Identification of Conus Peptidylprolyl Cis-Trans Isomerases (PPIases) and Assessment of Their Role in the Oxidative Folding of Conotoxins

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Peptidylprolyl cis-trans isomerases (PPIases) are ubiquitous proteins that catalyze the cis-trans isomerization of prolines. A number of proteins, such as Drosophila rhodopsin and the human immunodeficiency viral protein HIV–1 Gag, have been identified as endogenous substrates for PPIases. However, very little is known about the interaction of PPIases with small, disulfide-rich peptides. Marine cone snails synthesize a wide array of cysteine-rich peptides, called conotoxins, many of which contain one or more prolines or hydroxyprolines. To identify whether PPIase-associated cis-trans isomerization of these residues affects the oxidative folding of conotoxins, we identified, sequenced, and expressed three functionally active isoforms of PPIase from the venom gland of Conus novaehollandiae, and we characterized their ability to facilitate oxidative folding of conotoxins in vitro. Three conotoxins, namely μ-GIIIA, μ-SIIIA, and ω-MVIIC, derived from two distinct toxin gene families were assayed. Conus PPIase significantly increased the rate of appearance of the native form of μ-GIIIA, a peptide containing three hydroxyprolines. In contrast, the presence of PPIase had no effect on the folding of μ-SIIIA and ω-MVIIC, peptides containing no or one proline residue, respectively. We further showed that an endoplasmic reticulum-resident PPIase isoform facilitated folding of μ-GIIIA more efficiently than two cytosolic isoforms. This is the first study to demonstrate PPIase-assisted folding of conotoxins, small disulfide-rich peptides with unique structural properties.

Predatory marine cone snails (genus Conus) synthesize a great diversity (>50,000) of neurotoxic peptides commonly referred to as conotoxins. Conotoxins selectively target specific subtypes of receptors or ion channels throughout the nervous system, a characteristic that has lead to wide use of conotoxins in ion channel research and as therapeutic agents (for review see Refs. 1 and 2). It is now well understood that the vast diversity of conotoxins is driven by two major events: gene duplication followed by hypermutation of the mature toxin region (3) and the generation of posttranslational modifications (for review see Ref. 4). Posttranslational modifications in Conus generally aid in stabilizing the peptide structure (5, 6) as well as varying the structure and shape of the peptide to optimize target binding (7–9).

The most common posttranslational modification is the formation of disulfide bonds, a characteristic shared with neurotoxins from other venomous animals, antimicrobial peptides such as the defenses, and peptide proteinase inhibitors (Table 1). Conotoxins can be grouped into several superfamilies on the basis of their N-terminal signal sequence and their conserved disulfide framework. Interestingly, despite thousands of different toxin sequences biosynthesized there are only 12–18 structural scaffolds found in vivo, an obscurity recently referred to as the “conotoxin folding puzzle” (10). Even within the same disulfide scaffold, various conotoxins display an array of folding properties, suggesting that neither the Cys pattern nor the primary amino acid sequence is a key folding determinant (10). Discrepancies between in vivo and in vitro folding of conotoxins are emerging (10), with toxins that are difficult to synthesize chemically often being highly abundant in the snail venom gland (11, 12). These findings strongly suggest that the three-dimensional structure that conotoxins adopt in vivo is determined by specific interactions with folding enzymes and molecular chaperones. One apparent folding catalyst in the biosynthesis of cysteine-rich peptides is protein-disulfide isomerase (PDI).2 The oxidation and isomerization of disulfide bonds in the ER of Conus are now known to be catalyzed by PDI (13), one of the most abundant soluble proteins in the venom gland of Conus (14, 15). Given the structural complexity of conotoxins, mechanisms other than PDI-mediated folding are likely to occur.

A great number of conotoxins comprise one or more proline (Pro) residues, some of which are important for toxin-target interactions (16, 17). Hydroxylation of these Pro residues is a common modification in Conus and has recently been reported to affect the oxidative folding properties of several conotoxins including ω-MVIIC, a toxin with very poor in vitro folding.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Table 1.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) GU046310–GU046313 and GU067468.

The abbreviations used are: PDI, protein-disulfide isomerase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Cyp, cyclophilin; ER, endoplasmic reticulum; HPLC, high pressure liquid chromatography; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS/MS, tandem mass spectroscopy; pNA, p-nitroanilide; PPIase, peptidylprolyl cis-trans isomerase; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; Suc, succinyl.
**PPlase-assisted Folding of Conotoxins**

yields (18). Hydroxylation of Pro leads to a 2-fold increase in folding yields, whereas the neurotoxic activity of the toxin is maintained. For the μ-conotoxin GIla, the modification enhances bioactivity but does not affect folding. In contrast, Pro hydroxylation impairs activity but does not improve folding yields in the two α-conotoxins ImI and Gl, which naturally contain a conserved Pro residue (18). These findings emphasize the importance of Pro hydroxylation in the structure and bioactivity of conotoxins. Hydroxylation of Pro has been reported for a number of proteins including collagen, a well-studied substrate of the enzyme peptidylprolyl cis-trans isomerase (PPlase) (19–21).

PPlases are ubiquitous enzymes found in vertebrates, invertebrates, plants, and bacteria and are present in almost all cellular compartments (for review see Ref. 22). Today the terms PPlases, cyclophilins (Cyp), and immunophilins are often used synonymously. Genome analysis of the yeast *Saccharomyces cerevisiae* identified at least eight different isoforms (23), none of which are essential for its survival (24). The human genome comprises at least 16 genes encoding for Cyp-like proteins, eight of which have been identified in *vivo* (for review see Ref. 22). Many functions have been described for these diverse proteins including roles in cellular signaling (25) and the regulation of gene transcription (26, 27) and as chaperones and folding catalysts (28–30). Mammalian CypA is located in the cytosol where it is known to bind the immunosuppressive drug cyclosporine (31). Formation of the CypA-cyclosporine complex prevents T-cell proliferation via inhibition of the protein phosphatase calcineurin (32, 33). The two PPlase isoforms CypB and FKBP-13 reside in the ER (34, 35) and are overexpressed during heat shock, suggesting a major role in the folding and/or assembly of proteins (36, 37). Peptidylprolyl bonds can adopt two distinct conformations, cis or trans. PPlases catalyze the cis-trans isomerization of these bonds, an otherwise slow process that can impede protein folding. A number of proteins have been identified as *in vivo* (30, 38) and *in vitro* (19, 39) folding substrates for PPlases; however, little is known about their role in the folding of small cysteine-rich peptides. In the only study reported to date, the effects of the human cytosolic PPlase FKBP-12 on the oxidative folding of the scorpion toxin maurotoxin were evaluated (for sequence see Table 1) (40). The presence of PPlase accelerated the disappearance of intermediate folding species but does not change the overall folding kinetics of maurotoxin *in vitro* (40).

To address the paucity of information on conotoxin biosynthesis, the present study aimed to identify enzymes having the potential to assist conotoxin folding *in vivo*. We hypothesized that given the frequency of peptidylprolyl bonds in conotoxins and their importance for receptor binding, PPlase-mediated cis-trans isomerization of these bonds facilitates oxidative folding during toxin biosynthesis in the ER of *Conus*. To test this hypothesis we first identified and sequenced several PPlase isoforms in the venom gland of the Australian cone snail, *Conus novaehollandiae*, and recombinantly expressed functionally active enzymes. We demonstrate that PPlase-mediated isomerization of peptidylprolyl bonds significantly affects the rate of formation of the native disulfide bonds for the μ-conotoxin GIla, a peptide with three hydroxyprolines (Hyp) and three disulfide bonds. We further show that an ER-resident PPlase is more active in accelerating the folding of μ-GIla when compared with two cytosolic isoforms. This study furthers our knowledge on how cone snails efficiently synthesize such a great diversity of highly structured peptides and may shed light on the fundamental principals of oxidative folding of small cysteine-rich peptides.

**EXPERIMENTAL PROCEDURES**

**Specimen Collection, Tissue Preparation, and Histology—** Live specimens of *C. novaehollandiae* (Adams, 1854) were collected from Broome, Western Australia. For protein and RNA extractions, venom glands, venom bulbs, and muscle tissues were dissected, immediately snap-frozen in liquid nitrogen, and stored at −80 °C until further processing. For histological preparations, adult snails were transferred to seawater containing 2% MgCl₂ for 4 h. The shells were cracked, the viscera were removed, and overnight fixation was performed in 4% paraformaldehyde/phosphate-buffered saline. Fixed specimens were processed, sectioned (7 μm), and stained with Mallory’s trichrome stain (41) following routine histological procedures.

**Protein Extraction and Two-dimensional Gel Electrophoresis—** Frozen venom ducts were pooled (a total of two samples pooled from three snails each), ground under liquid nitrogen, resuspended in 1 ml of cell lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM Na₄P₂O₇, 1% Triton-X, 10% glycerol, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1× Complete protease inhibitor mixture (Roche Applied Science), pH 7.6), and incubated for 30 min on ice. Cell lysates were centrifuged, and proteins were precipitated from the supernatants and reconstituted in rehydration buffer (8 M urea, 2% CHAPS, 0.002% bromphenol blue, 20 mM dithiothreitol, 0.5% immobilized pH gradient buffer, pH 3–11 (GE Healthcare)) at a final concentration of 1 mg/ml in preparation for isoelectric focusing (IEF). Protein precipitation (2-D Clean-Up Kit, GE Healthcare) and quantitation (BCA assay kit, Thermo Fisher Scientific) were performed according to the manufacturer’s instructions. Two hundred μg of protein was applied onto a nonlinear, pH 3–11, immobilized pH gradient strip (Immobiline, GE Healthcare) and rehydrated overnight. IEF was performed on the Ettan IEGphor II IEF system (GE Healthcare). Running conditions were 500 V for 1 h at 0.5 kVh, 1000 V for 1 h at 0.8 kVh, 6000 V for 2 h at 7.0 kVh, and 6000 V for 40 min at 0.7–3.7 kVh. Following IEF, strips were reduced in equilibration buffer (75 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromphenol blue) containing 65 mM dithiothreitol for 15 min followed by alkylation for 15 min in the presence of 80 mM iodoacetamide. Second dimension gel electrophoreses were performed on 8–16% Tris-HCl SDS-PAGE (Criterion, Bio-Rad) for 50 min at 200 V. Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad). In-gel digestion and protein identification were performed as described previously (42). Briefly, two-dimensional gel electrophoreses spots were excised, washed in 50% acetonitrile/triethylammonium bicarbonate, and reduced with 20 mM dithiothreitol followed by alkylation in 100 mM iodoacetamide. In-gel digestion was performed using sequencing grade trypsin (Sigma-Aldrich) at a final concentration of 10 μg/ml in 25 mM triethylammonium.
bicarbonate. Peptides extracted after overnight digestion were separated on a C18 column (Zorbax 80SB-C18, 74 μm × 43 mm, Agilent, Forest Hill, Victoria, Australia) and analyzed using a linear ion trap mass spectrometer (LC/MSD Trap XCT Plus, Agilent). MS/MS data were used to search the UniProt nonredundant protein data base using Mascot, version 2.2 (Matrix Science, Boston) with the following settings: trypsin, one missed cleavage, carbamidomethyl as a fixed and oxidation of methionine as a variable modification, 1.2 Da peptide tolerance, 0.8 Da MS/MS tolerance, with error-tolerant search included.

cDNA Isolation and Identification of Conus PPIase Transcripts—Three frozen venom ducts were pooled and ground under liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was treated with Turbo DNase (Ambion, Scoresby, Victoria, Australia). RNA concentrations

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence / disulfide pattern</th>
<th>Species, common name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>conotoxin GS</td>
<td>ACSGRGSROODQCCMGLRCCRGNRFKC</td>
<td>Conus geographus, geography cone</td>
<td>Na$^+$ channel inhibitor</td>
</tr>
<tr>
<td>atracotoxin</td>
<td>LLACLFGNGCSNSRDCCELTVCKGRGSCSGLVG</td>
<td>Hadronyche infensa, funnel web spider</td>
<td>Na$^+$ channel inhibitor</td>
</tr>
<tr>
<td>α-conotoxin GI</td>
<td>EECNAPACGRHYSC*</td>
<td>Conus geographus, geography cone</td>
<td>nAChR inhibitor</td>
</tr>
<tr>
<td>kM-conotoxin RIIK</td>
<td>GCSCSLLNLRCSAVERNACRNCCCT*</td>
<td>Conus radiatus, rayed cone</td>
<td>K$^+$ channel inhibitor</td>
</tr>
<tr>
<td>Apamin</td>
<td>CNCKAPETACRCQQH*</td>
<td>Apis mellifera, honey bee</td>
<td>K$^+$ channel inhibitor</td>
</tr>
<tr>
<td>Maurotoxin</td>
<td>VSITGSKDCYACKKTGTPCAMKINCSCKCNYC*</td>
<td>Scorpio maurus palmatus, chactoid scorpion</td>
<td>K$^+$ channel blocker</td>
</tr>
<tr>
<td>PTU1 toxin</td>
<td>AEKDCIAAGACFGTDKPCCNPRAWGSYANKCL</td>
<td>Peirates turpis, assasin bug</td>
<td>Ca$^{2+}$ channel inhibitor</td>
</tr>
<tr>
<td>Huwentoxin-X</td>
<td>KCLPPGKGCYGATQKKGCCOVSHNKCT</td>
<td>Ornithoctonus huwena, chinese earth tiger</td>
<td>Ca$^{2+}$ channel inhibitor</td>
</tr>
<tr>
<td>Katala B1</td>
<td>CGETCVGGTCNCTGCCTSWPVCTRNLGFV</td>
<td>Oldenlandia affinis</td>
<td>plant defense</td>
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<td>Leginsulin</td>
<td>ADCNGACSPFEPPCRCRSRDCRCVPGFLVFGCIPHIG</td>
<td>Glycine max, soybean</td>
<td>plant hormone</td>
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<td>Guanylin</td>
<td>FGTCIECAYAACCTGC</td>
<td>Homo sapiens, human</td>
<td>intestinal hormone</td>
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<td>Defensin Hbd-2</td>
<td>PVTCLSKAIGHCVFCRRRRKQGKTCGLGRTKCCKKP</td>
<td>Homo sapiens, human</td>
<td>antimicrobial activity</td>
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<td>Bv-IWF4</td>
<td>SGECNMYGRCEFCGSCGCGYCGVGRAYCG</td>
<td>Eucomma ulmoides, hardy rubber tree</td>
<td>antimicrobial activity</td>
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<tr>
<td>EAFP1</td>
<td>QTCASRCRCCNAGLSIYGCSSGYNAGACGACRCCR</td>
<td>Alomyrina dichotoma, Japanese rhinoceros beetle</td>
<td>antimicrobial activity</td>
</tr>
<tr>
<td>EETH-II</td>
<td>GCPRILMRCKQDSDCLACGCVPNGFCSP</td>
<td>Eccallium elaterium, squirting cucumber</td>
<td>trypsin inhibitor</td>
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<tr>
<td>MCPI Ila</td>
<td>HADRCNKECKTHDOCSCGWFCQACWNSARTCGVYG</td>
<td>Solanum tuberosum, potato</td>
<td>carboxypeptidase inhibitor</td>
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<tr>
<td>AAI</td>
<td>CTKWNRCGKMDVGECPEYKTSDYVNGCS</td>
<td>Amaranthus hypochondriacus, amaranth plant</td>
<td>alpha-amylase inhibitor</td>
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<tr>
<td>Obtustatin</td>
<td>CTTGCCCCRQCKKLAPGTCWKTSTLTHYCTGKSCDCBLYRPG</td>
<td>Viperidae lebetina obtusa, blunt-nosed viper</td>
<td>integrin inhibitor</td>
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<tr>
<td>Gurmarin</td>
<td>QCCVKKDELCPFYLDCDEPLECKKVNDWDHCIG</td>
<td>Gymnema sylvestre, periploca of the woods</td>
<td>supresses sweetness response</td>
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</tbody>
</table>

TABLE 1
Diversity of proline/hydroxyproline-containing disulfide-rich peptides

* C-terminal amidation; O, hydroxyproline; γ, γ-carboxylation of glutamate. Prolines/hydroxyprolines are highlighted gray, and cysteine residues are shown in bold.
were determined using a spectrophotometer, and RNA integrity was verified by agarose-gel electrophoresis. For rapid amplification of cDNA ends (RACE), 5′ and 3′ universal primer-adapted cDNA was prepared from 1000 ng of total RNA using the SMART™ RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA). RACE was performed using primary and nested PCR proprietary universal oligonucleotides and three gene-specific oligonucleotides (supplemental Table 1) designed on the basis of conserved gene regions and/or on peptide matches obtained using two-dimensional gel electrophoresis coupled with LC/MS/MS (see above). Primary PCR reactions were performed in volumes of 50 µl containing 2.5 µl of 1:10 diluted cDNA, 0.5 µl of TITANIUM Taq DNA polymerase (Clontech), 1× Advantage 2 PCR buffer (Clontech), dNTPs at 200 µM, 5 µl of 10× universal primer A mix (Clontech), and a gene-specific oligonucleotide (0.2 µM) (supplemental Table 1). If the PCR reaction did not produce a visible PCR amplicon, a nested PCR using 5 µl of the primary PCR reaction was performed. To sequence the PPIase A-like isoforms, PCR was performed using redundant oligonucleotides designed to amplify the open reading frame specifically (supplemental Table 1). All PCR amplicons were analyzed by gel electrophoresis, cloned into pGEM-T plasmid vectors (Promega), and subsequently sequenced. Nucleotide sequencing was performed using the ABI Prism BigDye Terminator (version 3.1) cycle sequencing kit (ABI) with the SP6 promoter oligonucleotide (Promega). Samples were analyzed on an ABI 3730xl DNA analyzer (Applied Biosystems). All sequences analyzed in this study were deposited in GenBank™ (National Center for Biotechnology Information, U. S. National Library of Medicine, Bethesda, MD) with the accession numbers shown in supplemental Table 1. All nucleotide data were translated into the predicted amino acid sequences, and comparative alignment of the protein sequences were performed using MAFFT E-INS-i sequence alignment by means of local pairwise alignment vector containing a C-terminal His 6 tag (Novagen, Kilsyth, Victoria, Australia). Transcripts were PCR-amplified from venom gland cDNA prepared as described above (see supplemental Table 1 for oligonucleotide sequences used). PCR amplicons were ligated into pET22b+ using the BamHI (5′) and XhoI (3′) restriction sites (New England Biolabs, Arundel, Queensland, Australia), and sequences were verified as described above. Constructs were transformed into the Rosetta strain of Escherichia coli (Novagen). For expression of Conus PPIases, Luria broth medium (Sigma-Aldrich) containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml) was inoculated with overnight cultures and incubated at 37 °C with shaking until the A 600 spectrophotometric reading was 0.6. After the addition of Isopropyl-β-D-thiogalactopyranoside (final concentration 0.1 mM) the bacterial culture was further incubated for 3 h at 25 °C with shaking. Bacteria were harvested and resuspended in native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole (Sigma-Aldrich), pH 8.0) containing protease inhibitors (Complete protease inhibitor mixture, Roche Applied Science). Bacterial cells were lysed by incubating them with lysozyme (1 mg/ml, Sigma-Aldrich) on ice for 30 min followed by probe tip sonication. Cellular debris and insoluble protein were pelleted by centrifugation, and the supernatants were used for subsequent protein purifications.

Recombinant Conus PPIases were purified on a 1 ml immobilized metal affinity column under native conditions (Bio-Scale Mini Profinity IMAC cartridge, Bio-Rad). Protein purification was performed using the Bio-Rad Profinity protein purification system. Briefly, protein lysates were loaded onto the column at 0.5 ml/min, and nonspecifically bound proteins were removed with native buffer (NB: 300 mM KCl, 50 mM KH₂PO₄, pH 8.0) containing 5 mM imidazole at 2 ml/min for 6 min followed by a second wash with NB containing 10 mM imidazole for 3 min. His-tagged proteins were eluted with 250 mM imidazole in NB at 2 ml/min for 2 min. Further purification and buffer exchange into 0.1 M Tris-HCl, pH 8.0, containing 150 mM NaCl was accomplished by size exclusion chromatography (Superdex 200, HiLoad 16/60, GE Healthcare). Protein concentrations were determined spectrophotometrically using the molar absorption coefficients of the proteins (45). The purified recombinant proteins were analyzed by SDS-PAGE and sequence-verified by in-gel digestion and analysis of proteotypic tryptic peptides as described above.

Protease-coupled PPIase Assay—PPIase activity of recombinant proteins was determined using the coupled chymotrypsin assay (46) with modifications. Briefly, PPI (final concentrations 30 nM for PPI A (I) and (II) and 300 nM for PPI B) was added to 0.1 M Tris-HCl, pH 8, in the absence and presence of a 10× excess of the inhibitor cyclosporine (final concentrations 90 nM for PPI A (I) and (II) and 900 nM for PPI B (Sigma-Aldrich, stock made in ethanol)). After incubation at 25 °C for 2 min, reactions were transferred to 10 °C for 5 min before the addition of 30 µl of 0.6 M chymotrypsin (Sigma-Aldrich). After 5 min at 10 °C, the reaction was initialized with the substrate succinyl-Ala-Ala-Val-7-amido-4-methylcoumarin (Bachem AG, Bubendorf, Switzerland; stock made in trifluoroethanol in the presence of 0.45 M LiCl (Sigma-
TABLE 2  

Conotoxins used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Toxin</th>
<th>Sequence</th>
<th>Disulfide scaffold</th>
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<tr>
<td>Conus geographus</td>
<td>GIIIA</td>
<td>RDCCTODKKKCDQDQDKQRCQCA^</td>
<td>μ-conotoxin</td>
</tr>
<tr>
<td></td>
<td>SIITA</td>
<td>ZNCNGGSSRKWCDARHROCC^</td>
<td>C-C-C-</td>
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</table>

For MVIC two analogues were synthesized, containing either a proline (MVIC) or a hydroxyproline (MVIC OP7) in position 7. All peptides were produced as described previously (47). Briefly, peptides were synthesized on a solid support by an automated peptide synthesizer using Fmoc (N-(9-fluorenylethoxycarbonyl) protected amino acids, HBTU (O-(benzotriazol-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate), and diisopropylethylamine (48). Peptides were cleaved from the resin by treatment with reagent K (trifluoroacetic acid/thioanisole/ethanedithiol/water/phenol (90/5/5/2.5/7.5 by volume)) for 4–4.5 h. The peptides were subsequently filtered, precipitated, and washed with cold methyl tert-butyl ether. Linear peptides were purified by reversed-phase HPLC on a semipreparative C18 column (Vydac, 5 μm particle size, 10 × 250 mm; Grace, Deerfield, IL) using a linear gradient from 5 to 40% buffer B (90% acetonitrile, 0.1% trifluoroacetic acid) over 20 min. Absorbance was monitored at 220 nm. Concentrations of SIIIA and MVIC were determined spectrophotometrically using the molar absorption coefficient of the peptides at 280 nm. Absorbance was monitored at 220 nm. Concentrations of SIIIA and MVIC were determined spectrophotometrically using the molar absorption coefficient of the peptides at 280 nm.

RESULTS

Identification of Conus PPI Isoforms Using a Combination of Two-dimensional Gel Electrophoresis and cDNA Sequencing—Two-dimensional gel electrophoresis on venom gland proteins led to the identification of at least three different isoforms of PPIase excised from two distinct gel spots (Fig. 1A). Data base searches revealed sequence similarities to cyclophilin A from a number of different species including the German cockroach (GenBank™ accession number CAA60869) and the medicinal leech (GenBank™ accession number ABF18081) and to cyclophilin B from the channel catfish (GenBank™ accession number ABC75555) and the yellow fever mosquito (GenBank™ accession number ABF18081). A number of unique peptides derived from two proteins were identified for one gel spot indicating co-migration of two cyclophilin A isoforms, whereas the other gel spot contained one protein similar to cyclophilin B only. Using this unique sequence information, conventional RT-PCR, and RACE-PCR, a total of four PPIase isoforms were sequenced eventually (Fig. 2). Two cDNA transcripts showed high sequence similarities to cyclophilin A (PPI A) from a range of different organisms such as the Japanese scallop Chlamys farreri (identity, 81%; GenBank™ accession number AAR11779.1) and are hereafter referred to as Conus PPI A (I) and Conus PPI A (II). The A isoform of PPIase is located in the cytosol (49). The two transcripts have open reading frames of 495 bases encoding proteins with a predicted molecular mass of 39 kDa (Fig. 1B). The cis-trans isomerization was measured by following the absorbance at 390 nm in a Lambda 2 spectrophotometer (PerkinElmer Life Sciences). The mean and standard errors were calculated from three independent experiments.

Peptide Synthesis and Folding—Three conotoxins, μ-GIIIA, μ-SIIIA, and ω-MVIC, derived from two distinct toxin gene families were selected for oxidative folding studies (Table 2). For MVIC two analogues were synthesized, containing either a proline (MVIC) or a hydroxyproline (MVIC OP7) in position 7.

For MVIIC two analogues were synthesized, containing either a proline (MVIIC) or a hydroxyproline (MVIIC OP7) in position 7.

FIGURE 1. Proteomic identification of Conus PPIases. A, two-dimensional gel electrophoresis image of proteins extracted from the venom gland of C. novaehollandiae showing proteins subsequently identified as PDI, PPI A, and PPI B. 200 μg of total protein was loaded onto nonlinear pH 3–11 IEF strips and separated on 8–16% Tris-HCl SDS-PAGE. Gel spots were excised, reduced, and alkylated, and trypsin was digested and analyzed by ESI-ion trap LC/MS/MS. Mass spectrometry results were searched against the UniProt protein data base using Mascot software. B, protein sequences of Conus PPI A (I), A (II), and B obtained by cDNA sequencing. Peptide matches generated by the data base search of the trypsin-digested gel spots are highlighted in gray (Mowse score > 54). Unique peptide matches are depicted in bold. The percentage of sequence coverage is shown.
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17,523 and 17,542 Da. A third isoform with sequence identities of 73% to PPI B from Xenopus laevis (GenBank™ accession number AAH54168) and 72% to PPI B from Homo sapiens (GenBank™ accession number NP_000933) has an open reading frame of 621 bases encoding a protein with a predicted molecular mass of 22,614 Da. An N-terminal cleavage site between alanine 18 and aspartate 19 would release a mature peptide with an expected molecular mass of 20,821 Da as predicted by SignalP software (version 3.0). The presence of an N-terminal signal peptide and its homology to other B isoforms known to be located in the ER (Fig. 3) (49) strongly suggest that the mature protein resides in the ER. Thus, this isoform is hereafter referred to as Conus PPI B. The fourth cDNA transcript shares homology with peptidylprolyl isomerase-like 1 protein from a variety of organisms such as the copepod Caligus rogercresseyi (similarity, 78%; GenBank™ accession number AC011158) and Mus musculus (similarity, 74%; GenBank™ accession number BAE33862). The molecular mass of the predicted protein is 18,560 Da encoded by an open reading frame of 507 bases. This isoform was designated Conus PPI C. Protein sequence alignment by means of local pairwise alignment (43) reveals high conservation of the key residues involved in substrate binding between the four isoforms (Fig. 2) (50, 51), the conservation levels of conotoxins for this organ (Fig. 4).

**FIGURE 3. Comparative alignment of the ER-Resident B isoform of PPIases.** Alignment was performed using MAFFT E-INS-i by means of local pairwise alignment information (43). Identical amino acids are outlined in black. Dashes denote gaps. Amino acid conservations are denoted by asterisks, whereas colors and periods represent a high and low degree of similarity, respectively.

**FIGURE 2. Comparative alignment of PPIases identified in the venom gland of C. novaehollandiae (GenBank™ accession numbers: PPI A (I), GU046310; PPI A (II), GU046311; PPI B, GU046312; PPI C, GU046313).** Alignment was performed using MAFFT E-INS-i by means of local pairwise alignment information (43). Side chains forming the binding cleft are shown in black. Cysteine residues are shaded. The predicted signal sequence of PPI B is underlined (SignalP). Dashes denote gaps. Amino acid conservations are denoted by asterisks, whereas colors and periods represent a high and low degree of similarity, respectively.
All three enzymes exhibited peptidylprolyl isomerase activity that was reduced by preincubation of the reaction with the PPI inhibitor cyclosporine (Fig. 6). Interestingly, the two A isoforms were more active in catalyzing the prolyl cis-trans isomerization of the tetrapeptide Suc-Ala-Ala-Pro-Phe-pNA, and higher concentrations of PPI B (300 nM versus 30 nM for PPI A (I) and (II)) had to be used to obtain similar prolyl cis-trans conversion rates.

**FIGURE 4.** Differential expression of *Conus* PPIases and α-conotoxins across three different tissue types: the venom gland, the venom bulb, and the muscle tissue. A, RT-PCR showing high mRNA expression levels of the ER-resident folding enzyme PPI B in the venom gland when compared with the bulb and muscle tissue. PPI A and PPI C are constitutively expressed across the different tissue types. Ferritin was used as a reference gene. nRT, no reverse transcriptase control; NTC, no template control. B, schematic of cone snail and venom apparatus depicting orientation of histological section and histology showing the tissue used for RT-PCR, a 7-μm section (scale bar: 500 μm) stained with Mallory’s trichrome stain.

**FIGURE 5.** Expressed and purified PPIases cloned from the venom gland of *C. novaehollandiae*. C-terminal His-tagged fusion proteins were expressed in *E. coli* (Rosetta strain) and purified using immobilized metal affinity chromatography (IMAC) on the Profinia purification system (Bio-Rad) followed by size exclusion chromatography (Superdex 200, GE Healthcare). 1 μg of protein was loaded per lane. Ladder, unstained protein ladder (Invitrogen).

**FIGURE 6.** Peptidylprolyl cis-trans isomerase activity of *Conus* PPIase isoforms. Enzyme activity was tested using the coupled chymotrypsin assay with 30 nM PPI A (I) and (II) and 300 nM PPI B in the presence and absence of the PPIase inhibitor cyclosporine. Reactions containing chymotrypsin only served as base-line controls. Means ± S.E. were calculated from three independent experiments.
**PPlase-assisted Folding of Conotoxins**

![Graph showing HPLC analysis of oxidative folding of conotoxin μ-GIIIA](http://www.jbc.org/)

**DISCUSSION**

Combining proteomics with targeted cDNA sequencing proved to be a highly successful technique for the discovery of novel proteins in the venom gland of *Conus*. Sequence information obtained by two-dimensional gel electrophoresis and nucleotide sequencing was used for the functional characterization of a group of molecular chaperones, the peptidylprolyl cis-trans isomerases. This study describes for the first time the presence of multiple PPlase isoforms in the venom gland of *Conus* and evaluates PPlase-assisted oxidative folding of conotoxins *in vitro*. Further investigations using this integrated approach of proteomic and molecular analyses will facilitate the identification and characterization of additional venom gland proteins and provide insight into the generation of chemical diversity in *Conus*.

Despite their complex three-dimensional structure, which often comprises multiple disulfide bonds and posttranslational modifications (overview in Refs. 4 and 57), little is known about *in vivo* folding events in the ER of *Conus*. To date, the only ER-resident enzymes identified in *Conus* are γ-carboxylase (58) and PDI (14, 15). PDI was shown to be one of the major soluble proteins in the venom gland of *Conus textile* (14) and *Conus amadis* (15), and oxidative folding studies show PDI-assisted folding of a number of conotoxins *in vitro* including μ-GIIIA (13, 47, 59). Two-dimensional gel electrophoresis analysis of the venom gland proteome confirmed high abundances of this enzyme in the venom gland of *C. novaehollandiae* (Fig. 1A). As...
shown for a number of proline-containing proteins including ribonuclease T1, PPIase can improve the efficiency of PDI-mediated folding by providing partially folded protein chains with the correct proline isomers (56). As folding intermediates are highly susceptible to degradation, accelerating the time needed from translation at the ribosome to generation of the protein in its final three-dimensional structure is energetically advantageous. The epithelial cells of the venom gland are highly specialized in peptide biosynthesis and secretion and energetically rely upon fast turnover rates.

Here, we have shown that Conus PPIases can significantly accelerate the formation of the native disulfide bonds for conotoxin \( \mu \)-GIIIA, revealing that peptidylprolyl isomerization is a rate-limiting step in the oxidative folding of this conotoxin. The native peptide containing three disulfide bonds appeared \(~6\) times faster in the presence of PPI B. Concomitantly, both the disappearance of the linear form and the appearance and subsequent disappearance of other folding species were accelerated when Conus PPI B was added to the folding reaction (Figs. 7 and 8). Changes in the folding kinetics followed in a concentration-dependent manner with 10-fold higher folding rates for GIIIA in the presence of 2.5 \( \mu \)M PPI B (Fig. 9). No difference in GIIIA folding was observed when heat-treated enzyme was added to the folding reaction (data not shown).

As all three prolines in \( \mu \)-GIIIA are posttranslationally modified, we have further demonstrated that a 4-hydroxyproline-containing conotoxin can be recognized by Conus PPIase, a finding reported previously for collagen (19, 20). Hydroxylation of prolines is believed to be catalyzed by prolyl 4-hydroxylase. Interestingly, PDI is a subunit of prolyl 4-hydroxylase, and hydroxylation, formation of the native disulfide bonds, and cis-trans isomerization of Hyp residues may occur concurrently. Recently a complex

**FIGURE 8.** Folding kinetics of \( \mu \)-GIIIA in the presence of 0.1 mM GSH and 0.1 mM GSSG with and without Conus PPI B. The relative abundance of the correctly folded (A), linear (B), and other folding species (C) was determined by HPLC as shown in Fig. 7. The plotted values are averages from three independent experiments. Nonlinear regression analysis was performed on plotted data, and half-times for the appearance and disappearance were calculated for the native and linear forms, respectively (D).
PPIase-assisted Folding of Conotoxins

TABLE 3
Half-times for the appearance of μ-GIIIA containing native disulfide connectivity in the presence of Conus PPIases

Half-times were calculated by non-linear regression. The numbers in parentheses represent the upper and lower 95% confidence interval.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half-time for appearance of native form (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td>583 (471–766)</td>
</tr>
<tr>
<td>PPI B, 0.025</td>
<td>275 (190–495)</td>
</tr>
<tr>
<td>PPI B, 0.1</td>
<td>181 (140–257)</td>
</tr>
<tr>
<td>PPI B, 0.5</td>
<td>100 (77–141)</td>
</tr>
<tr>
<td>PPI B, 2.5</td>
<td>61 (50–78)</td>
</tr>
<tr>
<td>PPI A (I), 2.5</td>
<td>220 (156–375)</td>
</tr>
<tr>
<td>PPI A (II), 2.5</td>
<td>244 (173–414)</td>
</tr>
</tbody>
</table>

comprising prolyl 3-hydroxylase-1, cartilage-associated protein (CRTAP), and cyclophilin B was identified in chick embryos and shown to act as a chaperone for collagen (60). Similar complexes might be present in the ER of Conus, and their discovery would contribute greatly to our knowledge of the biosynthesis of conotoxins and other disulfide-rich small peptides.

Mature GIIIA is 22 amino acids in length and contains three Hyp residues in position 6, 7, and 17 (Table 2). NMR studies on GIIIA and its paralogue, GIIIB, strongly suggest a trans configuration of Hyp6 and Hyp17, whereas Hyp7 adopts a cis conformation (54, 55). Under unstrained conditions, e.g., during translation at the ribosomes, most prolyl peptide bonds adopt a trans configuration (61). Therefore, for proteins containing both cis and trans prolines, the folding kinetics are driven by the trans-to-cis isomerization, and the contribution of the trans prolines can be neglected (39). As a consequence, cis-Pro7 is likely to dominate the folding kinetics of GIIIA. However, slow intermediate trans-to-cis isomerizations of Pro6 and Pro17 during formation and/or reshuffling of disulfide bonds cannot be ruled out.

The presence of PPIase did not affect the folding kinetics of ω-MVIIC and μ-SIIIA under the various conditions tested. The inability of Conus PPIs to accelerate the folding of SIIIA is not surprising, as this peptide does not contain Pro or Hyp residues. MVIIC is a ω-conotoxin with one Pro/Hyp residue likely to be present as mixed cis/trans isomers (18). Isomerization of the Pro/Hyp residue might not represent a rate-limiting step in the oxidative folding of MVIIC. Alternatively, MVIIC may not represent a suitable substrate for Conus PPIases. Both MVIIC and GIIIA comprise three disulfide bonds, are similar in length, and have comparable charge states at pH 7.5 (pH +4.2 for GIIIA and +5.2 for MVIIC). Studies addressing the substrate specificities of the PPIases of the cyclophilin family reveal the broad specificities of these enzymes (62, 63). However, short tetrapeptides were the only substrates tested in these studies (62, 63), and differences in substrate recognition and affinity between GIIIA and MVIIC cannot be excluded, especially considering
the significant differences in their disulfide patterns and primary amino acid sequences (see Table 2). Future studies using a variety of conotoxins with sequence similarities to GIHIA and MVIIIC will shed light on substrate specificities of Conus PPlases.

RT-PCR on the venom gland, bulb, and muscle tissue revealed high expression levels of the ER-resident isofrom PPI B in the venom gland of C. novae-hollandiae (Fig. 4). As opposed to the bulb and the muscle, the venom gland is highly specialized for toxin biosynthesis, and up-regulation of PPI B strongly indicates a predominant role of this isoform in protein/peptide folding as reported for the human ER-resident PPlases CypB and FKBP-13 (36, 37). Furthermore, we have shown that Conus PPI B is approximately five times more efficient in accelerating the folding of GIHIA than the two cytosolic isoforms, PPI A (I) and A (II) (Fig. 10), but is less active in catalyzing the isomerization of the tetrapeptide Suc-AAPF-pNA (Fig. 6). These findings indicate a potential and specialized role of PPI B in the in vivo folding of conotoxins. Differences in catalytic efficacy between the A and B isoform have previously been reported for the trematode Schistosoma mansoni (64) and recently for human cyclophilins (65). Human CypA and CypB both recognize several proline residues on domain 2 of hepatitis C virus NS5A protein but with different catalytic efficiencies (65). For S. mansoni recombinant CypB was two to three times more active than CypA when tested against Suc-AAPF-pNA. As for Conus PPI A (I + II) and B, the amino acid residues important for substrate binding were highly conserved between the two isoforms with the only difference being the presence of serine 103 in PPlase B instead of the conserved alanine 103 for the A isoform. Although the authors (64) suggested that this amino acid replacement might cause the differences in substrate binding affinities, later studies demonstrated that the cytosolic A isoform can adopt two redox states, significantly impairing the enzyme activity (66). Under oxidizing conditions S. mansoni PPI A exhibits one disulfide bond, leading to a significant change in the binding pocket of the enzyme, thus impeding activity. A similar observation was made for cyclophilin CYP20–3 isolated from the chloroplasts of Arabidopsis thaliana (66, 68). CYP20-3, believed to be regulated by thioredoxin (68), comprises two disulfide bonds in its oxidized, inactive state (67). Conus PPI A (I) and (II) contain four cysteines in their primary amino acid sequence (Fig. 2). Formation of disulfide bonds between these cysteine residues may occur especially under the oxidizing conditions necessary for the conotoxin folding experiments. Only one cysteine remains conserved in the ER-resident Conus PPlase (Fig. 2), implying a potential adaptation of this isoform to the oxidizing conditions in the ER. Structural studies on the different PPlase isoforms under oxidizing and reducing conditions and in complex with Suc-AAPF-pNA, μ-GIHIA, and other potential conotoxin substrates will provide insight into the existence of redox states, differences in enzyme activities, and substrate recognition properties.

In summary, this study has identified the first members of the PPlase family in marine cone snails, animals that specialize in the biosynthesis of cysteine-rich peptides. We have demonstrated that a venom gland ER-resident isoform preferentially catalyzes the refolding of a conotoxin that contains three hydroxyprolines. These observations suggest that this isoform may have co-evolved with the toxin arsenal to efficiently produce the diverse venom repertoire of the Conus species. It is anticipated that Conus PPlases may act in concert with other foldases, such as PDI and prolyl 4-hydroxylase, to facilitate conotoxin biosynthesis. The characterization of these enzymes provides insights into the fundamental process of the folding of toxins and related polypeptides as well as having potential industrial application to the large scale production of toxins as therapeutics.

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PPIase-assisted Folding of Conotoxins


Identification of Conus Peptidylprolyl Cis-Trans Isomerases (PPIases) and Assessment of Their Role in the Oxidative Folding of Conotoxins
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