Propeptide Does Not Act as an Intramolecular Chaperone but Facilitates Protein Disulfide Isomerase-Assisted Folding of a Conotoxin Precursor†

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Received August 10, 2003; Revised Manuscript Received November 13, 2003

ABSTRACT: Conotoxins comprise a large and diverse group of peptide neurotoxins derived from *Conus* snail venoms; most contain multiple disulfide bonds. The conotoxin precursors consist of three distinct domains: the N-terminal signal sequence, an intervening propeptide region, and the C-terminal mature conotoxin. Formation of the native disulfide bonds during the oxidative folding of conotoxins is a prerequisite for their proper biological function, but in numerous in vitro folding experiments with mature conotoxins, a lack of specificity in formation of the native Cys−Cys connectivities is observed. The mechanisms that ensure that the native disulfide bonds are formed in venom ducts during biosynthesis remain unknown. To evaluate whether the propeptide could potentially function as an intramolecular chaperone, we studied the oxidative folding of a conotoxin precursor, pro-GI, belonging to the α-conotoxin family. Our results indicate that the propeptide sequence did not directly contribute to folding kinetics and thermodynamics. However, we found that the propeptide region of pro-GI played an important role when oxidative folding was catalyzed by protein disulfide isomerase (PDI). The PDI-assisted reaction was more efficient during the early folding in the context of the propeptide sequence (pro-GI), as compared to that of the mature conotoxin (α-GI). Taken together, our results suggest for the first time that the propeptide region may play a role in the PDI-catalyzed oxidative folding of conotoxin precursors.

Conotoxins are a highly specialized but structurally and functionally diverse group of disulfide-rich peptides. They are produced by venomous *Conus* snails to target prey, predators, and competitors; most appear to affect the function of a specific ion channel or receptor target (*1*−*3*). It is estimated that these peptide neurotoxins comprise over 50000 unique sequences in the venoms of living *Conus* species. Despite this impressive chemical diversity, there are only about 12−18 disulfide-rich scaffolds that are found in the great majority of conotoxins. Thus, a challenging biological and chemical puzzle is how the peptides with extremely diverse sequences that share the same arrangement of cysteine residues (the "Cys pattern") fold into the same three-dimensional scaffold (the "disulfide framework") with identical disulfide connectivity. Figure 1 illustrates the differences in the in vitro folding yields of conotoxins sharing the same Cys pattern and native disulfide framework. It is apparent from this figure that the conserved Cys pattern cannot be the only determinant that stabilizes the native disulfide framework. Similar observations are found for many other conotoxins, belonging to different structural groups, such as μ- or α-conotoxins (G. Bulaj and B. M. Olivera, unpublished results), further exemplifying the "conotoxin folding puzzle".

Conotoxins are initially translated as larger precursors (*4*) that share a similar organization, independent of the conotoxin gene superfamily (Figure 2), consisting of an N-terminal signal sequence, a propeptide, and the C-terminal cysteine-rich mature toxin region. Typically, when comparisons between different *Conus* species are made within a conotoxin superfamily, the signal sequences are very highly conserved, and propeptide regions are moderately conserved; in contrast, the mature toxin region is hypervariable but contains a conserved cysteine pattern. In combination, the highly conserved signal sequence and the Cys pattern serve as a reliable guide for identifying the superfamily a particular conopeptide belongs to.

Propeptide-facilitated oxidative folding has been described for a number of polypeptides, such as macrophage inhibitory cytokine-1 (MIC-1)[1] (*5*), human nerve growth factor (hNGF) (*6*), guanylyl cyclase activating peptide (GCAP) (*7*−*9*), or bovine pancreatic trypsin inhibitor (BPTI) (*10*). For example,

† This work was supported by NIH Program Project Grant GM 48677 (B.M.O.). ESI-MS analysis was performed in the Mass Spectrometry Facility in the Chemistry Department at the University of Utah, supported by the National Science Foundation (Grant CHE-9708413) and the University of Utah Institutional Funds Committee.

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[1] Abbreviatons: Acm, acetamidomethyl; BPTI, bovine pancreatic trypsin inhibitor; DIEA, diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; Fmoc, fluorenylmethoxycarbonyl; GCAP, guanylyl cyclase activating peptide; GSSG, oxidized glutathione; GSH reduced glutathione; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; hNGF, human nerve growth factor; k_{app}, apparent rate constant; k_{cat}, first-order rate constant; K_m, Michaelis constant; K_{ox}, thiol/disulfide exchange equilibrium constant; MIC-1, macrophage inhibitory cytokine-1; MTBE, methyl *t*-butyl ether; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid; Trt, trityl; $v_{initial}$, initial rate; v_{max}, maximum rate.

10.1021/bi0354233 CCC: $27.50
Published on Web 01/06/2004

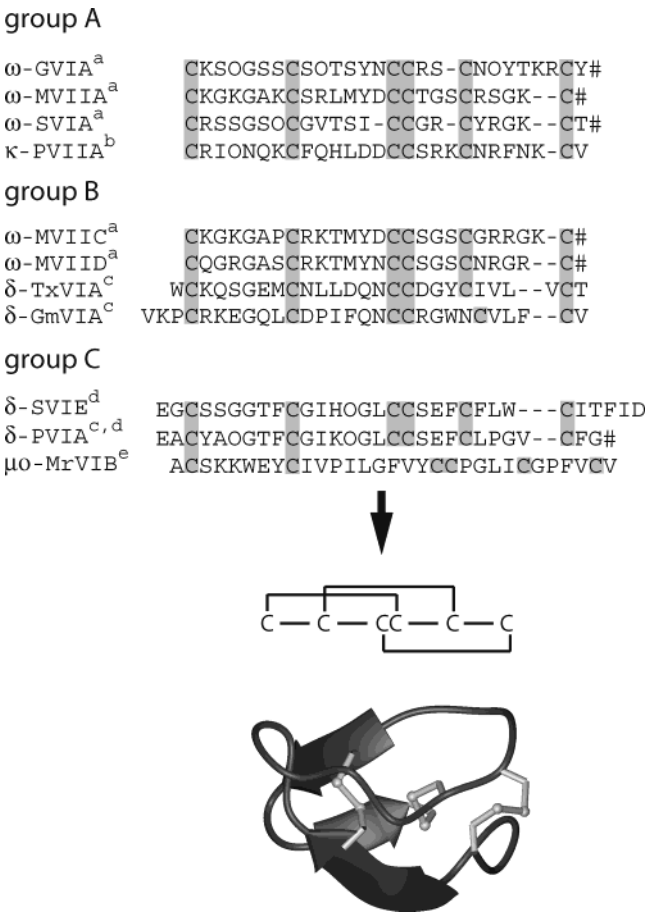

FIGURE 1: Conotoxins' folding puzzle illustrated by folding properties of conotoxins belonging to the O-superfamily. The cysteine arrangement C-C-CC-C-C is highly conserved among all members of this superfamily, with the native disulfide bond connectivity between cysteines 1−4, 2−5, and 3−6. The native disulfides stabilize the so-called ICK motif (inhibitory cystine knot). The ICK motif consists of three short antiparallel β-strands leading to a characteristic three-dimensional fold. The model representation of the ICK fold shown is based on ω-MVIIA (PBD ID: 1OMG). There are over 100 published sequences from this superfamily, with characteristic hypermutation of amino acids between conserved cysteines, but only few of them were synthetically produced. These peptides are grouped according to their in vitro folding properties: group A, folding yields higher than 50%, group B, folding yields between 5% and 50%, and group C, extremely low yields, <3%. C-Terminal amidation is denoted as #. Footnotes: a, ref *48*; b, ref *49*; c, ref *50*; d, ref *51*; e, S. Wei and G. Bulaj, unpublished results.

in the case of the two-disulfide-containing GCAP, folding yields increased from 7% to 95% in the presence of the propeptide (*9*). Similar studies on GCAP-II showed that two amino acids in the N-terminal fragment of propeptide were directly involved in a chaperone-like function during folding (*8*). For oxidative folding of bovine pancreatic trypsin inhibitor (BPTI), the propeptide substantially increased folding yields and kinetics through an additional N-terminal cysteine residue naturally present in the propeptide fragment (*10*). Thus, propeptides can act as intramolecular chaperones directly involved in the formation of native disulfide bonds in a wide variety of polypeptides and smaller peptides. The possibility of such a role for the conotoxin propeptide in folding was previously suggested (*4*).

In vivo, oxidative folding of polypeptides is catalyzed by protein disulfide isomerase (PDI), which can act both as a folding catalyst and as a molecular chaperone (*11*). PDI catalyzes protein thiol−disulfide exchange using a thioredoxin domain at the active site containing the sequence ...Cys-Gly-His-Cys.... This enzyme was shown to promote oxidative folding even in the absence of glutathione (*12*, *13*). PDI was also found to facilitate folding of proteins lacking disulfides, such as rhodanase or glyceraldehyde-3-phosphate dehydrogenase, leading to the suggestion that the enzyme serves as a molecular chaperone (*14*). This dual PDI function was recently shown in the oxidative folding of proinsulin (*15*); PDI both increased the rate of reaction and prevented proinsulin from aggregation. Since PDI is an abundant protein found in bacteria, fungi, plants, invertebrate, and vertebrate animals, it seems likely a priori that *Conus* snails have also employed this enzyme for facilitating proper oxidative folding of conotoxins. This possibility is strongly supported by the recent discovery that PDI is the most abundant polypeptide component of *Conus* venom duct extracts (*16*).

The role of the propeptide and PDI in the folding of one conotoxin, ω-MVIIA, was previously studied by Price-Carter and Goldenberg (*17*). The authors compared a disulfide-coupled folding of three peptides: (1) the mature toxin ω-MVIIA, (2) pro-MVIIA, which contained the propeptide sequence, and (3) ω-MVIIA-Gly with the C-terminal glycine (which is subsequently removed by monoamine oxidase to yield an amidated C-terminus in the mature toxin). The experimental results indicated that the C-terminal glycine residue was an important factor in ω-MVIIA folding but not the propeptide and PDI (*17*, *18*).

In this work, the role of the propeptide in the oxidative folding of conotoxins is further explored using the two-disulfide-containing α-conotoxin, GI (Figure 2). The results below indicate that although the propeptide does not directly affect oxidative folding of conotoxins, differences between α-GI and its precursor were observed when the reaction was catalyzed by PDI. Our results are consistent with the propeptide having a role in the PDI-catalyzed folding of conotoxins.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. All peptides were synthesized on a solid support by an automated peptide synthesizer using *N*-Fmoc-protected amino acids, HBTU, and DIEA (*19*). All cysteine residues were trityl (Trt) protected, unless specified otherwise. The coupling time was 1 h. Peptide cleavage/deprotection was accomplished with reagent K (82.5% TFA:5% phenol:5% H_2O:5% thioanisole:2.5% EDT) for 3 h at room temperature. Soluble crude peptide products were precipitated with cold MTBE, washed with MTBE, then dissolved in 25% aqueous acetonitrile and 0.1% TFA, and lyophilized. The linear peptides were purified on a Vydac C_{18} semipreparative HPLC column. The purified linear peptides were further used for folding experiments.

The α-GI and pro-GI used as HPLC standards of the native forms were chemically synthesized using two orthogonal protecting groups on Cys thiols. The first and third Cys residues were blocked with trityl groups and the second and fourth ones with the acetamidomethyl (Acm) groups. The α-GI was oxidized using a method described previously (*20*). The pro-GI was cleaved from the resin concurrent with removal of Trt groups, and the first disulfide bond was

signal sequence propeptide mature toxin

α-GI MGMRMMFTVFLLVVLATTVVS FPSERASDGRDDTAKDEGSDMEKLVEKK ECCNPACGRHYSCGR

FIGURE 2: Structure of the α-GI precursor. The cleavage site for the signal sequence was predicted using AnalyzeSignalase software (written by Ned Mentai, http://iubio.bio.indiana.edu/soft/molbio/mac). The cleavage site between propeptide and toxin was predicted from the sequence of the mature toxin. The mature toxin is posttranslationally amidated at the C-terminus (the last Cys residue is amidated).

formed in 0.1 M Tris-HCl, pH 8.7, containing 1 mM EDTA, 1 mM oxidized glutathione (GSSG), and 2 mM reduced glutathione (GSH) at 20 μM peptide concentration. After 1 h of oxidation at room temperature, the reaction was quenched with formic acid (8% final concentration). Peptide with the first disulfide bridge oxidized (Cys1−Cys3) was purified on a Vydac C_{18} semipreparative HPLC column to the 90% of purity. Solvents A (0.1% trifluoroacetic acid) and B (90% acetonitrile and 0.1% trifluoroacetic acid) were mixed to form a linear gradient of 10−30% solvent B for 60 min. The remaining two cysteines (Cys2 and Cys4) were deprotected and oxidized in a single step using iodine oxidation. The peptide was dissolved in 10% acetonitrile, 4% TFA, and 5 mM I_2 to the final concentration of 20 μM. After 5 min of the oxidation at room temperature, the reaction was quenched with formic acid (8% final concentration of formate). The correctly folded peptide was purified using reversed-phase C_{18} HPLC. Concentrations of α-GI and pro-GI were determined spectrophotometrically using the molar absorbance coefficient at 274.5 nm, $\epsilon = 1420\ M^{-1}\ cm^{-1}$ (*21*).

Mass Spectrometry. Electrospray mass spectrometry of all peptides used in this study was performed with a Quatro II Micromass mass spectrometer and Masslynx software. Samples were dissolved in methanol/water (1:1 v/v) containing 0.01% TFA. Molecular masses of all peptides were within 1.0 atomic mass unit from those calculated from amino acid sequences.

Oxidative Folding. Oxidative folding reactions for α-GI and pro-GI were performed in 0.1 M Tris-HCl, pH 8.7, containing 1 mM EDTA, 0.5 mM GSSG, and 5 mM GSH, at 22 °C. The folding experiments catalyzed by bovine protein disulfide isomerase (Sigma-Aldrich) were carried out in 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 2 μM PDI, and appropriate concentrations of glutathione at 0 °C. The folding reactions were initiated by adding the linear synthetic peptide to the folding mixture to a final concentration of 20 μM. The reactions were quenched by adding formic acid to the final concentration of 8%. The folding species were separated on a Vydac C_{18} analytical HPLC column. Solvents A (0.1% trifluoroacetic acid) and B (90% acetonitrile and 0.1% trifluoroacetic acid) were mixed to form a linear gradient of 5−30% solvent B for 40 min and 15−25% solvent B for 60 min for α-GI and pro-GI, respectively.

RESULTS

Synthesis of the Precursor for α-GI Conotoxin. Pro-GI was chemically synthesized on a solid support using a standard Fmoc protocol. Two versions of pro-GI were produced, where (1) all four cysteines were protected by trityl groups (Trt) or (2) the first Cys and third Cys were protected by the Trt groups and the second Cys and fourth Cys by the acetamidomethyl groups (Acm). The first variant of pro-GI containing all four deprotected cysteines was used in the folding experiments. The second variant was used to produce

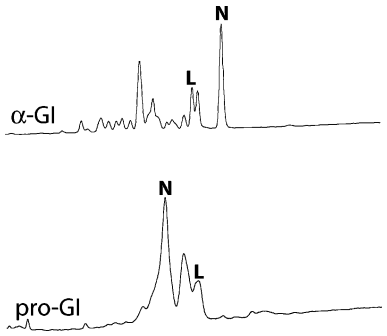

FIGURE 3: Effect of the propeptide sequence on the oxidative folding of α-GI. The steady-state distribution of the folding species for α-GI is compared to its precursor, pro-GI. Linear and correctly folded forms are denoted as L and N, respectively. Folding reactions were performed at 22 °C in 0.1 M Tris-HCl, pH 8.7, containing 1 mM EDTA, 0.5 mM GSSG, and 5 mM GSH. The steady state was observed after 1 h of the reaction.

peptide with the native disulfide connectivity and then to determine an HPLC retention time of the properly folded pro-GI. The identity of the peptides was confirmed by mass spectrometry: pro-GI linear, 4562.1 Da (calculated value 4563.0 Da), pro-GI correctly folded, 4559.0 (calculated value 4559.0 Da).

Role of the Propeptide in the Folding of α-GI. Oxidative folding of α-GI and pro-GI was carried out in buffered solutions containing oxidized and reduced glutathione as described in Experimental Procedures. On the basis of the time course of the appearance of the folding species, the folding reactions at pH 8.7 and at ambient temperature (22 °C) reached equilibrium after 1 h. The folding reactions were quenched after 2 h by acidification and analyzed by reversed-phase HPLC. The distribution of the folding species for pro-GI and mature α-GI is shown in Figure 3. The equilibrium accumulation of the native form was similar for the mature toxin and the propeptide-containing polypeptide: 20% yield of the native α-GI, compared to 27% yield for the native pro-GI. Interestingly, the native form of pro-GI was less hydrophobic relative to the linear form, whereas native α-GI appeared to be more hydrophobic when compared to the linear form.

To explore the possibility that the propeptide could influence oxidative folding under different experimental conditions, we screened several factors, such as temperature, redox potential, and presence of denaturants or osmolytes. These factors are typically tested in folding screens (*22*, *23*). As summarized in Figure 4, varying the folding environment in the presence of the N-terminal propeptide did not significantly change the steady-state distribution of the native species. The accumulations of the native α-GI and pro-GI were equally sensitive to the different conditions, despite changes in the temperature from 0 to 37 °C, redox potential from a GSSG/GSH molar ratio of 1:1 to 1:10, or the presence of other folding additives, such as urea, glycerol, nonionic detergents, or organic cosolvents. To assess whether adding protein disulfide isomerase (PDI) could affect the steady-

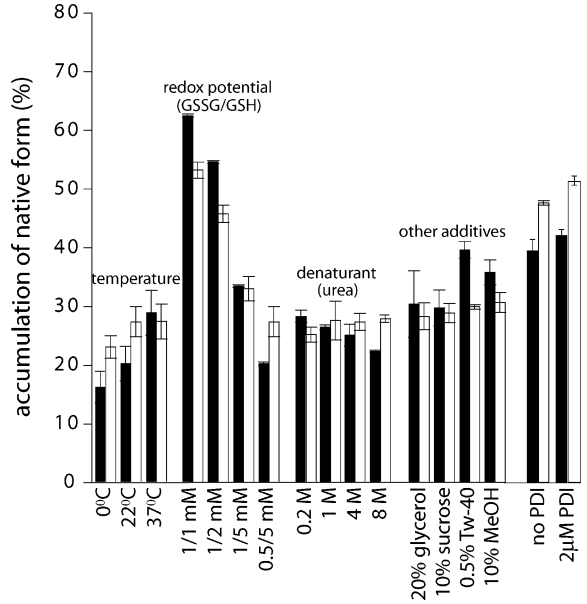

FIGURE 4: Effects of the propeptide on the stability of native α-GI. Changes in accumulation of the native forms for α-GI (black bars) and pro-GI (white bars). The percentage of the native form accumulation was averaged from three separate folding experiments, and the standard deviation is marked. Folding experiments were performed for 2 h in 0.1 M Tris-HCl, pH 8.7, and 1 mM EDTA at 20 μM peptide concentration. Key: Tw-40, detergent Tween-40; MeOH, methanol. Folding in the presence of 2 μM PDI was carried out in 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM GSSG, and 5 mM GSH at 20 μM peptide concentration at 0 °C for 2 h. The respective control experiments (in the absence of PDI) were carried out under conditions identical to those in the presence of the enzyme.

state distribution of the native α-GI and pro-GI, the equilibrium accumulation of the folding species for PDI-catalyzed reaction was compared. The folding experiments were carried out in the presence of 2 μM PDI, 0.5 mM GSSG, and 5 mM GSH at pH 7.5 and 0 °C for 2 h (under these conditions, the equilibrium was established after 45 min). As shown in Figure 4, the presence of PDI did not affect the equilibrium distribution of the native forms for both α-GI and pro-GI. These results confirm that the propeptide sequence does not directly participate in the stabilization of the native conotoxin as an intramolecular chaperone.

We also investigated whether the propeptide could influence the folding rates. Kinetics were studied in two different redox buffers (1) in the presence of 0.5 mM GSSG and 5 mM GSH, where the folding rates are determined by intramolecular rearrangement, or (2) in the presence of 0.1 mM GSSG, where folding rates are defined by the reactivity of the peptide Cys thiols (*24*). Under both conditions, the propeptide did not affect folding kinetics. The rate of formation of the first disulfide bond, assayed by the disappearance of the linear form, was comparable for both α-GI and pro-GI (Figure 5A). The rate of formation of the native form or the appearance of other folding species (one-disulfide intermediates and misfolded forms) was also comparable for the mature toxin and for the propeptide-containing variant.

Folding in the Presence of Protein Disulfide Isomerase. Since efficient formation of disulfide bonds in eukaryotic cells is mediated by protein disulfide isomerase, rather than by glutathione, we compared the folding of α-GI and pro-GI in the presence of bovine PDI. The PDI-catalyzed folding reactions were carried out under identical experimental conditions as described in the previous section, except that the enzyme was added to the folding mixture prior to the addition of the linear peptide. Under folding conditions with a mixture of 0.5 mM GSSG and 5 mM GSH, PDI was primarily present in the reduced form [estimated ratio of PDI_{red}/PDI_{total} was at least 97%, based on K_{ox} of 1.3 mM (pH 7.4) (*25*)]. In the presence of 0.1 mM GSSG, the enzyme was predominantly in the oxidized state. The enzyme concentration (2 μM) used in the experiments was approximately 100-fold lower compared to that estimated for the ER (*26*). However, this range of PDI concentrations (low micromolar) has been routinely used in other laboratories to characterize PDI-catalyzed folding reactions.

As illustrated in Figure 5A, the folding kinetics for both α-GI and pro-GI were different in the presence of 2 μM PDI and 0.1 mM GSSG, compared to the corresponding rates in the absence of the enzyme. The k_{app} for disappearance of the linear forms equaled 0.29 and 0.45 min^{-1} for α-GI and pro-GI, respectively. More significant differences between the precursor and the mature conotoxin were observed for the PDI-catalyzed formation of the native forms. After the first 10 min of the folding reaction in the presence of PDI, the amount of the native form of pro-GI increased 14.3-fold (relative to the uncalatyzed reaction), compared to only a 5-fold increase for α-GI. A single exponential fit of the experimental points yielded k_{app}= 0.046 min^{-1} (α-GI) and k_{app}= 0.145 min^{-1} (pro-GI). This 3-fold increase in the folding rates is further illustrated in Figure 5B.

It is apparent that the PDI-catalyzed reaction was more efficient during early folding when the propeptide was present. This effect was only observed when PDI was in the oxidized state (0.1 mM GSSG). In the presence of 0.5 mM GSSG and 5 mM GSH, the reactions for both α-GI and pro-GI were significantly faster when PDI was added, but the folding rates were comparable for the propeptide-containing form and the mature toxin (data not shown). These differences in the effects of the propeptide on the PDI-catalyzed folding under different redox conditions are discussed in the next section.

To better characterize α-GI and pro-GI as PDI substrates, folding kinetics was analyzed as a function of substrate concentration. Since the folding species were separated using HPLC and quantified by their absorbance at 220 nm, a limited range of low micromolar peptide concentration could be used in the study. The initial rates for appearance of native α-GI and pro-GI were measured for the first minute of the folding reaction in the presence of 5, 10, and 15 μM concentrations. The initial rate of oxidation appeared to be independent of substrate concentration for both α-GI and pro-GI (Figure 6A), suggesting that, for both variants of the peptide, K_m values are significantly lower than 5 μM. However, the v_{max}, and thereby k_{cat}, value for oxidation of the propeptide-containing variant was about 2 times higher than for the mature toxin. Figure 6B illustrates the oxidation kinetics for α-GI and pro-GI as a function of the substrate concentration. Within the range from 5 to 15 μM, the appearance of the native form of pro-GI was systematically faster than that of the native α-GI. The reaction half-times were calculated from each time course. The linear dependence of the half-times on the substrate concentration was

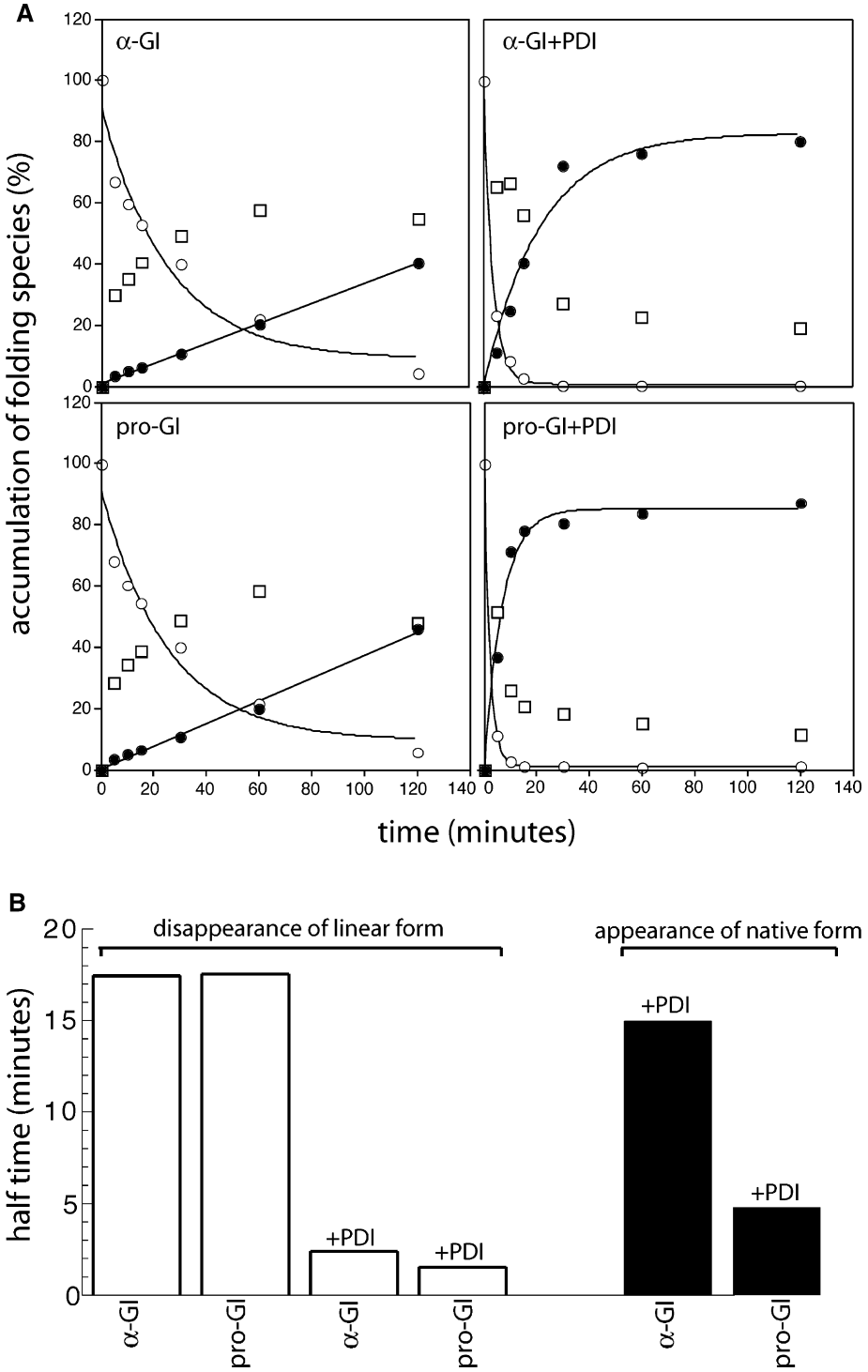

FIGURE 5: (A) Folding kinetics for uncatalyzed and the PDI-catalyzed oxidation of α-GI and pro-GI. The folding reactions were carried out at 0 °C in 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM GSSG, and 2 μM PDI. The filled and open circles denote native and linear forms, respectively, and the squares represent other folding species. The plotted values are an average from three experiments. The experimental points were analyzed by single exponential fit, and the k_{app} values for the appearance of the native form (only in the presence of PDI) and disappearance of the linear form (in the presence or absence of PDI) were calculated. For the appearance of the native form in the uncatalyzed reaction we did not determine rate constants, since the experimental points fit to the linear, rather than exponential, equation. (B) Bar graphs summarizing the half-times for PDI-catalyzed and uncatalyzed folding of α-GI and pro-GI (derived from fits to the folding kinetics shown in panel A). The white bars represent values for the linear form and the black bars for the native form.

different for α-GI (slope = 1.6) and pro-GI (slope = 1.0) (Figure 6C). We also tested a possibility that PDI could favor a rapid formation of the native disulfides in pro-GI. In the presence of more rapid oxidants, such as dithiobis(2-nitrobenzoic acid) (DTNB) or azodicarboxylic acid bis-(dimethylamide) (diamide), a distribution of the native forms of pro-GI and α-GI was comparable (data not shown), indicating that PDI did not favor the native pro-GI to partition kinetically upon oxidation.

DISCUSSION

To explore the mechanism of in vivo folding of conotoxins, we examined the role of the propeptide in folding for a model α-conotoxin precursor, pro-GI. Our results

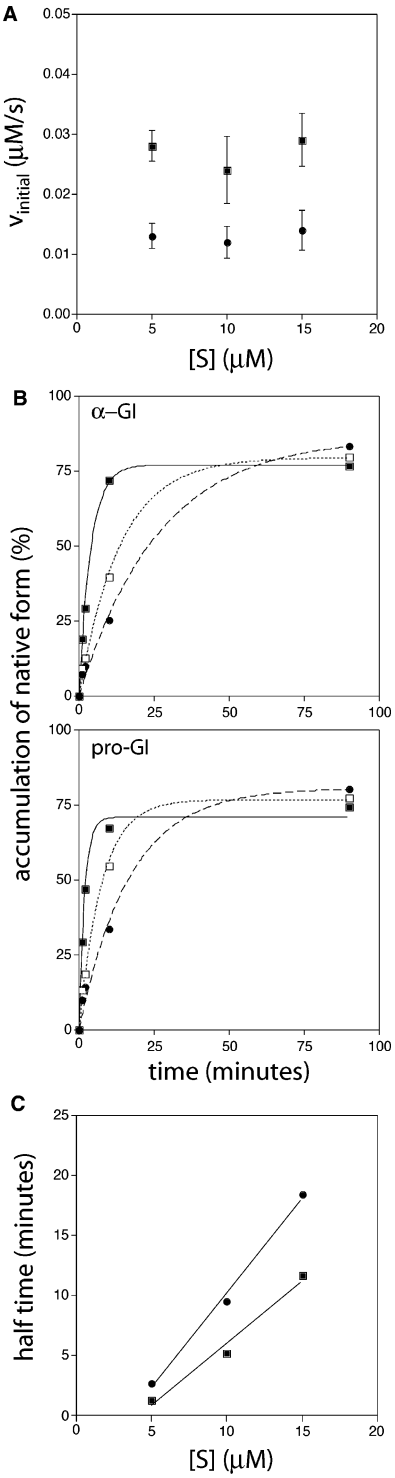

FIGURE 6: (A) Substrate concentration ([S]) dependence of the folding initial rate ($v_{initial}$). The oxidation reactions were performed in 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM GSSG, and 2 μM PDI at 0 °C. The initial rates were measured within the first minute of the folding reactions. The plotted values were averaged from three independent experiments, and the standard deviation is marked. The circles represent values for α-GI and the squares for pro-GI. (B) Folding kinetics for PDI-catalyzed oxidation of α-GI and pro-GI. The folding reactions were carried out at 0 °C in 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM GSSG, 2 μM PDI, and 5 μM (filled squares), 10 μM (open squares), or 15 μM (filled circles) peptide. The plotted values are an average from two experiments. The experimental points were analyzed by single exponential fit, and the half-times were calculated from each time course. (C) Dependence of the half-time of oxidation of α-GI (circles) and pro-GI (squares) on substrate concentration.

indicate that the propeptide region did not act as an intramolecular chaperone; under the experimental conditions, the propeptide did not directly stabilize the native conformation. However, our results suggest a role for the propeptide in the PDI-assisted folding.

The lack of propeptide-assisted folding has now been shown for two conotoxins, each belonging to a distinct family: ω-conotoxin MVIIA (*17*) and α-conotoxin GI. This finding is somewhat surprising in light of many well-documented examples of the propeptide-facilitated folding for other polypeptides. As mentioned earlier, propeptide sequences improved folding of polypeptides and peptides of different lengths and numbers of disulfide bonds: macrophage inhibitory cytokine-1 (MIC-1) (112 AA, 3 SS), human nerve growth factor (hNGF) (120 AA, 3 SS), guanylyl cyclase activating peptide, GCAP-I (16 AA, 2 SS) and GCAP-II (24 AA, 2 SS), or bovine pancreatic trypsin inhibitor (BPTI) (58 AA, 3 SS). Therefore, the propeptide-assisted folding seemed an attractive hypothesis. The experimental conditions were designed to detect even modest effects of propeptide on the folding yields. The redox buffer was used to favor a steady-state accumulation of many different folding species. Under given in vitro folding conditions, the propeptide affected neither kinetics nor thermodynamics for forming the native disulfide bonds, indicating that it does not act as an intramolecular chaperone. Thus the question remains unanswered whether there are any additional (besides noncovalent interactions within the mature toxin) intra- or intermolecular factors that stabilize the native folding species of conotoxins. For example, the C-terminal glycine was shown to stabilize the native conformation of ω-MVIIA (*17*, *18*), but other conotoxins lack this C-terminal residue. It is also possible that the in vitro folding conditions do not accurately reflect the folding environment within the ER. More folding screens are required to address this question.

Protein disulfide isomerase is an obligatory component of the oxidative folding pathway of polypeptides in the endoplasmic reticulum (*12*, *13*, *27*). In vivo, the oxidizing equivalents are directly transferred from Ero1p to PDI to protein/peptide thiolates. It has been postulated that glutathione maintains the oxidative environment in the ER, rather than playing a role in the disulfide-coupled folding of polypeptides (*12*). Therefore, the PDI-catalyzed folding reaction is more likely to mirror in vivo disulfide bond formation for conotoxins in the ER.

Our results indicate that propeptide improves early steps of the PDI-assisted oxidative folding of α-GI conotoxin. The apparent rate for forming the native disulfide bonds in pro-GI increased approximately 2−3-fold, compared to that for the mature α-GI. This finding is somewhat different from previous observations by Price-Carter and co-workers (*17*). The authors did not observe such differences between folding of the mature ω-MVIIA and the propeptide-containing pro-MVIIA (Table 1), despite using the identical concentration of the enzyme. It is important to mention that the ω-MVIIA folding experiments were carried out in the presence of 1 mM GSSH and 2 mM GSH, pH 7.3. We speculate that the differences in the PDI-catalyzed folding could be accounted for by redox systems used in the folding experiments. Different redox buffers influence PDI-assisted oxidative folding of RNase A (*28*); in vitro folding studies performed

Table 1: Comparison of Propeptides and Their Corresponding Mature Conotoxins from Different Families and from Different *Conus* Species[a]

conotoxin	*Conus* species	propeptide sequence	mature sequence	ref
α-conotoxins				
α-GI	*C. geographus*	FPSERASDGRDDTAKDEGSDMEKLVEKK	ECCNPACGRHYSCGR	*40*
α-ImIIA	*C. imperialis*	VTLDRASDGRNAAANAKTPRLIAPFIR	DYCCHRGPCMVWCG	*b*
α-SI	*C. striatus*	FPSDRASDGRDDEAKDERSDMHESDRKE	ICCNPACGPKYSCGR	*41*
δ-conotoxins				
δ-PVIA	*C. purpurascens*	DDSKNGLENHFWKARDEMKNREASKLDKK	EACYAPGTFCGIKPGLCCSEFCLPGVCFGG	*42*
δ-TxVIA	*C. textile*	DDPRNGLGNLFSNAHHEMKNPEASKLNKR	WCKQSGEMCNLLDQNCCDGYCIVLVCT	*43*
δ-KK-1	*C. textile*	DDSSNGLENLFSKAHHEMKNPEASKLNKR	CIEQFDPCEMIRHTCCVGVCFLMACI	*4*
δ-KK-2	*C. textile*	DDSGNGLENLFSKAHHEMKNPEASNLNKR	CAPFLHPCTFFFPNCCNSYCVQFICL	*4*
κ-conotoxins				
κ-PVIIA	*C. purpurascens*	DDSRRTQKHRALRSTTKLSLSTR	CRIPNQKCFQHLDDCCSRKCNRFNKCV	*44*
ω-conotoxins				
ω-GVIA	*C. geographus*	DDSRGTQKHRALGSTTELSLSTR	CKSPGSSCSPTSYNCCRSCNPYTKRCYG	*45*
ω-MVIIA	*C. magus*	DDSRGTQKHRALRSTTKLSTSTR	CKGKGAKCSRLMYDCCTGSCRSGKCG	*46*
ω-SVIA	*C. striatus*	EDSRGTQKHRTLRSTARRSKSESTTR	CRSSGSPCGVTSICCGRCYRGKCT	*47*
ω-SVIB	*C. striatus*	DDSRGTQKHRALRSDTKLPMSTR	CKLKGQSCRKTSYDCCSGSCGRSGKCG	*47*

[a] Translated sequences were not corrected for posttranslational modifications. [b] D. Zhao and P. Huang, direct submission to GenBank (Q9U619).

in Gilbert's group revealed that the catalytic efficiency of the enzyme varied significantly over a wide range of the reduced and oxidized glutathione concentrations (*28*, *29*).

Our attempts to compare α-GI and pro-GI as PDI substrates met with only limited success. Due to experimental limitations, K_m values for both peptides could not be determined (K_m is expected to be significantly lower than 5 μM). However, the oxidation of pro-GI was characterized by higher k_{cat} values, as compared to that for α-GI. The insufficient information about turnover parameters for α-GI and pro-GI did not allow us to infer a mechanism of the PDI-assisted folding of the propeptide-containing toxin. However, we believe that an increased length of the precursor, as compared to that of the mature conotoxin, may play an important role in interactions with PDI. The length of peptide substrates has been shown to contribute to binding to PDI (*30*, *31*). Different mature conotoxins may be considered as "poor" substrates for the PDI-catalyzed oxidative folding for a variety of reasons: (1) they are very short, ranging from 10 to 15 amino acids for α-conotoxins or 25−35 amino acids for δ- or ω-conotoxins, (2) the mature toxin sequences are hypervariable, differing in Cys patterns, hydrophobicity, and charge distribution, and (3) they exhibit marginal thermodynamic stability, forcing multiple redox cycles to achieve the native conformation. Therefore, the addition of a longer N-terminal propeptide sequence, which is significantly less variable, could offer an evolutionary advantage for maintaining efficient oxidative folding of conotoxins. Indeed, as shown in Table 1, the propeptides increase by approximately 2-fold the "folding" size for their corresponding mature conotoxins. Moreover, for different conotoxins belonging to the same family, the propeptide sequence is largely unchanged. On the other hand, propeptides from separate families (see δ-PVIA and κ-PVIIA) differ in their primary sequence. Thus, the presence of the more conserved propeptide region may "improve" properties of highly variable and smaller conotoxins as PDI substrates.

Our work provides the first biochemical insight into a role for the propeptide region in the folding pathway of conotoxins. Since the majority of conotoxins contain multiple disulfide bonds, *Conus* snails must have developed efficient oxidative folding pathways for "handling" small disulfide-rich peptides. To improve prey capture, these predatory snails must have evolved very efficient production of high concentrations of diverse disulfide-rich conotoxins in their venoms. More rapid PDI-assisted folding of conotoxin precursors may offer an evolutionary advantage for *Conus* snails, since it may lead to a lower accumulation of misfolded species, efficiently degraded in the ER through the quality control systems (*32*). Since the final folding yield of a disulfide-containing peptide is dependent on a number of PDI-catalyzed redox cycles [formation of disulfide bonds and reduction of non-native disulfides (*33*, *34*)], it is not surprising that cone snails may have adopted a more specialized folding mechanism, allowing more efficient production of conotoxins (*16*). Our previous results indicate that protein disulfide isomerase is a predominant polypeptide component of *Conus* venom ducts (*16*, *35*); it is likely that additional factors assist in conotoxin folding, since Hsp70 chaperones, such as BiP, were previously shown to cooperate with PDI in the ATP-dependent redox cycles (*33*). Thus, the mechanism of in vivo conotoxin folding may involve multiple intermolecular factors that (1) promote formation of disulfide bonds, (2) reduce the misfolded species, and (3) stabilize the native folding species and direct properly folded conotoxins into the secretory pathway.

Proteins involved in oxidative folding, other posttranslational modifications, and secretion of conotoxin precursors remain to be characterized. Studies on γ-carboxylation of conantokins resulted in identification, cloning, and expression of *Conus* γ-carboxylase (*36*). The propeptide derived from a precursor of conantokin-G was shown to enhance the efficiency of γ-carboxylation (*37*). Recently, Milne et al. (*38*) described a novel proteinase, isolated from *Conus textile* venom, which was shown to process a conotoxin precursor for a δ-conotoxin TxVIA. This proteinase, belonging to a group of CRISP proteins (cysteine-rich secretory proteins), exhibited a high selectivity for a cleavage between a propeptide sequence and the mature conotoxin. A role for a propeptide in a secretion of δ-TxVIA precursor via sorting receptors was also recently postulated (*39*).

In summary, our study provides an initial characterization of the PDI-catalyzed oxidative folding of conotoxin precursors. Since it is estimated that there are over 50000 unique conotoxins, produced in venom ducts of the 500 different *Conus* species, the long-term objective of our research is to understand how snails resolved many challenges in producing such a diversity of these disulfide-rich peptide neurotoxins.

ACKNOWLEDGMENT

We thank Drs. J. Michael McIntosh and Pradip K. Bandyopadhyay for critical reading of the manuscript and for their comments.

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BI0354233