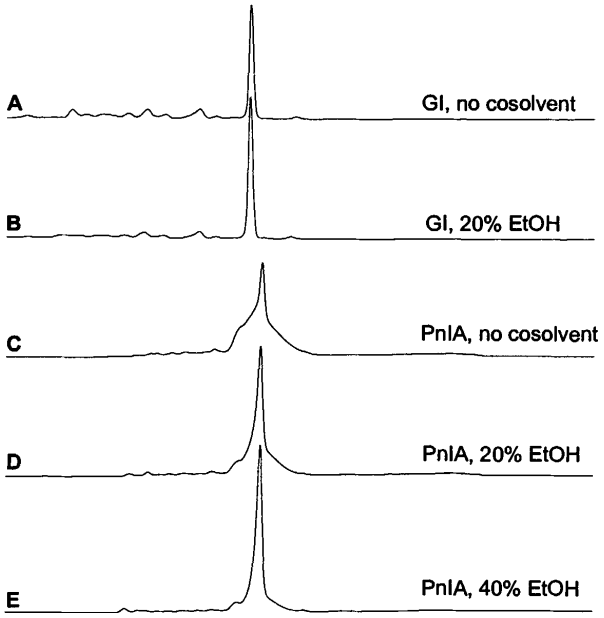

Figure 5 Effect of ethanol on the direct oxidative folding of α-GI and α-PnIA. The folding reactions were carried out in the presence of 1 mM oxidized and 2 mM reduced glutathione, pH 8.7 for 4 h at room temperature. The reactions were quenched by acidification and the folding mixtures were separated on the analytical C18 HPLC column. To determine HPLC retention times of the native α-GI and α-PnIA, reference standards were produced using a regioselective oxidation method. The major products detected in the direct oxidative folding were corresponding to the native α-GI or α-PnIA. **A**. Folding of α-GI without cosolvent. **B**. Folding of α-GI in the presence of 20% ethanol. **C**. Folding of α-PnIA without cosolvent. **D** and **E**. Folding of α-PnIA in the presence of 20% and 40% ethanol, respectively.

of native Cys pairs or (3) direct oxidative folding. The regioselective and direct oxidation strategies have advantages and disadvantages, as reviewed in the references [31,32]. The most common strategy for producing bicyclic α-conotoxins is a two-step strategy, in which native Cys pairs are protected by Trt and Acm groups and oxidation is achieved by subsequent use of $K_3[Fe(CN)_6]$ and iodine. Typically, cyclization yields vary from 10% to 30%, depending on the peptide sequence (J. Nielsen, S. Wei, G. Bulaj, unpublished data). In this work, direct oxidative folding and a recently described one-pot regioselective oxidation method were studied.

The one-pot strategy was previously described for α-conotoxin SI [9]. The authors reported high yields (53%) for producing the native isomer. This strategy has the advantage of involving fewer intermediate purification steps, compared with a traditional two-step oxidation. Our results indicated that α-ImI could not be effectively produced using this method. However, the regioselective one-pot method was successfully used in our laboratory to produce α-conotoxin GI with 36% folding yields. These differences in the cyclization yields may be dependent on individual peptide sequences and more work will be required to assess the general applicability to other α-conotoxins.

In contrast to the one-pot/two-step oxidation method, the direct oxidative folding method produced native α-ImI with high efficiency. The yields exceeded 70%, when organic cosolvent was present in the folding mixture. Only commonly used HPLC solvents, such as methanol, ethanol, and isopropanol (acetonitrile had no significant effects, data not shown) were tested. These effects of the cosolvents were observed within an α-ImI concentration ranging from 20 to 200 μM. The cosolvent-assisted folding might be applicable for a large-scale preparation of cyclized α-conotoxins, since higher peptide concentrations and a relatively low organic cosolvent concentrations (for example 20% ethanol) make this method compatible with preparative HPLC systems.

The mechanism by which cosolvents favour accumulation of native α-ImI is not clear. Positive effects of organic cosolvents were previously observed in the oxidative folding of hydrophobic conotoxins: ω-TxVII [25] and δ-PVIA [27]. In these two instances, organic solvents improved folding yields by protecting the peptides from aggregation and possibly by additional stabilization of the native conformation. Based on differences in the HPLC retention times, native α-ImI is relatively more hydrophobic than the misfolded form [2–12, 3–8], as illustrated in Figure 2A. The three-dimensional structure of the native α-ImI was determined by several groups using NMR spectroscopy [10,11,13,14]. α-ImI has a compact overall fold with the core of the molecule formed by two buried disulfide bonds. The surface is characterized by a relatively large non-polar area, comprising Cys3, Pro6, Ala9 and Cys12. In addition, the hydrophobic side chain of Trp10 is completely solvent-exposed and is located on the 'top' of the disulfide core. Therefore, the presence of organic cosolvent in the folding reaction may preferentially stabilize a more hydrophobic native form of α-ImI. The observed effects of ethanol on the folding of α-PnIA seem to confirm the above explanation, since this peptide is even more hydrophobic than α-ImI [11].

The maximum stability of α-ImI, as measured by the steady-state accumulation of the native form,

was observed between 0° and 25 °C (Table 2). When the reaction temperature increased from 25° to 45 °C, the folding yields decreased by 7% in the absence of any cosolvent and by as much as 24% in the presence of 60% isopropanol. Under the same experimental conditions, a decrease of temperature from 0° to −20 °C caused a drop in accumulation of the native form by 13%. This influence of temperature on the cosolvent-assisted folding may suggest that the native peptide could preferentially interact with the cosolvents. These interactions, most likely driven by hydrophobic effects, are known to follow a parabolic dependence on temperature, similar to that observed for α-ImI [33–35].

More studies are required to determine the mechanism of cosolvent-assisted oxidative folding. However, on a practical note, direct oxidative folding may be considered by peptide chemists as an efficient, empirical method for producing bicyclic conotoxins (or other peptides) when organic cosolvents are present in the folding reaction.

Acknowledgements

We would like to express many thanks to Drs J. Michael McIntosh, Baldomero M. Olivera and William R. Gray for helpful discussions and critical reading of the manuscript. This work was supported by the SBIR grant No AG18662-01 from the National Institute of Health.

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