Roles of Individual Disulfide Bonds in the Stability and Folding of an ω-Conotoxin†

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Received February 19, 1998; Revised Manuscript Received May 13, 1998

ABSTRACT: Although it contains only 25 amino acid residues, ω-conotoxin MVIIA folds into a well-defined three-dimensional structure that is stabilized by 3 disulfide bonds. To assess the contributions of the disulfides to folding and stability, three analogues, each with one pair of disulfide-bonded Cys residues replaced with Ala, were prepared and characterized. The analogues also contained a C-terminal Gly residue that is believed to be present when the peptide folds in vivo and has been shown previously to stabilize the native structure. Circular dichroism spectra and biological assays of the analogues indicated that removing any one of the disulfides greatly destabilized the native conformation. The two disulfides in each analogue were also reduced much more rapidly than in the native form with three disulfides. When the analogues were fully reduced and allowed to form disulfides in the presence of oxidized and reduced glutathione, the native disulfides were not formed in preference to non-native disulfides, further indicating that the forms with two-native disulfides are not significantly stabilized by noncovalent interactions. However, the measured equilibrium constants for disulfide formation indicate that forming any two of the three native disulfides leads to an effective concentration of approximately 25−50 M for the two remaining thiols. The two-disulfide analogues thus appear to represent a stage of folding in which the polypeptide is constrained to a distribution of relatively compact conformations that greatly favor formation of the third disulfide and the final folded structure.

Disulfide bonds between Cys residues are a common feature of many folded proteins (*1*), particularly those evolved to function extracellularly, where an oxidizing environment favors disulfide formation. Although these bonds generally contribute to the stability of the native proteins, it is often found that removing only one, or even two, disulfides frequently leaves the folded conformation intact, but measurably destabilized (*2*−*7*). There are, however, small proteins with unusually high densities of disulfides, and the individual disulfides of these proteins may be particularly important for their ability to fold. One class of such molecules are the ω-conotoxins, small peptide antagonists of presynaptic Ca^{2+} channels (*8*). These peptides are typically 25−30 amino acid residues long and are found in the paralytic venoms of *Conus* snails, along with numerous other peptide toxins. Although there is very little sequence similarity among the ω-conotoxins, they share a common three-dimensional conformation and characteristic pattern of disulfide bonds (*9*−*17*), as illustrated for one member of the family, ω-MVIIA, in Figure 1. The different members of the ω-conotoxin family have distinguishable, but overlapping, specificities for Ca^{2+} channel subtypes, and they have been widely used to analyze the distributions and functions of these subtypes (*8*). In addition, they have shown potential as therapeutic agents, particularly for the treatment of chronic pain (*18*).

As shown in Figure 1, the ω-conotoxin structure is characterized by a small three-stranded β-sheet and is stabilized by three disulfide bonds. The N-terminus of the polypeptide is held near the C-terminus by two disulfide bonds that link, in pretzel fashion, the termini to adjacent Cys residues in the central region. The third disulfide, between residues 8 and 20, links the N-terminal half of the molecule to the C-terminal half. The 8−20 disulfide also links together the first and second strands of the β-sheet, and that between Cys 15 and Cys 25 links the third strand to the approximate center of the sequence. Thus, the three disulfides form an integral part of the native structure, with the six Cys side chains representing approximately 25% of the total buried surface area (*19*).

Because of their small size and very limited sequence conservation, it has been suggested that the mature conotoxins may not contain sufficient sequence information to specify correct folding and disulfide formation (*20*, *21*). Analyses of cDNA clones indicate that ω-conotoxins are synthesized as precursors containing both an N-terminal propeptide and a C-terminal Gly residue that is posttranslationally cleaved to yield an amidated C-terminus (*22*, *23*). Although N-terminal propeptides are known to play a role in the folding of other proteins, especially bacterial proteases (*24*, *25*), studies with ω-MVIIA indicate that the mature sequence of this peptide is able to refold and form its correct disulfides with about 60% efficiency, and the propeptide does not enhance either the folding rate or the efficiency (*19*, *26*).

† Supported by NSF Grant MCB-9316065. Peptide synthesis and mass spectroscopy at the Protein/DNA Core Facility of the Utah Regional Cancer Center was supported by National Cancer Institute Grant 5 P30 CA 42014 and the Huntsman Cancer Institute.

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S0006-2960(98)00397-3 CCC: $15.00
Published on Web 06/17/1998

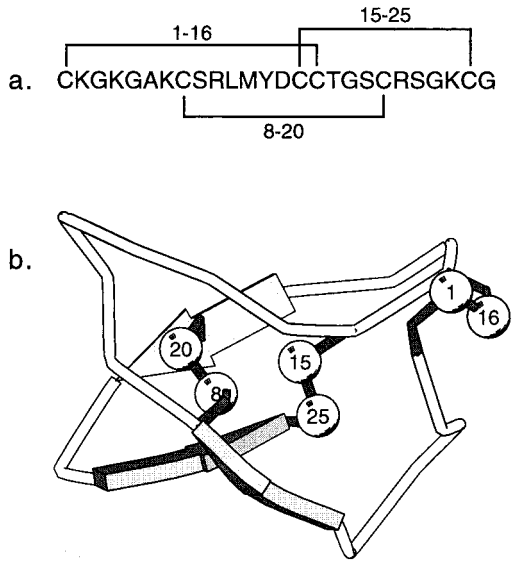


FIGURE 1: Primary structure of ω-MVIIA-Gly and the three-dimensional structure of mature ω-MVIIA. (a) The peptide used in this study, designated ω-MVIIA-Gly, consists of the mature sequence and the C-terminal Gly residue inferred from cDNA sequences (*23*). The disulfide bonds found in the native peptide are indicated by the solid lines linking the Cys residues (*17*). (b) The ribbon diagram of mature ω-MVIIA was drawn with the program MOLSCRIPT (*66*), from the atomic coordinates of one of the 13 NMR solution structures calculated by Kohno et al. (*15*).

The C-terminal Gly residue, however, does appear to favor folding, since a form of ω-MVIIA containing only the mature sequence plus the Gly residue, designated ω-MVIIA-Gly,[1] is about 1 kcal/mol more stable than the mature form and refolds with an efficiency of about 90% (*26*). The enzymes required for C-terminal amidation are located in secretory granules (*27, 28*), suggesting that the terminal Gly residue is probably present when the peptides fold in the endoplasmic reticulum. We have adopted ω-MVIIA-Gly as a model for the precursor form that folds in vivo and are studying its mechanism of disulfide-coupled folding in vitro. Solution NMR studies indicate that ω-MVIIA-Gly has a three-dimensional structure very similar to that of the mature toxin (D. P. Goldenberg, R. E. Koehn, D. E. Gilbert, and G. Wagner, in preparation).

To assess the contributions of the individual disulfides to folding and stability, we have prepared and analyzed a series of three ω-MVIIA-Gly variants in which the individual disulfides were eliminated by replacing the Cys residues with Ala. The conformations of the analogues containing two native disulfides were probed by circular dichroism spectroscopy, by a Ca^{2+} channel binding assay, and by measuring the rates of reductive unfolding. By each of these criteria, the two-disulfide forms appear to lack most of the specific structure found in the native protein. In addition, when the reduced analogues were allowed to refold in the presence of oxidized and reduced glutathione (GSSG and GSH, respectively), the native disulfides were not formed in preference to non-native disulfides. These results indicate that each of the three disulfides plays an essential role in stabilizing the native conformation and specifying the correct formation of the other two.

EXPERIMENTAL PROCEDURES

Synthesis of Analogues with Native Disulfide Bonds. Analogues of ω-MVIIA-Gly were synthesized on an HMP resin using Fmoc chemistry. To obtain products with the two native disulfide bonds, the peptides were synthesized using the selective protection scheme described previously (*26, 29, 30*). One pair of Cys residues that form a native disulfide was protected with trityl protecting groups (sensitive to deprotection by TFA), and the other pair was protected with acetamidomethyl (Acm) protecting groups (resistant to TFA). Upon cleavage and partial deprotection in TFA, the peptides had two Cys thiols capable of forming a native disulfide, with the other pair still protected by Acm groups. The cleaved peptides were purified by reversed-phase HPLC on a Vydac C_{18} column (2.5 cm $\times$ 30 cm) eluted with a gradient of acetonitrile in 0.1% TFA. To promote oxidation of the deprotected Cys residues, the peptide solutions were diluted 10-fold with H_2O, adjusted to pH 8.0 with Tris base, and stirred at room temperature. Oxidation reactions were analyzed by HPLC and judged to be complete when the reduced peptide was no longer detectable. The 8,20Ala and 15,25Ala analogues were fully oxidized within 16 h, but the disulfide in the partially deprotected 1,16Ala analogue (15−25) was slow to close via air oxidation, with a half-time of approximately 2.5 days. To facilitate the formation of this disulfide, the reduced form was incubated in a solution containing 1 mM GSSG and 2 mM GSH at pH 7.3. Under these conditions, formation of the 15−25 disulfide was complete after 1 h. After the first oxidation step, the peptides were purified by HPLC. To remove the ACM protecting groups and form the second native disulfide, the peptides were oxidized with I_2. In each case, only one species, with a shifted HPLC retention time, was formed. The final yields from 50 mg of the 1,16Ala, 8,20Ala, and 15,25Ala peptide resins were approximately 2, 6, and 2 mg, respectively.

ω-MVIIA-Gly with three native disulfide bonds was prepared by reducing fully deprotected synthetic peptide with DTT and then folding the peptide in 1 mM GSSG and 2 mM GSH, as described previously (*26*).

Preparation and HPLC Separation of Species with Other Disulfide Bonds. To generate the three possible two-disulfide forms of each four-Cys analogue, the peptides were fully reduced and then oxidized by I_2, as follows. The two native disulfides were first reduced by incubating 20−80 μM peptide with 50 mM dithiothreitol (DTT) in the presence of 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 0.001 M EDTA. After 45 min at room temperature, the pH of the solution

[1] Abbreviations: ω-MVIIA-Gly, a form of ω-conotoxin MVIIA composed of the mature sequence plus a C-terminal Gly residue with an unmodified carboxyl group; 1,16Ala, 8,20Ala, and 15,25Ala, analogues of ω-MVIIA-Gly with the indicated Cys residues replaced with Ala; forms of the analogues with specific disulfide bonds are indicated by the numbers of the disulfide-linked Cys residues enclosed in brackets and followed by a subscript indicating the analogue; thus, $[8-20,15-25]_{1,16Ala}$ is the form in which Cys 1 and Cys16 are replaced with Ala, and the other four Cys residues are disulfide-bonded as in the native structure; RCAM, reduced and carboxamidomethylated; GSSG and GSH, disulfide and thiol forms, respectively, of glutathione; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HMP, *p*-hydroxymethylphenoxymethyl; Fmoc, fluoromethoxy carbonyl; Acm, acetamidomethyl; TFA, trifluoroacetic acid; TCEP, tris(2-carboxyethyl)phosphine; CD, circular dichroism; HPLC, high-performance liquid chromatography; RNase, ribonuclease; BPTI, bovine pancreatic trypsin inhibitor.

was lowered to about 2 by the addition of 0.05 volume of concentrated phosphoric acid. The reduced peptide was then separated from the DTT and other buffer components by reversed-phase HPLC, lyophilized, and stored at −20 °C. To oxidize the reduced peptide, the lyophilized material was resuspended in 1% TFA, mixed with 0.1 volume of 5 mM I_2 (dissolved in methanol), and incubated for 30 s at room temperature. One-tenth volume of 0.3 M ascorbate was then added to quench the reaction, and the peptides were separated from the buffer components by HPLC. In each case, these highly oxidizing conditions led to the production of three species, one of which had the retention time of the form with two native disulfide bonds. When partially reduced at low pH (where disulfide rearrangement is minimal) with tris-(2-carboxyethyl)phosphine (TCEP) (*31*), each product of iodine oxidation gave rise to two intermediates as well as the fully reduced form, confirming that each contained two disulfide bonds.

The various disulfide-bonded forms were fractionated by reversed-phase HPLC using buffer systems optimized for each analogue. Forms of 15,25Ala were eluted using a gradient of acetonitrile (CH_3CN) in TFA, as described previously (*19*). The various forms of 1,16Ala were fractionated with a gradient prepared by mixing (A) 10 mM $NaPO_4$, pH 2.74, and (B) 30% CH_3CN (*32*). The disulfide-bonded forms of 8,20Ala were separated using a gradient prepared by mixing (A) 0.103% TFA and (B) 0.103% TFA, 40% 2-propanol. For optimal separation of the 8,20Ala forms, the column was warmed to 37 °C in a water bath. Elution profiles were monitored by the absorbance at 220 nm, and the absorption data were collected and analyzed using the Rainin Dynamax computer interface and software.

Identification of Non-Native Disulfide Bonds in Analogues. The disulfide bonds in the forms with two non-native linkages were identified by isolating and identifying disulfide-bonded tryptic fragments. For trypsin digestion, 20 nmol of lyophilized peptide was treated with 0.85 nmol of β-trypsin that had been affinity-purified using immobilized soybean trypsin inhibitor (Sigma). The digestions were carried out at room temperature in a total volume of 1 mL also containing 0.1 M Tris-HCl, pH 8.0, and 5 mM $CaCl_2$, and the progress of the reactions was monitored by reversed-phase HPLC. For most of the forms, digestion reached an apparent end point after 8 h, but in some cases a 16 h incubation was required. The major digestion products were purified by HPLC and analyzed by electrospray-ionization mass spectrometry. From their molecular masses, all of the isolated peptides appeared to be complete digestion products. The identities of the various two-disulfide forms were inferred by comparing the observed masses with those predicted for the various possible disulfide-bonded peptides.

Disulfide-Coupled Folding and Unfolding Reactions. For folding experiments, fully 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−NaOH, pH 7.3, 0.4 M KCl, 2 mM EDTA, and the oxidized and reduced forms of glutathione as appropriate for the experiment. In a typical experiment, 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 fractionated by reversed-phase HPLC, as described above, and the relative concentrations of the various species were calculated by integrating the appropriate HPLC peaks

Folding experiments in the presence of 8 M urea were carried out as described above, except that the peptide was dissolved in 10 mM HCl to a concentration of 200 μM and then mixed with 9 volumes of a solution containing 8.89 M urea, 0.11 M MOPS−NaOH, pH 7.3, 0.22 M KCl, and 1.11 mM EDTA.

Reductive unfolding experiments were initiated by mixing the various analogues with DTT (1 mM final concentration). These reaction mixtures also contained 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 1 mM EDTA.

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. Far-UV CD spectra were recorded with an AVIV Model 62D spectropolarimeter, using a bandwidth of 1.5 nm, a step size of 0.1 nm, and an averaging time of 0.8 s. The cell path length was 0.1 cm, and the sample temperature was 25 °C. Four spectra were collected for each sample and averaged.

Samples were prepared by dissolving lyophilized peptide to a concentration of approximately 0.2 mg/mL in 10 mM sodium phosphate buffer, pH 6.84. The protein concentrations were determined from the absorbance at 280 nm and an extinction coefficient calculated from the amino acid composition (*33*).

Ca^{2+} Channel Binding Assay. The protocol used to measure the binding of ω-MVIIA-Gly and the analogues to Ca^{2+} channels present in crude preparations of chick brain presynaptic membranes (synaptosomes) has been described previously (*19*, *34*, *35*). Briefly, binding was detected as the blocking of sites otherwise available to radiolabeled ω-conotoxin GVIA. To generate a binding curve, various amounts of peptide were mixed with a fixed quantity of receptors and incubated for 30 min on ice. After this incubation, ^{125}I-labeled ω-GVIA was added to each tube, and the solutions were allowed to incubate for another 30 min at room temperature. The synaptosomes were then separated from unbound ligands by filtration, and the total amount of γ-radioactivity retained by the filters was determined by counting.

RESULTS

To assess the contributions of each of the disulfides to the structure and stability of native ω-MVIIA-Gly, three synthetic analogues were prepared, each with one pair of the normally disulfide-bonded Cys residues replaced by Ala. Using a selective protection and deprotection strategy, the peptides were synthesized so as to contain only the two native disulfides. The three peptide sequences are designated here by the residues replaced by Ala: 1,16Ala, 8,20Ala, and 15,-25Ala. The forms with defined disulfides are indicated by the linked Cys residues, in brackets, with a subscript indicating the peptide sequence.

The conformations of the three analogues with native disulfide bonds were characterized by CD spectroscopy, a receptor binding assay, and reductive unfolding kinetics. The relative stabilities of these forms, with respect to forms with other disulfides, were then measured by allowing their

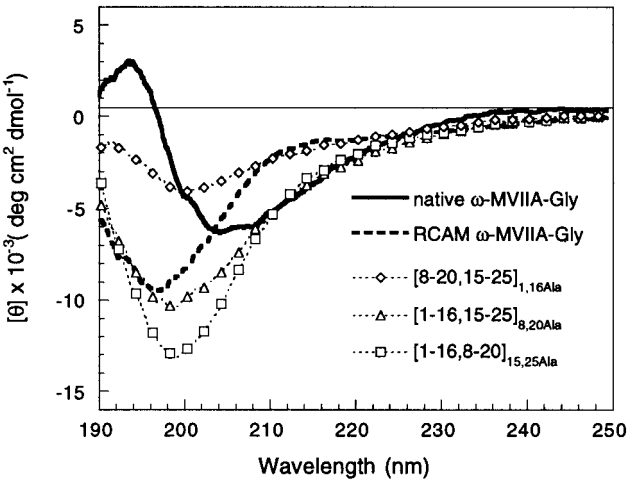


FIGURE 2: Circular dichroism spectra of ω-MVIIA-Gly containing zero, two, or three native disulfides. Spectra for the native and fully reduced (RCAM) forms of ω-MVIIA-Gly are shown as solid and dashed lines, respectively, and those for the three analogues with two native disulfides are identified by the symbols labeled in the key. All spectral intensities are expressed as mean residue ellipticities.

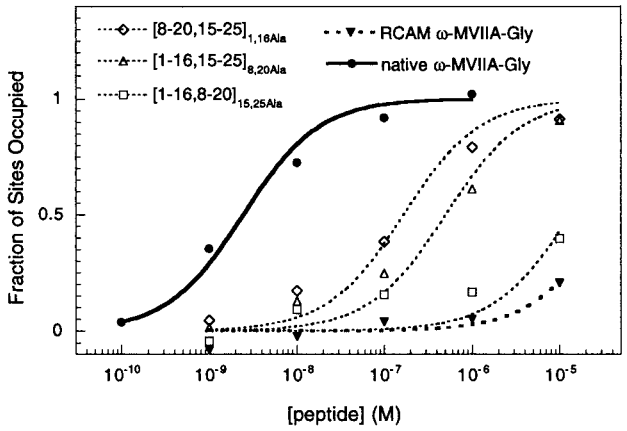


FIGURE 3: Calcium channel binding activity of ω-MVIIA-Gly containing zero, two, or three native disulfides. Peptides at the indicated concentrations were incubated with chick-brain synaptosomes, and the fraction of sites occupied was determined from the blocking of sites otherwise available for binding by radiolabeled ω-conotoxin GVIA. Each point represents the mean of two independent measurements, and the curves represent simple binding isotherms fit to the experimental data by the method of nonlinear least-squares. For the forms with native disulfides, the apparent dissociation constants were 2.5×10^{-9} M (native ω-MVIIA-Gly), 1.7×10^{-7} M ([8−20,15−25]$_{1,16Ala}$), 5.17×10^{-7} M ([1−16,15−25]$_{8,20Ala}$), and 1.3×10^{-5} M ([1−16,8−20]$_{15,25Ala}$). For the reduced and carboxamidomethylated form of ω-MVIIA-Gly, the dissociation constant was 3.7×10^{-5} M.

disulfides to undergo reversible exchange in the presence of oxidized and reduced glutathione.

Characterization of Analogues with Two Native Disulfides. The far-UV CD spectra of the three analogues with two native disulfide bonds are shown in Figure 2. For comparison, spectra of native ω-MVIIA-Gly (with three native disulfide bonds) and the fully reduced peptide (with the free thiols carboxamidomethylated) are also shown. The spectrum of the native form (thick solid curve) is characterized by a positive maximum at 194 nm and a relatively broad minimum around 205 nm, consistent with the β-sheet structure determined by NMR spectroscopy (*9*−*17*) (Goldenberg et al., in preparation). In contrast, the spectrum of the reduced and alkylated form (thick dashed curve) lacks a maximum and has a minimum at 196 nm. This spectrum is similar to that expected for a random coil and indicates that the form lacking disulfides has little regular secondary structure (*36*−*38*).

For two of the analogues with native disulfides, [1−16,-15−25]$_{8,20Ala}$ and [1−16,8−20]$_{15,25Ala}$, the CD spectra resembled that of the fully reduced and alkylated form. For these two forms, there was no maximum near 194 nm, and there was a deep minimum at about 199 nm, consistent with a conformation largely devoid of regular secondary structure. For the third form with native disulfides, [8−20,15−25]$_{1,16Ala}$, the far-UV CD spectrum had features intermediate between those of the native and fully reduced forms, suggesting that it may contain some residual regular structure. Some of the spectral differences observed upon removal of the disulfides may arise from direct contributions of these bonds to the spectrum of the native protein (*39*−*41*). However, because the CD bands associated with disulfides are generally positioned at wavelengths greater than 220, it is most likely that the differences in the far-UV spectra are due primarily to changes in backbone conformation. The spectra of the RCAM forms of the four-Cys analogues (data not shown) were all qualitatively similar to one another and to that of the form with six alkylated Cys residues, although the intensities of the spectra differed significantly.

As a functional test for the presence of nativelike structure in the two-disulfide analogues, their abilities to bind to neuronal Ca^{2+} channels were measured (Figure 3). In the binding assay used here, the peptides of interest were incubated at various concentrations with a fixed quantity of receptors, and binding was detected as the blocking of sites that would otherwise be available to bind radiolabeled ω-conotoxin GVIA. In Figure 3, the fraction of sites occupied is plotted as a function of total peptide concentration (which greatly exceeded the concentration of bound peptide). For comparison, the binding curves for the native and RCAM forms of ω-MVIIA-Gly are also shown. The data were fit to a simple binding isotherm to determine apparent dissociation constants.

Each of the two-disulfide analogues displayed much lower affinities for receptors than did the three-disulfide form, which has an apparent dissociation constant of approximately 2 nM (*26*). For two of the analogues, [8−20,15−25]$_{1,16Ala}$ and [1−16,15−25]$_{8,20Ala}$, the binding activity was significantly greater than that observed for fully reduced ω-MVIIA-Gly, with apparent dissociation constants of 1.7×10^{-7} and 5.1×10^{-7} M, respectively. For the third analogue, which lacked the 15−25 disulfide, however, fewer than half of the available sites were occupied at peptide concentrations as high as 10 μM. Following reduction and alkylation, all three of the four-Cys analogues displayed little or no specific binding (not shown). These results indicate that each of the three disulfides is necessary to maintain the conformation necessary for high-affinity binding, though the analogues lacking either the 1−16 or the 8−20 disulfide displayed significant affinities, corresponding to about 1% of that of the native form. This residual activity may reflect a small fraction of molecules with a conformation very similar to that of the native peptide, or the receptors may be able to bind, with reduced affinities, peptides in non-native confor-

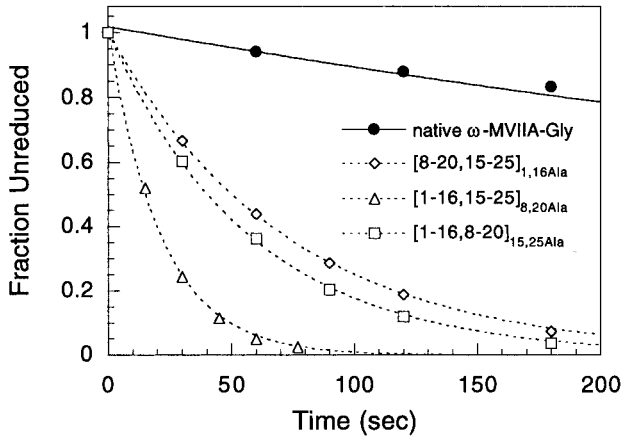


FIGURE 4: Kinetics of disulfide reduction in ω-MVIIA-Gly containing two or three native disulfides. The peptides with native disulfides were incubated with 1 mM DTT at 25 °C, pH 8.7. At the indicated times, samples of the reaction mixtures were withdrawn, acidified, and analyzed by reversed-phase HPLC to determine the fraction of unreacted peptide remaining. The curves represent exponential decay functions fit to the experimental data by least squares. The second-order rate constants for the reactions of the four proteins with DTT were 1.3 s^{-1} M^{-1} (native ω-MVIIA-Gly), 14 s^{-1} M^{-1} ($[8-20,15-25]_{1,16Ala}$), 47 s^{-1} M^{-1} ($[1-16,15-25]_{8,20Ala}$), and 17 s^{-1} M^{-1} ($[1-16,8-20]_{15,25Ala}$).

mations. These observations are consistent with those of Sabo et al., who found that removing any of the three disulfides from ω-conotoxin GVIA dramatically reduced the biological activity of this peptide (*42*).

The sensitivity of the disulfides to reduction by dithiothreitol (DTT) was used as a third measure of stable structure in the two-disulfide analogues. The disulfides in native ω-MVIIA-Gly are reduced in a single kinetic phase, with a second-order rate constant of 1 s^{-1} M^{-1} at pH 8.7, about 20-fold smaller than that for reduction of a fully exposed disulfide (*26*). The low rate of reduction is most likely a consequence of inaccessibility of the disulfides to the thiol reagent, in agreement with analysis of the solvent exposure of the three disulfide bonds in native ω-MVIIA (*19*).

As shown in Figure 4, each of the analogues with two native disulfides was reduced much more rapidly than was native ω-MVIIA-Gly. The second-order rate constants for the disappearance of the two-disulfide forms ranged from 14 s^{-1} M^{-1}, for $[8-20,15-25]_{1,16Ala}$, to 47 s^{-1} M^{-1}, for $[1-16,15-25]_{8,20Ala}$. Thus, removing any of the native disulfides makes the remaining two much more susceptible to reduction.

The reduction of the native form of ω-MVIIA-Gly is kinetically very cooperative, so that there is little or no accumulation of intermediates with one or two disulfides (*26*). Similarly, only low concentrations of intermediates were detected during the reduction of the 1,16Ala analogue. In contrast, during the reductive unfolding of $[1-16,15-25]_{8,20Ala}$ and $[1-16,8-20]_{15,25Ala}$, one-disulfide forms accumulated to detectable levels (not shown), indicating that the first native disulfide bond is broken at a rate that is as fast or faster than the rate for breaking the remaining disulfide. Five or six intermediates were observed during the course of each of the latter reactions, suggesting that intermediates with one native disulfide rapidly rearrange to yield several other one-disulfide forms, which are then reduced.

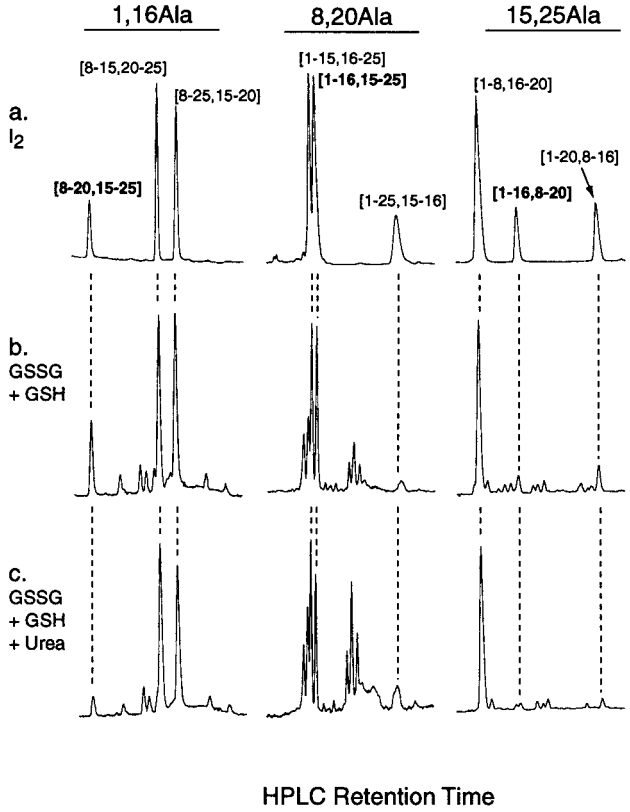


FIGURE 5: Distribution of disulfide-bonded species generated from the fully reduced four-Cys analogues by (a) iodine oxidation, (b) refolding in the presence of 1 mM GSSG and 2 mM GSH, or (c) refolding in the presence of 1 mM GSSG and 2 mM GSH plus 8 M urea. In (a), the fully reduced peptides were incubated with 0.5 mM I_2 for 30 s at room temperature, after which the reactions were quenched by the addition of ascorbate. In (b), the reduced peptides were incubated with 1 mM GSSG and 2 mM GSH for 90 min at pH 7.3, 25 °C, and the reactions were quenched by acidification. The reactions analyzed in (c) were identical to those in (b), except for the presence of 8 M urea. After quenching, each of the reaction mixtures was analyzed by reversed-phase HPLC, using solvent conditions optimized for each of the three analogues, as described under Experimental Procedures. The forms containing two native disulfides were identified by comparing the HPLC retention times with those determined for the peptides synthesized with the correct disulfides. The forms with non-native disulfides were identified by tryptic peptide mapping, as described in the text and summarized in Table 1.

In summary, the three different methods used to probe the conformations of the analogues revealed that each disulfide bond is required to maintain a stable nativelike conformation. In the absence of any one of the bonds, the analogues have less regular secondary structure and greatly reduced affinity for Ca^{2+} channels and are more susceptible to reduction than the form with three native disulfides.

Identification of Non-Native Two-Disulfide Forms. To facilitate the analysis of disulfide-coupled folding experiments with the four-Cys analogues, described in the following section, each of the three possible two-disulfide forms of each analogue was isolated and identified. To generate these species, the reduced forms of the analogues were oxidized by molecular iodine, which leads to efficient, rapid, and irreversible disulfide formation with relatively little specificity. The oxidation products were then fractionated by reversed-phase HPLC, as illustrated in Figure 5a.

For each of the three analogues, the reduced peptides were efficiently converted to three species, and in each case, one

Table 1: Identification of Two-Disulfide Forms of Four-Cys Analogues by Tryptic Peptide Mapping

Analog	Two-disulfide Isomer	Observed Mass of Tryptic Fragment	Assigned Fragment	Predicted Mass of Assigned Fragment
1,16 Ala	[8-15,20-25]	1757.7	C_8SR | $LMYDC_{15}ATGSC_{20}R$ | $C_{25}G$	1759.7
1,16 Ala	[8-25,15-20]	1217.5	$LMYDC_{15}ATGSC_{20}R$	1217.5
8,20Ala	[1-5,16-25]	1643.5	C_1K | $LMYDC_{15}C_{16}TGSAR$ | $C_{25}G$	1644.6
8,20Ala	[1-25,15-16]	1217.3	$LMYDC_{15}C_{16}TGSAR$	1217.5
15,25Ala	[1-8,16-20]	1217.3	$LMYDAC_{16}TGSC_{20}R$	1217.5
15,25Ala	[1-20,8-16]	1828.6	C_8SR | $LMYDAC_{16}TGSC_{20}R$ | C_1K	1830.8

of the products displayed the HPLC retention time of the peptide synthesized with the native disulfides. To identify the non-native disulfides present in the other species, these forms were subjected to tryptic peptide mapping. For each, a peptide was identified that clearly distinguished it from the other possible non-native isomer. The peptide fragments and the species they identified are listed in Table 1, and the HPLC peaks in Figure 5a are labeled to indicate the two-disulfide forms they contain.

Disulfide-Coupled Folding in the Presence of GSSG and GSH. When the reduced form of ω-MVIIA-Gly is allowed to re-form disulfides in the presence of a mixture of 1 mM GSSG and 2 mM GSH, approximately 85−90% of the molecules form the three native disulfides, with most of the remaining molecules accumulating as species with three disulfides, one or more of which is non-native (*26*). To determine whether the preference for forming native disulfides is also significant when only two disulfides can form, the analogues were fully reduced and then allowed to refold in the presence of GSSG and GSH.

After 90 min of refolding, the distribution of disulfide-bonded species reached an apparent equilibrium, illustrated by the HPLC traces in Figure 5b. For each of the analogues, the final distribution was dominated by one or more of the two-disulfide species. The relative equilibrium concentrations of the different forms are listed in Table 2. Of the three analogues, 8,20Ala displayed the strongest tendency to form the native disulfides, with [1−16,15−25] representing approximately 47% of the total two-disulfide forms. For the other two analogues, 1,16Ala and 15,25Ala, the forms with native disulfides were only 15% or 8%, respectively, of the total two-disulfide population. As indicated in Table 2, and discussed further below, the most favored forms were generally those in which one or both of the disulfides were between closely spaced Cys residues.

To determine whether any of the two-disulfide forms were stabilized significantly by noncovalent interactions, folding experiments were also carried out in the presence of 8 M urea. The resulting equilibrium distributions are illustrated in Figure 5c, and the relative yields of the two-disulfide forms are listed in Table 2. For each analogue, the yield of the form with native disulfides was decreased measurably, but the distributions were qualitatively very similar in the presence and absence of denaturant, with the forms containing small disulfide loops dominating.

Table 2: Equilibrium Populations of Two-Disulfide Forms of Four-Cys Analogues

analogue	two-disulfide isomer[a]	fraction of two-disulfide species −urea[b]	fraction of two-disulfide species +urea[c]	loop sizes[d]
1,16Ala	**[8−20,15−25]**	0.15	0.05	11, 9
1,16Ala	[8−15,20−25]	0.42	0.50	6, 4
1,16Ala	[8−25,15−20]	0.43	0.45	16, 4
8,20Ala	**[1−16,15−25]**	0.47	0.37	14, 9
8,20Ala	[1−5,16−25]	0.45	0.44	13, 8
8,20Ala	[1−25,15−16]	0.08	0.19	23, 0
15,25Ala	**[1−16,8−20]**	0.08	0.03	14, 11
15,25Ala	[1−8,16−20]	0.8	0.93	6, 3
15,25Ala	[1−20,8−16]	0.12	0.04	18, 7

[a] The forms with native disulfides are indicated by boldface type. [b] The relative concentrations of the three two-disulfide forms of each analogue were determined after equilibration with 1 mM GSSG and 2 mM GSH for 90 min at pH 7.3, 25 °C. [c] The relative concentrations of the three two-disulfide forms of each analogue were determined after equilibration with GSSG and GSH under conditions as above, but with 8 M urea also present. [d] The loop sizes are defined as the number of intervening residues between the disulfide-bonded Cys residues.

Table 3: Equilibrium Constants for Forming Two Native Disulfides

two-disulfide form	[reduced][a] (% total)	[II][a] (% total)	K_{II}[b] (M2)
$[8-20,15-25]_{1,16Ala}$	7.6 ± 1.1	13.2 ± 2.1	5.7×10^{-3}
$[1-16,15-25]_{8,20Ala}$	4.9 ± 0.1	11.1 ± 1.3	4.4×10^{-3}
$[1-16,8-20]_{15,25Ala}$	3.3 ± 0.8	8.9 ± 0.1	3.7×10^{-3}

[a] The relative concentrations of the fully reduced forms of the four-Cys analogues and the forms with two native disulfides were determined by reversed-phase HPLC after equilibration in the presence of 1 mM GSSG and 10 mM GSH at pH 7.3, 25 °C. For each analogue, two independent measurements were made, one in which the reaction was initiated with the fully reduced polypeptide and other starting with the form with two native disulfides. For each reaction, the concentrations of reduced and two-disulfide forms were determined at three times after the reactions appeared to have reached equilibrium, ranging from 30 to 150 min. The values reported are the means and standard errors for the six measurements. [b] K_{II} is the equilibrium constant for forming the two-disulfide forms from the fully reduced form, as defined by eq 1 in the text.

These results indicate that the forms with two native disulfides are not significantly stabilized by noncovalent interactions and that all three disulfides must be able to form in order for the native pairings to be favored.

Equilibration under Reducing Conditions. To measure stabilities of the two-disulfide forms relative to the fully reduced peptides, the analogues were allowed to equilibrate with 1 mM GSSG and 10 mM GSH. The reactions were initiated with either the fully reduced peptide or the form with two native disulfides, and the resulting distributions were analyzed by HPLC. The results of these measurements are summarized in Table 3.

From the relative concentrations of native and reduced peptide present at equilibrium, the overall equilibrium constants (K_{II}) for forming the three sets of two native disulfides were calculated according to

$$K_{II} = \frac{[II_N][GSH]^4}{[R][GSSG]^2} \quad (1)$$

where II_N represents the form with two native disulfide bonds and R represents the fully reduced form. The calculated equilibrium constants were similar for the three analogues: 0.0057 M^2 (1,16Ala), 0.0044 M^2 (8,20Ala), and 0.0037 M^2 (15,25Ala).

Equilibrium constants for forming single disulfides in disordered polypeptides are typically in the range of 0.001−0.1 M (*43*−*45*). Thus, the equilibrium constants measured here lie within the range expected for forming two such disulfides independently, further suggesting that there is relatively little stabilization of the two-disulfide forms.

One potential concern in studies of this type is the possibility that conformations that are favored in the authentic disulfide-bonded intermediates may be destabilized by the Cys to Ala replacements. Because folding and disulfide formation are thermodynamically linked, any destabilization from the substitutions would also tend to decrease the equilibrium constant for forming the two-disulfide forms from the fully reduced state. To test for such destabilization, it is useful to compare the equilibrium constants for forming the two-disulfide forms in the four-Cys peptides with those for the analogous reactions in the peptide with all six Cys residues.

Incubating ω-MVIIA-Gly in the presence of 1 mM GSSG and 20 mM GSH results in an equilibrium distribution in which 6% of the molecules are fully reduced and 17% contain the three native disulfides (*26*). The remaining molecules have HPLC retention times intermediate between those of the native and fully reduced forms. If approximately half of these molecules contain 2 disulfides, the overall equilibrium constant for forming the total population of 2-disulfide intermediates, of which there are 45 possible, is approximately 1 M^2. For comparison, the total equilibrium constant calculated for forming the nine two-disulfide species that can be formed by the three four-Cys analogues is approximately 0.1 M^2. If, on average, these 9 species are as stable as the other 36 possible 2-disulfide forms, the expected overall equilibrium constant for forming all 45 species would be about 0.5 M^2, i.e., about half that estimated for the peptide with all 6 Cys residues.

Although these calculations do not provide an exact quantitative comparison, they suggest that the Cys to Ala replacements probably do not greatly destabilize the two-disulfide species that can be formed by the four-Cys analogues. It is thus likely that the species with two native disulfides make up only a small fraction of the two-disulfide intermediates that accumulate during the folding of the peptide with all six Cys residues. In addition, these species probably have relatively little of the folded conformation found in the native protein with all three disulfides.

DISCUSSION

Contributions of the Individual Disulfides to the Folded Conformation. The results presented here demonstrate that each of the three disulfides in ω-MVIIA-Gly is necessary to maintain the three-dimensional structure characteristic of the ω-conotoxins. Removing any of the three disulfides led to pronounced changes in the far-UV spectrum, indicative of decreased β-strand content. In addition, removal of any of the disulfides reduced the affinity of the peptide for Ca^{2+} channels, by factors ranging from 68- to 5200-fold. The analogues with two native disulfides were also more susceptible to reduction by DTT than is the native form, suggesting that the remaining disulfides are more fully exposed to solvent.

These observations are in striking contrast with the results of similar studies with larger proteins containing multiple disulfides, such as BPTI, RNase A, and RNase T1. In the case of BPTI, the folded conformation of the native protein remains almost fully intact if any one of the three disulfides is removed by genetic or chemical modification (*2*−*4*). Similarly, two different forms of RNase A, each lacking one of the four disulfides, have been shown to have very nativelike conformations (*6*, *7*). The two disulfides of native RNase T1 can be removed individually without causing the protein to unfold, and even forms lacking both disulfides are folded under favorable conditions (*5*).

The much greater effect of removing the individual disulfides of ω-MVIIA-Gly is most likely a consequence of the small size of this molecule and its marginal stability. Amide−hydrogen exchange experiments indicate that the most highly protected hydrogen atoms in the native conformation have exchange rates that are approximately 1000-fold lower than expected for fully exposed amide groups (D. P. Goldenberg et al., in preparation). If the exchange of these hydrogen atoms reflects complete unfolding, the free-energy change for unfolding can be estimated to be about 4 kcal/mol. The contributions of individual disulfides in other proteins are often greater than 5 kcal/mol (*46*, *47*), so that it is quite reasonable that removing any one disulfide in ω-MVIIA-Gly would make folding thermodynamically unfavorable.

Although all three of the disulfides appear to be essential for the stability of ω-MVIIA-Gly, one of the disulfides has been found to be at least partially dispensable for the stability of another protein with a similar structure. The small trypsin inhibitors isolated from the seeds of squash plants have three-dimensional structures and disulfide patterns very similar to those of the ω-conotoxins (*48*, *49*). For one of the trypsin inhibitors isolated from *Ecballium elaterium*, EETI II, the disulfide between Cys 2 and Cys 19 (corresponding to 1−16 in ω-MVIIA) is much more readily reduced than are the two more buried disulfides (*50*). The resulting two-disulfide form is very stable to further reduction, and NMR studies indicate that it contains a large fraction of the native conformation. It thus appears that the stability of EETI II is significantly less dependent on at least one of its three disulfides than is ω-MVIIA-Gly.

Relative Stabilities of Two-Disulfide Forms Containing Native and Non-Native Disulfides. By allowing the fully reduced four-Cys analogues to refold in the presence of both GSSG and GSH, the tendencies to form the various possible disulfide bonds were compared. As shown in Figure 5b, the forms with two native disulfides each accumulated to detectable levels, but these species were not favored with respect to the forms with non-native disulfides. The addition of urea to the reactions caused only small decreases in the relative stabilities of the native forms (Figure 5c), suggesting that they are not greatly stabilized by noncovalent interactions.

In the absence of stabilizing interactions, the tendencies of alternative disulfide bonds to form are expected to be influenced primarily by the lengths of the polypeptide

segments separating the various Cys pairs. From statistical arguments, forming a disulfide between closely spaced Cys residues is predicted to cause a smaller decrease in conformational entropy than is forming a larger loop (*51, 52*), and this trend has been observed experimentally (*44, 45, 53*). As summarized in Table 2, the most favored 2-disulfide forms were generally those containing at least one disulfide between residues separated by fewer than 10 residues. Species containing loops with fewer than five intervening residues were particularly favored.

Even the species with a disulfide between adjacent Cys residues, $[1-25,15-16]_{8,10Ala}$, was found to accumulate to significant levels. Although disulfides between adjacent Cys residues are often thought to be strongly disfavored, experiments by Zhang and Snyder (*44*) have shown that the equilibrium constant for forming a disulfide of this type is not greatly different from that for forming other small loops. Although the disulfide requires that the intervening peptide bond have a cis conformation, this conformation apparently positions the two sulfur atoms in an orientation very favorable for disulfide formation.

The disulfide-bonded loops found in the native ω-conotoxins are among the longest that can be formed given the spacings between Cys residues. As a consequence, forming the native disulfides is generally disfavored entropically. On the other hand, once a disulfide is formed, its contribution to the stability of the folded conformation is expected to be inversely related to its tendency to form in the unfolded state, since the contribution of a disulfide, or any other interaction, depends on the extent to which its formation is more favorable in the folded state than in the unfolded (*46, 54, 55*). It is possible that the sequences and structures of the ω-conotoxins have evolved so as to maximize the loop sizes, thereby gaining the maximum entropic destabilization of the unfolded state. Harrison and Sternberg have noted that this general pattern is frequently observed in disulfide-bonded proteins shorter than about 75 residues (*56*). Because of the relatively small number of noncovalent interactions in the ω-conotoxins, the initial formation of the native disulfides is unfavorable. Once two native disulfides are present, however, forming the third is highly favored, as discussed below.

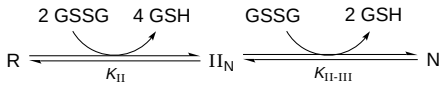
Effective Concentrations of Thiols in Intermediates Containing Two Native Disulfides. The tendency of two chemical groups to form an intramolecular interaction can be usefully expressed as an "effective concentration" (C_{eff}), which is defined as the equilibrium (or rate) constant for forming the intramolecular interaction (K_{intra}) divided by the corresponding constant for a chemically equivalent intermolecular reaction (K_{inter}). In the case of disulfide formation, the equilibrium effective concentration is simply the equilibrium constant for forming the intramolecular disulfide via exchange with an intermolecular disulfide reagent, such as GSSG:

$$C_{eff} = K_{GSSG} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} \quad (2)$$

where P_S^S and P_{SH}^{SH} represent the forms of the protein with and without the disulfide, respectively (*54*). This equilibrium constant has units of concentration and represents the relative stabilities of the intra- and intermolecular disulfides.

The effective concentrations of thiol pairs in proteins have been found to cover a range of several orders of magnitude. In unfolded polypeptides, observed values range from 0.001 to 0.1 M, depending on the number and type of intervening residues (*43−45, 53*). In folded proteins, effective concentrations of thiols are often greater than 10^2 M and as large as 10^5 M (*54, 57*). The stability of a disulfide in a folded protein can be very sensitive to perturbations of the surrounding environment, as demonstrated by the ability of single amino acid replacements to dramatically decrease the effective concentration for the Cys 14 and 38 thiols in native BPTI, from 150 M to as low as 30 mM (*58*).

The measurements of the equilibria between the fully reduced four-Cys analogues and the forms with two native disulfides can be used to estimate the effective concentrations of the two free thiols in the ω-MVIIA-Gly folding intermediates with two native disulfides. For this purpose, it is useful to envision the process of forming the three native disulfide bonds as occurring in two steps:



(3)

The first step represents the formation of an intermediate with two native disulfides (II_N), with equilibrium constant K_{II}, followed by the formation of the third disulfide, with equilibrium constant K_{II-III}. Although not all of the possible paths may actually contribute to the kinetically most important folding mechanism, three separate paths of this type exist in principle. Assuming that the Cys to Ala replacements have minimal effects on the structure and stability of the two-disulfide forms, the overall equilibrium constant for forming the first two native disulfides (K_{II}) should be approximately equal to that for forming the two disulfides in the analogues. The equilibrium constant for forming the third disulfide, which corresponds to the effective concentration of the thiols in the two-disulfide intermediate, can then be calculated as

$$K_{II-III} = C_{eff} = \frac{K_{III}}{K_{II}} \quad (4)$$

where K_{III} is the overall equilibrium constant for forming the three native disulfides. This last equilibrium constant has previously been determined to be 0.18 M^3 for ω-MVIIA-Gly (*26*).

From the values of K_{II} reported in Table 3, the effective concentrations calculated in this way are 27 M for the 1−16 disulfide, 41 M for 8−20, and 51 M for 15−25. All of these values are much greater than typically observed in disordered polypeptides, demonstrating that formation of each of the three native disulfides is greatly favored by the presence of the other two.

As discussed in the previous sections, the three analogues with two native disulfides all appear to be largely devoid of the native conformation. It is thus somewhat surprising that the effective concentrations calculated for the thiols in the corresponding two-disulfide intermediates are so high. One possible explanation is that the two disulfides present in the intermediates constrain the polypeptide in a compact distribution of conformations with a topology favorable for

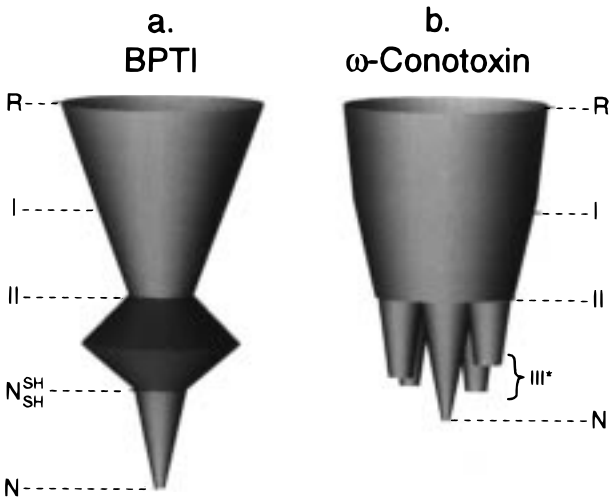


FIGURE 6: Conformational funnels representing the disulfide-coupled folding mechanisms of (a) BPTI at pH 7.3 and (b) ω-MVIIA-Gly. The funnels are drawn so that the distance along the vertical axis represents the progress of the folding reaction, defined by the disulfide formation steps, and the cross-sectional areas represent the conformational entropy of the polypeptide chain. As described in (*62*), the relative conformational entropies of the BPTI intermediate populations were estimated, very approximately, from the characterization of these species by solution NMR. In the case of ω-MVIIA-Gly, even the forms with two native disulfides appear to be largely devoid of folded structure, and only modest decreases in conformational entropy, due to the formation of the cross-links, are indicated for the formation of intermediates with one or two disulfides. In addition to the native form, three-disulfide species with at least two non-native disulfides (indicated as III*) also form early in folding and later are converted to the native form (*19, 26*). The paths leading from the population of two-disulfide intermediates to the various three-disulfide forms are shown as isolated sub-funnels, since each of these species must be at least partially reduced before being converted to another three-disulfide form.

forming the third disulfide, without any of the specific interactions found in the native protein. Alternatively, a large fraction of the molecules may contain some specific substructures that favor the third disulfide but are not detectable by the low resolution methods used here. A third possibility is that the conformational distributions for the two-disulfide intermediates include very low concentrations of molecules with nativelike conformations that greatly favor forming the third disulfide. For instance, if only 1% of the molecules have a nativelike conformation, but the effective concentration of the thiols in this conformational substate was 5000 M, the overall effective concentration calculated for the intermediate would be 50 M. To help distinguish among these possibilities, high-resolution NMR studies of the two-disulfide analogues are currently in progress.

Implications for the Folding Mechanisms of ω-Conotoxins. Previous studies have shown that the folding of the ω-conotoxins is characterized by the accumulation of a much broader distribution of disulfide-bonded intermediates than is seen, for instance, in the refolding of BPTI (*19, 26*). In addition, non-native three-disulfide species accumulate in preference to the native form at early times in folding, but can be converted to the native form provided a thiol reagent is present to promote reduction of at least one disulfide. These differences in folding mechanism can be illustrated using "conformational funnels", as drawn in Figure 6. In this representation, which has recently been adopted to describe the results of both computational and experimental studies of protein folding (*59*−*62*), the conformational entropy of the polypeptide is indicated by the cross-sectional area of a funnel. The top of the funnel represents the unfolded state, and the narrow bottom represents the native conformation with its greatly reduced entropy. The distance along the axis of the funnel represents the progress of the folding reaction. In the funnels shown for the folding of BPTI and an ω-conotoxin, the vertical axis represents the disulfide formation or rearrangement steps, which are linked, to varying degrees, with the formation of other stabilizing interactions.

As described previously, the funnel for BPTI was drawn using information from NMR studies of the intermediates to estimate, very approximately, the conformational entropies of the intermediate populations with one or two disulfide bonds (*62*). For this protein, particularly at neutral pH, the formation of the first disulfide is associated with a quite rapid narrowing of the conformational distribution. Before the third disulfide is incorporated, however, the two-disulfide intermediates that are formed most readily must undergo intramolecular rearrangements to generate the immediate precursor of the native protein, designated N^{SH}_{SH}. This rearrangement is the slowest intramolecular process in the BPTI folding pathway and is associated with extensive disruption of interactions already present in the other two-disulfide intermediates, as indicated by the bulge in the funnel.

In contrast, there appears to be relatively little specificity in the formation of the initial disulfides during the folding of the ω-conotoxins, and the funnel in Figure 6b is drawn to represent only a small initial decrease in conformational entropy. In addition, at least some of the non-native two-disulfide intermediates are able to form non-native three-disulfide species, collectively labeled "III*". The reactions leading to the various three-disulfide forms, which cannot interconvert directly, are drawn as separate sub-funnels emerging from a manifold representing the numerous possible two-disulfide intermediates. We refer to this type of funnel as a "folding udder", despite the nonbovine origin of the protein studied here.

The results with the four-Cys analogues provide additional insights into the nature of the ω-conotoxin folding mechanism. As discussed above, the intermediates with two-native disulfides are not significantly more stable than the other six two-disulfide forms that can collectively be formed by the analogues. In addition, 36 other two-disulfide forms can, in principle, be formed by the protein with all 6 Cys residues. As a consequence, the intermediates with native disulfides are likely to represent only a few percent of the total population of two-disulfide intermediates. The tendency for non-native three-disulfide species to form in preference to the native form may, then, simply reflect the relative concentrations of the different precursors. In terms of the conformational funnel shown in Figure 6b, the potential precursors of the native protein correspond to only a small fraction of the cross-section representing all of the two-disulfide forms, and there is a high probability that the polypeptide will follow one of the sub-funnels leading to a non-native three-disulfide form. Once formed, these species must be partially reduced to enter the sub-funnel leading to the native state. However, because the native form is more

stable than the other three-disulfide species, it dominates the final distribution. Similar disulfide-coupled folding pathways have been described for other small proteins, including hirudin (*63*), potato carboxypeptidase inhibitor (*64*), and human epidermal growth factor (*65*).

Although the ω-MVIIA-Gly forms with two native disulfides show very little evidence of nonrandom structure, the presence of these disulfides is expected to restrict the polypeptide to compact conformations and strongly bias the chain toward the topology of the native structure. These species may thus represent a stage of folding in which the chain has collapsed but has not yet formed a large fraction of the nativelike interactions and structure. Further studies of the analogues described here may provide new insights into relatively early events in protein folding.

ACKNOWLEDGMENT

We thank Drs. W. R. Gray and B. M. Olivera for numerous suggestions during the course of this work, Dr. D. F. Blair for helpful comments on the manuscript, Dr. Robert Schackmann for synthesizing the peptides used in this study, Dr. Pamela Crain for mass spectrometric analyses, and Lita Imperial for assistance with the binding assays.

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BI9803978