The conotoxins are a family of small, naturally occurring γ-carboxyglutamate (Gla)-rich peptides that specifically antagonize the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptor. One member of this family, conantokin-G (con-G), undergoes Ca\(^{2+}\)-mediated self-assembly to form an antiparallel helical dimer. Subunit interactions in this complex are incumbent upon intermolecular Ca\(^{2+}\) bridging of Gla residues spaced at \(i, i + 4, i + 7, i + 11\) intervals within the monomer. Herein, we further probe the molecular determinants governing such helix-helix interactions. Select variants were synthesized to evaluate the contributions of non-Gla residues to conantokin self-association. Con-G dimerization was shown to be exothermic and accompanied by positive heat capacity changes. Using positional Gla variants of conantokin-R (con-R), a non-dimerizing conantokin, \(i, i + 4, i + 7, i + 11\) Gla spacing alone was shown to be insufficient for self-assembly. The Ca\(^{2+}\)-dependent antiparallel heterodimerization of con-G and con-T(K7γ), two peptides that harbor optimal Gla spacing, was established. Last, the effects of covalently constrained con-G dipeptides on NMDA-evoked current in HEK293 cells expressing combinations of NR1a, NR1b, NR2A, and NR2B subunits of the NMDA receptor were investigated. The antiparallel dipeptide was unique in its ability to potentiate current at NR1a/2A receptors and, like monomeric con-G, was inhibitory at NR1a/2B and NR1b/2B combinations. In contrast, the parallel species was completely inactive at all subunit combinations tested. These results suggest that, under physiological Ca\(^{2+}\) concentrations, equilibrium levels of con-G dimer most likely exist in an antiparallel orientation and exert effects on NMDA receptor activity that differ from the monomer.

The predatory snails of genus Conus are abundant sources of pharmacologically diverse peptides ("conotoxins") that target numerous neural and neuromuscular ion channels (reviewed in Ref. 1). It is estimated that the Conus venom library may contain as many as 100,000 different peptides distributed among 500–700 species. Hundreds of conotoxins have been identified to date, the majority of which are relatively short (10–30 amino acids) peptides containing 1–4 intramolecular disulfide bonds that conform to a limited array of bridging frameworks. Conus snails also possess the enzymatic machinery to effect numerous post- and co-translational peptide modifications, including C-terminal amidation, epimerization, O-glycosylation, hydroxylation of proline, lysine, and valine, and γ-carboxylation of glutamate. The latter modification occurs to a high degree in a small family of conotoxins known as the conantokins. To date, four conantokins have been identified: conantokin (con)-G, con-T, con-R, and con-L (2–5). These possess γ-carboxyglutamate (Gla) and total amino acid content ratios of 5:17, 4:21, 4:27, and 4:19, respectively, and are unusual in the conotoxin realm because of their lack of disulfide bonds (excepting con-R, which has a single heptapeptide disulfide loop). Although γ-glutamyl carboxylase activity has been detected in Drosophila (6) and Leptospira (7), Conus remain the only non-mammalian organisms that contain Gla in a polypeptide context. In the conantokins, the Gla residues fulfill both structural and functional roles. In the former category, a high degree of conformational rigidity is imposed through intramolecular metal ion bridging among the malonate head groups of Gla side chains. Specifically, metal ion binding to optimally spaced \(i, i + 3\), and/or \(i, i + 4\) Gla residues promotes the formation of end-to-end helices (8). Functionally, the conantokins are specific and potent antagonists of the N-methyl-D-aspartate (NMDA) receptor, a ligand-operated ion channel with high Ca\(^{2+}\) permeability that, in its overactivated state, has been implicated in a host of chronic and acute neuropathologies (9). Studies with synthetic peptide variants have ascribed high functional importance to the conserved Gla residues at conantokin sequence positions 3 and 4 (8). On the other hand, Gla residues located in the C-terminal segment tend to maximize the metal cation-mediated helix content of the conantokins, but are not crucial to the NMDAR activity of the peptides.

In addition to the contribution of conantokin Gla residues to NMDAR antagonism and secondary structure, we have recently established that Gla residues, as arranged in con-G,
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allow for the adoption of helical dimers in the presence of Ca\(^{2+}\) (10). This conclusion, based on sedimentation equilibrium studies with con-G and con-G derivatives, and thiol-disulfide rearrangement assays with Cys-containing versions of con-G, suggested a model for the dimer in which helix-helix interactions are primarily maintained through interhelical Gla-Ca\(^{2+}\) coordination between peptide strands aligned in antiparallel fashion. Because it appears driven solely by electrostatic contacts, independent of any hydrophobic contributions, our dimerization model is unique in the realm of helix-helix interactions in both naturally occurring and designed peptide/protein supersecondary structural contexts. These assumptions have now found definitive support from x-ray crystallographic studies on Ca\(^{2+}\)-complexed con-G and Ca\(^{2+}\)-complexed con-T[K7γ], peptides that share the i, i + 4, i + 7, i + 11 format of Gla spacing upon which self-assembly is contingent (11, 12). However, because con-T[K7γ] dimerizes to a greater extent than con-G, it is apparent that amino acids other than Gla must contribute to Ca\(^{2+}\)-triggered self-association. These previous studies also raise the possibility that heterodimer formation between appropriately designed conantokin-based monomers may be viable. Furthermore, because the dimeric form of con-G exists under physiological Ca\(^{2+}\) concentrations, the biological relevance of this species is of interest. The current study addresses these questions and, whereas confirming that optimal Gla spacing is the principal molecular feature driving dimerization, reveals that non-Gla residues impart subtle effects on helix-helix interactions.

**EXPERIMENTAL PROCEDURES**

*Peptide Synthesis, Purification, and Characterization—*The peptides employed in this study, which will be referred to by the numbers designated in Fig. 1, were synthesized as previously described (13), on a model 433A peptide synthesizer (Applied Biosystems, Foster City, CA) at 0.1 mmol scale using PAL resin (Applied Biosystems). All Fmoc-derivatized amino acids were obtained from either Sigma or Novabiochem (San Diego, CA) with the exception of Fmoc-γ,γ′-di-o-tBu-L-Gla, which was synthesized as reported earlier (14). The purification of non-Cys and Cys-containing peptides has been described previously, as has the oxidation of the Cys-containing variants to provide the disulfide-linked species (10). All peptides were characterized by analytical HPLC and delayed extraction matrix-assisted laser desorption ionization-time-of-flight mass spectrometry on a Voyager-DE spectrometer (PerSeptive Biosystems, Framingham, MA).

*Circular Dichroism—*CD spectra were recorded between 195 and 260 nm on an AVIV model 202SF spectrometer using either 1- or 0.2-cm path length cells. Each spectrum represents the average of three scans collected at 1.0-nm intervals using a 1.0-nm bandwidth. Corrections for non-peptidic contributions to the ellipticity were made by subtracting the averaged spectrum of the peptide-free sample. The α-helical content of peptides in solution was determined from the mean residue ellipticities at 222 nm using the empirical relationship, % helix = \((-0.2\times 222 \times 2340)/30300\) × 100 (15).

*Sedimentation Equilibrium Ultracentrifugation—*Sedimentation equilibrium experiments were performed on an Optima XL-I analytical ultracentrifuge in an An-60 Ti rotor equipped with a standard two-channel cell (10). The initial peptide concentration for all runs was 150 μM. The buffer consisted of 10 mM sodium borate, 100 mM NaCl, pH 6.5. The apparent molecular weight (M\(_{app}\)) was obtained by fitting the data to a single ideal species using the sedimentation analysis software supplied by Beckman. The partial specific volumes of the peptides analyzed were calculated from the mass average of the partial specific volumes of the individual amino acids (16). The partial specific volume of Gla was assigned that of Glu. The fractional dimer content (f\(_{d}\)) was calculated using the equation:

\[
\text{f}_{d} = \frac{M_{r(app)} - M_{r(mono)} - n M_{r(metal)}}{M_{r(mono)} + n M_{r(metal)}}
\]

where M\(_{r(app)}\), M\(_{r(mono)}\), and M\(_{r(metal)}\) denote the partial specific volumes of the optimal species, the monomer, and the metal complex, respectively. The stoichiometries (n) of Ca\(^{2+}\) binding to peptides 1, 5, 6, 8, 9, and 10 were determined from titration calorimetry (17). All other peptides were assigned a Ca\(^{2+}\) stoichiometry of 3, based on the values empirically determined for 1 and 6.

**Calorimetrically Monitored Peptide Dimer Dissociation—**The enthalpies attending the dissociation of the dimeric form of 1 were monitored at 15, 25, and 35 °C using a VP-ITC calorimeter (MicroCal, Inc., Northampton, MA). Peptide was introduced into the syringe at a concentration of 2.4 mM in buffer consisting of 50 mM Heps, 150 mM NaCl, and 20 mM CaCl\(_2\), pH 7.4. At 3-min intervals, 8 μl of this solution was diluted into the sample cell containing 1.4 ml of buffer alone, and the enthalpy changes were recorded. The dilution of con-G[γ7P], a non-associating conantokin variant, was used to assess the contribution of heats of peptide dilution to the combined enthalpies of dilution and dimer dissociation observed with 1.

**Determination of the Heterodimerization Tendency of 1 and 6—**The capacity of 1 and 6 to form heterodimers was determined through previously employed thiol-disulfide oxidation and rearrangement experiments using Cys-containing variants of 1 and 6 (10). The progress of oxidation or rearrangement was monitored by analytical reverse-phase HPLC as follows. Reaction samples (20 μl) were injected onto an HPLC equipped with a Vydac C\(_{18}\) column (218TP, 4.6 mm × 250 mm) equilibrated in 95% of 0.1% trifluoroacetic acid and 5% of 0.1% trifluoroacetic acid/CH\(_3\)CN at a flow rate of 1.0 ml/min. At a time of 3 min post-injection, a 40-min gradient of 5–45% 0.1% trifluoroacetic acid/CH\(_3\)CN was implemented to elute the peptides. Absorbance detection was performed at 220 nm. Individual peaks were collected, lyophilized, and characterized by delayed extraction matrix-assisted laser desorption ionization-time-of-flight.

**Surface Plasmon Resonance-monitored Heterodimerization—**Real time analysis of Ca\(^{2+}\)-dependent heterodimerization between 1 and 6 was monitored by surface plasmon resonance (18) using a Biacore instrument, model 3000 (Biacore Inc., Piscataway, NJ). The Cys-containing peptides were immobilized on CM5 sensor chips using the standard ligand thiol coupling protocol outlined by the manufacturer (18). The carboxymethylated dextran surface was activated by the injection of 45 μl of a solution containing 0.2 M N-ethyl-N-′-(3-dimethylamino-propyl)carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide, and modified by injection of 40 μl of 80 mM 2-(2-pyridyldithio)ethanethiolamine in 0.1 M borate buffer, pH 8.5. Either 13 or 14 was dissolved in 10 mM sodium citrate, pH 2.8 (280 μg/ml), and injected under manual control until the desired amount of peptide was coupled (500 to 2000 RU). The

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**References**

1. T[K7γ], A.N. and H.A. (1983) J. Biol. Chem. 258, 12642
2. Dimerization of Gla-containing Peptides
3. Sedimentation equilibrium ultracentrifugation
4. Analytical reverse-phase HPLC
5. Biacore instrument
6. Surface plasmon resonance
thiol-reactive surface was subsequently quenched with 50 mM cysteine, 1 M NaCl. Sensorgrams were obtained at 25 °C at a flow rate of 5 µl/min and were corrected using double referencing. The running buffer was 10 mM NaBO₃, 100 mM NaCl with or without 20 mM CaCl₂. The dissociation constant (K₈) for each peptide was determined by plotting the RU at the extrapolated plateau of the binding curve versus the peptide concentration using the BLAevaluation 3.0 software package (Biacore).

Electrophysiology—Whole cell patch clamp recordings were conducted on HEK293 cells transiently expressing combinations of NR1 (α or β) and NR2 (A or B) NMDARs as detailed in a prior study (19). Cells were maintained at a holding potential of −70 mV. The extracellular solution consisted of 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 10 mM Na-Hepes, and 20 mM dextrose, pH 7.35. Recording pipettes contained 140 mM CsF, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM Cs-Hepes, 2 mM tetraethylammonium chloride, and 4 mM Na₂ATP, pH 7.35. Currents were evoked with the application of extracellular solution containing 100 µM NMDA and 10 µM glycine. The peptides were applied at a concentration of 40 µM. Solutions were applied using a nine-barrel rapid solution changer (Biologic, Calix, France) positioned 200–300 µm from the cell being perfused. Data were filtered at 5 kHz, digitized, and acquired on a personal computer using pClamp software (Axon Instruments, Foster City, CA).

**RESULTS**

Effects of Non-Gla Residues and Gla Arrangement on Self-assembly in the Conantokins—The primary sequences of the peptides synthesized for this study, as well as their putative α-helical heptad repeat assignments are shown in Fig. 1. The helix content and fractional dimer distribution of the nominal monomers, in the absence or presence of Ca²⁺, are summarized in Table 1. Several peptides were designed to evaluate the role of e, g, and e', g' residues in the stabilization of the dimeric form of 1. These include the replacement of Arg¹³ with Ala, of Leu¹¹ with Gla, and a trio variant (peptides 4, 2, and 3, respectively). Whereas all of the con-G variants displayed a reduction in helicity comparable with that of 4, yet could dimerize to nearly the same extent as 1, the full-length parent. In 2, the presence of a Lys in heptad position e was predicted to increase dimer stability through electrostatic

![TABLE 1](image)

| Peptide | Metal ion | % α-Helix | M(α-app) | f_d
|---|---|---|---|---|
| 1 | None | 2 | 2550 | 0 |
| 2 | Ca²⁺ | 50 | 3950 | 0.48 |
| 3 | Ca²⁺ | 2 | 2610 | 0 |
| 4 | Ca²⁺ | 42 | 4170 | 0.53 |
| 5 | None | 2 | 2490 | 0 |
| 6 | Ca²⁺ | 47 | 4240 | 0.62 |
| 7 | None | 0 | 2550 | 0 |
| 8 | Ca²⁺ | 0 | 3000 | 0.13 |
| 9 | None | 0 | 2130 | 0 |
| 10 | Ca²⁺ | 17 | 2320 | 0.46 |
| 11 | None | 49 | 3160 | 0 |
| 12 | Ca²⁺ | 72 | 3240 | 0 |
| 13 | None | 12 | 3390 | 0 |
| 14 | Ca²⁺ | 49 | 6670 | 0.88 |
| 15 | None | 19 | 3550 | 0.08 |
| 16 | Ca²⁺ | 35 | 2580 | 0.08 |
| 17 | None | 22 | 2810 | 0 |
| 18 | Ca²⁺ | 47 | 2840 | 0.26 |

*For both CD and ultracentrifugation experiments, the metal ion concentrations were 20 mM for Ca²⁺ and Mg²⁺. In all instances, the buffer was 10 mM NaBO₃, 100 mM NaCl, pH 6.5. The chloride salts of the metals were used.

*Percentage of helix was derived from CD measurements as described under Experimental Procedures.* Peptide concentrations were 35 mM.

Values for M(α-app) represent the average of from two to four separate analyses (3 scans per analyses). Variability, in all cases, was ≤5%.

*Fractional dimer (f_d) content was calculated using the equation, f_d = (M(α-app) - M(α-mon) - nM(α-mono))/M(α-mono), as described previously (10).
interactions with Gla in position e' of the antiparallel partner helix. A similar strategy was invoked in the case of \( \text{Gla}^5 \) with a 3-fold greater tendency to those within \( \text{Gla}^3 \). The replacement of Glu with Lys was designed to decrease the negative interactions between residues 2 and 6. The small increase in dimer content associated with the L11K replacement \( (f_d = 0.53) \) was further increased in \( \text{Gla}^3 \), the E2K,Q6E,L11K variant \( (f_d = 0.62) \), suggesting that amino acids peripheral to the Ca-bridged helix-helix interface can influence the stability of the dimeric species.

In the case of con-T, peptide 7 was used to confirm the contribution of Gla to the Ca-mediated self-association. Within con-G, this position was found to be crucial to the dimerization event \( (10) \). The large decrease in fractional dimer content observed with 7 points toward a similar role for Gla in the context of the con-T peptide.

Like 5, peptide \( 8 \) contains four Gla residues and fails to undergo self-association in the presence of Ca\(^{2+} \) (Table 1). However, unlike 5 and 1, the two C-terminal Gla residues of 8 occupy sequence positions 11 and 15, rather than positions 10 and 14 as occurs in the other conantokins. The Met \( \rightarrow \) Gla variant, 9, was synthesized to determine whether an intervening Gla residue between Gla and Gla would be sufficient to permit Ca-mediated dimerization in a manner homologous to 6. Whereas 9 displayed a tendency to dimerize, the fractional dimer content was modest \( (f_d = 0.08) \) compared with the amount of dimer present in 1 and 6 under identical conditions. However, in 9 the \( i \)th residue in the \( i, i + 4, i + 7, i + 11 \) sequence is assumed by Gla rather than Gla. Hence the \( i \)th residue in the con-R variant is flanked by its neighboring Gla on the N-terminal rather than C-terminal side (as occurs in 1 and 6). To provide for a con-R analog that more fully mimics the Gla pattern in 1 and 6, peptide \( 10 \) was synthesized. This variant contains five Gla residues that are arrayed identically to those within 1 and 6. These sequence alterations yielded a con-R-based peptide with a 3-fold greater tendency to dimerize than 9.

**Heat Changes Accompanying Con-G Dimer Dissociation**—The Ca\(^{2+}\)-triggered dimerization of the conantokins appears to rely heavily on the electrostatic interactions that underlie interstrand metal ion bridging \( (10–12) \). To investigate the thermodynamics involved in the monomer-dimer equilibrium, isothermal titration calorimetry was used to monitor heat changes accompanying small injections of a high concentration \( (2.4 \text{ mM}) \) of Ca\(^{2+}\)-complexed 1 into a large volume of matching buffer at 15, 25, and 35 °C. Assuming a \( K_d \) of 170 \( \mu \text{M} \) for the monomer-dimer equilibrium \( (10) \), a concentration of 2.4 mM would provide for a fractional dimer content of 0.82 in the syringe at 25 °C. Dilution of \( 8 \mu \text{l} \) of syringe sample into 1.4 ml of buffer results in a solution with substantially less dimer content \( (f_d = 0.12) \). Hence, the heat changes accompanying these dilution injections correspond to the dissociation process (as well as small heat changes stemming from sample dilution). Examples of the raw calorimetric data from the injection schedule are shown in Fig. 2. At each temperature examined, the dilutions gave rise to a series of endothermic peaks. Because dimer dissociation is endothermic, this requires the association process to be exothermic. In a parallel experiment, a non-dimerizing con-G variant, con-G[γ7P], was subjected to the identical dilution series. The con-G[γ7P] injections resulted in endothermic pulses much reduced in comparison to those deriving from the dilution profile of 1 and were attributed solely to heats of dilution. Despite the quality of the raw data, after subtracting these background heats from the data for 1, the integrated heats could not be fit to a simple model of monomer-dimer equilibrium \( (20) \).

Qualitatively, it is clear that an inverse relationship exists between the magnitude of the first several enthalpies and the temperature at which the experiments were conducted. This corresponds to a negative heat capacity change for dimer dissociation and a positive change for subunit association. Because negative heat capacity changes are diagnostic of the displacement of solvent-exposed hydrophobic groups to more hydrophobic environments, the calorimetric data suggest that the formation of the con-G dimer complex is driven by electrostatic, not hydrophobic, interactions \( (21) \).

**Heterodimerization between Conantokin Peptides**—It has been amply demonstrated that conantokin-based peptides undergo Ca\(^{2+}\)-mediated self-association, provided that an optimal arrangement of Gla residues is present. This phenomenon raises the additional question of whether two different conantokins can associate to form a heterodimer. This possibility is not readily amenable to examination by sedimentation equilibrium approaches, because hetero- versus homomeric interactions are difficult to distinguish using this technique. As an alternative to sedimentation equilibrium analysis, we employed HPLC-monitored oxidative folding of thiol-containing peptides \( (10, 11, 22, 23) \) to assess the likelihood of strand alignment between heteromeric species. The two peptides recruited for this study were 11 and 14 that, if able to reversibly associate in a manner similar to 1 and 6, would allow for the positioning of Cys thiol groups at positions \( a \) and \( d' \) in the...
antiparallel helix-helix interface (Fig. 1). The distribution of folding products obtained from a 1-h incubation of a 1:1 molar ratio of 11 and 14 under a variety of oxidizing conditions are shown in Fig. 3. In the presence of Ca\(^{2+}\), the heterodimeric disulfide product, 21, was the predominant species formed. A chromatogram obtained after 4 h of incubation was unchanged, reflecting the stability of the heterodimeric product initially formed (data not shown). However, in the presence of Mg\(^{2+}\), or in buffer alone, a mixture of homodimers, heterodimer, and reduced monomeric peptides were observed after 1 h of incubation. After 4 h of incubation, the chromatograms of the Mg\(^{2+}\)-containing sample and buffer alone were similar and reflected the preferential formation of 21. Whereas random collision predicts a distribution of peptides 16, 19, and 21 of 1:1:2, respectively, the ratios obtained in Mg\(^{2+}\)-containing and metal-free buffer were 1:1:1:3 (as shown for buffer, bottom trace of Fig. 3). These data indicate that whereas Ca\(^{2+}\) directs heterodimer formation considerably faster than Mg\(^{2+}\)-containing or metal-free buffers, the antiparallel heterodimer is more stable than either of the parallel-oriented homodimers in all milieus examined. The thermodynamic stability of the disulfide-linked heterodimer, in comparison to the individual homodimers, was further examined through thiol-disulfide exchange experiments in which 16 and 19 were separately incubated with monomeric 14 and 11, respectively (Fig. 4). Beginning with 16 and 14 in a 1:2 molar ratio in the presence of Ca\(^{2+}\), the heteromeric disulfide product was the most prevalent species at equilibrium (Fig. 4, a and b). The heterodimer was also detected, albeit in lesser quantity, in the complement experiment in which 11 and 19 were the initial reactants (Fig. 4, c and d). These results indicate that the antiparallel heterodimer is a stable product in systems originating with parallel homodimeric reactants. Furthermore, in the presence of Ca\(^{2+}\) and reduced 11 or 14, peptide 21 is completely refractory to scrambling, affirming the Ca\(^{2+}\)-linked thermodynamic stability of the heterodimer (data not shown).

The previous oxidative folding and exchange experiments require the oxidative trapping of Cys-containing conantokin variants to provide disulfide-linked products amenable to HPLC elution conditions. To obviate the covalent aspects of this methodology and to demonstrate the autonomous, reversible nature of the complex between 1 and 6, surface plasmon resonance experiments were conducted. Peptide 13 was immobilized onto a CM5 sensor chip through thiol-disulfide exchange of the N-terminal Cys of the peptide with the disulfide-modified chip surface. Representative sensograms depicting the concentration-dependent binding of 1 to immobilized 13 in the presence of Ca\(^{2+}\) are displayed in Fig. 5a. These results clearly indicate that Ca\(^{2+}\)-mediated higher order species can form between heterologous peptides. In the absence of Ca\(^{2+}\), no response was observed at any of the concentrations examined (data not shown). The association and dissociation phases associated with these interactions were rapid and could not provide reliable on and off rate constants. The affinity of 1 for immobilized 13 was therefore evaluated on the basis of steady-state responses and was compared with the values obtained with other peptide analytes, namely 5 and 6 (Fig. 5b). The interaction of immobilized 13 was most robust for 6. Data fitting for these binding partners yielded a \(K_D\) of 260 ± 40 \(\mu M\) and a maximal response of 530 ± 30 RU. The \(K_D\) and \(RU_{max}\) values obtained for analyte 5 were 565 ± 55 \(\mu M\) and 530 ± 25 RU, whereas the corresponding results for 1 as analyte were 519 ± 85 \(\mu M\) and 195 ± 15 RU. These data suggest that whereas the Ca\(^{2+}\)-mediated reversible association of 1 and 5 is permitted, the self-association of 6 is preferred by ~2-fold. The results also reveal that 5 is capable of binding immobilized 13 with an affinity comparable with that of con-G and a maximal response that is similar to the homomeric interaction of peptides 6 and 13.
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The Effect of Con-G Chain Orientation on NMDAR Activity—Of the naturally occurring conantokins, only con-G (peptide 1) is capable of self-association in the presence of Ca\(^{2+}\). Previous work suggests that at physiological Ca\(^{2+}\) concentrations (about 2 mM), the fractional dimer content of a 150 \(\mu\)M solution is \(-0.1\) (10). At such a low distribution, the effect of noncovalent, dimeric 1 on NMDAR activity cannot be readily discerned from the monomeric contribution. Furthermore, because the NMDAR is a Ca\(^{2+}\)-permeable ion channel, increasing the Ca\(^{2+}\) concentration to shift the equilibrium toward dimer formation has a confounding effect on NMDAR activity. To circumvent these impediments, disulfide-linked con-G dipeptides, in both parallel and antiparallel strand orientations, were evaluated for their effects on NMDA-elicited currents in HEK293 cells expressing various combinations (NR1a/NR2A, NR1b/NR2A, NR1a/NR2B, NR1b/NR2B) of NMDAR subunits. As shown in Fig. 6, monomeric 1 (40 \(\mu\)M) was inhibitory toward NR2B-containing receptors, with \(\tau_{on}\) values of 4.5 \(\pm\) 0.5 and 1.9 \(\pm\) 0.4 s at NR1a/NR2B and NR1b/NR2B combinations, respectively. As reported previously (19), the rate of recovery was slow (>100 s, data not shown). In contrast, a high concentration (40 \(\mu\)M) of 16, the N-terminal disulfide-linked parallel dimer, was without effect at any of the four subunit combinations examined. The C-terminal disulfide parallel counterpart, 17, was also inactive at each receptor combination tested (data not shown). Unlike the parallel-oriented dimers, 40 \(\mu\)M applications of 20, the antiparallel species, resulted in activity at three of the four NMDAR subunit combinations tested. The effects included slow onset of potency of NMDA-elicited currents at NR1a/2A receptors \((\tau_{on} = 2.7 \pm 0.4\) s), slow, weak inhibition at NR1a/2B receptors \((\tau_{on} = 4.3\) s), and fast, complete inhibition at NR1b/2B combinations. The \(\tau_{off}\) value for 20 at the NR1a/2A combination is 1.7 \(\pm\) 0.3 s. As seen with 1, the offset of inhibition of 20 at NR1a/2B receptors is very slow (>100 s). However, at NR1b/2B receptors, the onset and offset of inhibition were so fast (<0.1 s) that \(\tau_{on}\) and \(\tau_{off}\) values could not be reliably assessed. No inhibition was observed at receptors consisting of NR1b/2A subunits.

DISCUSSION

Results from this study reveal that Arg\(^{13}\), despite being peripheral to the Gla-rich helix-helix core of con-G (10, 12), contributes to peptide self-association. However, it is unlikely that this is due to elimination of interhelix g-g’ contacts, because the crystal structure of the dimer does not divulge any inter-helix contacts involving Arg\(^{13}\) (12). More probable is that the R13A variant has compromised secondary structure in the vicinity of the replacement. This could disrupt the monomeric helix such that a stable interface between interacting peptide strands cannot be supported. This hypothesis is grounded in both the crystal and NMR-derived structures, which indicate that an intrapeptide salt bridge likely exists between Arg\(^{13}\) and Gla\(^{10}\) (12, 25). The helix stabilizing effect of this electrostatic interaction is reinforced by the CD-derived helicity of Ca\(^{2+}\)-saturated 4, which is greatly diminished compared with con-G (15 versus 50%). However, reduced helicity is not a predictor of dimerization tendency because the truncation variant 15, with a Ca\(^{2+}\)-induced helix content of 17%, exhibits a capability for self-association that is comparable with the full-length parent peptide (Table 1). Clearly, the capacity for Gla\(^{10}\)–Arg\(^{13}\) salt bridge formation is preserved in this peptide and the reduced helicity, as is well documented for shorter peptides strands, reflects a global effect on peptide structure. Also, the stabilization of the negative C-terminal helix macrodipole imparted by Lys\(^{15}\) is eliminated in 15, further diminishing the overall helicity.

Further evidence that e-e’ and g-g’ interactions are of marginal consequence to the helix-helix interface is provided by variants 2 and 3, which were designed to allow for the possibility of interstrand (e-e’) electrostatic contacts between Lys\(^{11}\) and Gla\(^{4}\) (for 2) and between Glu\(^{6}\)–Arg\(^{13}\) and Lys\(^{11}\)–Gla\(^{4}\) (g-g’ and e-e’) in the case of 3. For the latter peptide, the replacement of Glu\(^{2}\) with Lys was designed to decrease the negative repulsion between thi and i + 4 residues Glu\(^{2}\) and Glu\(^{6}\) that could compromise helix formation. The increase in fractional dimer content associated with the Ca\(^{2+}\)-complexed forms of these peptides corresponds to \(K_d\) values for the monomer-dimer equilibrium of 125 \(\mu\)M for 2 and 70 \(\mu\)M for 3. With a \(K_d\) value of 170 \(\mu\)M for con-G dimer dissociation, the L11K substitution reduces the free energy of dimerization by 0.18 kcal/mol, whereas the triple substitutions combine for a free energy decrease of 0.52 kcal/mol. These values are qualitatively similar to those obtained for a single interhelical Glu-Lys ion pair (about 0.3 kcal/mol) in both parallel and antiparallel coiled-coils (26, 27). The crystal structures of the noncovalent, Ca\(^{2+}\)-complexed dimers of 1 and 6 reveal that neither assembly assumes a coiled-coil motif (12). Additionally, in the case of 1, the helical axes are skewed by \(-30^\circ\) from a parallel geometry, whereas the two axes are strictly parallel in 6 (12). As such, it
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seems unlikely that e-e'/g-g' ion pairing interactions are mediating the increase in dimerization observed with 3, but that these replacements are participating in intrapeptide interactions that stabilize the Ca\(^{2+}\)-binding helix-helix core of the dimer. However, in the absence of structural data, it remains possible that the axes of dimeric 3 assume a strictly parallel geometry. In this case, favorable e-e'/g-g' electrostatic interactions could augment dimer formation.

The reduced dimer content of Ca\(^{2+}\)-complexed 7 compared with 6 confirms the importance of Gla\(^3\) in peptide self-association. Yet, the sedimentation equilibrium results obtained with 10 suggest that a Gla residue at position 3 is not imperative for dimerization as long as an i, i + 4, i + 7, i + 11 arrangement of Gla residues is preserved. Whereas this motif also exists in 9, the presumed “non-participating” Gla in the self-assembly is Gla\(^3\), rather than Gla\(^4\), as occurs in 1 (10, 12) and 6 (12). We have amply demonstrated, through \(^{13}\)C NMR-monitored Ca\(^{2+}\) titrations employing selectively \(^{13}\)C-labeled con-G peptides (28) and by potentiometric metal ion titrations with individual Gla → Ala mutants (25), that metal ion binding to Gla\(^4\) differs sharply from Gla\(^3\). Hence, Gla\(^4\) in 9 may be unable to bind ligand Ca\(^{2+}\) in a manner that allows for helix-helix bridging. By replacing Gla\(^4\) with Ala and Val with Gla, the resulting variant, 10, retains i, i + 4, i + 7, i + 11 Gla spacing, but places the ith Gla, Gla\(^4\), as the first Gla in the primary sequence. This provides the opportunity for Gla\(^4\) to be flanked on the C-terminal side by a second Gla. These alterations enhanced the fractional dimer content from 0.08 for 9 to 0.26 for 10. However, these results, as well as those obtained with 4, indicate that the mere presence of the optimal i, i + 4, i + 7, i + 11 Gla motif is not entirely sufficient for biasing the monomer-dimer equilibrium toward dimerization, because 1, 6, and 10 each manifest different self-associative tendencies under identical conditions. Clearly, non-Gla residues, via subtle and complex intra- and interhelical interactions, assist in modulating the propensity of the conantokins for self-association.

Based on the calorimetrically monitored dilution profile of 1, it can be concluded that con-G dimer dissociation is endothermic, whereas the association process is exothermic. The favorable enthalpy accompanying self-assembly is consistent with the formation of noncovalent contacts, primarily Ca\(^{2+}\)-mediated interhelical Gla-Gla bridging. Based on a qualitative assessment of the heat pulses resulting from dilution experiments conducted at 15, 25, and 35 °C, which correspond to a slightly negative heat capacity change, the self-association of con-G is attended by a positive heat capacity change. This is rarely encountered in the realm of protein folding and protein-protein interactions, wherein attainment of the final state is typically driven by the hydrophobic effect (21). In these processes, the magnitude of the negative heat capacity changes are directly correlated with the area of hydrophobic surface that is buried. The positive heat capacity change for con-G self-association is supportive of a predominant role for electrostatics in this process. This interpretation is consistent with our model for helix-helix binding and has been corroborated by the crystallographic data (10, 12).

Neither the cumulative nor the individual heats derived from the raw calorimetric data could be fit to a model of monomer-dimer equilibrium (20). Our previous studies convincingly point to a dimer as the highest order structure capable of forming in the presence of Ca\(^{2+}\) (10, 11). Direct evidence for the dimer form is also provided by the crystallographic data (12). However, distinct differences may exist between the structure of con-G in monomeric form and as it occurs as a subunit of the dimer, including the disposition of side chains and the mode of Ca\(^{2+}\) chelation. If such structural rearrangements occur during the monomer-dimer transition, the raw enthalpies would necessarily be influenced, complicating interpretation of the data in terms of a simple monomer-dimer equilibrium. Confirmation of this supposition awaits high-resolution structural data for Ca\(^{2+}\)-complexed monomeric con-G. Whereas an NMR-derived structure for nominally monomeric Ca\(^{2+}\)-con-G has been solved, the concentrations of peptide (5.8 mM) and Ca\(^{2+}\) (58 mM) in the sample suggest that a substantial fraction of con-G existed in the dimer form during data acquisition (29).
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The results of the HPLC-monitored thiol oxidation experiments clearly indicate that the formation of an antiparallel heterodimer between 1 and 6, as probed with Cys-containing variants, is the kinetically and thermodynamically favored product in the presence of Ca\(^{2+}\). At equilibrium, the antiparallel adduct also predominates in Mg\(^{2+}\)-containing buffer and in metal-free conditions at levels higher than those prescribed by random collision. Hence, of the possible folding products that could result from the incubation of the reduced monomeric species, 21 prevails over the parallel-stranded homodimeric products 16 and 19. This suggests a general preference for an antiparallel versus parallel alignment of two peptide species bearing an i, i + 4, i + 7, i + 11 Gla spacing pattern (Fig. 7a). Similarly, in thiol-disulfide exchange experiments, a marked preference for 21 at equilibrium resulted from the incubation of 14 and 16. However, when 11 and 19 were the reactants, the product distribution at equilibrium was dominated by 19. A previous investigation of strand orientation showed that 19 was more refractory to exchange with 13 than in the complement experiment, i.e. exchange of 18 with 14. We have also determined that, in the presence of Ca\(^{2+}\), 16 and 17 form higher order structures (10), as do the N-terminal and C-terminal linked disulfide-bonded versions of con-T(K7). Possible configurations for the Ca\(^{2+}\)-mediated dimerization of disulfide-linked parallel-oriented peptides, as shown for a C-terminal-linked dipeptide, are depicted in Fig. 7b. If 19 adopts the interlocking motif of the structure represented in the right panel, the accessibility of the disulfide bridge to the attacking free Cys of 11 could be compromised. Whereas a similar interlocking arrangement of peptide strands is also possible for 16 and 18, the observation that these species undergo nearly full exchange with reduced peptides may reflect a greater instability of such assemblies compared with 19.

In contrast to self-associating systems, the reversible association of 1 and 6 cannot be straightforwardly evaluated using sedimentation equilibrium because of the difficulty in distinguishing between homomeric and heteromeric contributions to the apparent molecular weight. Our surface plasmon resonance results clearly point to complex formation between immobilized 1 and 6, although the self-association of 6 is preferred. Our results also reveal that 5 and 6 assemble in a Ca\(^{2+}\)-dependent manner, in violation of the proposed requirement for i, i + 4, i + 7, i + 11 Gla spacing in both constituents of the dimer complex. The interaction of 5 and 6 likely occurs in a manner analogous to that proposed for Ca\(^{2+}\)-mediated dimerization of conantokin with i, i + 4, i + 7, i + 11 Gla spacing. If so, a salt bridge between Lys\(^{7}\) of 5 and Glal\(^{10}\) of 6 could effectively substitute for the Ca\(^{2+}\)-bridged Glal\(^{10}\)-Gla contact that stabilizes the dimer interface. This particular mode of dimerization is also exclusively dependent upon electrostatic interactions, and suggests new avenues for the design of heteromeric helix superstructures.

Lastly, we have modeled a stable version of the noncovalent dimeric form of Ca\(^{2+}\)-complexed 1 with a disulfide-constrained antiparallel dimer. This dipetide is able to antagonize NMDA-elicited current at NR1a/NR2B- and NR1a/NR2B-containing receptors, whereas the disulfide-containing parallel species is completely inactive at the four NMDAR subunit combinations examined. This implies that in the Ca\(^{2+}\)-containing physiological milieu, in which a minor amount of noncovalent dimeric 1 exists in equilibrium with the monomer, both of these species are inhibitory at NR2B-containing NMDARs. Additionally, the potentiating effect of 20 at NR1a/NR2A subunit combinations, compared with the null effect of con-G, suggests that the anti-parallel dimer adopts a receptor-bound conformation that cannot be assumed by the monomer at this specific NMDAR form. Whereas this potentiation of the steady-state current was an unexpected finding, it is not unprecedented in the conantokin field. Con-G and con-G[γ7A] potentiation of glutamate-evoked responses at NR2A-containing receptors has been noted before in combination with either NR1a or NR1b subunits expressed in Xenopus oocytes (30). In that study, as

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peptide concentration was increased (\( \geq 50 \, \mu M \)), stimulation was reversed and the inhibition of currents was eventually attained. This effect was more pronounced at low (3 \( \mu M \)) versus high (30 \( \mu M \)) glutamate concentrations. (We have not observed this biphasic effect in our concentration-response studies with con-G in embryonic murine hippocampal neurons (31).) This discrepancy may stem from the difference in NMDAR source between the two studies and/or because potentiation would be expected to be negligible at the high concentration of NMDA (100 \( \mu M \)) we employed.) In contrast to the biphasic response at NR2A-containing receptors, con-G effects at NR2B-containing NMDARs were exclusively inhibitory in \textit{Xenopus}. This biphasic behavior was interpreted in terms of two distinct sites in NR2A-containing receptors that are differentially responsive to G binding. Consequently, the stimulatory effect elicited by 20 at NR1a/NR2A receptors may reflect preferential occupancy of the antiparallel dimer at the putative stimulatory site. Because this effect occurs in the presence of saturating concentrations of NMDA and glycine, binding of 20 to agonist receptor sites is an unlikely mechanism for rationalizing the stimulatory response. It also bears mentioning that some derivatives of con-G have been described that potentiate, rather than inhibit, \([3H]MK-801\) binding to rat brain membrane suspensions with characteristics suggestive of polyamine-like modulation (24).

Whereas the mechanism underlying the enhancement, by 20, of NR1a/NR2A steady-state responses remains to be elucidated, it is clear that con-G dimerization provokes receptor responses that differ significantly from the monomeric parent. Furthermore, compared with 1, 20 exhibits extremely rapid onset and offset of block at the NR1b/NR2B receptor form. This, again, indicates a mode of antiparallel dimer interaction at the putative stimulatory site in this receptor form.

In summary, the current investigation expands upon the determinants for the dimerization of conantokin-based peptides containing an \( i, i + 4, i + 7, i + 11 \) arrangement of Gla residues and demonstrates that the \( \text{Ca}^{2+} \)-mediated heteromeric assembly of such peptides is viable. As with the self-assembling conantokins, heterodimer formation appears to be driven exclusively by electrostatics and is accompanied by the antiparallel alignment of constituent subunit strands. Our results demonstrate that the intermolecular interactions guiding the dimerization of naturally occurring con-G can also be applied to designed peptides for both homo- and heteromeric systems. This property may find utility in engineered polypeptides for effecting precise modes of folding. In the case of naturally occurring con-G, helix-helix association may confer NMDAR activity that differs significantly from that of the monomeric species. This suggests the possibility that different physiological effects can be exerted by one peptide, depending upon its local monomer-dimer distribution.

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