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Forum Review

Folding of Conotoxins: Formation of the Native Disulfide Bridges During Chemical Synthesis and Biosynthesis of *Conus* Peptides

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ABSTRACT

Conopeptides from >700 species of predatory marine *Conus* snails provide an impressive molecular diversity of cysteine-rich peptides. Most of the estimated 50,000–100,000 distinct conopeptides range in size from 10 to 50 amino acid residues, often with multiple posttranslational modifications. The great majority contain from two to four disulfide bridges. As the biosynthetic and chemical production of this impressive repertoire of disulfide-rich peptides has been investigated, particularly the formation of native disulfide bridges, differences between *in vivo* and *in vitro* oxidative folding have become increasingly evident. In this article, we provide an overview of the molecular diversity of conotoxins with an emphasis on the cysteine patterns and disulfide frameworks. The conotoxin folding studies reviewed include regioselective and direct oxidation strategies, recombinant expression, optimization of folding methods, mechanisms of *in vitro* folding, and preliminary data on the biosynthesis of conotoxins in venom ducts. Despite these studies, how the cone snails efficiently produce properly folded conotoxins remains unanswered. As chemists continue to master oxidative folding techniques, insights gleaned from how conotoxins are folded *in vivo* will likely lead to the development of the new folding methods, as well as shed some light on fundamental mechanisms relevant to the protein folding problem. *Antioxid. Redox Signal.* 10, 141–155.

INTRODUCTION

Formation of native disulfide bridges in polypeptides is coupled to protein folding; since the classic work on ribonuclease (2, 3), numerous studies defining key factors that govern disulfide-coupled folding of proteins have been carried out. The role of the primary amino acid sequence, the position of Cys residues, and their reactivity have been investigated (4, 37, 104, 145). The relations between conformational folding and formation of the native disulfide bridges have been studied in several polypeptides, including bovine pancreatic trypsin inhibitor and ribonuclease A, reviewed recently in (9). Despite significant progress in understanding protein folding, how the amino acid sequence of a protein determines its three-dimensional conformation remains perhaps the most fundamental unresolved issue in biology. This applies also to the folding of polypeptides coupled to the formation of specific disulfide crosslinks.

What minimal amino acid sequence information determines the native fold in a short peptide sequence? The problem of properly folding small, disulfide-rich peptides, such as neurotoxins, plant cyclotides, or peptide proteinase inhibitors, would appear to be even more conceptually challenging than folding polypeptides longer than 100 AA (10, 19, 26–28, 36, 40, 44, 52, 58, 64, 128, 130). These peptides have a well-defined three-dimensional conformation stabilized by highly crosslinked disulfide bridges; one could regard these peptides as "mini-" or "microproteins" (140, 141). The mechanism by which these small peptides form the native disulfide bridges remains unclear; how much sequence information is "used' during the

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disulfide-coupled folding, both *in vitro* and *in vivo*, must be addressed. For the oxidative folding of small, disulfide-rich peptides, a definition of the relation between the primary amino acid sequence, the spacing of Cys residues, reactivity of Cys thiolates, and the oxidative folding mechanism remains far from understood. For peptides containing four Cys, three different potential disulfide connectivities exist, and for a six-Cys–containing peptide, 15 are known. The critical factors that favor formation of the native disulfide bridges of the final folded peptide and how much the arrangement and spacing between Cys residues contribute to the relative stability of the folded conformation are questions that must be addressed. The peptide-folding problem is illustrated by apamin and endothelin-1; despite having identical patterns and spacing of four Cys residues, these peptides fold into two distinct conformations, stabilized by a different pattern of disulfide bridges (142). Even more remarkable are the families of small, disulfide-rich peptides in which hundreds, or even thousands of distinct amino acid peptide sequences adopt essentially the same general three-dimensional fold. Such is the case for the venom-derived peptide toxin families from spiders and cone snails (50, 111, 134).

Conotoxins are neurotoxins produced by >500 species of predatory marine *Conus* snails (113, 115); >50,000 different conotoxins range in size from 10 to 50 amino acid residues, often with many posttranslational modifications and generally containing two or more disulfide bridges. Despite this impressive molecular diversity, conotoxins apparently adopt only a limited number of conformations, stabilized by the native disulfide bridges. Understanding the principles that underlie the formation of the native disulfides in conotoxins has been a focus of research for more than two decades. This review describes the efforts of peptide chemists to reproduce the oxidative folding of conotoxins. Little is known about the native mechanisms that lead to the efficient oxidative folding of conotoxins *in situ*, in the cone snail venom duct. As peptide chemists continue to investigate conotoxin folding, more-sophisticated oxidative folding methods are likely to be developed.

BIOLOGIC CONTEXT

All cone snails are carnivorous predatory animals that use a venom-based prey-capture strategy. They have evolved to prey on a plethora of different marine animals that fall into at least four different phyla. However, as they have evolved the deadly combinations of disulfide-rich toxins used to immobilize their prey, these snails have either coopted previously existing oxidative folding machinery, or they rapidly evolved specialized proteins to facilitate the biosynthesis of conotoxins. While peptide chemists continue to struggle to fold efficiently many conotoxins, the cone snails have somehow managed to synthesize and specifically fold at least 50,000 different disulfide-rich peptides in their venom ducts.

An estimated 500–700 different cone snail species prey on fish, worms, or other snails (111, 112). As shown in Fig. 1, cone snails exhibit a morphologic diversity in their shell patterns, which mirrors the molecular diversity of the conotoxins they produce in their venom ducts. Each snail's venom contains a mixture of ~50–200 conotoxins. Essentially no molecular redundancy exists in the complement of conotoxins between two different *Conus* species. Therefore, a conservative estimate of the conotoxin molecular diversity is that ~50,000 different peptides exist. To date, only a small fraction of these have been identified, and <200 have been functionally characterized (7, 77, 87, 111, 116). Therefore, despite more than two decades of intensive conotoxin research, this field can be regarded as still at an early stage.

The impetus for the cone snails to generate such a diversity of conotoxins is based on each conotoxin targeting a specific macromolecule (most of the time an ion channel or receptor) in a target animal (prey, predator, or competitor) with exquisite potency and selectivity. In one well-studied interaction, conotoxins affect the nervous system of the prey to cause a massive depolarization, resulting in instant immobilization, followed by an irreversible neuromuscular block (137). Table 1 provides examples of conotoxins and their the molecular targets.

Three key factors contribute to the molecular diversity of conotoxins. First, the snails have evolved a limited number of structural frameworks (equivalent to the "privileged structures" of medicinal chemists) that are stabilized by disulfide bridges (77). Some of these disulfide scaffolds [such as the ICK motif (110, 118) or Kunitz domains (13, 47)] have been found in other systems, but most disulfide scaffolds have not yet been found outside *Conus*. Next, the snails have in effect used these scaffolds for generating combinatorial libraries, in which hypermutation of the primary amino acid sequence generates molecular diversity as new *Conus* species evolve. Like medicinal chemists, the snails have modified their lead compounds by using numerous posttranslational modifications that improve the stability, potency, and selectivity of the conotoxins (17, 35). Thus, each disulfide scaffold provides the structural basis for generating a "*Conus* combinatorial library."

CONOTOXIN DISULFIDE SCAFFOLDS

The great majority of conotoxins belong to one of a small number of gene superfamilies, each comprising several pharmacologic families [recent review (136)]. All peptides in a gene superfamily are a highly conserved in their signal sequences, as well as in the arrangement of the cysteine residues in the mature toxin that form disulfide bridges [for the latest work on characterizing two gene superfamilies, see (34, 131)]. Thus, each conotoxin family has one (or a few) characteristic disulfide scaffolds, summarized in Fig. 2. The largest diversity of scaffolds is apparent for conotoxins containing six Cys residues: three distinct Cys arrangements yield seven different disulfide scaffolds. Remarkably, three identical patterns of six-Cys–containing conotoxins from the M-superfamily produced three distinct disulfide scaffolds (34, 45, 65, 95). For the four-Cys–containing peptides, three Cys arrangements produce five different scaffolds. Interestingly, conotoxins containing Cys framework 14 can adopt two distinct conformations, stabilized by differently connected disulfide bridges (71, 100). The disulfide connectivity in the 10-Cys–containing conotoxin, σ-GVIIIA, remains unknown (49). In isolated instances, cone snails have

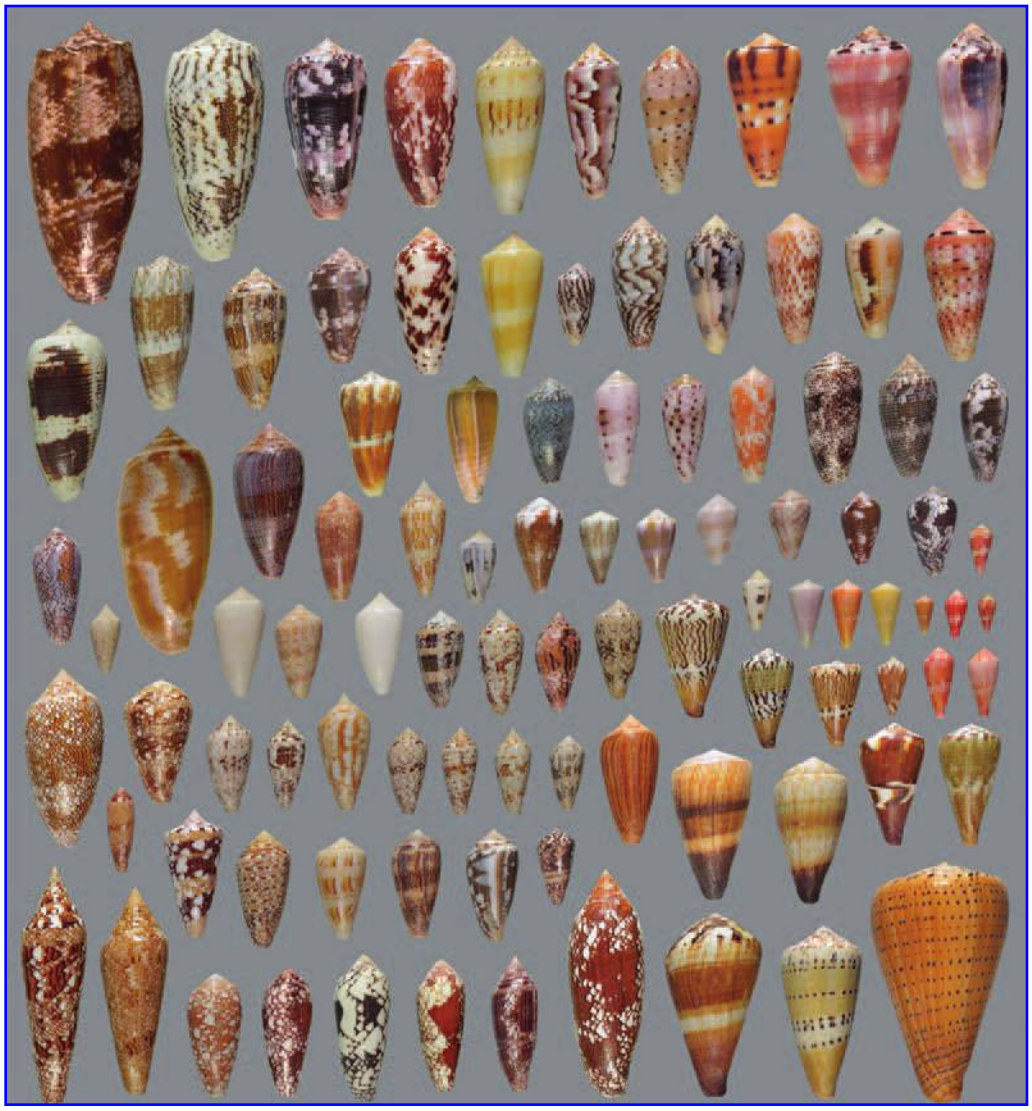

FIG. 1. Diversity of cone-snail shell patterns mirrors the molecular diversity of *Conus* peptides produced by these venomous animals. It is estimated that each of >700 distinct *Conus* species produces a repertoire of 50–200 distinct conotoxins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

deleted an apparently nonessential disulfide bridge without compromising bioactivity; an example is conkunitzin-S, an otherwise standard Kunitz domain conopeptide that has two instead of three disulfide crosslinks (47). No known examples of conotoxins contain a vicinal disulfide bridge, although many Cys residues in conotoxins are adjacent [and a precedent exists for a vicinal disulfide bridge in spider toxins (144)].

The native conformation of most conotoxin scaffolds has been determined by using NMR spectroscopy methods (59, 91, 98, 109). As illustrated in Fig. 3, conotoxins have well-defined compact structures, stabilized by disulfide bridges. For two-disulfide–bridged conotoxins, different disulfide connectivity determined either a ribbon or globular shape (46). For three-disulfide–containing conotoxins, scaffolds vary from a globular shape for the ICK motif to compact, cagelike structures. Relatively little is known about the backbone dynamics of conotoxins; although they are ideal for NMR relaxation experiments, few studies have addressed this important issue.

ω-Conotoxin MVIIA was shown to exhibit slow conformational exchange in the second loop that was associated with changing conformation of the Cys8-Cys20 disulfide bridge (11). With NMR relaxation experiments on uniformly labeled ^{15}N MVIIA-Gly analogue, Goldenberg (56) showed that the second loop (residues 9–15) underwent a slow conformational exchange on a microsecond time scale. An interesting aspect of these findings is that the second loop, comprising Tyr13, is critical for binding to the voltage-gated calcium channel, suggesting a role for conformational flexibility in interactions of conotoxins with their target ion channels or receptors. Two interconverting conformations of α-conotoxins GI or MI were observed (61, 92). Slow-exchanging conformations of contryphans could be monitored by HPLC separation (72, 73). In-

TABLE 1. DIVERSITY CONOTOXINS AND THEIR MOLECULAR TARGETS

	Conotoxins	*Molecular targets*
A-superfamily		
α-GI	ECCNPACGRHYSC#	nAChRs
α-ImI	GCCSDPRCAWRC#	nAChRs
α-SI	ICCNPACGPKYSC#	nAChRs
T-superfamily		
χ-MrIA	NGVCCGYKLCHOC	NET
M-superfamily		
μ-GIIIA	RDCCTOOKKCKDRQCKOQRCCA#	VGSCs
μ-PIIIA	ZRLCCGFOKSCRSRQCKOHRCC#	VGSCs
μ-SmIIIA	ZRCCNGRRGCSSRWCRDHSRCC#	VGSCs
μ-SIIIA	ZNCCNGGCSSKWCRDHARCC#	VGSCs
μM-RIIIK	LOSCCSLNLRLCOVOACKRNOCCT	VGKCs
O-superfamily		
ω-MVIIA	CKGKCAKCSRLMYDCCTGSCRSGKC#	VGCCs
ω-MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGKC#	VGCCs
ω-GVIA	CKSOGSSCSOTSYNCCRSCNOOYTKRCY#	VGCCs
μ-O-MrVIB	ACSKKWEYCIVPILGFVYCCPGLICGPFVCV	VGSCs
δ-SVIE	EACSSGGTFCGIHPGLCCSEFCFLWCITFID	VGSCs
δ-PVIA	EACYAPGTFCGIKPGLCCSEFCLPGVCFG#	VGSCs
δ-TxVIA	CKQSGEMCNLLDQNCCDGYCIVLVCT	VGSCs

NAChRs, nicotinic acetylcholine receptors; VGSCs, voltage-gated sodium channels; VGCCs, voltage-gated calcium channels; VGKCs, voltage-gated potassium channels; NET, norepinephrine transporter; O is hydroxyproline; Z is puroglutamatae; # is amidated C-terminus.

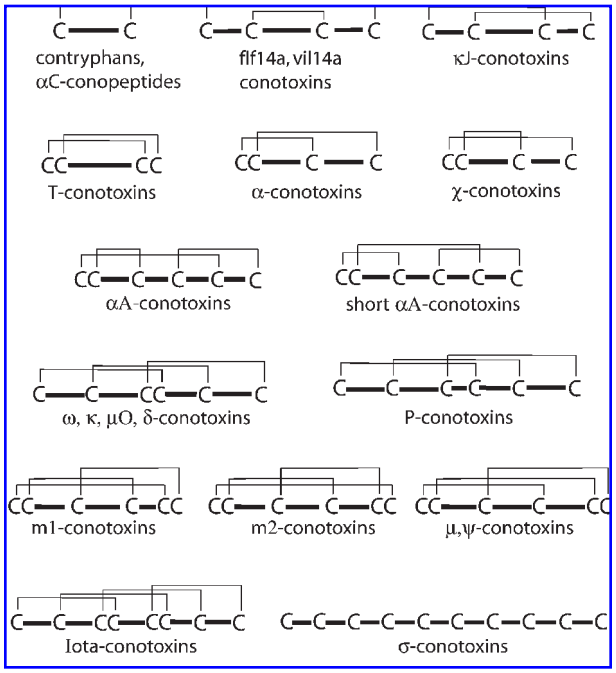

FIG. 2. Examples of cysteine patterns and disulfide scaffolds in conotoxins. Please note that identical cysteine patterns may lead to three different disulfide scaffolds, making the predictions of disulfide connectivity very difficult for some cases. In addition to those shown in the figure, several more cysteine patterns are known, for which the disulfide connectivity is not yet determined.

terestingly, the native conformation of α-conotoxin BuIA has multiple conformations, whereas the misfolded form exhibits a single, well-defined conformation (76). Thus, despite a relatively compact structure stabilized by disulfide bridges, conotoxin scaffolds exhibit a degree of conformational flexibility, the functional consequences of which remain undefined.

CHEMICAL SYNTHESIS OF CONOTOXINS

The first chemical syntheses of conotoxins were carried out in the early to mid 1980s: α-conotoxins GI and MI (60, 61, 108), as well as ω-conotoxin GVIA (107, 126). Since then, at least a hundred conotoxins have been synthesized by using either Fmoc or Boc chemistry. For two-Cys–containing conotoxins such as contryphans, the oxidation of a single pair of Cys residues has been a relatively easy task; the S-trityl (S-Trt) protection has been the most widely used during synthesis (66, 74, 75, 129). However, for peptides containing multiple disulfide bridges, a technical challenge exists: forming native disulfide bridges, identical to those found in venom-derived toxins. Depending on the number of Cys residues to be oxidized, distinct synthetic strategies are used.

For four-Cys–containing conotoxins, such as α-, χ-, or τ-conotoxins, the orthogonal protection of Cys pairs with S-trityl and S-acedamidomethyl (S-Acm) protection groups is routinely used, although, as described later, other protection strategies are also very efficient. The orthogonal protection of Cys pairs allows regioselective formation of subsequent disulfide bridges,

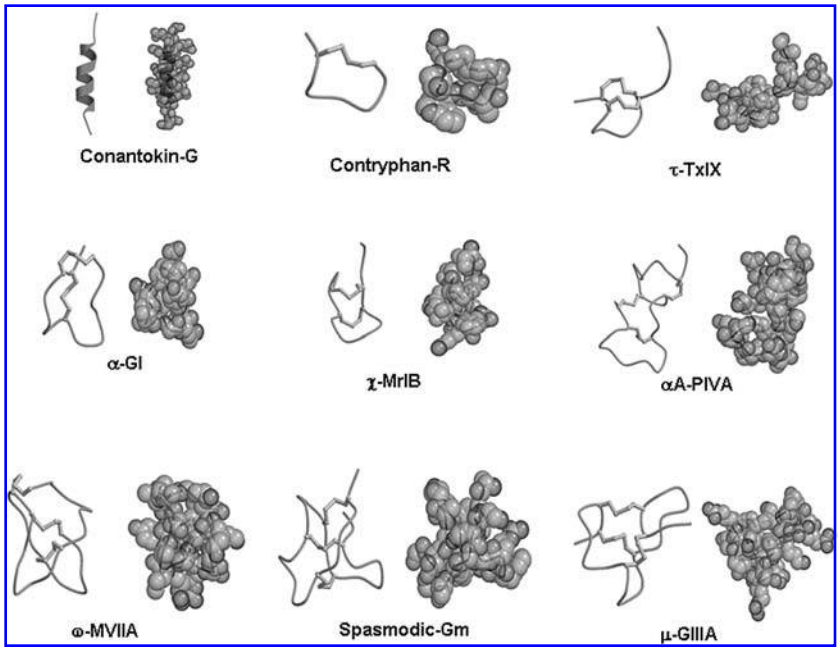

FIG. 3. Model structures of representative conotoxins. Three-dimensional models of disulfide scaffolds and a space-filling models of conotoxins illustrate compact and well-defined native conformations of conotoxins. These peptides may be considered "microproteins."

as shown in Fig. 4. Excellent reviews on the oxidative folding strategies are available (1, 6, 15, 101, 102). Despite the fact that for two-disulfide–containing conotoxins, a relatively "simple" scheme to form native disulfide bridges can be used, producing some of these conotoxins continues to be a great challenge for chemists (79).

Barany's group (103) tested several Cys-protecting groups and oxidation strategies with the aim of establishing efficient chemical synthesis for two-disulfide–bridged α-conotoxins. First, they evaluated distinct protection and oxidation strategies and compared yields of folding in solution and on-resin of α-conotoxin SI. The main conclusions were that (a) one-step oxidation of the peptide with deprotected Cys thiols (after removal of S-Tmob groups; 2,4,6-trimethoxybenzyl) was efficient, with yields of 39% (1% DMSO, 25°C for 7 hr); (b) two-step oxidation of the peptide containing S-Tmob protection on one Cys pair and S-Acm protection on the other Cys pair resulted in 38% overall yield, but the yield was sensitive to the oxidation strategies; (c) two-step oxidation was more efficient when the small loop was closed first, followed by closing the larger loop; and (d) on-resin oxidation of S-Tmob/S-Acm–protected peptide produced correctly folded conotoxin with 14% overall yields. This group was also the first to develop and introduce immobilized Ellman's reagent to oxidize two-disulfide–containing conotoxins (described later).

An alternative, one-pot strategy was introduced for conotoxins by Cuthbertson and Indrevoll (38) by using α-SI. The selective deprotection of Cys pairs was accomplished by changing temperature: the *t*-Butyl (tBu)–protected pair of Cys was deprotected and oxidized at ambient temperature, whereas 4-methylbenzol (MeBz)-protected Cys pairs were removed by elevating the temperature to 70°C. The same strategy was applied to produce α-conotoxin GI (106). Cuthbertson and Indrevoll (39) also used a double one-pot oxidation strategy to produce a dimer of α-conotoxin SI containing four disulfide bridges. Four different S-protecting groups were used (Trt, Acm, tBu and MeBz) to achieve regioselective formation of all four disulfide bridges with very appreciable yields. This work was the first example of using four distinct protection groups to produce conotoxin analogues.

For synthesis of conotoxins containing three or more disulfide bridges, all Cys residues are usually protected by an acid-labile trityl group. The simultaneous formation of the disulfide brides is promoted by either air oxidation, a mixture of oxidized/reduced glutathione, cysteine, or other thiol/disulfide-ex-

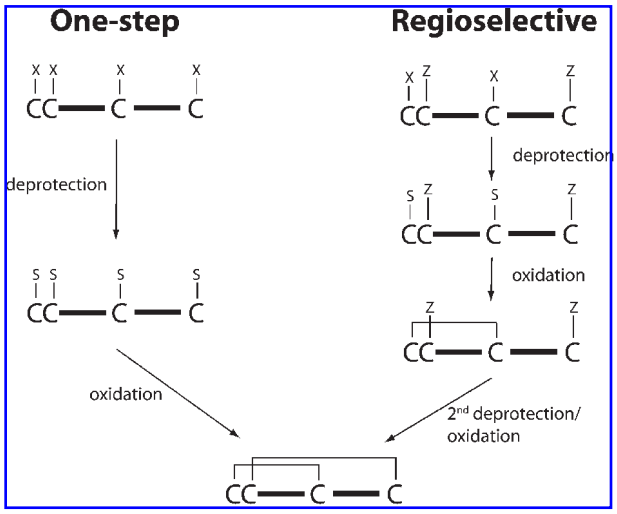

FIG. 4. Oxidative folding strategies for conotoxins. The regioselective strategy is a method of choice for conotoxins containing two disulfide bridges, whereas the direct-oxidation strategy is preferred for conotoxins containing three or more disulfide bridges.

changing reagents. The inherent problem with this strategy is that the formation of disulfide bridges is a random process, and the final yield greatly depends on the thermodynamic stability of the native conformation of a given conotoxin. As described later, the thermodynamic stability of many conotoxins (in particular hydrophobic ones) is marginal, making the production of larger quantities of correctly folded peptides a challenge (23, 42). To obtain higher yields during the direct oxidative folding, the empiric optimization approach is still the method of choice (21). The variables most often manipulated to improve folding yields are: temperature, folding additives (enhancers), oxidizing strategy (including varying the redox potential), peptide concentration, and duration of the folding reaction.

IMPROVING OXIDATIVE FOLDING OF CONOTOXINS

Several strategies have been developed to improve the oxidative folding of conotoxins.

Immobilized Ellman's reagent was introduced by Barany's group (5) to oxidize one- and two-disulfide–bridged peptides, including oxytocin, somatostatin, α-conotoxin SI, and apamin. The immobilized Ellman's reagent was applied to fold α-conotoxin SI in one step with overall yields of 90% ($t_{1/2}$, 3 min at pH 6.6) and 79% ($t_{1/2}$, 54 min at pH 2.7). The immobilized Ellman's reagent also proved superior in a two-step oxidation strategies in which Cys pairs were protected with xanthyl (Xan) and Acm groups (68). Recently, the Ellman's reagent immobilized on CLEAR resin was developed (41). The use of Clear-Ox to produce two-disulfide–containing α-conotoxin GI and three-disulfide–containing μ-conotoxin PIIIA has been evaluated (62). In a one-step oxidation, the Clear-Ox–mediated oxidative folding was significantly improved at higher peptide concentrations, consistent with pseudo-dilution effects. Even at 5 m*M* concentration of α-GI, the folding yield in the presence of Clear-Ox was $>80\%$, as compared with less than 10% for the glutathione-promoted folding in solution.

An assisted-folding strategy has been used to improve yield by stabilizing the native conformation of the peptide during folding. The first successful attempt to improve folding of ω-conotoxin MVIIC by using this strategy was described in 1996 (85). Folding of MVIIC was significantly improved when the oxidation was carried out at low temperature and in the presence of high salt concentration. High concentrations of salt were also efficient in improving folding of μ-SmIIIA (52). Addition of nonionic detergents or organic cosolvents to the folding mixture improved folding yields of the hydrophobic δ-conotoxins (23, 42) or α- and μO-conotoxins (25, 106), respectively.

Selenocysteines replacing cysteine residues was recently used by the Alewood group (8) to improve folding of conotoxins. In this work, the authors produced three analogues of α-ImI with a single bridge or two disulfide bridges replaced by diselenide bridges. Diselenide-containing analogues proved very stable in a redox buffer and maintained full bioactivity when compared with the wild-type, Cys-containing peptide. This approach was previously successful for other Cys-rich peptides (119). Selenocysteine-containing conotoxins are likely to become very useful analogues with simplified folding patterns. The N-to-C backbone cyclization was recently shown to improve formation of the native disulfide bridges in two conotoxins: α-MII and χ-MrIA (31, 88). Other synthetic strategies to produce conotoxins with a simplified cross-linking included replacements of the disulfide bonds with lactam, thioether, or dicarba bridges (14, 69, 127). The application of the safety-catch amide linker that allowed DMSO-mediated oxidation of conotoxins in a 96-well format (16) provides a promising strategy for a high-throughput synthesis of conotoxin-based combinatorial libraries (48, 132).

MECHANISM OF *IN VITRO* OXIDATIVE FOLDING

Given the extreme hypervariability of the primary amino acid sequence between the highly conserved Cys residues, the fundamental question related to folding mechanism is what factors are critical for the formation of the native disulfide bridges. *In vitro* folding yields of conotoxins vary from $<1\%$ to 50–60% (52). How much does the Cys pattern and spacing between Cys residues (loop size) contribute to the folding and stability of the native toxins? As pointed out earlier, an identical arrangement of Cys residues in conotoxins from the M-superfamily resulted in three distinct connectivities of the native disulfide bridges. What is the role of noncovalent interactions? The differences between disulfide structures of the two-disulfide–bridged apamin and endothelin are mediated only by one to two distinct amino acid residues (142). Thus, the hypervariability of amino acid sequence in conotoxins belonging to a single gene family imposes a major challenge for the formation of native disulfide bridges. We define this as the "conotoxin folding puzzle" (19).

In the pioneering work of Zhang and Snyder (150, 151), the authors attempted to define the rules for forming disulfide bridges in conotoxins. Equilibria were measured for forming disulfide bridges in α-GI, as well as in several analogues that differed in either primary AA sequence or the Cys positions (151). Formation of the disulfide bridges was primarily determined by the position of Cys residues. Because the folding yield for α-GI was lower under denaturing conditions, a role of tertiary interactions was postulated. Kaerner and Rabenstein (78) measured equilibria for forming disulfide bonds in α-GI analogues missing the second pairs of Cys residues and calculated equilibria for forming the second disulfide bridge. Formation of the second disulfide bridge was characterized by an increased cooperativity (relative to forming the first disulfide bridge); this conclusion was confirmed in NMR experiments suggesting some amount of secondary structures in single-disulfide folding intermediates (78). Conformational heterogeneity was observed for misfolded α-GI conotoxin analogues, consistent with a key role of the native disulfide bridges in the folding and stability of this peptide (54).

The mechanism of the oxidative folding of ω-MVIIA was investigated by the Goldenberg group (121–124). MVIIA was shown to fold with relatively high yields (50%) in the redox buffer containing GSSG and GSH (122). The folding was sensitive to a ratio of GSSG to GSH. Two observations suggested that the overall folding yield is not determined by spacing of

Cys residues, but rather by noncovalent interaction of amino acid residues: (a) folding was sensitive to urea, and (b) ω-conotoxin MVIID folded with 28% yield despite having identical spacing to that of MVIIA, whereas other ω-conotoxins with different loop sizes folded with 50% yield. The C-terminal glycine, but not the N-terminal propeptide sequence was shown to increase the folding yield, presumably by providing additional stabilizing interactions within the native conformation (56, 123). Folding studies with the MVIIA analogues containing only four Cys residues indicated that no preference existed in forming any of the native disulfide bonds (124). The analogues containing two native disulfide bonds were greatly destabilized, as judged by CD spectra and a lack of bioactivity (124). Similar destabilization effects caused by deletion of individual pairs of Cys residues were also reported for ω-conotoxin GVIA (51). In another study (121), the equilibria for forming individual disulfide bridges in ω-MVIIA were measured, confirming no preference in forming native versus non-native disulfide bridges. The effective concentrations of the Cys pairs forming SS bridges were largely independent of the presence or absence of denaturant, but were correlated with the loop size, confirming the studies by Zhang and Snyder (151). Interestingly, once the two native disulfide bonds were formed, a high degree of cooperativity was found in forming the third native disulfide bond: the effective concentration for forming any of the third native disulfide bond (in preexisting species containing two native disulfide bridges) was denaturant sensitive and increased several orders of magnitude, compared with those for forming the first or the second disulfide bridge.

Taken together, the *in vitro* folding studies with conotoxins α-GI and ω-MVIIA suggest that the formation of the initial disulfide bridge is a random process: no preference exists between native and non-native disulfides, but equilibria are governed by the spacing of the Cys residues. However, formation of the last native disulfide bond in the native-like intermediates greatly depends on noncovalent interactions and is characterized by a high degree of the cooperativity, as schematically illustrated in Fig. 5. Thus, the early folding events are determined by the Cys patterns, but the formation of the final folding product is determined by noncovalent interactions among noncysteine residues.

A role for the primary AA sequence in the folding of conotoxins was investigated for peptides from the M-superfamily (52) and in MrIA (also named CMrVIA) (80). Four M-superfamily conotoxins having identical spacing between Cys, but otherwise differing in primary AA sequence, exhibited two distinct folding mechanisms: rapid, but low-yield folding, or slow-rearrangement folding with relatively higher yields (52). The steady-state accumulation of the rapidly folded μ-SmIIIA was sensitive to ionic strength, but not to denaturing conditions, suggesting that electrostatic interactions mediated by multiple positively charged residues play a predominant role in determining low folding yields. Conversely, folding of slowly rearranging μ-GIIIA was sensitive to urea, but not ionic strength, emphasizing a role of noncovalent interactions in forming the native disulfide bonds in this peptide. In another μ-conotoxin, SIIIA, the replacement of two adjacent glycine residues located between two Cys residues with flexible backbone spacers did not significantly affect the oxidative folding of these chimeric peptides, suggesting a minor role of amino acid residues in the first loop (63). Recent work of Kang and co-workers (80) showed that an interchange of a single amino acid residue (Lys $\leftrightarrow$ Pro), along with C-terminal amidation in χ/λ-MrIA, resulted in switching the disulfide connectivity between α- and χ/λ-scaffolds. Future studies on structure–folding relations in various conotoxins will likely identify critical amino acid residues that determine their folding patterns. To this end, a more extensive cladistic analysis of conotoxin sequences may prove useful for identifying such residues.

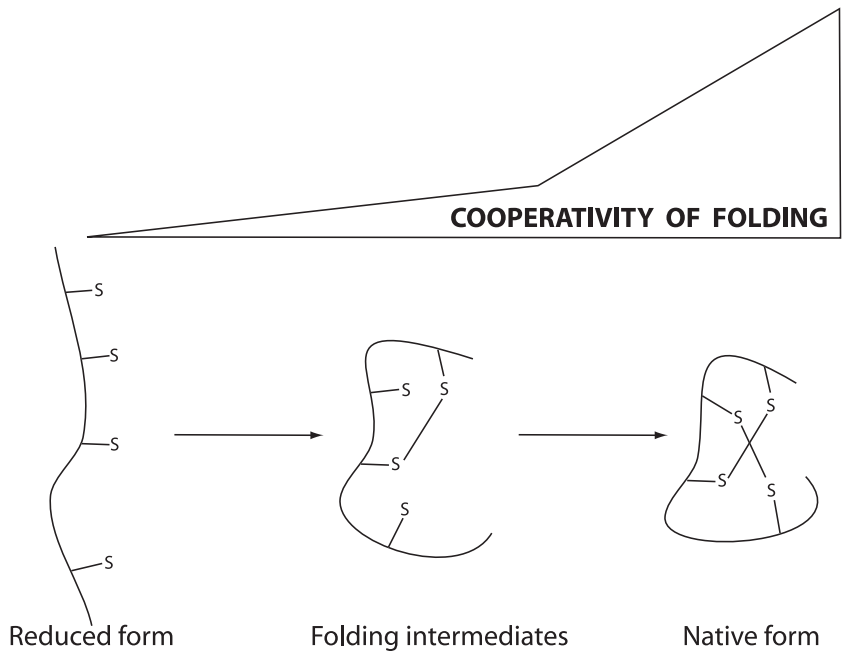

FIG. 5. The *in vitro* oxidative folding of conotoxins is characterized by a tendency not to form native disulfide bridges at the early folding steps. The position of Cys residues in the sequence, rather than non-covalent interactions, is the major determinant for forming the first disulfide bridge. However, the role of non-covalent interactions and the cooperativity of folding increases at the final stages of forming the native disulfide bridges.

ROLE OF POSTTRANSLATIONAL MODIFICATIONS IN FOLDING

The role of posttranslational modifications (PMs) in the oxidative folding of conotoxins is largely unexplored territory. The order in which folding and the various posttranslational modifications occur is unknown. Thus, whether a posttranslational modification occurs before, concurrent with, or after the formation of the disulfide bonds has not yet been definitively determined. It is conceivable, however, that PMs may affect the stability of the native disulfide bonds. The presence of γ-carboxyglutamate (Gla) in the tx9a conotoxin was shown to facilitate the oxidative folding when calcium ions were present in the reaction mixture (22). Furthermore, the interactions between calcium ions and γ-conopressin-vil, a conopeptide containing Gla and a single disulfide bridge, were recently shown by using NMR experiments (99). Because Ca ions are present in the ER at concentrations comparable to those used in these experiments (millimolar range), these findings suggest that γ-carboxylation of glutamate may stabilize the native conformation of the folded toxin *via* chelation of calcium ion by the modified residues.

A role for the C-terminal amidation in folding was investigated in two conotoxins, ω-MVIIA and α-ImI (81, 123). As mentioned previously, the C-terminal glycine significantly improved folding of the mature toxin (56, 123). In contrast, the C-terminal Gly residue had a negative effect on folding yields (81). Because NMR studies did not reveal any major conformational differences between ImI and the free carboxyl–containing analogue (81), one possible explanation is the effect of electrostatic interaction (provided by the C-terminal negative charge) on the formation of the disulfide bridge between the pair of Cys residues that involved a C-terminal Cys. Such electrostatic interactions were previously shown to play a significant role in the thiol/disulfide exchange reactions in peptides containing charged residues (24). However, the presence or absence of C-terminal amidation did not affect folding of μ-conotoxin SmIIIA (52).

Recent experimental results (Lopez-Vera *et al.,* unpublished data) suggest that 4-hydroxylation of prolines may affect the folding of conotoxins. Interestingly, protein disulfide isomerase (PDI) is a subunit of prolyl-4-hydroxylase (P4H); thus, the two enzymes may work in a concerted way to produce 4-hydroxyproline (Hyp) and disulfide bonds concurrently. Thus, although the role of posttranslational modifications in the folding and stability of conotoxins is clearly unexplored territory, it is likely from these fragmentary observations that PMs may facilitate proper folding of conotoxins.

ROLE OF THE PROPEPTIDE, PROTEIN DISULFIDE ISOMERASE, AND MACROMOLECULAR CROWDING

In vivo, conotoxins are first translated as precursors containing signal and propeptide sequences. Woodward *et al*. (147) suggested that the propeptide might facilitate conotoxin folding. However, the propeptide was subsequently shown not to play a direct role as an intramolecular chaperone in the oxidative folding of ω-MVIIA, α-GI, and δ-PVIA (19, 20, 123). Folding yields for pro-GI and GI were comparable under different folding conditions (including variation in redox buffers and temperature) (19). The ultimate test whether the propeptide region could serve as an intramolecular chaperone was performed by using a highly hydrophobic conotoxin PVIA, previously shown to fold with extremely low yields (23). The 58-AA precursor of PVIA, pro-PVIA was synthesized by using the native chemical ligation (20). The folding yields were very low (>4% without Tween-40; >6% in the presence of Tween-40) and were comparable for pro-PVIA and PVIA. The results of all three studies (on MVIIA, GI, and PVIA), led to the suggestion

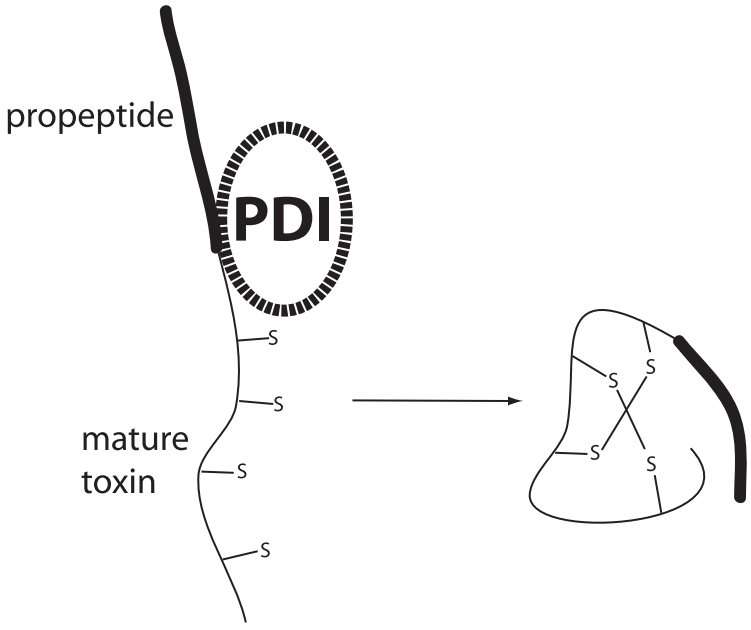

FIG. 6. The N-terminal propeptide facilitates protein-disulfide-isomerase (PDI)–catalyzed folding of conotoxin precursors. The propeptide does not act as an intramolecular chaperone during the folding of conotoxins, but it improves substrate properties of conotoxin precursors for PDI, the main protein component of *Conus* venom ducts.

that conotoxin propeptides may belong to the so-called class II propeptides that may play a role in interactions with folding enzymes and receptors in the ER (20). Interestingly, when a conotoxin precursor of GI (pro-GI) was folded in the presence of bovine PDI, the enzyme-catalyzed folding of the propeptide-containing conotoxin was significantly faster than PDI-catalyzed folding of mature toxin (19). As illustrated in Fig. 6, the presence of propeptide altered conotoxin as a substrate for PDI-catalyzed folding.

PDI appears to be a major protein component of the venom duct of *Conus textile* (22). When venom duct extract was analyzed with SDS-PAGE, a single major band was detected; mass spectroscopy and microsequencing were consistent with a sequence of PDI. Analysis of a *Conus* PDI cDNA clone revealed that the *Conus* enzyme had strong sequence similarity to other PDIs (22). With a proteomics approach, the Balaram group (57) recently identified PDI as a major component of the *C. amadis* venom duct. Based on these and other findings [for example, PDI from *C. marmoreus*, accession number ABF 48564 (89)], it is likely that more folding catalysts from various cone snails will be identified. Folding studies with *Conus* PDI and conotoxin precursors have not yet been reported in the literature.

Macromolecular crowding is an integral component of the folding environment in the cell: concentrations of "neutral" molecules easily exceed 100 mg/ml. Theoretical studies predicted that the excluded volume effect, produced by high concentrations of crowding molecules, might affect folding of ω-conotoxin MVIIA (55). Recent work (18) showed that the macromolecular crowding did not influence folding rates or equilibria for forming the native disulfide bridges in three conotoxins: GI (13 AA), PVIIA (25 AA), and r11a (46 AA). Surprisingly, if albumin was used as the crowding agent, a dramatic increase was observed in the folding rates, even at concentrations that did not produce excluded volume effects, suggesting that this protein could act as an oxidant for disulfide bond formation (18).

RECOMBINANT EXPRESSION OF CONOTOXINS

Because of their small size and a high degree of posttranslational modification, conotoxins have not been ideal candidates for recombinant expression. Interestingly, successful overexpression of $^{15}N/^{13}C$-labeled conopeptide was recently reported (86). The first recombinant conotoxin precursor, pro-ω-MVIIA-Gly, was expressed as a fusion protein with TrpLe protein in *Escherichia coli* strain BL21 (123). The insoluble material was dissolved, and the TrpLE fragment was cleaved by chemical methods. After HPLC purification, the conotoxin precursor was used for folding studies. By using the same recombinant strategy, Goldenberg (56) produced ^{15}N-labeled ω-MVIIA-Gly for NMR relaxation studies (56). A recombinant ω-MVIIA was recently produced in *E. coli* by using a fusion with thioredoxin and a His-tag (149) or with glutathione-*S*-transferase (148). The purified products exhibited the analgesic activity when tested in mice (148). A similar strategy was recently used to produce another O-superfamily conotoxin, lt7a, as a fusion protein with thioredoxin (120). Recombinant conkunitzin-S1 was overexpressed in an insoluble form as a fusion construct with intein (12). Subsequent solubilization, cleavage, and refolding produced sufficient material for NMR experiments (13). Recombinant expression methods will continue to be an attractive alternative strategy for producing conotoxins, especially those that are very difficult to obtain in high yield by using chemical synthesis (25).

BIOSYNTHESIS OF CONOTOXINS

Relatively little is known about biosynthetic and secretory pathways for conotoxins *in vivo.* Conotoxins are produced in a long, convoluted venom duct. The morphology of vesicles stored in the lumen of the duct was noted in early studies (84, 114). A more recent anatomic study of the venom duct of *Conus californicus* suggested that two types of endothelial cells may be present (90). Most biochemical and cellular events that occur in venom ducts have not been characterized (Figs. 7 and 8). Although conotoxins are synthesized in the form of a precursor, the extraction and characterization of venom-duct components did not detect the presence of propeptide-containing conotoxins (53, 90, 105). However, interactions between propeptide-containing conotoxin proTxVIA and secretory receptors from the sortilin Vps10p family have been described (32). The same group showed that the propeptide was important for efficient secretion of the toxin to media when conotoxin was expressed in COS7 cells (32). It remains unclear when and where the propeptide is proteolytically separated from the mature toxin. One proteinase, Tex31, that processed the precursor of TxVIA was isolated and cloned from venom of *C. textile*

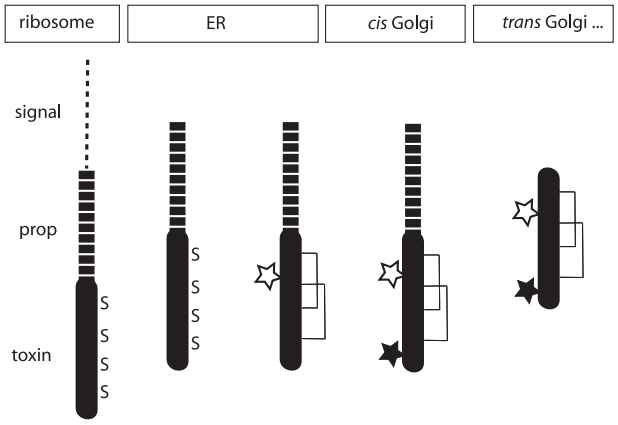

FIG. 7. Biosynthesis pathways for conotoxins is largely unexplored territory. The conotoxin precursors start their secretory pathway in the *Conus* ER, where they form disulfide bridges and are likely to undergo the first set of posttranslational modifications (*open star*). The only characterized *Conus* ER-resident proteins are γ-glutamyl carboxylase and protein disulfide isomerase. More posttranslational modifications (*solid star*) occur in the Golgi apparatus. The subcellular and molecular aspects of proteolytic processing of the precursor sequences remain largely unknown. How conotoxins are packed into insoluble secretory vesicles has not been characterized.

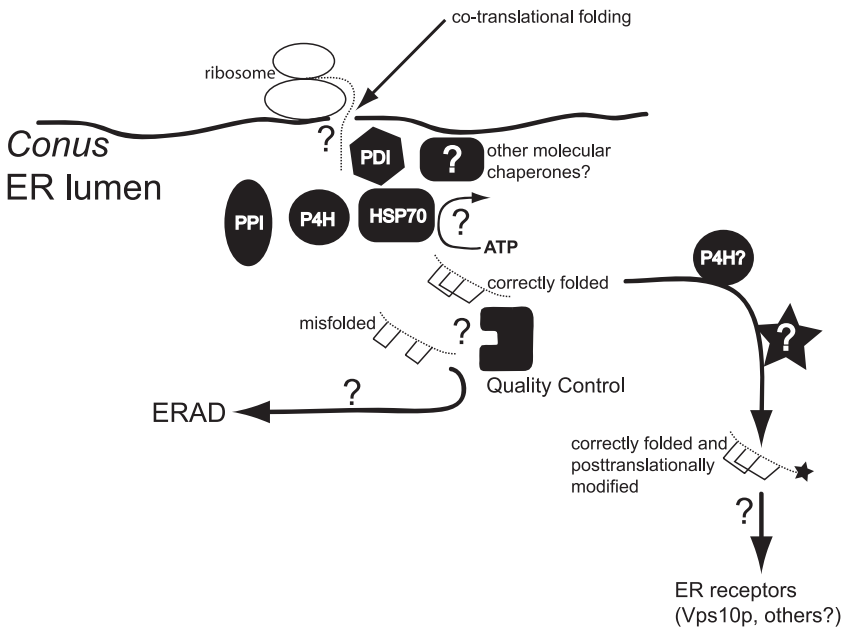

FIG. 8. Mechanism of the oxidative folding of conotoxins in the *Conus* ER remains largely unknown. What key factors contribute to the formation of the native disulfide bridges in conotoxins? Neither molecular chaperones nor, other than PDI, folding catalysts have been purified or cloned from the *Conus* ER. Although the diversity of cysteine patterns, disulfide scaffolds, and propeptide sequences is well known, no studies addressed the substrate specificity for *Conus* PDI isoforms. Potential relations between formation of the disulfide bridges during the oxidative folding of conotoxins and ATP consumption and the total energetic costs also remain unspecified. The ER quality-control system that can discriminate between the correctly folded and misfolded conformations is unknown. *Star with a question mark*, a group of other enzymes that posttranslationally modify conotoxins. *Question marks*, Key steps in the conotoxin folding that remain to be characterized. PPI, peptidyl prolyl isomerase; P4H, prolyl-4-hydroxylase; ERAD, ER-associated degradation.

(97). This enzyme was characterized by high substrate specificity. The sequence analysis of Tex31 suggested that it belonged to the cysteine-rich secretory proteins (CRISP) family (97). Recently, a new member of CRISP protein family was cloned from *C. marmoreus*; however, this γ-carboxyglutamate–containing protein did not exhibit any proteolytic activity (67). Whether CRISP proteins in cone snails' venom ducts play a role as proteolytic enzymes or toxins, as has been found in snake venoms (133, 143), will require more studies.

As mentioned previously, the presence of PDI as a major protein component of the *C. textile* venom duct was reported (22). Two closely related PDI isoforms were cloned from *C. textile* venom duct: both *Conus* proteins shared 75% sequence homology to human PDI. *Conus* PDI consisted of four thioredoxin domains with two [bond]CGHC[bond] active sites and the ER retention signal. Because it has been shown that PDI catalyzes folding of the propeptide-containing conotoxins more efficiently (19), it seems likely that the high concentration of *Conus* PDI is critical for the efficient production of conotoxins in venom ducts. Recently, biochemical and gene-expression analysis of conotoxins in *C. textile* venom ducts revealed that members of different superfamilies are secreted in distinct regions of the venom duct (53). Conotoxins that are very difficult to synthesize chemically and efficiently fold *in vitro* [such as δ-TxVIA, δ-SVIE, or μO-MrVIB (25, 42)] can often be abundant components of snail venom ducts, further emphasizing the gap between *in vitro* and *in vivo* folding of conotoxins. Based on these findings, it is tempting to hypothesize that discrete parts of venom duct comprise superfamily-specific secretory cells, with specialized folding catalysts and molecular chaperones that facilitate the oxidative folding of various conotoxin precursors in a geographically specific fashion.

Several additional important questions about the *in vivo* oxidative folding of conotoxins remain unanswered. As summarized in Fig. 8, questions relevant to understanding the most basic principles of protein folding are as follows.

1. What is the role of molecular chaperones and ATP in the conotoxin folding? Does *Conus* Hsp70 (BIP) cooperate with PDI in the ATP-dependent, iterative cycle of binding and release of conotoxins during formation of the disulfide bridges in the ER, as described for other proteins or peptides (93, 94)?
2. Does *Conus* ER contain multiple folding catalysts belonging to the PDI family? Numerous members of the PDI family varying in a number of thioredoxin-like domains have been discovered in other organisms: their role and substrate specificity remain undefined.
3. What is a role of peptidyl prolyl isomerase (PPIs) or prolyl-4-hydroxylase (P4H) in assisting formation of the native disulfide bridges? Because PDI and P4H form functional heterotetramers (PDI is a β-subunit of P4H), is oxidative folding coupled to proline hydroxylation in Hyp-containing conotoxins? Do *Conus* folding catalysts and chaperones form multiprotein complexes, as observed in the ER of other eukaryotic cells (96)?
4. Do molecular chaperones and folding catalysts provide a different pathway for forming native disulfide bridges, from the *in vitro* folding pathway (30)? To what degree do *intermolecular* interactions between conotoxin precursors and the ER residents influence the formation of the native disulfide bridges? In other words, how do energy landscapes for folding of conotoxins differ when folding occurs in *Conus* cells or a test tube?
5. Is co-translational folding important for the correct formation of the native disulfide bridges in conotoxin precursors? Does the extremely high degree of position-specific codon use for Cys residues (33) contribute to regulating rates for

forming disulfide bridges by silent polymorphism mechanisms (83)?

6. Do ER-resident proteins recognize correctly folded conotoxins, facilitating further translocation through the secretory pathway? What types of molecular interactions during ER quality control underlie discrimination between the native and nonnative conformations of conotoxins? Do *Conus* venom duct cells use the ER-associated degradation (ERAD) system for removing misfolded conotoxins?

To answer these and related questions, new experimental strategies combining peptide chemistry and molecular biology must be developed. Fortunately, because of their small size and their accessibility to recombinant methods and chemical synthesis, conotoxins provide tractable experimental system for further folding research.

CONCLUSIONS AND FUTURE DIRECTIONS

Conotoxin folding research is at an intersection between chemistry and biology; it provides a challenge for peptide chemists to develop more efficient oxidative folding methods, while biologists and biochemists continue to elucidate how more-general protein folding mechanisms are adapted to the *in vivo* folding of conotoxins. A relatively large number of studies involve *in vitro* conotoxin folding; the current evidence suggests that early folding steps are determined primarily by the positions of Cys residues, rather than by specific non-covalent interactions of non-Cys amino acid residues. This stage is characterized by a lack of cooperativity. However, the role for non-covalent interactions in forming native disulfide bonds appears to be greater in the later stages of folding. The N-terminal propeptide does not act as an intramolecular chaperone, but it may facilitate the PDI-assisted oxidative folding of conotoxin precursors.

Much less is known about the oxidative folding of conotoxins that occurs in the *Conus* ER. Although *Conus* PDI is a major component of the venom duct and multiple isoforms are likely to be present, no additional published studies characterize the efficiency and substrate specificity of the *Conus* enzyme. No reported studies address a potential role of *Conus* molecular chaperones and their interactions with folding catalysts and conotoxin precursors; this aspect of the conotoxin folding research is completely unexplored. To bridge *in vitro* and *in vivo* folding studies on conotoxins, novel strategies that allow studying the formation of the native disulfide bridges in complex biologic milieus may have to be developed (70, 125). For example, recently synthesized derivatives of α-conotoxin MI (139) provide strong support for using fluorescence-based assays to study the disulfide-bond formation in cells, cell extracts, or microsomes (29, 82, 117, 135, 146). In summary, a combination of biochemical, genetic, and chemical methods is likely to dissect the molecular mechanisms for forming the native disulfide bridges in conotoxins, as it occurs in the *Conus* ER.

Conotoxin folding will continue to be a challenge for peptide chemists to develop efficient strategies to yield larger quantities of these peptides. Replacing cysteines with selenocysteines, or optimizing one-pot regioselective or assisted oxidation strategies may provide the means for improving rates and equilibria for forming the native disulfide-bridging pattern in conotoxins. Less traditional strategies for improving oxidative folding are likely to emerge from the conotoxin folding research. For example, peptide chemists might learn from cone snails how to apply artificial chaperones and small-molecule folding catalysts to improve folding by means of kinetic partitioning (43, 138). What becomes obvious is that the formation of the native disulfide bridges in conotoxins will continue to be a challenge for biochemists and peptide chemists in the years to come.

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ABBREVIATIONS

AA, amino acid; CD, circular dichroism; CRISP, cysteine-rich secretory proteins; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; Hyp, 4-hydroxyproline; ICK, inhibitory cystine knot; MeBz, 4-methylbenzol; PDI, protein disulfide isomerase; P4H, prolyl-4-hydroxylase; PMs, posttranslational modifications; PPI, peptidyl prolyl isomerase; S-Acm, S-acedamidomethyl; SDS-PAGE, denaturing polyacrylamide gel electrophoresis; S-Tmob, 2,4,6-trimethoxybenzyl; tBu, *t*-butyl; Trt, trityl.

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