Folding of ω-Conotoxins. 2. Influence of Precursor Sequences and Protein Disulfide Isomerase†

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ABSTRACT: The peptide Ca^{2+} channel antagonists found in the venoms of *Conus* snails, ω-conotoxins, are synthesized as precursors that include a leader peptide, presumed to direct the polypeptide to the endoplasmic reticulum, and a propeptide of unknown function. In addition, the precursors are synthesized with a C-terminal Gly residue that is posttranslationally converted to a terminal amide group. In order to determine whether the precursor sequences contain information that helps direct folding of the mature sequences, the disulfide-coupled folding of mature ω-conotoxin MVIIA was compared with that of two putative precursor forms: pro-ω-MVIIA-Gly, which contains the propeptide and the C-terminal Gly residue, and ω-MVIIA-Gly, which differs from the mature form only at the C-terminus. The three forms folded with similar kinetics, but the folding efficiency of ω-MVIIA-Gly was greater than 80%, versus approximately 50% for both mature ω-MVIIA and the form containing the propeptide. The enzyme protein disulfide isomerase was found to catalyze disulfide formation and folding of all three forms similarly. The affinity of ω-MVIIA-Gly for receptors in chick brain synaptosomes was approximately 10-fold lower than that of the mature peptide, and the N-terminal propeptide of pro-ω-MVIIA-Gly was found to decrease binding further, by approximately 100-fold. These results suggest that the ω-conotoxins do not rely on the propeptide region of their precursors to facilitate folding. Rather, the mature sequence contains most of the information required to specify the native disulfide pairings and three-dimensional conformation. The C-terminal Gly may enhance the folding efficiency by forming interactions that stabilize the native conformation with respect to other disulfide-bonded forms.

Although nearly all proteins contain within their amino acid sequences information sufficient to direct folding into biologically active conformations, additional factors often facilitate folding in vivo. The enzymes protein disulfide isomerase (Freedman et al., 1994) and prolyl *cis*−*trans* isomerase (Schmid, 1993, 1995) have been shown to enhance folding rates in vitro, and it is generally believed that they play this role in vivo. Other proteins, collectively referred to as molecular chaperones, improve folding yields by decreasing the tendency of unfolded or partially folded molecules to enter nonproductive pathways such as those leading to aggregation (Hendrick & Hartl, 1993; Landry & Gierasch, 1994). In addition, some proteins are synthesized with precursor sequences that facilitate folding (Baker et al., 1993; Shinde et al., 1993). These "intramolecular chaperones" are then cleaved from the folded functional forms.

Peptides found in the venoms of *Conus* snails (conotoxins) may represent another class of proteins that rely on precursor sequences or cellular cofactors for efficient folding in vivo. These peptides are typically only 12−30 amino acid residues long but fold into well-defined three-dimensional structures, often stabilized by disulfide bonds (Gray et al., 1988; Olivera et al., 1990). Results presented in the accompanying paper indicate that at least some conotoxins are capable of folding to their native conformations with efficiencies significantly greater than expected for random disulfide formation but that the stabilities of the native peptides are only marginally greater than those of other disulfide-bonded forms. Analyses of cDNA sequences have shown that conotoxins are synthesized as precursors that are approximately 3 times as large as the mature forms (Olivera et al., 1990; Woodward et al., 1990; Colledge et al., 1992). Interestingly, the excised portions of the precursor sequences are often more highly conserved than are the mature sequences among a family of conotoxins with similar activities and three-dimensional structures. Olivera and colleagues have proposed that the conserved precursor sequences may help direct the disulfide-coupled folding of the conotoxins and then be removed to leave small well-structured functional forms (Olivera et al., 1990).

Conotoxin precursor sequences might promote folding in at least two distinct ways. Like the propeptides of some proteases, the precursor sequences could lower kinetic barriers between folding intermediates and the native conformations by forming specific interactions with portions of the mature sequences (Baker et al., 1992; Shinde et al., 1993; Bryan et al., 1995). Alternatively, precursor sequences could act indirectly by interacting with cellular proteins that catalyze folding or prevent nonproductive interactions. It is very likely that the disulfide-coupled folding of conotoxins is catalyzed by the enzyme protein disulfide isomerase (PDI)[1] in vivo. Recent studies indicate that PDI contains a peptide binding site (Novia et al., 1993) and that larger peptides bind to PDI with higher affinities than do smaller peptides (Morjana & Gilbert, 1991). The conotoxin precursor se-

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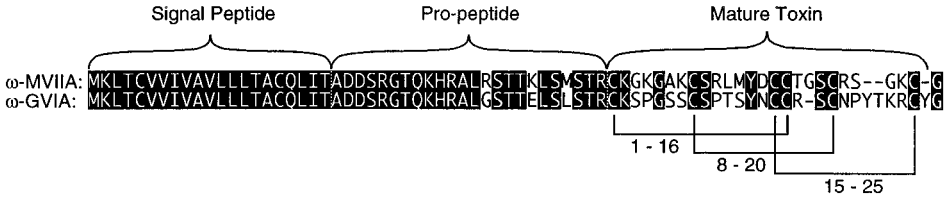

FIGURE 1: Sequences of predicted precursors of conotoxins ω-MVIIA (D. R. Hillyard and B. M. Olivera, personal communication) and ω-GVIA (Colledge et al., 1992), inferred from cDNA sequences. The boundary indicated between the leader sequence and the propeptide was predicted by the method of von Heijne (1986). Amino acid residues that are conserved between the two sequences are shaded. The disulfide bonds found in the native forms of the mature peptides are indicated by the lines below the sequence of the ω-GVIA precursor.

quence might facilitate binding to PDI, thereby enhancing the efficiency of catalysis.

The precursor sequences of two conotoxins, isolated from two different *Conus* species, are shown in Figure 1 (Colledge et al., 1992; D. R. Hillyard, unpublished data). These two toxins are members of the ω-class of peptides and, like other members of this family, bind to and inhibit presynaptic Ca^{2+} channels (Olivera et al., 1994). The various members of the family have similar, but not identical, biological activities, and the mature forms of three of these peptides have been shown by high-resolution NMR spectroscopy to have very similar three-dimensional structures (Davis et al., 1993; Pallaghy et al., 1993; Skalicky et al., 1993; Basus et al., 1995; Farr-Jones et al., 1995; Kohno et al., 1995). In spite of this functional and structural similarity, however, few residues in the mature sequences are conserved other than the disulfide-bonded Cys residues. In contrast, the predicted precursor sequences are highly conserved.

The precursor sequences include an N-terminal signal sequence and a "propeptide" positioned between the signal sequence and mature region (Colledge et al., 1992). In addition, the peptides are synthesized with a C-terminal Gly residue that is converted to a terminal amide group. The signal sequences are presumed to direct translocation of the peptides into the endoplasmic reticulum and to be then cleaved from the molecules by signal peptidase. The propeptides, on the other hand, may be attached to the mature sequences when they undergo disulfide-coupled folding within the lumen of the endoplasmic reticulum. Since the enzymes responsible for converting C-terminal Gly residues to amide groups are generally localized in secretory granules (Oyarce & Eipper, 1995), it is also likely that the unmodified Gly residue is present during folding. Either the N-terminal propeptide or the C-terminal Gly residues might influence folding in vivo.

To test the possible roles of the precursor sequences in folding, we have compared the folding of three forms of ω-conotoxin MVIIA: (1) the mature peptide with an amidated C-terminus, (2) the mature sequence with a C-terminal Gly residue (designated here as ω-MVIIA-Gly), and (3) the putative precursor containing both the highly conserved propeptide and the C-terminal Gly residue (designated pro-ω-MVIIA-Gly). In addition, the ability of PDI to catalyze the folding of each form was examined.

EXPERIMENTAL PROCEDURES

Production of Pro-ω-MVIIA-Gly as a Fusion Protein in Escherichia coli. A synthetic gene encoding the predicted ω-MVIIA precursor, as shown in Figure 1, was assembled from four oligonucleotides and cloned into an expression vector derived from the pET plasmids of Studier (Studier et al., 1990; Doering, 1992; Staley & Kim, 1994). The conotoxin gene was expressed from this vector as a C-terminal fusion to the *TrpLE* Δ1413 fragment, which is itself a fusion of portions of the *E. coli TrpL* and *TrpE* genes (Miozzari & Yanofsky, 1978; Kleid et al., 1981). To facilitate chemical cleavage of the fusion protein, a tryptophan codon was introduced between the *TrpLE* sequence and the pro-ω-MVIIA-Gly gene. The sequence of the synthetic gene was confirmed by dideoxy sequencing.

For protein production, *E. coli* strain BL21 containing the pro-ω-MVIIA-Gly expression vector was grown at 37 °C to midlogarithmic phase, and transcription was induced by the addition of isopropyl β-D-thiogalactoside as described by Studier et al. (1990). Cells were harvested by centrifugation 2−3 h after induction, and the cell pellets were stored at −20 °C.

Frozen bacterial cell pellets from a 1-L culture were resuspended in 75 mL of 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10% (w/v) sucrose, 1 mM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride and lysed by passage through a French pressure cell. The lysate was centrifuged for 20 min at 13 000 rpm in a Beckman JA-14 rotor. Electrophoresis of the supernatant and pellet fractions indicated that nearly all of the fusion protein was in the pellet fraction. The pellet was washed three times by resuspension in 75 mL of 1% (w/v) Triton X-100 and 1 mM EDTA, pH 8.0, followed by centrifugation (Fischer et al., 1993). To dissolve the fusion protein, the washed pellet was resuspended in 75 mL of 8 M urea, 25 mM dithiothreitol (DTT), 0.1 M Tris-HCl, pH 8.0, and 1 mM EDTA. Insoluble material was removed from the solution by centrifugation at 13 000 rpm for 20 min in a Beckman JA 14 rotor. SDS−polyacrylamide gel electrophoresis of the resulting pellet and supernatant fractions indicated that approximately half of the fusion protein was solubilized in the urea/DTT solution. Urea and DTT were

[1] Abbreviations: ω-MVIIA, the mature form of ω-conotoxin MVIIA containing an amidated C-terminus, as isolated from the venom of *Conus magus*; ω-MVIIA-Gly, a putative precursor form of ω-MVIIA containing a C-terminal Gly residue, rather than the amide group; pro-ω-MVIIA-Gly, a putative precursor form containing an N-terminal propeptide sequence inferred from cDNA sequences, as well as the C-terminal Gly residue; PDI, protein disulfide isomerase; RNase A, ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; RCAM, reduced and carboxamidomethylated; GSSG and GSH, the disulfide and thiol forms, respectively, of glutathione; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; BNPS-skatole, 2-(2′-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine; HMP, [*p*-(hydroxymethyl)phenoxy]methyl; Fmoc, (fluoromethoxy)-carbonyl; Acm, acetamidomethyl, TFA, trifluoroacetic acid; TCEP, tris-(2-carboxyethyl) phosphine; CD, circular dichroism; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

separated from the peptide by dialysis against 0.1% (v/v) acetic acid. The protein precipitated during dialysis but redissolved upon addition of 73 g of solid urea, 15 mL of 1 M Tris-HCl, pH 8.0, 1.5 mL of 0.1 M EDTA, and water to yield a final volume of 150 mL. To protect free cysteine thiols during the subsequent chemical cleavage reaction, glutathione mixed disulfides were formed by adding oxidized glutathione to a final concentration of 5 mM and incubating for 2 h at room temperature. This solution was dialyzed against 0.1% acetic acid at 4 °C, during which some of the protein again precipitated. The protein was then lyophilized.

Chemical Cleavage of the Fusion Protein and Purification of Pro-ω-MVIIA-Gly. The fusion protein was cleaved at the tryptophan residue between the TrpLE fragment and the pro-ω-MVIIA-Gly peptide by treatment with BNPS-skatole as follows: The lyophilized protein (approximately 150 mg) was dissolved in 12.5 mL of 25% (v/v) acetic acid and then mixed with 14 mg/mL BNPS-skatole (Pierce) that had been dissolved in 12.5 mL of glacial acetic acid. The solution was incubated for 3−3.5 h at room temperature in the dark (Fontana, 1972). The cleavage efficiency, as estimated from SDS−polyacrylamide gels (Schägger & Von Jagow, 1987), was approximately 50−65%. Most of the excess reagent precipitated upon 10-fold dilution of the cleavage solution into 10 mM HCl and was pelleted by centrifugation for 20 minutes at 10 000 rpm in a JA-14 rotor. The peptide solution was concentrated 10-fold by tangential flow ultrafiltration using a Filtron 25A miniultrasette unit with a nominal molecular mass cutoff of 1000 Da. The peptide solution was then diluted 10-fold with H_2O and reconcentrated by ultrafiltration. The remaining BNPS-skatole and the uncleaved fusion protein were separated from the cleaved peptide by gel filtration chromatography on a 2.5-cm × 40-cm column of Sephadex G-50 equilibrated and eluted with 0.1 M acetic acid. Column fractions containing pro-ω-MVIIA-Gly were identified by SDS−polyacrylamide gel electrophoresis, pooled, and lyophilized. The glutathione mixed disulfides were reduced by dissolving the lyophilized peptide in 50 mM DTT, 0.1 M Tris, pH 8.7, 1 mM EDTA, and 6 M guanidinium chloride and incubating the solution for 1 h at room temperature. The reduced peptide was then purified from the TrpLE fragment by reversed-phase HPLC on a Vydac C_{18} column (1 cm × 30 cm) eluted with a gradient of acetonitrile in 0.1% TFA.

Electrospray ionization mass spectroscopy of pro-ω-MVIIA-Gly purified as described above revealed that the protein had a mass 32 Da greater than expected, suggesting that the two Met residues in the peptide had been oxidized to the sulfoxide form. To reduce the putative sulfoxides, a sample of partially purified peptide was treated with methyl sulfide prior to reduction of the mixed disulfides, as follows: Lyophilized peptide (∼1 mg) was dissolved in 200 μL of 12 N HCl and mixed with 50 μL of methyl sulfide (Savige & Fontana, 1977). The solution was incubated for 5 min at room temperature and then mixed with 1.05 mL of 3 M Tris base, 8 μL of 0.25 M EDTA, 1.15 g of guanidinium chloride, and 16 mg of DTT. After incubating for 3 h at 25 °C to reduce mixed disulfides, the peptide was purified by reversed-phase HPLC. The mass spectrum of methyl sulfide-treated pro-ω-MVIIA-Gly indicated that the peptide had the molecular weight expected if the sulfoxides had been reduced. All of the pro-ω-MVIIA-Gly used in the experiments described here was prepared in this fashion. Fully reduced peptide was lyophilized and stored at −20 °C. The final yield of purified, fully reduced pro-ω-MVIIA-Gly was approximately 4.5 mg/L of bacterial cell culture.

Chemical Synthesis of ω-MVIIA-Gly. ω-MVIIA-Gly was synthesized on a HMP resin using Fmoc chemistry. To simplify synthesis of the form with native disulfides, a selective protection scheme, described by Monje et al. (1993), was utilized in which Cys residues with trityl protecting groups (sensitive to deprotection by TFA) were incorporated at positions 1, 15, 16, and 25, and Cys residues with acetamidomethyl (Acm) protecting groups (resistant to deprotection by TFA) were used at positions 8 and 20. By first deprotecting the four Cys residues blocked with trityl groups and allowing disulfides to form among only these residues, and then forming the third disulfide under conditions where intramolecular rearrangements are minimized, this strategy is expected to result in three different disulfide-bonded isomers, as compared to the 15 possible species expected for fully random disulfide formation. Following synthesis, the peptide was cleaved from the resin and partially deprotected with TFA as described by Shon et al. (1995). The released peptide was purified by reversed-phase HPLC on a Vydac C_{18} column (2.5 cm × 30 cm). To promote oxidation of the deprotected Cys residues, the peptide solution was diluted 10-fold with H_2O, adjusted to pH 8.0 with Tris base, and stirred for 16 h at room temperature. Three different two-disulfide forms were expected from this oxidation, but when the oxidized peptides were fractionated by HPLC, only two peaks were detected, a relatively sharp major peak and a broader minor peak. To remove the Acm protecting groups and form the third disulfide bridge, the peptides in the HPLC peaks (diluted 10-fold in H_2O) were each mixed with TFA and then I_2, to final concentrations of 10% and 0.5 mM. respectively, and incubated for 3 min in the dark at room temperature. The oxidation reaction was stopped by adding 0.1 volume of 0.3 M ascorbic acid. The solution was diluted 4-fold with H_2O and the peptides were purified by reversed-phase HPLC.

The material derived from the major peak following air oxidation gave rise to two different species (A and B) after iodine oxidation, while the minor peak yielded a single product (C). The molecular masses of the three oxidized forms were 2697.6−2697.7, very close to that expected for a three-disulfide form of ω-MVIIA-Gly (2698.1), indicating that the three peptides obtained were the expected disulfide isomers. The final yield of peptide with native disulfides, identified as described below, was 2 mg from 50 mg of peptide−resin.

Identification of ω-MVIIA-Gly Containing the Native Disulfides. The strategy used to determine which of the three-disulfide forms described above contained the native disulfide bonds is outlined in Figure 2. The unresolved two-disulfide forms generated by air oxidation (A′ and B′), were partially reduced at low pH (where disulfide rearrangement is minimal) with tris(2-carboxyethyl) phosphine (TCEP) (Gray, 1993). The resulting one-disulfide species were then separated from one another by reversed-phase HPLC. An aliquot of one of the purified one-disulfide forms was reacted with iodoacetamide to block its two free thiols. This peptide, with Acm groups on Cys 8 and 20 and carboxyamidomethyl groups on two other Cys residues, was purified by HPLC. The peptide's remaining disulfide was reduced in 10 mM DTT and the resulting thiols were blocked with vinyl pyridine

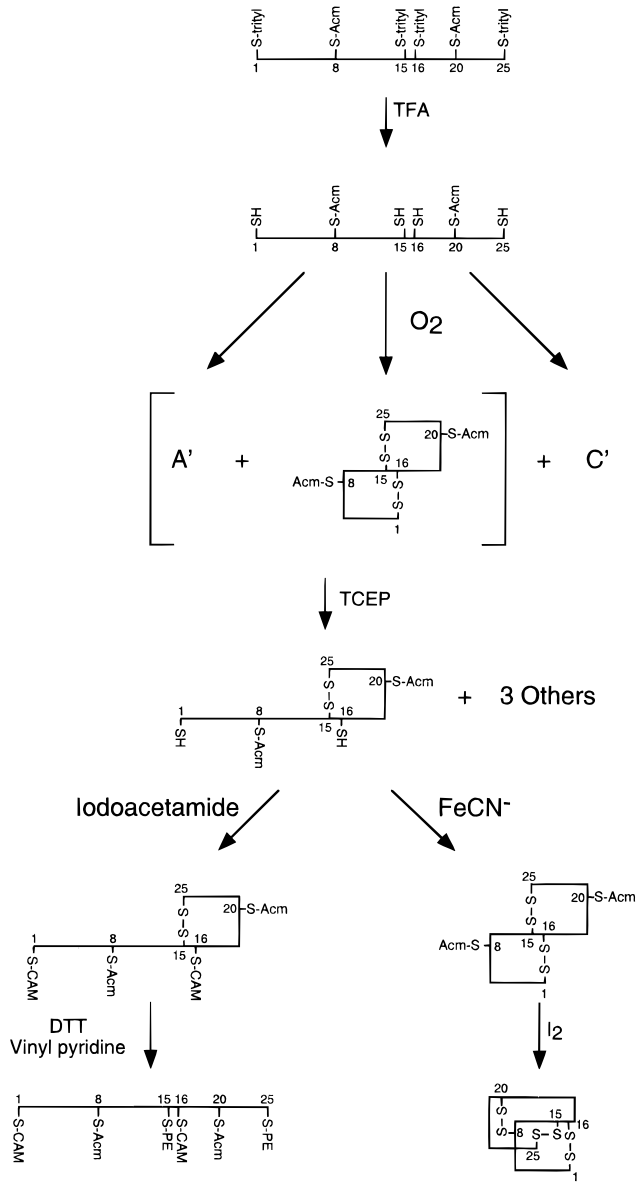

FIGURE 2: Strategy used to identify the form of ω-MVIIA-Gly with native disulfides. As described under Experimental Procedures, ω-MVIIA-Gly was chemically synthesized using two sets of Cys thiol-protecting groups. Four of the thiols (at Cys 1, 15, 16, and 25) were protected with trityl groups, which were removed by treatment with TFA, leaving Cys 8 and 20 protected with Acm groups. This material was subjected to oxidation by molecular oxygen to generate three species, one of which (labeled C′) was chromatographically resolved from the other two (A′ and the species later shown to contain the 1−16 and 5−25 disulfides). The mixture of unresolved species was partially reduced with TCEP under acidic conditions, where rearrangements are minimized. One of the four resulting one-disulfide species was then blocked with iodoacetamide, further reduced with dithiothreitol, and finally, blocked with vinyl pyridine. Sequencing by Edman degradation revealed the positions of the various blocking groups, as shown, and demonstrated that the initial one-disulfide form contained the 15−25 disulfide, which is found in the native mature peptide. Another sample of this one-disulfide form was oxidized with ferrous cyanide to form the 1−16 disulfide and then treated with iodine to produce ω-MVIIA-Gly with the three native disulfides.

(Gray 1993). This peptide, with three pairs of differentially derivatized Cys residues, was sequenced by automated Edman degradation. Pyridylethyl groups were found at positions 15 and 25, which form a native disulfide, indicating that this was the disulfide present in the purified one-disulfide species. Another aliquot of this same one-disulfide species was oxidized in 10 mM potassium ferrous cyanide at low pH to form the second disulfide (1−16), purified by HPLC, and oxidized with iodine to remove the Acm protecting groups and form its third bridge (8−20), thus resulting in a sample containing the three disulfides of the natural peptide (1−16, 8−20, and 15−25) (Chung et al., 1995). This material was indistinguishable from isomer B derived by direct oxidation of the material in the major two-disulfide fraction.

The three different isomers were characterized by circular dichroism, a receptor-binding assay, and reduction kinetics. In all of these analyses, ω-MVIIA-Gly with the native disulfides behaved more like native mature ω-MVIIA than either of the other three-disulfide forms, and is, accordingly, referred to as "native" ω-MVIIA-Gly in the text and figures.

Disulfide-Coupled Folding and Unfolding Reactions. For folding experiments, reduced and lyophilized peptide was dissolved in 10 mM HCl to a final concentration of 40 μM. The dissolved peptide was then mixed with an equal volume of 0.2 M MOPS, pH 7.3, 0.4 M KCl, 2 mM EDTA, and thiol and disulfide reagents as appropriate for the experiment. In a typical experiment, a total of 20 nmol of peptide was used in a reaction volume of 1 mL, and 100−200-μL samples were withdrawn and quenched with 0.05 volume of H_3PO_4. The trapped disulfide-bonded species were then fractionated by reversed-phase HPLC using a Vydac C_{18} column (4.6 mm i.d. × 25 cm long) eluted with a gradient of acetonitrile (CH_3CN) at a flow rate of 1 mL/min. Gradients were prepared by mixing two buffer solutions: A (0.103% TFA) and B (0.96% TFA and 60% CH_3CN). Refolding and unfolding reaction mixtures involving mature ω-MVIIA were fractionated using a linear gradient of 15−32% buffer B. For ω-MVIIA-Gly, a linear gradient of 15−35% buffer B was used. For these separations, the concentration of buffer B was increased at a rate of 0.5%/min. For pro-ω-MVIIA-Gly, a 25−37% gradient was used, with the concentration of buffer B increased at a rate of 0.25%/min. Elution profiles were monitored by absorbance at 220 nm.

Folding experiments in the presence of protein disulfide isomerase were carried out as described above, except that one-third volume of 12 μM rat PDI [produced in recombinant *E. coli* (Gilbert et al., 1991) and kindly provided by Dr. H. F. Gilbert] in 50 mM Tris-HCl, pH 7.0, was added to the buffer components and glutathione, and the resulting solution was bubbled with N_2 for 5 min at 25 °C prior to mixing with the peptide in HCl. Samples from the reaction mixtures were quenched with phosphoric acid and fractionated by HPLC as described above, except that the gradient segments used to separate the folding intermediates were followed by a steep linear gradient to 100% B (in 20 min), so as to elute the PDI.

Reductive unfolding experiments were initiated by mixing the various peptides with DTT (5 mM final concentration). The reactions also contained 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 1 mM EDTA. Samples were withdrawn and analyzed as above.

All solutions used for folding and unfolding experiments were flushed with N_2 and the reactions were carried out in septum vials under an N_2 atmosphere.

Circular Dichroism Spectroscopy. Circular dichroism spectra were collected on an Aviv Model 62DS spectropolarimeter using a band width of 1.5 nm, a step size of 0.5 nm, and an averaging time of 0.8 s. Four spectra were

collected for each sample and averaged. All spectra were collected at 25 °C. A cell with a path length of 0.1 cm was used for far-UV scans, and a 1.0-cm path-length cell was used for near-UV scans. Samples were prepared by dissolving lyophilized peptide to a concentration of approximately 0.2 mg/mL in 10 mM phosphate buffer, pH 6.84. Protein concentrations were calculated from the absorbance at 280 nm and an extinction coefficient was calculated from the amino acid composition using the method described by Gill and von Hippel (1989).

Ca^{2+} Channel Binding Assay. The protocol used to measure the binding of ω-MVIIA forms to Ca^{2+} channels in chick brain membranes (Cruz & Olivera, 1986; Olivera et al., 1987) is described in the accompanying paper.

RESULTS

Analyses of cDNA sequences indicate that ω-conotoxins are synthesized as larger precursors, containing a leader peptide for targeting to the endoplasmic reticulum, where they presumably fold and form their disulfides, a propeptide located between the signal sequence and mature sequence, and a Gly residue at the C-terminus of the peptide (Figure 1). Because the propeptide and C-terminal glycine may be attached to MVIIA while it folds, we prepared forms of MVIIA corresponding to the putative precursors and compared their folding kinetics and efficiencies with those of the mature peptide.

Synthesis of Predicted Conotoxin Precursors. Pro-ω-MVIIA-Gly is a predicted precursor form of ω-MVIIA that contains the conserved propeptide and C-terminal glycine. A gene encoding this molecule was synthesized and cloned into an expression vector from which it was expressed to produce a fusion to an *E. coli* polypeptide. After solubilization in 8 M urea and DTT, the fusion protein was cleaved with the reagent BNPS-skatole at a Trp residue placed between the bacterial polypeptide and pro-ω-MVIIA-Gly. The identity of the peptide was confirmed by mass spectrometry and Edman degradation of the first four amino acid residues.

ω-MVIIA-Gly is identical in sequence to the form that is isolated from *C. magus* venom (Olivera et al., 1987), except that the amide group attached to the C-terminal Cys residue of the mature peptide is replaced by the genetically encoded Gly residue. This peptide was chemically synthesized by standard solid-phase techniques. The strategy used to prepare and confirm the identity of the form with native disulfides (Chung et al., 1995) is outlined in Figure 2 and described in the Experimental Procedures section. Two nonnative forms of ω-MVIIA-Gly, each inferred to contain one native disulfide (between Cys 8 and Cys 20) and two nonnative disulfides, were obtained as byproducts of the synthesis and are identified here as forms A and C.

Disulfide-Coupled Folding of Precursor ω-MVIIA Forms. Because their predicted precursors contain leader sequences (Colledge et al., 1992), conotoxin peptides are presumed to fold within the lumen of the ER. Experiments described in the accompanying paper demonstrated that the mature form of ω-MVIIA was capable of folding, with modest efficiency, to its native form in the presence of oxidized (GSSG) and reduced (GSH) glutathione in ratios similar to that reported to exist in the ER lumen (Hwang et al., 1992). The same conditions were used here to study the folding of the precursor forms. Figure 3 illustrates chromatograms of intermediates trapped during the disulfide-coupled folding of the precursor and mature forms.

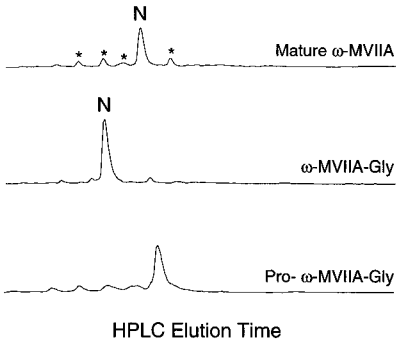

FIGURE 3: Refolding of mature ω-MVIIA, ω-MVIIA-Gly, and pro-ω-MVIIA-Gly monitored by reversed-phase HPLC. For each experiment, the peptide was fully reduced by dithiothreitol, separated from the reductant, lyophilized, and then allowed to refold at 25 °C in the presence of 1 mM GSSG, 2 mM GSH, 0.1 M MOPS, pH 7.3, 0.2 M KCl, and 1 mM EDTA. At times ranging from 5 to 240 min, samples of the reactions were withdrawn and mixed with 0.05 volume of phosphoric acid and fractionated by reversed-phase HPLC as described under Experimental Procedures. The separated disulfide-bonded species were detected by monitoring absorbance at 220 nm. The HPLC traces shown represent the three reactions 90 min after the initiation of refolding. The chromatograms representing earlier time points in the three reactions were qualitatively similar to those shown in Figure 2 of the accompanying paper. The peaks labeled N correspond to the native forms of mature ω-MVIIA and ω-MVIIA-Gly. Those labeled with asterisks in the chromatogram of the mature ω-MVIIA folding mixture are nonnative three-disulfide forms, as discussed in the accompanying paper.

The distribution of intermediates that formed during the folding of ω-MVIIA-Gly was quite similar to that seen with the mature peptide. After 90 min of folding in the presence of 1 mM GSSG and 2 mM GSH, 65% of the ω-MVIIA-Gly was found in one peak (Figure 3). After 240 min, the yield of this form reached a final level of 85%. The major form had an HPLC retention time identical to that of ω-MVIIA-Gly known to contain the three disulfides of the natural peptide, and as described in detail below, the CD spectra, biological activity, and reductive unfolding rates of these two forms were indistinguishable. Thus, ω-MVIIA-Gly appeared to fold with significantly greater efficiency than the mature peptide, for which the maximal yield was approximately 50%. The major effect of the C-terminal Gly residue on the final distribution of disulfide-bonded species appeared to be a markedly reduced accumulation of the nonnative forms believed to contain three disulfide bonds each (marked with asterisks in Figure 3 and discussed in the accompanying paper).

In order to determine if the ω-MVIIA-Gly folding rates and efficiency, like those of the mature form, were sensitive to the thiol−disulfide redox environment, folding was examined in the presence of 1 mM GSSG and GSH concentrations ranging from 0 to 8 mM. As was seen for the mature peptide, when ω-MVIIA-Gly was folded in the absence of GSH, there was rapid accumulation of nonnative species that were only slowly converted to the native form, with a half-time of approximately 500 min. The final yield of native peptide, however, was the same in the presence of 0 or 2 mM GSH. In the presence of 4 or 8 mM GSH, the final yield was reduced to 75% and 60%, respectively. Under the same conditions, refolding of the mature sequence

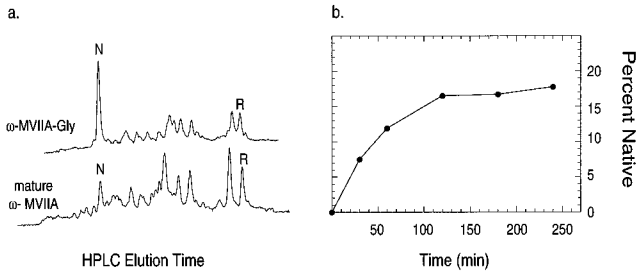

FIGURE 4: Equilibration of ω-MVIIA-Gly in the presence of oxidized and reduced glutathione. (a) Reversed-phase HPLC separations of disulfide-bonded species generated by equilibrating the native forms of either ω-MVIIA-Gly (upper trace) or mature ω-MVIIA (lower trace) for 3 h in the presence of 1 mM GSSG and 20 mM GSH at pH 7.3, 25 °C. The reactions were quenched and the intermediates were separated by reversed-phase HPLC as described in the legend to Figure 3. (b) Concentration of native ω-MVIIA-Gly, expressed as percent of total peptide, after various times of incubation under the conditions described above.

resulted in yields of 40% and 30%. Thus, the presence of the C-terminal Gly residue significantly enhanced the folding efficiency under each set of conditions examined.

Very similar distributions of disulfide-bonded molecules were generated when either the native or reduced forms of ω-MVIIA-Gly were incubated with mixtures of GSSG and GSH. This observation suggests that the final yields observed reflect the thermodynamic stability of the native peptide relative to those of other forms. The equilibrium distributions obtained with mature ω-MVIIA and ω-MVIIA-Gly in the presence of 1 mM GSSG and 20 mM GSH are compared in Figure 4a. Under these conditions the equilibrium concentrations of the fully reduced and native forms of ω-MVIIA-Gly were 6% and 17%, respectively, as compared to 5% and 6% for the corresponding forms of the mature peptide. From these measurements, the overall equilibrium constant for formation of the three native disulfides in ω-MVIIA-Gly was estimated to be 0.18 M^3, vs 0.04 M^3 for the mature form. Thus, the C-terminal Gly residue of the putative precursor stabilizes the native conformation with respect to the fully reduced peptide by about 1 kcal/mol.

Although ω-MVIIA isolated from snail venom is monomeric (Basus et al., 1995; Kohno et al., 1995), it is possible that intermolecular interactions are an important factor in folding, and therefore, the folding rate might depend on the concentration of peptide in solution. Folding experiments were performed with 2, 5, and 20 μM ω-MVIIA-Gly, with no apparent change in the folding rate, suggesting that monomeric MVIIA is the folding unit.

Folding of Pro-ω-MVIIA-Gly. Intermediates in the refolding of pro-ω-MVIIA-Gly were separated using reversed-phase HPLC conditions similar to those employed in the analysis of the mature peptide and ω-MVIIA-Gly. As observed during the refolding of the smaller forms, the disappearance of fully reduced pro-ω-MVIIA-Gly was accompanied by the appearance of forms with shorter retention times, consistent with the formation of structures with decreased exposure of nonpolar surface area. After 90 min of folding (Figure 3), approximately 48% of the total protein was found in a single peak, and the yield of this form increased to approximately 63% at later times. Because a sample of pro-ω-MVIIA-Gly known to contain the native disulfides was not available as a standard, it was not possible

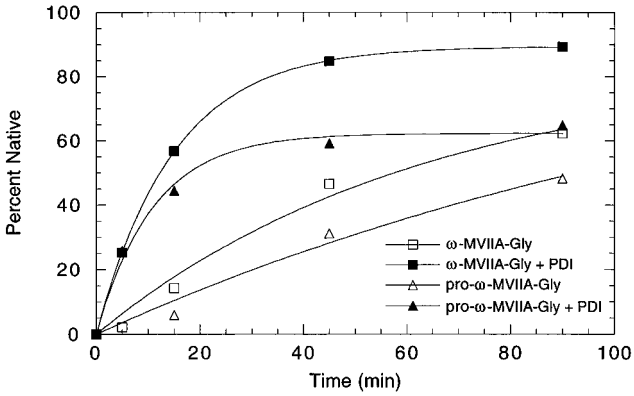

FIGURE 5: Catalysis of folding of ω-MVIIA-Gly and pro-ω-MVIIA-Gly by protein disulfide isomerase. Fully reduced ω-MVIIA-Gly (squares) and pro-ω-MVIIA-Gly (triangles) were refolded under the conditions described in the legend of Figure 3 in either 2 μM (filled symbols) or 0 μM (open symbols) protein disulfide isomerase. The resulting disulfide-bonded species were separated by reversed-phase HPLC, and the fraction of native peptide, identified by its HPLC elution time, present at the indicated times was determined by integration of the resulting chromatograms. The curves drawn represent a fit of the data to an integrated first-order rate expression. The half-time for the appearance of fully folded ω-MVIIA-Gly was approximately 50 min in the absence of PDI and 8 min in the presence of the enzyme. Similar half-times were observed for the appearance of the major product in the folding of pro-ω-MVIIA-Gly in the absence and presence of PDI.

to determine directly whether the major product was correctly folded. As described in subsequent sections, however, characterization of this form by CD spectroscopy and reduction kinetics suggested that it had a conformation similar to that of the native mature peptide.

The half-time for the appearance of the major product was approximately 50 minutes in the presence of 1 mM GSSG and 2 mM GSH, similar to those observed for the mature peptide and ω-MVIIA-Gly under these conditions. Thus, the propeptide did not appear to enhance either the folding rate or efficiency above those seen for the mature sequence.

Folding Kinetics with PDI. Protein disulfide isomerase is found in high concentrations in the ER lumen and has been shown to catalyze the folding of several disulfide-bonded proteins in vitro (Freedman 1994). Although the mechanism of this enzyme is not yet fully understood, it is known to contain at least one peptide binding site (Morjana & Gilbert, 1991; Novia et al., 1993), and by analogy with results obtained with the related *E. coli* protein DsbA (Darby & Creighton, 1995), binding of polypeptide substrates to the PDI active sites is likely to contribute to catalysis by stabilizing the transition states for thiol−disulfide exchange reactions. A possible role for the ω-MVIIA propeptide might be to optimize interactions between PDI and the precursor polypeptide. In order to explore this possibility, folding experiments were carried out with each of the three forms of ω-MVIIA in the presence of a 0.1 molar ratio of PDI.

In Figure 5, the folding kinetics of ω-MVIIA-Gly and pro-ω-MVIIA-Gly with and without PDI are compared. Under these conditions, PDI increased the folding rates of both forms, resulting in half-times of approximately 10 min, versus 50 min for the uncatalyzed reactions. A similar degree of rate enhancement was seen for mature ω-MVIIA. The enzyme did not significantly affect the folding efficiencies of any of the forms examined. These effects are similar to the 5-fold rate enhancement observed for RNase A (Lyles

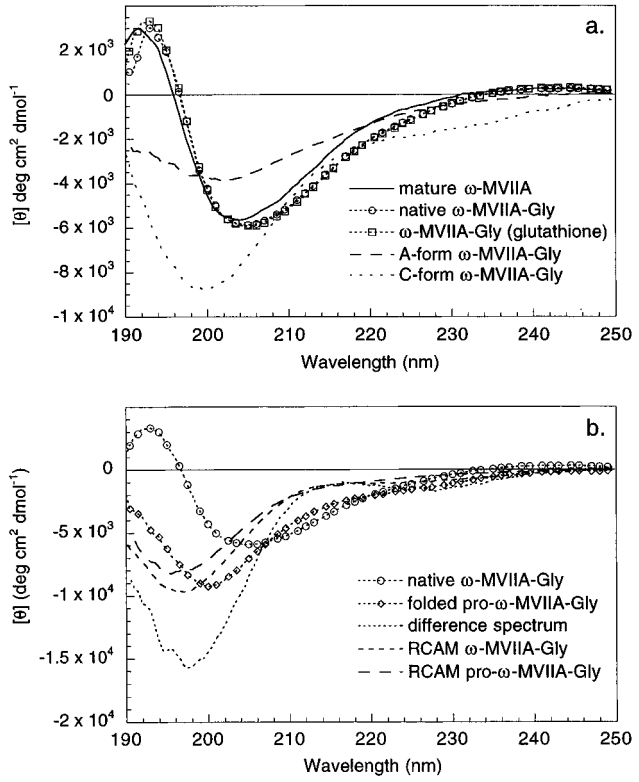

FIGURE 6: Far-UV circular dichroism spectra of different forms of ω-conotoxin MVIIA. (a) CD spectra of native mature ω-MVIIA, ω-MVIIA-Gly known to contain the three native disulfides, ω-MVIIA-Gly folded in glutathione, and two forms, A and C, known to contain two nonnative disulfides each, as indicated by the key in the figure. (b) CD spectra of native ω-MVIIA-Gly, the major folding product of pro-ω-MVIIA-Gly, and reduced and iodoacetamide-blocked (RCAM) forms of ω-MVIIA-Gly and pro-ω-MVIIA-Gly. Also shown is a difference spectrum generated by subtracting the spectrum of native ω-MVIIA-Gly from that of glutathione-folded pro-ω-MVIIA-Gly. All of the spectral intensities are expressed as mean residue elipticities.

& Gilbert, 1991) and the 10-fold rate enhancement of the folding of BPTI (Creighton et al., 1993) with similar concentrations of PDI. The distribution of intermediates that accumulated during the PDI-catalyzed folding reactions were similar to the distributions observed in the absence of the enzyme, suggesting that PDI catalyzed many of the disulfide formation and rearrangement steps in each folding pathway to similar extents.

Since PDI had similar effects on the folding of ω-MVIIA-Gly and pro-ω-MVIIA-Gly, the propeptide does not appear to play a significant role in optimizing interactions between the enzyme and the folding polypeptide.

Characterization of Folded Products by Circular Dichroism Spectroscopy. To determine if the major products generated during the disulfide-coupled folding of ω-MVIIA-Gly and pro-ω-MVIIA-Gly had conformations similar to that of the native mature peptide, they were characterized by UV circular dichroism spectroscopy. For comparison, the native mature (amidated) form, the A and C forms of ω-MVIIA-Gly (each inferred to have one native and two nonnative disulfides), and reduced and alkylated forms were also analyzed.

Far-UV circular dichroism spectra of the various forms of ω-MVIIA-Gly are shown in Figure 6a. The spectrum of native mature ω-MVIIA, shown as a solid line in the figure, is characterized by a positive maximum at 192 nm and a minimum at 206 nm. This spectrum is similar to that reported by Kim et al. (1995) for ω-MVIIA and is consistent with the high content of β-strand structure seen in NMR solution structures of this peptide (Kohno et al., 1995; Basus et al., 1995). The form of ω-MVIIA-Gly known to contain the three disulfides of the natural peptide and the major product obtained from folding ω-MVIIA-Gly in glutathione had nearly identical far-UV CD spectra, which closely resembled that of the native mature form, indicating that all three had similar conformations. In contrast, the spectra of the A and C forms lacked the maximum between 190 and 200 nm and displayed only a broad minimum near 200 nm, suggesting the absence of regular secondary structure.

The far UV CD spectrum of the major product generated by folding pro-ω-MVIIA-Gly in glutathione is shown in Figure 6b, along with the spectra of ω-MVIIA-Gly and the two forms with their disulfides reduced and the thiols blocked by iodoacetamide (RCAM forms). The spectra of RCAM ω-MVIIA-Gly and RCAM pro-ω-MVIIA-Gly each display a single minimum at approximately 196 nm, as expected for random-coil polypeptides (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Johnson, 1988). The spectrum of folded pro-ω-MVIIA-Gly had features qualitatively intermediate between those of the RCAM forms and that of folded ω-MVIIA-Gly. A difference spectrum was calculated by subtracting the spectrum of folded ω-MVIIA-Gly from that of folded pro-ω-MVIIA-Gly and was found to be similar to that expected for a random coil (Figure 6b). Thus, the observed CD spectrum for the folded form of pro-ω-MVIIA-Gly is consistent with the mature region having a folded conformation with a high β-strand content and the propeptide either being disordered or having a fixed conformation lacking extensive regular secondary structure.

Near-UV spectra were also collected for the various forms of ω-MVIIA but were very weak, presumably because the peptide contains only a single aromatic residue (Tyr 13 in the mature sequence), which is largely exposed to solvent in the native conformation. Although the spectra of the folded forms of ω-MVIIA-Gly had discernible minima at approximately 275 nm (with intensities of approximately -100 deg cm^2 dmol^{-1}), those of the A, C, and RCAM forms were essentially flat in this region. The spectrum of pro-ω-MVIIA-Gly that had been folded in glutathione was similar to those of the smaller native forms but less intense on a per-residue basis.

Reduction Kinetics of ω-MVIIA-Gly and Pro-ω-MVIIA-Gly. As an additional probe of conformation in the various forms of ω-MVIIA, the rates at which the disulfides were reduced by dithiothreitol (DTT) were measured (Figure 7). As shown in the accompanying paper, the three native disulfides in mature ω-MVIIA are reduced in a single kinetic phase with a second-order rate constant of 3 s^{-1} M^{-1} at pH 8.7, a rate that is approximately 5-fold slower than that for the reduction of oxidized glutathione (Creighton, 1975; Szajewski & Whitesides, 1980; Rothwarf & Scheraga, 1992). When ω-MVIIA-Gly with the three native disulfides was treated with DTT, the native form disappeared with a rate constant of 1 s^{-1} M^{-1}, and as for the mature form, there was very little accumulation of partially reduced species. In contrast, the A and C forms of ω-MVIIA-Gly were reduced much more rapidly, with rate constants of 14 and 28 s^{-1} M^{-1}, respectively, for the disappearance of the three-disulfide forms. When the ω-MVIIA-Gly obtained from folding the

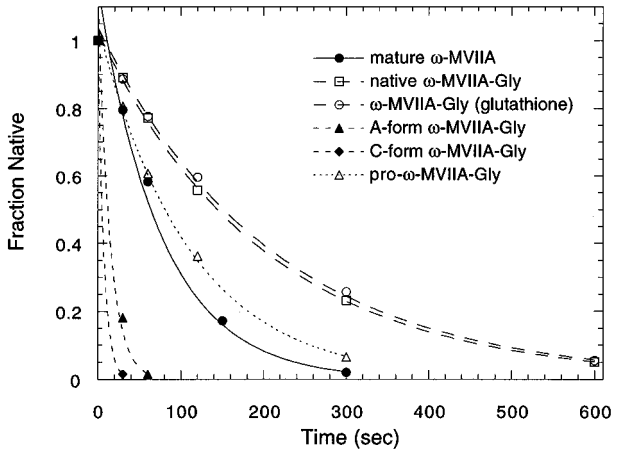

FIGURE 7: Kinetics of reductive unfolding of different forms of ω-conotoxin MVIIA. Native mature ω-MVIIA, native ω-MVIIA-Gly prepared as outlined in Figure 2, ω-MVIIA-Gly folded in glutathione, pro-ω-MVIIA-Gly folded in glutathione, and the nonnative forms A and C, as indicated by the key, were treated with 5 mM DTT at pH 8.7, 25 °C. The reactions were quenched by the addition of phosphoric acid and the various forms were separated by reversed-phase HPLC. All of the reduction reactions, except those of forms A and C, appeared to be kinetically cooperative, with no significant accumulation of intermediate species. A small amount of an intermediate, presumably containing one or two disulfides, was detected in the reduction of form A, while the reduction of form C was too rapid to have detected any intermediates. The fraction of native peptide remaining at the indicated times was determined by integration of the resulting chromatograms. The curves represent fits of the data to a first-order exponential decay. The resulting pseudo-first-order rate constants were used to calculate second-order rate constants of 3 s^{-1} M^{-1} (mature ω-MVIIA), 1 s^{-1} M^{-1} (both forms of ω-MVIIA-Gly), 2 s^{-1} M^{-1} (pro-ω-MVIIA-Gly), 14 s^{-1} M^{-1} (nonnative form A), and 28 s^{-1} M^{-1} (nonnative form C).

peptide in the presence of glutathione was treated with DTT, the observed reduction kinetics were indistinguishable from that of the peptide known to contain the three native disulfides, providing further evidence that the major folding product contained the correct disulfides.

Reductive unfolding kinetics were also measured for the major product obtained from folding pro-ω-MVIIA-Gly in the presence of glutathione. As for the native forms of the mature peptide and ω-MVIIA-Gly, this form was reduced without significant accumulation of intermediates. In addition, the rate constant for reduction, 2 s^{-1} M^{-1}, was similar to that of the other native forms, consistent with all three forms having the same set of disulfides and similar conformations.

Biological Activities of Folded Peptides. The receptor binding assay described in detail in the accompanying paper was also used to characterize the precursor forms of ω-MVIIA. In Figure 8a, the abilities of various forms of ω-MVIIA-Gly to bind to presynaptic Ca^{2+} channels are compared. The form of ω-MVIIA-Gly with native disulfides was found to bind to receptors with an apparent dissociation constant of 2×10^{-8} M, as did the ω-MVIIA-Gly that was folded in the presence of glutathione, consistent with the two forms having very similar conformations. In contrast, there was no detectable binding of the reduced and alkylated form of ω-MVIIA-Gly at concentrations as high as 1 μM. Although the A and C forms, with nonnative disulfides, appeared to bind with affinities only about 10-fold less than that of the native peptides, the binding activity of the A form

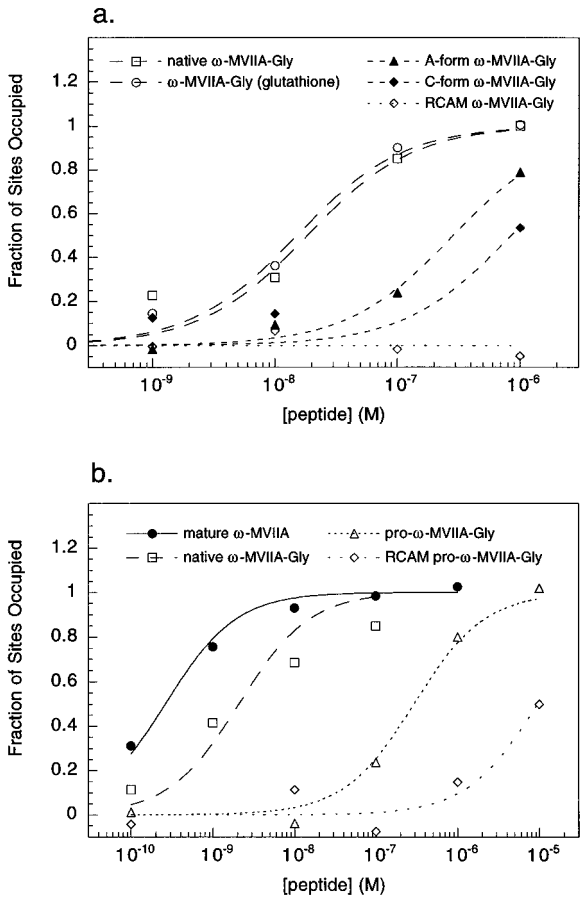

FIGURE 8: Binding of different forms of ω-conotoxin MVIIA to receptors in chick brain synaptic membranes. Peptides at the indicated concentrations were incubated with chick brain synaptosomes, and the fraction of sites occupied was determined as the blocking of sites for subsequent binding by radiolabeled ω-GVIA, as described in the accompanying paper. (a) Receptor binding curves of native ω-MVIIA-Gly prepared as outlined in Figure 2, ω-MVIIA-Gly folded in glutathione, the nonnative forms A and C, and reduced and carboxamidomethylated ω-MVIIA-Gly. (b) Receptor binding curves for native mature ω-MVIIA, ω-MVIIA-Gly folded in glutathione, pro-ω-MVIIA-Gly folded in glutathione, and reduced and iodoacetamide-blocked pro-ω-MVIIA-Gly. For the assays in panel a, the peptides were dissolved in 10 mM phosphate buffer, pH 6.8, while for the assays shown in panel b, the peptides were dissolved in a buffer optimized for this assay (SHTL buffer, containing 0.32 M sucrose, 5 mM Hepes−Tris, pH 7.4, 2 mg/mL hen egg white lysozyme, and 0.01 M NaCl). This difference in buffer composition resulted in consistently lower apparent affinities for the peptides dissolved in phosphate buffer. The data were analyzed as described in the accompanying paper. The apparent dissociation constants calculated for the peptides dissolved in phosphate buffer (panel a) were 2×10^{-8} M (both native forms of ω-MVIIA-Gly), 3×10^{-7} M (nonnative form A), and 9×10^{-7} M (nonnative form C). The apparent dissociation constants for peptides dissolved in SHTL buffer were 2.5×10^{-10} M (native mature ω-MVIIA), 3×10^{-9} M (ω-MVIIA-Gly folded in glutathione), 3×10^{-7} M (pro-ω-MVIIA-Gly folded in glutathione), and 9×10^{-6} M (RCAM pro-ω-MVIIA-Gly).

may have been significantly overestimated by small amounts of contaminating native peptide, which was not completely separated by HPLC.

Figure 8b compares the binding activities of glutathione-folded ω-MVIIA-Gly and pro-ω-MVIIA-Gly with that of native mature ω-MVIIA. The assays shown in this panel were performed using a buffer composition somewhat different from that used in Figure 8a, and, as a consequence, the dissociation constants obtained from the two sets of

experiments are not directly comparable. Under the conditions of Figure 8b, the apparent K_d for ω-MVIIA-Gly with native disulfides was 2×10^{-9} M, as compared to 2×10^{-10} M measured for native amidated ω-MVIIA. The major product obtained from the folding of pro-ω-MVIIA-Gly displayed a much lower affinity than either of these forms, with 3.5×10^{-7} M peptide required for half-saturation. Although the greatly reduced affinity observed for pro-ω-MVIIA-Gly may arise from a conformational difference between this form and the smaller peptides, it is also possible that the presence of the propeptide inhibits binding for steric reasons. There was very little specific binding of reduced and alkylated forms of either ω-MVIIA-Gly or pro-ω-MVIIA-Gly detected at micromolar peptide concentrations.

DISCUSSION

The disulfide-bonded conotoxins represent some of the smallest known peptides with well-defined three-dimensional structures. Although the disulfides contribute to the stabilities of these structures, additional stabilizing interactions appear necessary to direct formation of the correct covalent bonds. In the case of the peptide studied here, ω-MVIIA, studies described in the accompanying paper demonstrated that the mature sequence is able to fold with approximately 50% efficiency in the presence of optimal concentrations of thiol−disulfide reagents. While this yield is substantially greater than the 5−10% expected for random disulfide formation and observed in the presence of 8 M urea, it represents only a marginal stability difference (1−2 kcal/mol) between the native form and other disulfide-bonded species. The discovery that many of the conotoxins are synthesized as larger precursors has led to the suggestion that additional information needed to specify the native structures may be contained in precursor sequences (Olivera et al., 1990), as has recently been found to be the case for some other proteins. To explore this possibility, we have compared the in vitro folding reaction of the mature form of ω-MVIIA with those of two putative precursors predicted from cDNA sequences. One of these forms, ω-MVIIA-Gly, differs from the mature form only by the presence of the α-carbon and carboxyl group of the C-terminal Gly residue but was found to fold with significantly greater efficiency than the mature peptide. The other putative precursor, pro-ω-MVIIA-Gly, includes a 24-residue N-terminal propeptide. Although this propeptide is highly conserved among the ω-conotoxin precursors, it did not appear to influence folding favorably.

Because mapping disulfides between closely spaced Cys residues is extremely difficult, no attempt was made to establish directly that the major species produced during the disulfide-coupled folding of reduced ω-MVIIA-Gly did, in fact, contain the correct disulfides. Instead, its properties were compared with those of a sample known to contain the three disulfides found in the native peptide, as established by the scheme outlined in Figure 2. In addition to having the same HPLC retention times, the two forms displayed indistinguishable far-UV circular dichroism spectra, receptor binding activities, and disulfide reduction kinetics. In contrast, two forms known to contain two nonnative disulfides each were easily distinguished by each of these criteria. Collectively, these results provide strong evidence that the major product obtained from folding ω-MVIIA-Gly in the presence of glutathione contains the three disulfides of the mature natural peptide and has a very similar conformation.

Disulfide-coupled folding of pro-ω-MVIIA-Gly also generated a single major species, with a yield of about 65%. In this case, however, the evidence that this species contains the three correct disulfides is less conclusive. Since a reference sample of this precursor form with the native disulfides was not available, the properties of the folding product could be compared only with those of the native mature and ω-MVIIA-Gly forms. The circular dichroism spectrum of folded pro-ω-MVIIA-Gly was qualitatively consistent with that expected if part of the peptide had a conformation similar to that of the mature peptide and a portion was disordered. This form also displayed significant receptor binding activity, but the apparent affinity for Ca^{2+} channels was approximately 100-fold lower than that seen for ω-MVIIA-Gly containing the correct disulfides. Finally, the rate at which the folded pro-ω-MVIIA-Gly was reduced by dithiothreitol was very similar to that observed for the native mature peptide, and like the forms known to contain the correct disulfides, there was no accumulation of intermediates during reductive unfolding. Overall, we believe it most likely that the major product seen in the folding of pro-ω-MVIIA-Gly contains the disulfides and folded structure of the mature region and irregular or disordered structure in the propeptide region. It is difficult from these data, however, to rule out the possibility that the major product contains nonnative structure or disulfides. Nonetheless, since the yield of this major form was actually lower than that for ω-MVIIA-Gly, it can be safely concluded that the propeptide does not enhance either the efficiency or rate of in vitro folding.

Role of the C-Terminus in Folding and Function. Amidation by oxidative cleavage of a C-terminal Gly residue is a quite common posttranslational modification, particularly among peptide hormones, and frequently results in increased biological activity and stability against proteolytic digestion (Eipper et al., 1992,1993). The two enzymatic activities responsible for this conversion, collectively referred to as peptidyl-glycine α-amidating monooxygenase (PAM), have been shown to be localized in secretory granules (Oyarce & Eipper, 1995), suggesting that the form of the peptide that folds in the endoplasmic reticulum probably contains the unmodified Gly residue at the C-terminus.

For both mature ω-MVIIA and ω-MVIIA-Gly, the final folding yield appears to reflect the relative thermodynamic stabilities of the native form and other disulfide-bonded species. Thus, the greater folding efficiency seen for ω-MVIIA-Gly suggests that the Gly residue selectively stabilizes the native form. The presence of the Gly residue was also found to increase the overall equilibrium constant for folding by approximately 4.5-fold, corresponding to a free energy difference of about 1 kcal/mol, a small but significant energetic effect. These observations suggest that the Gly residue may participate in specific interactions within the folded conformation.

Interestingly, a Gly residue is also found at the C-termini of members of the squash family of protease inhibitors (Otlewski, 1993), which have folded conformations very similar to that of the ω-conotoxins (Pallaghy et al., 1994). Unlike the ω-conotoxins, however, the Gly residue is present in the mature sequences of the squash inhibitors. X-ray crystal structures have been determined for two of the squash

inhibitors (Bode et al., 1989; Huang et al., 1993). In these two molecules, the carboxyl group of the C-terminal Gly residue forms a network of hydrogen bonds with the guanidino group of an Arg side chain and a backbone amide group. Solution structures of some of the squash inhibitors have also been determined by NMR (Heitz et al., 1989; Holak et al., 1989, 1991), but the positions of the C-terminal atoms do not appear to be defined well enough in these structures to allow unambiguous identification of specific interactions. Another molecule with a very similar conformation is the 29-residue circular peptide kalata B1 (Pallaghy et al., 1994; entry 1KAL in the Brookhaven Protein Data Bank). In this case, the residue corresponding to Gly 26 of the ω-MVIIA precursor does not, of course, have a terminal carboxyl group, but the corresponding carbonyl oxygen forms a β-sheet hydrogen bond. Examination of the solution structure of the amidated form of ω-MVIIA (Kohno et al., 1995) suggests that the extra atoms of the Gly residue could be accommodated without extensive structural perturbations, and, conceivably, could form hydrogen bonds with nearby backbone amide groups and basic side chains.

Although ω-MVIIA-Gly appears to fold more efficiently than the mature peptide, its affinity for Ca^{2+} channels is approximately 10-lower. The decreased affinity may arise either from an electrostatic effect caused by the extra negative charge or from steric effects caused by the extra atoms. Mutational and chemical modification studies of both ω-MVIIA and ω-GVIA have shown that binding of these peptides to their receptors is very sensitive to many modifications that decrease the net charge of the peptides, suggesting that electrostatics may play a major role in the interaction (Haack et al., 1993; Lampe et al., 1993; Sato et al., 1993; Nadasdi et al., 1995). The extra atoms of the Gly residue could also interfere with binding sterically, either directly or by promoting a structural difference in other regions of the peptide.

Role of the N-Terminal Propeptide. Precursor sequences have now been implicated in the folding of several proteins, including subtilisin (Zhu et al., 1989; Eder et al., 1993; Shinde & Inouye, 1993; Bryan et al., 1995), α-lytic protease (Silen & Agard, 1989; Baker et al., 1992, 1993), carboxypeptidase Y (Winther & Sørensen, 1991), cathepsin L (Tao et al., 1994), and neurophysin (Deeb & Breslow, 1996). In the case of the bacterial proteases, subtilisin and α-lytic protease, the N-terminal propeptides have been shown to greatly enhance the rate of folding in vitro, apparently by binding to and stabilizing the rate-determining transition state (Baker et al., 1992; Eder et al., 1993; Bryan et al., 1995). Neurophysin is synthesized with either oxytocin or vasopressin attached at the N-terminus, and the two polypeptides remain non-covalently attached after cleavage, with the neurophysin acting as a carrier for the peptide hormone. The efficiency of disulfide-coupled refolding of reduced mature neurophysin is very low but approaches 100% in the presence of either of the peptide hormones or small peptides. Recent studies indicate that peptide ligands favor folding thermodynamically by stabilizing the native conformation with respect to other disulfide-bonded forms (Deeb & Breslow, 1996), as described here for the C-terminal Gly residue of ω-MVIIA.

Although a role in folding was anticipated for the ω-conotoxin propeptide, the experiments reported here failed to reveal such an effect. The rate and efficiency of folding were, if anything, lower for the putative precursor containing the propeptide than they were for the mature sequence containing the C-terminal Gly residue. Since protein disulfide isomerase increased the folding rates for ω-MVIIA-Gly and pro-ω-MVIIA-Gly to very similar extents, it also appears unlikely that the propeptide promotes interactions between the peptide and enzyme significantly.

It is possible, however, that in vivo the propeptide could play a role in folding, for instance by enhancing the solubility of the nascent chain or by promoting interactions with other cellular factors. Other possible roles for the propeptide include targeting to enzymes required for post-translational modifications, or directing intracellular trafficking to secretory granules, where the toxins are stored before being secreted into the venom.

Conclusions. In contrast to other recently described precursor sequences, the propeptide region of pro-ω-MVIIA-Gly does not appear to promote folding, either thermodynamically or kinetically. The C-terminal Gly residue, however, does measurably increase the stability of the native form, leading to a folding efficiency greater than 80% under optimal conditions, versus about 50% observed for the mature form. It thus appears that ω-MVIIA-Gly contains most, if not all, of the information required to direct correct folding, in spite of its small size and the low level of sequence conservation seen among members of the ω-conotoxin family. As discussed in the accompanying paper, efficient burial of the non-polar disulfides may be an important determinant of folding specificity in these molecules. The ω-conotoxins may prove to be very interesting subjects for more detailed folding studies, particularly since their small size may facilitate comparisons between experimental results and those obtained from theoretical treatments. In addition, further analysis of the factors required for efficient folding may make it possible to use this disulfide-linked motif as a framework for the construction of new peptides with the same overall conformation but with new functions.

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