Solution structure and backbone dynamics of an ω -conotoxin precursor

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(RECEIVED July 27, 2000; FINAL REVISION November 29, 2000; ACCEPTED December 7, 2000)

Abstract

Nuclear magnetic resonance spectroscopy was used to characterize the solution structure and backbone dynamics of a putative precursor form of ω -conotoxin MVIIA, a 25-amino-acid residue peptide antagonist of voltage-gated Ca^{2+} channels. The mature peptide is found in the venom of a fish-hunting marine snail *Conus magus* and contains an amidated carboxyl terminus that is generated by oxidative cleavage of a Gly residue. The form examined in this study is identical to the mature peptide except for the presence of the unmodified carboxy-terminal Gly. This form, referred to as ω -MVIIA-Gly, has previously been shown to refold and form its disulfides more efficiently than the mature form, suggesting that the presence of the terminal Gly may favor folding in vivo. The nuclear magnetic resonance (NMR) structure determination indicated that the fold of ω -MVIIA-Gly is very similar to that previously determined for the mature form, but revealed that the terminal Gly residue participates in a network of hydrogen bonds involving both backbone and side chain atoms, very likely accounting for the enhanced stability and folding efficiency. ^{15}N relaxation experiments indicated that the backbone is well ordered on the nanosecond time scale but that residues 9–15 undergo a conformational exchange processes with a time constant of ~ 35 microseconds. Other studies have implicated this segment in the binding of the peptide to its physiological target, and the observed motions may play a role in allowing the peptide to enter the binding site

Keywords: Conotoxin; SNX-111; Ziconotide; peptide amidation; disulfide bonds

The ω -conotoxins are a family of small disulfide-bonded proteins that bind to and inhibit presynaptic voltage-gated Ca^{2+} channels, thereby blocking synaptic transmission (Olivera et al. 1994). They are found in the venoms of marine

snails of the genus *Conus*, along with numerous other peptides, generally referred to as conopeptides, that collectively display a remarkably wide range of activities (Olivera et al. 1990, 1991). Like most of the other conopeptides characterized so far, the ω -conotoxins fold into well-defined three-dimensional structures. The solution structures of several members of the ω -conotoxin family have been determined by high-resolution nuclear magnetic resonance (NMR) spectroscopy, and they have been found to share a common fold stabilized by three conserved disulfide bonds (Davis et al. 1993; Basus et al. 1995; Kohno et al. 1995; Nemoto et al. 1995; Nielsen et al. 1996). This structure is characterized by a β -sheet composed of three short strands, along with loop segments. Because the various ω -conotoxins display specificity for different Ca^{2+} channel subtypes, they have been widely adopted as reagents for studying the distributions and biological roles of these channels (Olivera et al. 1987, 1994). In addition, they are being actively studied as poten-

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Abbreviations: ω -MVIIA-Gly, a form of ω -conotoxin MVIIA composed of the mature sequence plus a carboxy-terminal Gly residue with an unmodified carboxyl group; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single-quantum correlation; HNHA, amide-proton to α -proton correlation; HNHB, amide-proton to β -proton correlation; RMS, root-mean-square; HPLC, high performance liquid chromatography; GSSG and GSH, the disulfide and thiol forms, respectively, of glutathione; BNPS-skatole, 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine; TMSP, 3-(trimethylsilyl) propionate.

Article and publication are at www.proteinscience.org/cgi/doi/10.1110/ps.30701.

tial therapeutic agents, and the subject of the present study, ω -conotoxin MVIIA, has proven especially promising for the treatment of chronic pain (Miljanich and Ramachandran 1995; Bowersox and Luther 1998). (Synthetic ω -MVIIA is also referred to as SNX-111 and by the drug name Ziconotide.)

All of the ω -conotoxins that have been characterized at present possess an amidated carboxyl terminus. Analysis of cDNA clones encoding these molecules shows that they are synthesized with an additional carboxy-terminal Gly residue, indicating that the amide group is generated by oxidative cleavage (Woodward et al. 1990; Colledge et al. 1992). This post-translational modification is found widely in animal peptides, especially peptide hormones and neuropeptides, and has been shown to play an important role in biological activity or stability (Eipper et al. 1993; Merkle 1994). Although the enzymes responsible for the modification in *Conus* snails have not yet been identified, they are likely to be related to the well-characterized mammalian enzymes, which have been shown to modify the carboxy-terminal Gly residue in a two-step process shown below:

Recent studies suggest that the carboxy-terminal Gly residues in the precursors of ω -conotoxins may play a significant role in the folding of these molecules. When the mature amidated forms of these peptides were unfolded by reducing their disulfides and then allowed to reoxidize by thiol-disulfide exchange, the native forms were regenerated with yields in the range of 25–60%, depending on the particular sequence, versus ~5–10% expected if the three disulfides were to form randomly (Price-Carter et al. 1996a). For one member of the family, ω -MVIIA, the folding of the mature form was compared with that of a form containing the carboxy-terminal Gly, designated ω -MVIIA-Gly (Price-Carter et al. 1996b). Although the mature form refolds with an efficiency of ~50%, the yield is >80% for the Gly terminal form. The carboxy-terminal Gly residue appears to favor folding by stabilizing the native conformation with respect to both the fully reduced protein and other forms containing non-native disulfides. The presence of this residue also

slightly decreases the affinity of ω -MVIIA for Ca^{2+} channels, resulting in a dissociation constant of ~3 nM, versus 0.3 nM for the mature peptide.

Although the effect of the Gly residue on stability is estimated to be only ~1 kcal/mole, this small effect could be of considerable biological significance as the native conformation of the mature peptide is only marginally more stable than the forms with non-native disulfides. If the amidating enzymes are located in secretory granules, as is the case for mammalian cells (Oyarce and Eipper 1995), the Gly residue would be present when the conotoxins fold and form their disulfides in the endoplasmic reticulum.

Although the conversion of carboxy-terminal Gly residues to amide groups has been shown to influence the function and stability of peptides from many organisms, very little is known about the structural bases for these effects. To learn how the terminal residue contributes to the stability of an ω -conotoxin precursor, we have used NMR spectroscopy to determine the three-dimensional conformation of ω -MVIIA-Gly and have compared this structure to those determined in other laboratories for mature ω -MVIIA. The family of calculated ω -MVIIA-Gly structures was found to be very similar to that of the mature form. However, the precursor form is distinguished by the presence of the two carboxyl oxygen atoms of the terminal Gly residue, each of which was found to be well positioned to form hydrogen bonds with other atoms. These interactions may well be responsible for the enhanced stability of ω -MVIIA-Gly.

The backbone dynamics of the precursor form were studied using ^{15}N relaxation measurements. These experiments indicate that all of the backbone amide groups are well ordered with respect to motions on the nanosecond to picosecond time scale. However, a significant fraction of the chain, residues 9–15, appears to undergo slower motions, with a time constant of ~35 microseconds. This segment includes the residues that have been most strongly implicated in binding to Ca^{2+} channels, and it is possible that the motions indicated by these studies may play a role in this interaction, perhaps facilitating entry into the binding site.

Results and discussion

Sample preparation and resonance assignments

The NMR studies described here used both ^1H homonuclear and ^1H - ^{15}N heteronuclear methods. For the homonuclear experiments, a sample of chemically synthesized ω -MVIIA-Gly was used. The identity and correct folding of the synthetic peptide was confirmed by its molecular mass and its activity in a channel-binding assay. Peptide uniformly labeled with ^{15}N was prepared by expression of a synthetic gene in *Escherichia coli*. The resulting protein had the expected molecular mass and biological activity that was indistinguishable from that of the chemically synthesized ma-

terial. Except where noted, all of the NMR experiments were carried out at pH 6, to ensure that the terminal carboxyl group, as well as that of Asp-14, would be in the ionized state.

Complete assignments of the backbone ^1H resonances, as well as those of most side chain protons, were made using homonuclear nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) and the standard sequential assignment procedure (Wüthrich 1986). Resonance assignments for the amide ^{15}N nuclei were made from a two-dimensional ^1H - ^{15}N heteronuclear single-quantum correlation (HSQC) spectrum and the amide proton assignments.

The chemical shifts of the protons of ω -MVIIA-Gly were generally very similar to those of the mature peptide (Basus et al. 1995). The major exceptions were the amide protons of Ser-9 and Ser-19, which had chemical shifts that were larger than those of the mature form, by 0.46 and 0.33 ppm, respectively. In addition, the chemical shift of the amide proton of Gly-26 was ~ 0.8 ppm greater than that of the terminal amide of mature ω -MVIIA, reflecting the different chemical structures of the two amide groups. The close correspondence between the chemical shifts observed for the two forms of the peptide indicate that their overall conformations are very similar and that the few differences in resonance frequency are likely to reflect local structural differences.

Amide hydrogen exchange kinetics

To facilitate identification of hydrogen bonds in ω -MVIIA-Gly, as well as provide information about the energetics of the structure, the kinetics of amide hydrogen exchange were measured. The ^{15}N -labeled peptide dissolved in H_2O was transferred to D_2O by gel filtration, and the kinetics of exchange were monitored in a series of HSQC spectra. This experiment was carried out at pH 4 to decrease the exchange rates, after determining that there were only very small chemical shift differences between pH 6 and pH 4.

Of the 25 amide protons, 13 exchanged slowly enough to remain detectable after 1 h. For these protons, the intensities of the HSQC cross-peaks were quantified, and first-order rate constants for exchange were estimated by fitting the data to a single exponential. The measured rates are plotted in Figure 1 as "protection factors," which were calculated by dividing the observed rate constants by those predicted for the same residues in a fully exposed environment (Bai et al. 1993). For six amide groups, those of Lys-2, Ala-6, Cys-8, Cys-16, Lys-24, and Cys-25, the calculated protection factors were >100 , and these amides were considered the most likely candidates for forming hydrogen bonds, as discussed in the following section.

The calculated protection factors also provide tentative information about the conformational stability of ω -MVIIA-

Fig. 1. Hydrogen exchange protection factors for the amide protons of ω -MVIIA-Gly at 10°C and pH 4.0. First-order rate constants for amide hydrogen exchange were determined from ^1H - ^{15}N HSQC spectra collected at various times after transfer of the protein from H_2O to D_2O . Protection factors were calculated by dividing the observed rate constants by those calculated for a fully exposed amide group. The sequence of ω -MVIIA-Gly and its disulfide connectivity are shown above the figure.

Gly. If the exchange of the most highly protected amide hydrogens is assumed to take place from the fully unfolded state, then the protection factor can be interpreted as the equilibrium constant for folding, and the free energy change for unfolding is estimated to be ~ 4.5 kcal/mole at 10°C . Although this value should probably be considered a minimum estimate of stability, as exchange may take place by way of partially unfolded forms, it is consistent with other evidence demonstrating that ω -MVIIA-Gly is only marginally more stable than either the fully reduced form or forms with non-native disulfides (Price-Carter et al. 1996b).

Solution structure of ω -MVIIA-Gly

A total of 249 nonredundant H-H distance constraints, 45% of which were between atoms separated by two or more residues in the sequence, were derived from a two-dimensional homonuclear NOESY spectrum (Table 1). In addition, 20 dihedral angle constraints were derived from coupling constants measured in amide-proton to α -proton correlation (HNHA) and amide-proton to β -proton correlation (HNHB) experiments, which also facilitated stereospecific assignments for the β -protons of eight residues. The three disulfides in ω -MVIIA-Gly have been identified by chemical experiments (Chung et al. 1995; Price-Carter et al. 1996b), and this information was also used in the structure calculations. From these constraints, an initial family of structures was calculated by simulated annealing and energy minimization using the program DYANA (Güntert et al. 1997).

Table 1. Analysis of ω -MVIIA-Gly structure calculation

A. Constraint types	Number	
Total inter-proton NOEs	249	
Intra-residue NOEs	51	
Sequential NOEs	86	
Medium and long range NOEs	112	
Hydrogen bonds	16 (2 per hydrogen bond)	
Disulfide bonds	9 (3 per disulfide bond)	
ϕ -dihedral constraints	10	
χ_1 -dihedral constraints	9	
Total restraints per residue	11.3 (10.6 excluding hydrogen bonds)	
B. Residual constraint violations	Mean $\pm$ sd	Range
Total DYANA target function ($\text{\AA}^2$)	0.36 ± 0.02	0.33–0.42
Max. upper distance violation ($\text{\AA}$)	0.18 ± 0.01	0.17–0.20
Max. lower distance violation ($\text{\AA}$)	0.12 ± 0.01	0.10–0.15
Max. van der Waals violation ($\text{\AA}$)	0.07 ± 0.01	0.06–0.08
Max. dihedral angle violation (degree)	1.0 ± 0.1	0.8–1.1
C. Average pairwise RMS deviations of atomic coordinates ($\text{\AA}$)		
N, C $^\alpha$, CO	0.44 ± 0.15	
All non-hydrogen atoms	1.38 ± 0.27	

The initial structures were used to identify potential hydrogen bonding partners for the six amide groups that displayed hydrogen exchange protection factors >100 . Two other amide hydrogens, those of Gly-5 and Gly-26, both showed modest protection factors (>10) and were frequently found to be well positioned to form hydrogen bonds in the calculated structures.

The eight hydrogen bonds were then used to derive distance constraints. These constraints were all compatible with the experimental constraints and were incorporated in the final set of structure calculations. A total of 50 structures were calculated, and the 20 with the lowest DYANA target function values were used for subsequent analysis. Among these 20 structures, which are illustrated in Figure 2A, the largest violation of the experimental distance constraints was $0.2 \text{ \AA}$. The mean pairwise root-mean-square (RMS) deviation among backbone atoms was $0.44 \text{ \AA}$, and that for all of the non-hydrogen atoms was $1.38 \text{ \AA}$ (Table 1).

The overall fold observed in the calculated structures (Fig. 2B) is very similar to that described previously for mature ω -MVIIA (Fig. 2C), as well as for other members of the ω -conotoxin family. Along with the β -sheet made up of three short strands, one of the most distinctive features of the structure is the exposure of almost all of the side chains on the protein surface. The important exceptions to this pattern are the disulfide-bonded Cys residues, which are largely buried. The similarities and differences among the various ω -MVIIA structures are discussed further in a later section.

The conformations and positions of the 1–16 and 15–25 disulfides were well defined in the structures, whereas the disulfide between Cys-8 and Cys-20 displayed considerable heterogeneity. More generally, the positions of residues 8 through 13 were much less well defined than the rest of the

structure. For these residues, the average RMS deviation of backbone atoms was $0.57 \text{ \AA}$, versus an average of $0.18 \text{ \AA}$ for residues 1–7 and 14–25. Residues 21 and 22 also had larger than average RMS deviations among the calculated structures. Thus, the segments that appear least well defined are those that are linked by the 8–20 disulfide, as has also been observed in structures determined for mature ω -MVIIA (Basus et al. 1995; Kohno et al. 1995; Atkinson et al. 2000).

One of the motivations for determining the structure of ω -MVIIA-Gly was to identify possible stabilizing interactions that might account for the enhanced stability and folding efficiency of this molecule, as compared to the mature amidated form. Although Gly-26 displayed significant conformational heterogeneity among the calculated structures, this residue was in a very similar position in 15 of the 20 structures with the lowest target functions (Fig. 3A). In many of these structures, one or both of the carboxyl oxygen atoms of Gly-26 were well positioned to form a hydrogen bond, with the backbone amide group of Ser-19 or the side chain hydroxyl of Thr-17. These hydrogen bonds were not used as constraints in the structure calculation. To confirm that these interactions were compatible with the experimental constraints, an additional family of structures was calculated in which these constraints were included. The presence of these additional constraints did not lead to violation of any of the experimental constraints, and had no significant effect on the average target function.

The proposed Gly-26 hydrogen bonds are illustrated in Figure 3B, which was drawn from one of the original structures that was compatible with both interactions. As indicated in Figure 3B, the carboxyl group is positioned to bridge the hydroxyl group of Thr-17 and the amide group of Ser-19. Although the Ser-19 amide hydrogen did not display protection from exchange, this is most likely due to the

Fig. 2. Solution structure of ω -MVIIA-Gly. (A) Wire-frame representation of the 20 structures with lowest DYANA target function values. Backbone atoms are drawn in dark blue and side chain atoms in light blue, except for the side chains of the disulfide-bonded Cys residues, which are orange. (B) Ribbon diagram of the calculated structure with the smallest RMS deviation of backbone atom positions from those of a mean structure calculated from the 20 structures shown in A. The sulfur atoms of the disulfide bonded Cys residues are drawn as yellow spheres and are labeled with the Cys residue numbers. (C) Comparison of the backbone conformation of the ω -MVIIA-Gly structure calculated here (thick blue coil) with three independent structures of ω -MVIIA. For each family of solution structures, the backbone of the structure with the smallest RMS deviation from the mean is shown as a smooth coil drawn through the α -carbon positions. The three structures of ω -MVIIA were drawn using the coordinates in protein database files 1DW4 (green; Atkinson et al. 2000), 1MVI (magenta; Nielsen et al. 1996), and 1OMG (red; Kohno et al. 1995). All of the molecular structure representations in this figure and Fig. 3 were drawn with the program MOLMOL (Koradi et al. 1996).

proximity of this atom to the protein surface, and more direct evidence for an interaction with the terminal carboxyl group is provided by the pH titration experiment described in the following section. Figure 3B also illustrates a hydrogen bond between the amide hydrogen of Gly-26 and the

carbonyl oxygen of Ser-19, an interaction that was indicated by hydrogen exchange data and the initial structure calculations. Because individual hydrogen bonds often contribute 1–3 kcal/mole to the stabilities of folded proteins (Byrne et al. 1995; Myers and Pace 1996; Takano et al. 1999), this

Fig. 3. Proposed hydrogen bond interactions between Gly-26 and surrounding residues. (A) Wire-frame representation of the 20 calculated ω -MVIIA-Gly structures with lowest target function values. (B) Ball-and-stick representation of one structure in which the carboxyl group of Gly-26 is well positioned to form hydrogen bonds with the amide hydrogen of Ser-19 and the side chain hydroxyl of Thr-17. These hydrogen bonds were not used as constraints in the structure calculation.

network of bonds could readily account for the observed stability difference between ω -MVIIA-Gly and the mature peptide.

pH titration

To obtain further information about the interactions of the terminal carboxyl group with nearby amides, chemical shift changes were monitored as the pH was adjusted from 5.9 to 2.3. For 6 of the 25 backbone amide protons, changes of 1.5 ppm or greater were observed and are plotted in Figure 4. Consistent with the hydrogen bond proposed from the calculated structure, the chemical shift of the amide proton of Ser-19 decreased substantially, by 0.24 ppm, as the pH was lowered.

The largest chemical shift change, however, was that for Ser-9, which decreased by 0.72 ppm. This effect was most likely due to an interaction between the amide hydrogen and the side chain carboxyl group of Asp-14, which were located 3.2–4.8 Å apart in the calculated structures. When constraints corresponding to a hydrogen bond between these

groups were introduced in a subsequent structure calculation, they caused only small structural changes, primarily affecting the position of the Asp-14 side chain, and only slightly increased in the average target function (from 0.36 to 0.41 Å²). Thus, all of the data appear to be compatible with an interaction between the amide of Ser-9 and the side chain of Asp-14. Decreases in chemical shift were also observed for the amide protons of Lys-2 and Gly-5, although these groups do not appear to interact directly with either carboxyl group. Two other residues, Tyr-13 and Cys-25, displayed chemical shift *increases* as the pH was lowered. The changes in the chemical shifts of these four amide protons most likely reflect indirect effects of protonating the carboxyl groups.

For each of the amide protons displaying a significant chemical shift change, the observed changes were well fit by a simple model assuming independent single protonation processes, with apparent pK_a in the range from 2.43 to 2.68 (Fig. 4). These values are significantly lower than those reported for carboxyl groups in unstructured polypeptides (with pK_a of ~3.5–4.5), but are comparable to those measured for carboxyls interacting with amide groups in other peptides or proteins (Bundi and Wüthrich 1979; Oliveberg et al. 1995; Pérez-Cañadillas et al. 1998).

The pK_a differences indicate that the conformation predominating at neutral pH favors the ionized states of the carboxyls and must, by thermodynamic linkage, be stabilized by the interactions of these groups with the surrounding amide groups. The observed pK_a shifts correspond to interaction energies in the range of 1.5–2.5 kcal/mole.

Fig. 4. pH titration of ω -MVIIA-Gly monitored by changes in amide proton chemical shifts. The chemical shifts were determined from ¹H-¹⁵N HSQC spectra recorded at 10°C after adjusting the sample pH to the indicated values. Data are shown only for those amide protons displaying a chemical shift difference of 1.5 ppm or more over the pH range from 2.3 to 6. The curves shown represent nonlinear least squares fits to the data for each proton resonance, assuming independent protonation equilibria. The apparent pK_a ranged from 2.43 to 2.68.

Given the marginal stability of ω -MVIIA-Gly relative to forms with non-native disulfides, these electrostatic interactions are likely to be particularly significant.

Backbone dynamics

As discussed earlier, the atomic positions of residues 8–13 were significantly less well defined by the distance and dihedral angle constraints than were those for the rest of ω -MVIIA-Gly. To determine whether the lack of consensus in the calculated structures was due to conformational flexibility, ^{15}N relaxation measurements were used to characterize the backbone dynamics of the folded peptide. Following an approach now used widely (Kay et al. 1989; Clore et al. 1990; Dayie et al. 1996; Mandel et al. 1996), three ^{15}N relaxation parameters were measured: The longitudinal relaxation rate (R_1), the rotating frame relaxation rate ($R_{1\rho}$, equivalent for the purposes of these studies to the transverse relaxation rate, R_2), and the ^1H – ^{15}N heteronuclear nuclear Overhauser effect (NOE).

Both R_1 and the heteronuclear NOE displayed remarkably little variation along the polypeptide sequence, suggesting that all of the backbone atoms undergo similar degrees of motion on the nanosecond to picosecond time scale (Fig. 5). In contrast, the measured $R_{1\rho}$ values varied over a wide range (Fig. 5A), with the amide groups of residues 9–15 displaying particularly large values. Slightly elevated $R_{1\rho}$ values were also observed for residues 21 and 22.

To interpret the relaxation data in terms of molecular motions, the experimental parameters were analyzed using the “model-free” formalism of Lipari and Szabo (1982a,b). In this treatment, the spectral density function describing the motions of the N–H bond vector is assumed to have a simple functional form with three parameters: a correlation time for molecular tumbling (τ_m), a correlation time for internal motions faster than tumbling (τ_e), and an order parameter describing the degree of spatial restraint for the faster internal motions (S^2). Exchange processes contributing to transverse relaxation are not treated explicitly in this analysis, but give rise to a residual term, R_{ex} , which represents the contribution to $R_{1\rho}$ (or R_2) that is not accounted for by the relaxation mechanisms involving tumbling and fast internal motions. Estimates for the Lipari–Szabo parameters were determined by least-squares fitting, using the software developed in the laboratory of A.G. Palmer (Mandel et al. 1995). The overall correlation time τ_m was estimated to be 1.8 ns, consistent with the size of the molecule and with previous estimates from ^{13}C relaxation measurements (Bassus et al. 1995; Atkinson et al. 2000).

The calculated values of the order parameter, S^2 , all lay between 0.75 and 0.95 (Fig. 6A). These values are similar to those measured for the well-ordered regions of other proteins, whereas disordered segments at chain termini and loops typically give rise to order parameters of 0.7 or less

Fig. 5. ^{15}N relaxation rates and ^1H – ^{15}N NOE for the backbone amide groups of ω -MVIIA-Gly. (A) Longitudinal and rotating frame relaxation rates. R_1 (open symbols) and $R_{1\rho}$ (filled symbols) rates were measured at 10°C and pH 4.6 and a field strength of 9.4 T. The $R_{1\rho}$ measurements used a spin-lock field strength of 5.8×10^{-4} T. (B) Heteronuclear NOE, measured under the same conditions.

(Kay et al. 1989; Clore et al. 1990; Mandel et al. 1996). The experimental data were not sufficient to define accurately the internal correlation time τ_e for any of the residues, and the data could be well fit by assuming only that τ_e was much smaller than τ_m , that is, <100 ps.

For many of the residues, the observed values of $R_{1\rho}$ were too large to be accounted for by the rapid internal motions, leading to significant R_{ex} terms (Fig. 6B), with residues 9–15 displaying the largest values. Although the R_{ex} values are not directly interpretable in terms of molecular motions, under favorable conditions the rate of exchange can be estimated by measuring $R_{1\rho}$ at different spin-lock fields (or, for R_2 experiments, using different delay times in the pulse sequence used to refocus the transverse relaxation) (Deverell et al. 1970; Szyperski et al. 1993; Orekhov et al. 1994; Mandel et al. 1996; Beeser et al. 1998). $R_{1\rho}$ was measured at spin-lock fields of 1.8, 3.2, and 5.8×10^{-4} T, and the resulting R_{ex} terms for the lowest and highest field strengths are plotted in Figure 6B.

The R_{ex} terms measured at the highest and lowest spin-lock fields were used to estimate a time constant for the

Fig. 6. Lipari-Szabo model-free parameters derived from experimental relaxation data. (A) Order parameters, based on $R_{1\rho}$ measurements with a spin-lock field strength of 4.8×10^{-4} T, together with R_1 and NOE measurements. (B) R_{ex} residuals derived from $R_{1\rho}$ measurements with spin-lock field strengths of 5.8×10^{-4} T (filled symbols) and 1.8×10^{-4} T (open symbols), and the same R_1 and NOE data as used in A. The error bars associated with the S^2 and R_{ex} values were derived from the nonlinear least squares fit. (C) The expected relationship between the time constant for conformational exchange τ_{ex} and the ratio of R_{ex} terms measured at two field strengths, 1.8×10^{-4} T and 5.8×10^{-4} T. The single point plotted on the curve represents the average measured value of this ratio for the amide groups of residues 9–15. The vertical error bar represents the total range of these values, and the horizontal bar the corresponding range of inferred τ_{ex} values.

conformational exchange process, using an approach similar to that described by Deverell et al. (1970) and Szyperski et al. (1993). As shown by these researchers, the contribution to $R_{1\rho}$ from an interconversion between two states is given by:

$$R_{ex} + p_A p_B \Delta\Omega^2 \frac{\tau_{ex}}{1 + (\omega_1 \tau_{ex})^2}$$

where p_A and p_B are the fractional populations of the two states, $\Delta\Omega$ is the difference in ^{15}N chemical shifts for the two states, τ_{ex} is the correlation time for the exchange process, and ω_1 is the spin-lock field expressed as the ^{15}N Larmor frequency in rad/sec. If the R_{ex} terms are determined at two different spin-lock fields but otherwise identical conditions, their ratio is expected to depend only on the spin-lock fields and τ_{ex} . The predicted relationship between this ratio and the time constant is plotted in Figure 6C (for spin-lock fields of 5.8 and 1.8×10^{-4} T, the highest and lowest fields used in this study). For these spin-lock fields, significant changes in R_{ex} are expected only if τ_{ex} is >20 microseconds.

For the amide groups of residues 9–15, the ratios of R_{ex} values were all in the range of 0.68–0.88, with a mean of 0.78. That all of the amide groups in this segment gave rise to similar ratios indicates that they all undergo motions on a similar time scale, perhaps in a concerted process. Using the mean R_{ex} ratio and the theoretical relationship graphed in Figure 6C, the time constant for these motions was estimated to be ~35 microseconds. For the other amide groups, the R_{ex} terms were too small to make reliable estimates of their ratios at the two spin-lock fields.

Recent ^{13}C relaxation measurements by Atkinson et al. (2000) demonstrate that the segment between Cys-8 and Cys-15 in mature ω -MVIIA-Gly undergoes motions similar to those described here for the precursor. Interestingly, the polypeptide segment that displays the greatest mobility, in both the precursor and mature form, also contains some of the residues that have been most strongly implicated in determining the Ca^{2+} channel-blocking activity of ω -MVIIA and related ω -conotoxins (Kim et al. 1995; Nadasdi et al. 1995; Flinn et al. 1999; Nielsen et al. 1999). As suggested previously by Gray, Olivera, and colleagues, motions in the binding regions of conotoxins and other peptide ligands may play a role in enabling these molecules to enter their binding sites (Gray et al. 1983; Jacobsen et al. 1999).

Effects of carboxy-terminal amidation on the structure of ω -MVIIA

A major goal of this study was to determine whether modification of the carboxy-terminal Gly residue, to generate mature ω -MVIIA, causes significant structural changes. In

Figure 2C, the backbone conformation of ω -MVIIA-Gly is compared with three previously published structures of the mature form. For each family of structures, the member closest to the mean structure is drawn, with the structure representing the backbone of ω -MVIIA-Gly drawn as the thicker blue coil. As indicated in the Figure, there are quite substantial differences between the three independent structures of the mature peptide. These differences are illustrated more quantitatively in Figure 7A, where the pairwise RMS deviations among the three closest-to-mean structures are plotted as a function of residue number.

The pairwise RMS deviations between the ω -MVIIA-Gly structure and each of the three structures for the mature peptide are plotted in Figure 7B. Strikingly, there is a quite good match between the structure calculated here and one of the structures for the mature form, which was recently published by Atkinson et al. (2000) (the green backbone ribbon in Fig. 2C). When the closest-to-mean structures from these two families are compared, the average RMS deviation of backbone atoms is only 0.8 Å, a much better fit than seen for any of the other structure pairs. Although the differences among the independent ω -MVIIA structures introduces considerable uncertainty in any comparisons, the good fit between the structure calculated for ω -MVIIA-Gly and the structure determined by Atkinson et al. suggests that the modification does not cause extensive structural rearrangement, consistent with the very limited chemical shift differences observed.

When all four of the structures are compared, the regions displaying the smallest deviations include residues 24 and 25, and there is very good superposition of the 15–25 disulfide (Fig. 2C). Thus, the presence of Gly-26 in ω -MVIIA-Gly does not appear to influence the conformation of its most immediate covalent neighbors. It is more difficult, however, to determine whether the terminal Gly residue influences the positions of the residues it interacts with non-covalently, especially residues 17 and 19, as there is not a consensus for the position of these residues in the published structures of the mature form.

Perhaps the strongest indication of a structural difference between the two forms of the peptide is the evidence for a direct interaction between the carboxyl group of Asp-14 and the amide group of Ser-9 in ω -MVIIA-Gly. As discussed earlier, the chemical shifts of the amide proton of Ser-9 differ by more than 0.4 ppm in the two forms. In both forms, there is only a small change in the chemical shift of this proton over the pH range of 3.5–6 (Fig. 7; Nielsen et al. 1999), indicating that this difference is not a result of the different pH values used for the various studies. Although these observations suggest that the interaction between Asp-14 and Ser-9 might be influenced by the nature of the carboxyl -terminus, it is difficult to define these differences precisely, especially in light of the relaxation data indicating that these residues undergo conformational exchange in

Fig. 7. Comparison of three independent NMR structure determinations of mature ω -MVIIA and one of ω -MVIIA-Gly. (A) RMS deviations among backbone atoms of ω -MVIIA structures. For each of the three structure families, the structure closest to the calculated mean structure was used for comparison. The backbone atoms of these representative structures were superimposed pairwise, and the graph represents the average RMS deviations for the backbone atoms for each residue (amide nitrogen, α -carbon, and carbonyl carbon atoms). The key indicates the pairs of structures compared using the protein databank accession codes, along with the overall average RMS deviations for the backbone atoms. (B) Comparisons between each ω -MVIIA structure and the structure of ω -MVIIA-Gly determined in this study. As in A, RMS deviations were calculated using representative structures identified as being closest to the mean structure for each family. (C) Schematic representation of the ω -MVIIA sequence, showing the positions of the three disulfide bonds and short β -strands in the folded conformation.

both forms of the peptide. Also, it is not clear how the terminal Gly residue might exert its influence on residue 9 or 14, neither of which it contacts directly. One possibility is that the presence of the additional carboxyl group changes the electrostatic balance of the molecule and indirectly favors the interaction of the Asp-14 side chain with the Ser-19

amide group. Alternatively, the effect might be mediated through a structural change in the segment including residues 17 and 19. In either case, however, the reduced affinity of ω -MVIIA-Gly for Ca^{2+} channels may be associated with the presence of the Asp-14-Ser-9 interaction, as Asp-14 lies within the segment that is most likely to interact directly with the channel.

Conclusion

One of the most striking features of the ω -conotoxins is their ability to fold into well-defined three-dimensional structures in spite of their small size and high sequence variability. The structures are highly dependent on the presence of the three disulfide bonds, such that removal of any one leads to the loss of the native conformation and activity (Price-Carter et al. 1998). However, noncovalent interactions are also necessary to specify formation of the correct structure, as the addition of denaturants greatly reduces the folding efficiency (Price-Carter et al. 1996a). In this respect, these molecules resemble larger disulfide-bonded proteins, such as bovine pancreatic trypsin inhibitor (BPTI) and ribonuclease A, which are stabilized through the cooperative effects of covalent and noncovalent interactions (Creighton 1992; Wedemeyer et al. 2000). In contrast to these larger molecules, however, the native ω -conotoxins are only marginally more stable than forms with non-native disulfides, and their folded conformations appear to be much more dynamic. The NMR studies described here provide new information about both the folding mechanisms of ω -conotoxins and the nature of their native conformations.

In the context of the folding process, the direct interactions between Gly-26 and residues 17 and 19 (Fig. 3) may be of particular significance. Because the conversion of the terminal Gly residue to an amide group probably occurs after the peptide is transported from the endoplasmic reticulum (Oyarce and Eipper 1995), these interactions are likely to contribute to stability when the peptide folds and forms its disulfides. Although the stability difference between the precursor and mature forms is only ~ 1 kcal/mole, the biological significance of a 50% increase in folding efficiency could be high. Interestingly, a Ser or Thr residue is found at position 17 in 27 of 62 ω -conotoxin sequences compiled by Olivera and colleagues (G. Bulaj and B.M. Olivera, pers. comm.), suggesting that interactions with the terminal carboxyl group may play an evolutionarily conserved role in folding.

The results described here also support the view that the folded conformations of the ω -conotoxins have unusually small net stabilities and are more dynamic than more typically sized protein domains. The exchange rates for the amide hydrogens (Fig. 1) indicate that even the most protected regions of the peptide undergo either local or global unfolding transitions with free energy changes of only 4–5

kcal/mole, versus 10–15 kcal/mole usually observed for larger molecules. In addition, the ^{15}N relaxation experiments indicate that 6 of the 26 residues undergo conformational exchange processes on the microsecond time scale. On the other hand, the relaxation experiments do not reveal faster motions that are characteristic of highly disordered loop or terminal segments.

The low stability and pronounced motions observed in this and other ω -conotoxins may simply be a reflection of their small size. It is possible, however, that some aspects of the observed flexibility have evolved under natural selection for functional properties. Motions in the free peptides are likely to increase the entropic penalty for binding to a receptor, but they might also allow binding to a broader range of targets or facilitate the process of entering a highly constrained binding site. Understanding the relationships between flexibility and binding is likely to be particularly important both for elucidating structure–function relationships for the naturally occurring peptides and for further development of conotoxin analogs for novel applications.

Materials and methods

Protein samples

Unlabeled ω -MVIIA-Gly used for homonuclear ^1H NMR experiments was chemically synthesized, as described previously (Price-Carter et al. 1996b). After cleavage from the resin and deprotection of side chains, the protein was folded in the presence of 1 mM GSSG and 2 mM GSH (disulfide and thiol forms, respectively, of glutathione). The identity and purity of the resulting material was confirmed by reversed-phase high performance liquid chromatography (HPLC), by mass spectrometry and a Ca^{2+} channel-binding assay (Price-Carter et al. 1996b).

^{15}N -labeled protein was produced in *E. coli* using a modified form of an expression system previously developed to prepare a precursor form of ω -MVIIA containing an amino-terminal propeptide, as well as the carboxy-terminal Gly residue (Price-Carter et al. 1996b). In these constructs, the sequence encoding the conotoxin precursor was fused to a fragment of the *E. coli* Trp operon and placed under the control of a phage T7 promoter (Studier et al. 1990; Staley and Kim 1994). In the plasmid used here, the fusion was constructed to encode a Trp residue immediately before the ω -MVIIA-Gly sequence, allowing it to be released by chemical cleavage with BNPS-skatole (2-[2'-nitrophenylsulfenyl]-3-methyl-3'-bromoindolenine). The plasmid was introduced into *E. coli* strain BL21. The bacteria were grown in minimal M9 medium containing $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source, and transcription of the fusion gene was induced by the addition of isopropyl β -D-thiogalactoside. After lysis, the fusion protein was found in the insoluble fraction of the cell extract, but was solubilized in 6 M guanidinium chloride. The thiols were protected by forming mixed disulfides with glutathione and the fusion protein was cleaved with BNPS-skatole as described previously (Price-Carter et al. 1996b). The peptide was treated with methyl sulfide to reduce any methionine sulfoxide and then with dithiothreitol to reduce the mixed disulfides. After these treatments, the reduced protein was purified by reversed-phase HPLC and allowed to fold and form disulfides for 5 hr in the presence of 1 mM GSSG and 2 mM GSH at pH 7.3,

25°C. The folded protein was repurified by HPLC, and its identity was confirmed by mass spectrometry and the Ca^{2+} channel-binding assay. The samples used for NMR spectroscopy contained ~2 mM peptide, 6.7% D_2O , 1.7 mM TMSP (3-(trimethylsilyl) propionate), and 0.2 mM Na-azide. The pH of the sample was adjusted to 6.0 with NaOH.

NMR spectroscopy

All of the NMR experiments were carried out at 10°C. Two-dimensional homonuclear TOCSY and NOESY spectra used to make ^1H resonance assignments were recorded on a Bruker AMX 500 spectrometer and a Varian UnityPlus 500 spectrometer, respectively. To determine NOE intensities for structure calculations, an additional two-dimensional NOESY spectrum was recorded on a Varian UnityPlus 750 spectrometer, with a mixing time of 300 msec. All of the homonuclear experiments incorporated the WATERGATE pulse sequence for solvent signal suppression (Piotto et al. 1992). A three-dimensional ^1H - ^{15}N NOESY-HSQC spectrum (recorded on a Bruker AMX 600 spectrometer, with a 200-msec mixing time) was also used to resolve some ^1H assignment ambiguities and to make ^{15}N resonance assignments.

Three-dimensional ^1H - ^{15}N heteronuclear experiments, HNHA (Vuister and Bax 1993) and HNHB (Archer et al. 1991), were used to establish constraints on ϕ and χ_1 dihedral angles, respectively. Both of these spectra were recorded on a Bruker AMX 500 spectrometer.

Amide hydrogen exchange and pH titration experiments were monitored using ^1H - ^{15}N HSQC spectra collected on a Bruker AMX 600 spectrometer. To confirm the assignments of the HSQC peaks, the titration experiment was also monitored in parallel by a series of two-dimensional homonuclear TOCSY spectra recorded on the same spectrometer.

Longitudinal (R_1) and transverse ($R_{1\rho}$) ^{15}N relaxation rates were measured using pulse sequences incorporating pulsed-field gradients to preserve coherence (Dayie and Wagner 1994) and the WATERGATE sequence for solvent suppression. The ^1H - ^{15}N heteronuclear NOE was measured using the sensitivity-enhanced pulse sequences described by Farrow et al. (1994). All of the relaxation measurements were made with a Varian UnityPlus 400 spectrometer.

NMR data were processed using the NMRPipe computer program (Delaglio et al. 1995) and the resulting spectra were displayed and analyzed with the program XEASY (Bartels et al. 1995).

Structure calculation

NOE intensities were determined by integrating the cross-peaks in the 750-MHz two-dimensional NOESY spectrum and were converted to distance constraints using the "caliba" routine in the program DYANA (Güntert et al. 1997). Additional distance constraints were derived from the known disulfide connectivity of ω -MVIIA-Gly and from hydrogen bonds inferred from amide hydrogen exchange kinetics and preliminary structure calculations. $J_{\text{HN-HA}}$ coupling constants were determined from the ratios of the diagonal and cross-peak intensities in the HNHA spectrum (Vuister and Bax 1993). Six residues were identified for which the $J_{\text{HN-HA}}$ coupling constant was >7 Hz, and the ϕ dihedral angles for these residues were constrained to lie between -80° and -160° . Four other residues displayed $J_{\text{HN-HA}}$ values <4 Hz, and the ϕ dihedral angles for these residues were constrained to lie between

-40° and -90° . For residues with two β protons, the relative values of the $J_{\text{N-HB}}$ coupling constants were determined from the cross-peak intensities in the HNHB spectrum, and a homonuclear TOCSY spectrum collected with a short mixing time (20 msec) was used to estimate the relative values of the $J_{\text{HA-HB}}$ coupling constant. From these measurements, stereospecific assignments were made for the β protons of seven residues, and the χ_1 dihedral angles of these residues were constrained to lie either between -30° and -90° or between 30° and 90° (Wagner et al. 1992). For two other residues, there was sufficient information to place constraints on the χ_1 dihedral angle, but not to establish stereospecific assignments. After initial structures were calculated, relative NOE intensities were used to make additional stereospecific assignments for the β protons of Cys-1, and for the α protons of three Gly residues (3, 5, and 23).

The distance and dihedral constraints were used to calculate several families of structures by simulated annealing in torsion angle space, using the program DYANA (version 1.3). The standard DYANA residue library was modified to include Cys and Gly residues with the terminal amino and carboxyl groups, respectively, using the geometries specified in the Amber library distributed with DYANA. For each structure, a total of 10,000 molecular dynamics steps were performed at decreasing "temperature," followed by 1000 steps of conjugate-gradient minimization. From the initial structures, violations of the NOE constraints were used to reevaluate assignments in the NOESY spectrum and the NOE calibration parameters. After this process, hydrogen bonds were identified and introduced as additional constraints for a final set of calculations.

Calculated structures were visualized and analyzed using the computer program MOLMOL (Koradi et al. 1996). Atomic coordinates have been deposited in the Protein Data Bank with accession code 1FEO.

Amide hydrogen exchange

The sample used for the hydrogen exchange experiment was buffered at pH 4.0 with 20 mM Na-acetate. After collecting an initial ^1H - ^{15}N HSQC spectrum, the sample was exchanged into D_2O by centrifugation through a 3-mL column of Sephadex G-15 equilibrated with 20 mM Na-acetate at pH 4.0 (uncorrected measurements with a glass electrode) in D_2O . An HSQC spectrum was recorded ~15 min after the exchange. The sample was maintained at 10°C, and a total of 27 spectra were recorded over a period of 76 h.

Rate constants for the exchange of individual amide hydrogen ions were determined by fitting the HSQC peak volumes to a single exponential decay by the method of least squares. Expected rate constants for exchange of amide hydrogen ions in the same sequence in a fully exposed environment were predicted using the parameters of Bai et al. (1993) and the program Sphere (<http://dino.fold.fccc.edu:8080/sphere.html>) (Zhang 1995).

pH titration

The pH of an unbuffered solution of uniformly ^{15}N -labeled ω -MVIIA-Gly was adjusted to 6.0 with NaOH. After recording initial ^1H - ^{15}N HSQC and ^1H - ^1H TOCSY spectra, small volumes of 0.1 N HCl were added to lower the pH successively to 5.1, 3.9, 3.4, 3.0, and 2.3, as measured with a glass electrode. After each pH adjustment, HSQC and TOCSY spectra were recorded, and the ^1H chemical shifts were determined relative to TMSP.

Analysis of ^{15}N relaxation data

For the R_1 and $R_{1\rho}$ experiments, peak volumes in the two-dimensional ^1H - ^{15}N correlation spectra were fit to a single exponential decay by the method of least squares. The uncertainties in the relaxation rates were assumed to be 5% of the measured values. The ^1H - ^{15}N NOE was calculated as the ratio of the peak volumes in the presence and absence of ^1H saturation. Because the NOE values were all close to zero (Fig. 5), these values could not be determined as precisely as the other relaxation parameters, and the uncertainty for each NOE was assumed to be ± 0.1 .

The MODELFREE program suite (version 3.1) from the laboratory of Dr. Arthur Palmer (Columbia University) was used to interpret the relaxation data in terms of the Lipari-Szabo formalism. To identify those amides for which exchange contributions were significant, the data were fit to a form of the Lipari-Szabo model in which the adjustable parameters were the overall tumbling rate, τ_m , the order parameter for each amide group, S^2 , and the exchange residual term, R_{ex} , for each amide. The data from three $R_{1\rho}$ experiments (with spin-lock fields of 1.8, 3.2, and 5.8×10^{-4} T) were analyzed in conjunction with the same R_1 and NOE data sets. The three resulting estimates for τ_m were very similar (1.747, 1.749, and 1.751 ns), as were the estimates of S^2 for each residue, as expected if the strength of the spin-lock field affects only the exchange contribution. For each residue, the resulting values of τ_m , S^2 , and R_{ex} were able to reproduce the measured relaxation parameters to within 95% confidence limits calculated from Monte Carlo simulations. Introducing explicit values for the internal correlation time τ_c did not significantly improve the fit to the experimental data, and removing R_{ex} terms smaller than $\sim 0.5 \text{ sec}^{-1}$ did not noticeably harm the fit. Rather than using arbitrary criteria to select residues for which an R_{ex} term was appropriate, the calculated values of this parameter for all of the residues are plotted in Figure 6B.

Acknowledgments

We thank especially Drs. Kwaku Dayie and Volker Dotsch for help with the NMR experiments, as well as the entire Wagner laboratory for making D.P. Goldenberg's sabbatical visit an enjoyable and educational experience. We also thank Drs. Marian Price-Carter, William R. Gray, Baldomero M. Olivera, and Grzegorz Bulaj for helpful discussions and advice. Synthetic oligonucleotides and mass spectrometry analyses were provided by the Protein/DNA core facility of the Utah Regional Cancer Center (supported by National Cancer Institute Grant No. 5 P30 CA 42014). This study was supported by National Science Foundation grant number MCB-9316065 (to D.P.G.) and National Institutes of Health grant number GM47467 (to G.W.). D.P.G. was the recipient of a fellowship from the John Simon Guggenheim Memorial Foundation.

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