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The crystal structure of the calcium-bound con-G[Q6A] peptide reveals a novel metal-dependent helical trimer

Sara E. Cnudde[‡], Mary Prorok[§], Xiaofei Jia, Francis J. Castellino^{§,✉}, and James H. Geiger^{‡,✉}

[‡]Department of Chemistry, Michigan State University, East Lansing, MI 48824

[§]Department of Chemistry and Biochemistry and W.M. Keck Center for Transgene Research, University of Notre Dame, Notre Dame, IN 46556

Abstract

The ability to form and control both secondary structure and oligomerization in short peptides has proven to be challenging due to the structural instability of such peptides. The conantokin peptides are a family of gamma-carboxy-glutamic acid containing peptides produced in the venoms of predatory sea snails of the *conus* family. They are examples of short peptides that form stable helical structures, especially in the presence of divalent cations. Both monomeric and dimeric conantokin peptides have been identified and represent a new mechanism of helix association, “the metallo-zipper motif” that is devoid of a hydrophobic interface between monomers. In the present study, a parallel/anti-parallel three helix bundle is identified and its crystal structure determined at high resolution. The three helices are almost perfectly parallel and represent a novel helix-helix association. The trimer interface is dominated by metal chelation between the three helices, and contains no interfacial hydrophobic interactions. It is now possible to produce stable monomer, dimer, or trimeric metallo-zippers depending on peptide sequence and metal ion. Such structures have important applications in protein design.

Keywords

Crystallography; Conantokins; γ -carboxy-glutamic acid; peptide α -helix

Introduction

The α -helix is one of two ubiquitous structural motifs on which virtually all protein and peptide structures are based. However, it has been notoriously difficult to produce short α -helical peptides because the structural stability of virtually all such helices depends upon hydrophobic packing of the helix against an adjacent structure.[1] Alternative strategies for stabilizing the α -helical conformation find broad interest in a number of fields, including de novo protein and peptide drug design. There is also significant interest in the production of stable, predictable and homogeneous peptide helix oligomers. [2–10] The use of metal ions in stabilizing peptide helices[11–14] and helix oligomers[15–18] has been applied with significant success, but all of these structures still largely rely on the hydrophobic effect for their stability, and high resolution structural information has been lacking for most. Clearly,

Corresponding author: Dr. James H. Geiger, Department of Chemistry, Michigan State University, East Lansing, MI 48824; Telephone 517-355-9715 X234; Telefax 517-353-1793; geiger@chemistry.msu.edu and Francis J. Castellino, Department of Chemistry and Biochemistry and W.M. Keck Center for Transgene Research, University of Notre Dame, Notre Dame, IN 46556; fcastell@nd.edu.

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a system that produces highly ordered, predictable helical oligomers in the absence of the hydrophobic effect could find wide use in protein design applications.

Conantokin-G (con-G) is a 17 residue peptide found in the venom of the predatory marine snail *Conus geographus*. [19] In mammals, con-G antagonizes ion flow through the glutamate/glycine activated N-methyl-D-aspartate receptor (NMDAR) ion channels, the dysfunction of which is linked to several chronic and acute neuropathologies. [20] Con-G has γ -carboxyglutamic acid (Gla) residues at positions 3, 4, 7, 10, and 14, although only Gla4 is absolutely required for NMDA receptor activity (Table 1). [21] In the absence of metal ions, con-G has no significant secondary structure. However a variety of metal ions (Cd^{2+} , Mn^{2+} , Hg^{2+} , Mg^{2+} , Ca^{2+} , and others) induce con-G to adopt a stable helical conformation throughout its length. [22] Ca^{2+} binding additionally results in the formation of an antiparallel dimeric structure. [23] We have called such metal-dependent helix interaction motifs “metallo zippers.” [24, 25]

Another member of the conantokin family, conantokin-T (con-T), shares some sequence identity to con-G and contains Gla residues at positions 3, 4, 10, and 14 (Table 1). However, several primary and secondary structural differences exist between the two peptides. Position 7 is occupied by a Lys in con-T as opposed to a Gla in con-G. While con-T does not undergo Ca^{2+} -induced self-assembly, replacement of the Lys at position 7 with a Gla, to yield the variant con-T[K7 γ], results in a peptide that does form an antiparallel helix dimer in the presence of Ca^{2+} . [23]

Even though con-G and con-T share significant sequence homology and behave similarly in biochemical assays, our crystal structures of the peptides bound to Ca^{2+} have shown that the dimerization interface of Ca^{2+} /con-T[K7 γ] is not similar to that of Ca^{2+} /con-G. [24] Both peptides form antiparallel helical dimers that are stabilized by Ca^{2+} -mediated chelation, but the relative orientation of the helices, as well as the Ca^{2+} coordination in the interface, are completely different. The two helices of con-T[K7 γ] are almost parallel and make significantly more cross-helix interactions, consistent with the higher Ca^{2+} binding affinity of con-T[K7 γ], whereas the two helices of con-G are rotated significantly, more reminiscent of a coiled-coil structure. We hypothesized that this radical difference is due to the conformation of the conserved residue Gln6. Therefore, in this study, the critical residue, Gln6, is mutated to Ala in con-G to test this hypothesis. We report herein the x-ray crystallographic structure and metal binding properties of the Ca^{2+} /con-G[Q6A] complex, which forms a unique, parallel/antiparallel three helix bundle structure stabilized exclusively by metal coordination, and devoid of any hydrophobic interactions in its interface. This further demonstrates the potential of these peptides to produce very novel helical structures.

Materials and Methods

Peptide Synthesis, Purification, and Characterization

Con-G[Q6A] was synthesized, purified, and characterized analogously to previously described methods. [22]

Crystallization

The lyophilized solid of con-G[Q6A] was dissolved in 50 mM CaCl_2 /100 mM Tris-HCl, pH 8.0, to a concentration of 15 mg/ml. Crystals were grown at room temperature by the hanging drop vapor diffusion method by mixing equal volumes of the protein solution described above with the reservoir solution containing 30% PEG monomethyl ether 5000/0.1 M MES pH 6.5/0.2 M AmSO_4 . The crystals appeared within 4 days.

Data Collection

The crystals were briefly soaked in a solution containing 30% PEG monomethyl ether 5000/0.1 M MES pH 6.5/0.2 M AmSO_4 /30% glycerol at room temperature and flash frozen by immersion in liquid nitrogen. Data were collected at the Advanced Photon Source LS-CAT 21-DG at Argonne National Laboratory using radiation with a wavelength of 0.9785 Å and processed and scaled using the HKL2000[26] suite of programs to a resolution of 1.4 Å in the monoclinic space group $P2_1$. The crystal-to-detector distance was 120 mm and 360° of data were collected with an oscillation of 1°. The crystal parameters and data collection statistics are listed in Table 2.

Structure Determination

The phases were determined by molecular replacement using the program Phaser[27] and the Ca^{2+} -con-G structure as search model (PDB ID 2DPQ). Using three molecules per asymmetric unit, a Matthews coefficient of 1.9 and solvent content of 33.4% was calculated, consistent with the existence of three molecules per asymmetric unit.

Refinement of con-G[Q6A]

Rigid body refinement using the Refmac5[28] (CCP4[29] suite of programs) resulted in an R_{work} of 45.74 and R_{free} of 43.07%. Six Ca sites were located in the $F_o - F_c$ map contoured at 8σ (Figure 1). Subsequent refinements of all three molecules of con-G[Q6A] after adding six Ca^{2+} and 75 waters using anisotropic refinement produced a final R_{work} of 14.54% and R_{free} 18.26%. Refinement statistics are listed in Table 2.

Analytical ultracentrifugation

The average molecular weights (M_w) of apo, Ca^{2+} -, and Cd^{2+} -saturated con-G[Q6A] were determined by sedimentation equilibrium in a Beckman XL-I analytical centrifuge. All experiments were conducted at a con-G[Q6A] concentration of 200 μM in the absence and presence of CaCl_2 (40 mM) or CdCl_2 (5 mM). The buffer consisted of 10 mM sodium borate/100 mM NaCl, pH 8.0. Samples were rotated at 20 °C at speeds of 42,000 and 52,000 rpm. Scans were conducted in absorbance mode at 237 nm. Based on amino acid composition, a partial specific volume of 0.701 ml/g was calculated for con-G[Q6A]. For this purpose, Glu residues were assigned the partial specific volume of Glu (0.66 ml/g). M_w determinations ($\pm$ SD) from the data were obtained using the single ideal species model included in the Beckman XL-I software and represent the average of duplicate runs of 4 scans each at the two indicated rotor speeds.

Isothermal Titration Calorimetry (ITC)

Heat changes accompanying the binding of Ca^{2+} and Cd^{2+} to con-G[Q6A] were measured using a VP-ITC calorimeter (MicroCal, Inc, Northampton, MA). Peptide (0.1–0.4 mM) was dissolved in 10 mM sodium borate/100 mM NaCl, pH 8.0 and added to the reaction cell. Solutions of titrant CaCl_2 or CdCl_2 (in 25–50 fold molar excess of the peptide sample) were prepared in the same buffer and delivered into the reaction cell at 3 min intervals in 5–10 μL increments. To correct for heats of dilution of titrant, the heat changes resulting from addition of the titrant to buffer were subtracted from the raw heats. The integrated heats were then deconvoluted for the best-fit model using the ORIGIN software supplied by MicroCal. Parameters (n , K_d , ΔH) thus obtained represent the average of at least two experiments. Error estimates were 15% for replicate experiments.

Circular Dichroism

Wavelength spectra of apo, Ca^{2+} -, and Cd^{2+} -bound con-G[Q6A] scans were recorded between 200 and 250 nm on an AVIV 202SF spectrometer using a 1 cm path length cell.

Spectra represent the average of three scans collected at 1.0 nm intervals at a 1.0 nm bandwidth at a concentration of 35 μ M peptide. The buffer used was 10 mM sodium borate/100 mM NaCl, pH 8.0. Titrations of the change in ellipticity of con-G[Q6A] with added CaCl_2 or CdCl_2 were monitored at 222 nm. The helical content of con-G[Q6A] under free and metal ion-bound conditions was determined from the mean residue ellipticities at 222 nm using the equation, % helix = $(-(\Theta)_{222}-2,340)/30,300 \times 100$. [30]

Results

The overall structure of the Ca^{2+} /con-G[Q6A] complex is shown in Figure 1. The length of all three con-G[Q6A] helices is approximately 26 Å. A total of six Ca^{2+} and 75 water molecules were included in the model. Alternative side-chain conformations were modeled for residues Ile12, Lys15, and Ser16 of Helix A and Leu5 and Ser16 of Helix B. The C-terminal amide is present in all three helices.

The structure of the helical trimer

The high-resolution crystal structure shows that instead of the expected antiparallel helical dimer, a parallel/antiparallel trimer is formed instead. Figure 1a shows that two of the helices (Helix A and Helix B) are in an antiparallel orientation whereas Helix A and Helix C have a parallel orientation. Helix B and Helix C are necessarily also orientated in an antiparallel fashion. The axes of the three helices in the trimer are orientated almost parallel to one another. Four of the Gla residues (3, 7, 10, 14) are present on one face of the helix, specifically the Ca^{2+} coordination interface, while Gla4 resides on the opposite side of the helix and is not involved in Ca^{2+} coordination. In the presence of Ca^{2+} , wild-type con-G forms an antiparallel dimeric structure with Helix A and Helix B where Helix B is rotated reminiscent of a coiled-coil. [24] The same naming convention is used here where the antiparallel helices are labeled Helix A and Helix B, with the third helix designated Helix C. If each helix is viewed in isolation, the Ca^{2+} coordination is also very similar to that seen in both the con-G/ Ca^{2+} and con-T[K7 γ]/ Ca^{2+} structures, with 2 Ca^{2+} bound to each helix by bidentate coordination via Gla3, Gla7 and Gla10, Gla14, respectively, and we will therefore preserve the Ca labeling as previously described for the Ca^{2+} /con-T[K7 γ] and Ca^{2+} /Con-G/ structures (Ca1A is coordinated by Gla3 and Gla7 of Helix A, Ca2A is coordinated by Gla10 and Gla14 of Helix A and Ca1B, Ca2B, Ca1C and Ca2C are similarly coordinated by Helix B and Helix C, respectively (Figure 2)). [24] Figure 1a shows that the Calcium ions from the three helices interdigitate perfectly with a helix B calcium, Helix A calcium and Helix C calcium following in order. It is thus the Calcium ions that interlock to produce the metallo-zipper motif.

The trimer interface is dominated by intermolecular Ca^{2+} coordination between the three helices (Figure 2). All six Ca^{2+} are seven-coordinate and all six coordinate at least one water molecule. Figure 2c shows that Ca1B coordinates with only two helices, Helix A (Gla14) and Helix B (Gla3 and Gla7), and with two waters (W1 and W2) (Figure 2c). Ca1C coordinates exclusively with the two parallel helices (Gla3 of Helix A and Gla3 and Gla7 of Helix C) and two water molecules (W8 and W29) (Figure 2e). Therefore, the calcium ions that define each end of the interface, Ca1B and Ca1C, coordinate with only two of the three helices and both coordinate to Helix A. The four calcium ions that define the interior of the interface, Ca1A, Ca2A, Ca2B, and Ca2C, coordinate with all three helices in the trimer. Ca1A coordinates with Helix A (Gla3 and Gla7), Helix B (Gla14), Helix C (Gla7), and with water W4 (Figure 2a). Ca2A coordinates with Helix A (Gla10 and Gla14), Helix B (Gla7), Helix C (Gla14) and water W7 (Figure 2b). Ca2B coordinates with Gla7 of Helix A, Gla10 and Gla14 of Helix B, Gla10 of Helix C, and with water W2 (Figure 2d). Figure 2f shows that Ca2C coordinates with Gla14 of Helix A, Gla10 of Helix B, Gla10 and Gla14 of Helix C, and one water (W7). In addition to the metal coordination, Arg13 from each of the three

helices forms a salt bridge with one of the adjoining helices (Arg13 of Helix A with Glu3 of Helix B, Arg13 of Helix B with Glu3 of Helix A and Arg13 of Helix C with Glu14 of Helix A).

Figure 3a shows all three helices overlayed. Although a few subtle changes are noticeable, viz., E2 in Helix B has a different conformation from that seen in Helix A and Helix C, Glu10 moves slightly between all three helices, and Leu5 and Gln9 of Helix B are also different, these conformational differences are all outside the trimer interface. Figure 3b shows all three helices overlayed with native con-G. Again, though con-G residues Glu2 and Q9 have different conformations than that seen in con-G[Q6A], all other residues, including all the residues within the interface, are similar. Arg13 is in exactly the same position in all structures.

Determination of the aggregation state in solution by analytical ultracentrifugation

To determine if the forces mediating the trimeric form of Ca^{2+} -complexed con-G[Q6A] in the crystal also operate in solution, we performed sedimentation equilibrium on con-G[Q6A] in free and Ca^{2+} -saturated states. The apo form of the peptide was determined to have a M_w of 2560 ± 150 , in good agreement with the calculated molecular weight of 2208 that was obtained from the primary sequence. In the presence of 40 mM Ca^{2+} , the M_w increased to 4380 ± 80 . This value is clearly not reflective of the trimeric form of the peptide, but is consistent with the presence of a dimer, as has been observed for the Ca^{2+} -saturated forms of other conantokins, including con-G and con-T[K7 γ].[22, 23] This discrepancy between the molecularity of Ca^{2+} -bound con-G[Q6A] in the solution and crystalline states may be reflective of packing interactions in the crystal lattice that cannot be recapitulated in solution. To ascertain if con-G[Q6A] has the capability of forming higher order complexes, we performed sedimentation equilibrium ultracentrifugation on the peptide in the presence of Cd^{2+} (Figure 4). In the case of con-T[K7 γ], this metal ion, like Ca^{2+} , was shown to trigger dimer formation of the peptide.[25] However, the binding affinity of Cd^{2+} for con-G[K7 γ] is nearly 40-fold greater than that displayed by Ca^{2+} . Hence, we reasoned that if comparable differences in the relative binding affinities of Ca^{2+} and Cd^{2+} were also occurring with con-G[Q6A], then the stronger Glu-metal ion interpeptide bridging interactions existing in the Cd^{2+} -con-G[Q6A] complex (compared to the Ca^{2+} -con-G[Q6A] complex) could be sufficient to mediate trimer formation. In the presence of 5 mM Cd^{2+} , the M_w of con-G[Q6A] was found to be 6250 ± 330 , indicative of a species of higher order than a dimer. Representative scans of the apo, Ca^{2+} -, and Cd^{2+} -complexed states of the peptide are shown in Figure 4.

Calorimetric measurements of Ca^{2+} and Cd^{2+} -bound Con-G(Q6A) peptide

Insofar as the higher M_w associated with the Cd^{2+} -complexed form of con-G[Q6A] could represent numerous Cd^{2+} ions bound to the dimer, we conducted isothermal titration calorimetry (ITC) on the peptide to determine the stoichiometries and binding constants attending the binding of Ca^{2+} and Cd^{2+} (Figure 5). The sequential binding model provided the best-fit for the isotherms accompanying both metal ion titrations from which binding parameters were obtained by constraining the metal ion stoichiometries to 2.0 and 3.0 for Ca^{2+} and Cd^{2+} , respectively (Figure 5). In the case of Ca^{2+} , this would correspond to an n of 6 in the trimer state of con-G[Q6A], in sound agreement with the 6 Ca^{2+} ions observed in the crystal structure. For the experiments performed with Ca^{2+} , K_d values of 590 μM and 720 μM were calculated for sites 1 and 2, corresponding to ΔG values of approximately -4.4 kcal/mol for occupation of each site. These were further characterized by molar enthalpies (ΔH) of 0.77 kcal/mol and -0.44 kcal/mol, respectively, revealing that the binding of Ca^{2+} to con-G[Q6A] is primarily driven by favorable entropies in order to compensate for the unfavorable enthalpy associated with site 1 and the weakly favorable ΔH

value accompanying Ca^{2+} binding to site 2. In contrast to Ca^{2+} , much tighter binding was observed for the complexation of Cd^{2+} to con-G[Q6A]. K_d values for Cd^{2+} were estimated at 20 μM for Cd^{2+} sites 1 and 2 and 55 μM for a third, lower affinity Cd^{2+} site. Favorable molar enthalpies of -2, -6 and -0.8 kcal/mol were determined for Cd^{2+} sites 1, 2, and 3, respectively. The relative differences in ΔH observed for Ca^{2+} and Cd^{2+} complexation with con-G[Q6A] in solution are diagnostic of much stronger binding between Cd^{2+} and the side-chains within the peptide binding interface than occurs in the case of Ca^{2+} binding. Furthermore, the stoichiometry of 3 mol Cd^{2+} /mol peptide determined from the ITC experiments translates to an n of 6 mol Cd^{2+} /mol dimer and 9 mol Cd^{2+} /mol trimer. As such, the M_w of 6250, obtained under sedimentation equilibrium conditions for Cd^{2+} -saturated con-G[Q6A], are supportive of a metal ion-mediated trimer, as a Cd^{2+} stoichiometry of 6 for the dimeric form is insufficient to account for the observed M_w . The thermodynamic parameters are tabulated in Table 3.

A possible contributor to the apparent stability of the Cd^{2+} -induced trimeric solution state of con-G[Q6A] may relate to the increased stability of the Cd^{2+} -bound helical form compared with the Ca^{2+} -bound counterpart. As shown in the CD spectra in Figure 6, both metal ions induce helix formation in the unstructured apo-peptide. However, the helical content of con-G[Q6A] in the presence of saturating Cd^{2+} (330 μM) is 85%, whereas in the presence of saturating Ca^{2+} , this value is 37%. As shown in the inset to Figure 6, the concentration of Cd^{2+} required to effect maximal helical content is much less than that observed with Ca^{2+} . Fits of these titration data to obtain the effective total concentrations of Ca^{2+} and Cd^{2+} corresponding to 50% of the maximal change in ellipticity at 222 nm (i.e., EC_{50}) yielded values of $560 \pm 70 \mu\text{M}$ for Ca^{2+} and $51 \pm 13 \mu\text{M}$ for Cd^{2+} . This 10-fold concentration difference for helix induction roughly parallels the K_d values obtained from the direct binding experiments conducted by ITC.

Discussion

Our original assumption was that removal of the Gln6 side chain would allow con-G to adopt a con-T[K7 γ]-like antiparallel helix interface, which would be preferred over the lower stability con-G-like interface. An overlay between Helix A of the con-G[Q6A] trimer and helix A of the con-G dimer structures (Figure 7a) shows that indeed helix B does not overlay, nor do the calcium ions predominantly coordinated to the Helix B Glu residues (Ca1B and Ca2B). Only Ca1A and Ca2A, the calcium ions associated with the Glu residues of Helix A overlay relatively well. On the other hand, when Helix A of con-G[Q6A] is overlayed on Helix A of con-T[K7 γ], the Helix B's also overlay quite well (overall rmsd of 0.62 Å for all Ca atoms), as do all four Calcium ions (Ca1A, Ca2A, Ca1B and Ca2B) found in this interface (Figure 7b), proving that Con-G[Q6A] does indeed produce an antiparallel helix-helix interaction that is very similar to that of con-T[K7 γ], as we expected. It is therefore clear that the presence of the Gln6 sidechain prevents the con-T[K7 γ]-like interface from forming in the case of con-G and that removal of this sidechain allows the formation of the more stable anti-parallel helix interface. As described previously, the reason for this selectivity is that Gln6 flips into the interface, coordinating Ca2B, and thus interfering with the con-T[K7 γ]-like interface by colliding with Arg13 (Figure 7c). Gln6 is flipped out of the interface in con-T[K7 γ], which allows the more stable interface. We suspect that it is this dimer, between helix A and B that constitutes the Ca-bound dimer seen in solution.

Although our prediction regarding the importance of the conformation of Q6 is accurate, we were surprised to find that the mutant forms a trimer in the crystal. Because the interface between Helix A and Helix B of both con-G[Q6A] and con-T[K7 γ] is similar, the question is: why doesn't con-T[K7 γ] form a trimer in the presence of Ca^{2+} ? The Ca^{2+} /dimer

interface of con-T[K7 γ] contains an extensive spine of water molecules that are chelated by Ca²⁺. This spine of hydration is not seen in Ca²⁺-bound wild-type con-G or con-G[Q6A]. Helix C of the trimer fulfills some of the hydration contacts with Ca²⁺. Figure 7d shows that Helix C Glu residues have displaced most of the water molecules that are involved in the water spine seen in Ca²⁺/con-T[K7 γ], specifically, W71, W79, W53, W80, W37, W142, W68, and W95. It is possible that the stability of this water network in the con-T[K7 γ]/Ca²⁺ complex inhibits the binding of the third helix in this structure.

Interestingly, though con-G[Q6A] only forms a dimer in solution when bound to Ca²⁺, Cd²⁺ binding results in a stable trimer in solution. ITC measurements indicate that Cd²⁺ binds with much higher affinity than does Ca²⁺, and that Cd²⁺ binding is both enthalpy and entropy favored while Ca²⁺ binding is only entropically favored. Further, the binding of the second Cd²⁺ ion leads to a significantly larger negative enthalpy change and a much smaller entropy change than binding of the other metal ions. Given that the peptides are at 0.2 mM concentration, the same concentration used in the AUC experiments, and the fact that the structure shows intimate involvement of 2 metal ions from each helix in both the trimer and dimer, the binding of the second metal ion may correspond to oligomerization in each case. Therefore, the increased stability of the trimer with Cd²⁺ is likely due to the more negative enthalpy of the metal/peptide interaction, and that entropy favors metal binding far more than it favors trimerization. This seems to indicate that Cd²⁺ prefers coordination to the negatively charged carboxylate groups of the Glu residues over water, giving a negative enthalpy change upon peptide binding while Ca²⁺ has little preference of water over Glu, resulting in very small enthalpy change upon binding. This is not unexpected as the electronegativity of Cd is higher than that of Ca (1.6 versus 1.0) and thus may interact more strongly with the negatively charged carboxylate than with the neutral water ligand. Figure 8 schematically represents con-G, con-T[K7 γ], and con-G[Q6A] dimer and trimer interfaces and illustrates the larger number of carboxylate-metal interactions in the trimer relative to the dimers.

Virtually all helix-helix interfaces are predominantly hydrophobic in nature, consisting of hydrophobic groups inserting into the empty spaces in the partner helix. Though there are examples of helix dimers that are partially stabilized via metal ion coordination, the interfaces are still predominantly hydrophobic.[13, 14] The conantokin peptides have provided a new paradigm in helix-helix association, the “metallo zipper” that consists of an interface that is almost exclusively due to metal ion coordination, and has no hydrophobic contacts between helices. The resultant helix dimers are remarkably stable, especially given the length of peptide, and are readily amenable to high-resolution structural analysis, which is rare for short peptides. In addition, we have found that single amino acid changes in these peptides can give rise to quite different helix orientation and altered metal coordination interfaces. Here we have shown that the metallo-zipper motif can be expanded to produce a parallel/antiparallel helix trimer. This helix trimer consists of three helices that are in an almost perfectly parallel orientation, which to our knowledge is completely novel in three helix bundles.

The parallel/antiparallel three helix bundle we have produced is held together almost exclusively by metal coordination, with no hydrophobic interactions in the helix interface. The metal ions from each of the three helices perfectly interdigitate forming an almost linear sequence of metal ions that define the helical interface. The fact that the helices are almost perfectly parallel (or antiparallel) to one another, and the interdigitation of metal ions leave open the possibility that longer tri-helical metallo zippers could be made by extending the peptide motif over more turns of helix. This is, to our knowledge, a novel type of peptide trimer. Our ability to control helix orientation and oligomerization state via amino acid changes in the metallo-zipper assemblies make them of great use to the protein design

community. The ease with which high-resolution crystal structures can be produced also makes this system ideal for rational design applications.

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Abbreviations used

Con conantokin. **Gla**, γ -carboxyglutamate. **γ** , Gla (abbreviation only used in figures). **NMDAR**, N-methyl-D-aspartate receptor. **RMSD**, root mean square deviation

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Fig. 1.

The overall structure of the $\text{Ca}^{2+}/\text{con-G}[\text{Q6A}]$ complex. a) An F_o-F_c map, calculated in the absence of metal ions, contoured at 8σ is shown (blue cage). Helix A is colored cyan, Helix B is green, and Helix C is colored magenta. The red crosses are Ca^{2+} . Helix A and Helix B are antiparallel. Helix B and Helix C are antiparallel. Helix A and Helix C are parallel. b) Top view of the trimer showing all Glu residues. Calcium ions are blue spheres and side chain carbon atoms are colored by helix and oxygens are colored red.

Fig. 2. Metal coordination in the con-G[Q6A] calcium complex. a) Ca1A is seven-coordinate. Ca1A coordinates with all three helices in the trimer. It coordinates with Gla3 and Gla7 of Helix A (magenta), Gla14 of Helix B (green), and Gla7 of Helix C (cyan) as well as with one water, W4. b) Ca2A is seven-coordinate. Ca2A coordinates with all three helices within the trimer, including Gla7 of Helix B (green), Gla14 of Helix C (cyan), and Gla10 and Gla14 of Helix A (magenta), as well as with W7. c) Ca1B coordinates with only helix A and B within the trimer, specifically Gla3 and Gla7 of Helix B (green), and Gla14 of Helix A (magenta), along with W1 and W72. d) Ca2B is seven-coordinate. Ca2B coordinates with all three helices in the trimer specifically Gla7 of Helix A (magenta), Gla14 and Gla10 of Helix B (green), and Gla10 of Helix C (cyan), as well as W2. e) Ca1C is seven-coordinate. Ca1C coordinates with Gla3 of Helix A (magenta) and Gla3 and Gla7 of Helix C as well as with two waters W8 and W29. It does not coordinate with molecule B. Ca1C is strictly involved in chelating parallel helices A and C. f) Ca2C is seven-coordinate. Ca2C coordinates with all three helices, specifically Gla10 and Gla14 of helix C (cyan), Gla 14 of helix A (magenta), gla10 of helix B (green) and additionally W7. All distances are shown in Angstroms.

Fig. 3.
 a) The three helices in the trimer are overlaid. b) All three helices overlaid with con-G. The view is rotated approximately 1/3 of a helical turn relative to a. Con-G is colored yellow, con-G[Q6A] Helix A is magenta, Helix B is green, and Helix C is cyan. All side chains are colored by atom save carbon, which is colored by helix.

Fig. 4. Sedimentation equilibria data showing the effects of Ca^{2+} and Cd^{2+} on the M_w of con-G[Q6A]. Each depicted absorbance scan was collected at 237 nm at a rotor speed of 45,000 rpm at a peptide concentration of 200 μM . The buffer was 10 mM sodium borate/100 mM NaCl, pH 8.0. The data were obtained under the following conditions: (O) no added metal ion, (□) 40 mM CaCl_2 , (Δ) 5 mM CdCl_2 . For simplification in assessing the effects on M_w of added metal ions, the data were fit to a single ideal and are shown as lines as follows: (—) no added metal ion, (----) 40 mM CaCl_2 , (.....) 5 mM CdCl_2 . The M_w values derived from these specific scans are 2500, 4490, and 6590 for free, Ca^{2+} -, and Cd^{2+} -bound con-G[Q6A], respectively. The *inset* represents the distribution of residuals for the indicated fits. Symbols are the same as those used for the primary absorbance data.

Fig. 5.

Calorimetric data representing the raw heat changes (upper panels) and integrated heats (lower panels) for the titration of con-G[Q6A] with Ca²⁺ and Cd²⁺. a) Titration of 0.4 mM con-G[Q6A] with 10 mM CaCl₂ (10 μ L injections). b) Titration of 0.1 mM con-G[Q6A] with 5 mM CdCl₂ (7 μ L injections). The buffer was 10 mM sodium borate/100 mM NaCl, pH 8.0. The best fits (solid lines) for the integrated heats were obtained with a sequential binding sites model in which metal ion stoichiometries of 2.0 and 3.0 were employed for the Ca²⁺ and Cd²⁺ isotherms, respectively.

Fig. 6. Circular dichroism wavelength scans of con-G[Q6A] in free and Ca^{2+} - or Cd^{2+} -saturated forms. The buffer used was 10 mM sodium borate/100 mM NaCl, pH 8.0. The spectra shown represent con-G[Q6A] in (—) the absence of metal ion, and in the presence of (-----) 30 mM CaCl_2 or (.....) 330 μM CdCl_2 . The inset shows the change in mean residue ellipticity at 222 nm as a function of (○) CaCl_2 or (□) CdCl_2 concentration, with the lines representing the fit to the equation for a one-site hyperbola.

Fig. 7.

Structural comparisons. a) Comparison of the con-G and con-G[Q6A] structures. Con-G[Q6A] (Helix A and B of con-G[Q6A] are shown in green with 6 Ca^{2+} colored as green spheres) and con-G (blue helices with five Ca^{2+} colored as blue spheres) Helix A's are overlayed. Helix C has been omitted for clarity. con-G[Q6A] antiparallel helices A and B are orientated parallel similar to the interface seen in con-T[K7 γ] whereas in con-G helices A and B assume a more coiled-coil conformation. Some of the Ca^{2+} positions in con-G are similar to con-G[Q6A] especially Ca1A, and Ca2A. However, the rest of the calcium ions do not overlap. b) Comparison of the G[Q6A] (Helix A is magenta and Helix B is green with 6 blue spheres as Ca^{2+}) and con-T[K7 γ] (blue helices with four Ca^{2+} colored as orange spheres) structures. Helix C has been omitted for clarity. con-G[Q6A] antiparallel helices A and B are orientated similar to the interface seen in con-T[K7 γ]. The Ca^{2+} positions in con-T[K7 γ] are almost identical to con-G[Q6A]. c). Role of Q6. R13 makes a salt bridge with Gla3. Gln6 of con-G (yellow) would crash with R13. Helix A is magenta and Helix B is green of con-G[Q6A]. d). Helix C Gla residues have displaced water molecules in Ca^{2+} /con-T[K7 γ] that are involved in the water spine. con-T[K7 γ] is colored blue and its Ca^{2+} are shown as blue spheres and the waters are shown as red spheres. Helix A is orientated at the top and Helix B is on the bottom. Helix C of the trimer is shown in cyan. Residues are colored by atom.

Fig. 8.
A schematic comparing all Ca²⁺ bound structures

Table 1

Amino acid sequences of some Conantokins.

Con-G	GE $\gamma\gamma$ LQ γ NQ γ LIR γ KSN
Con-G(Q6A)	GE $\gamma\gamma$ LA γ NQ γ LIR γ KSN
Con-T	GE $\gamma\gamma$ YQKML γ NLR γ AEVKKNA
Con-T(K7Gla)	GE $\gamma\gamma$ YQ γ ML γ NLR γ AEVKKNA
Con-R	GE $\gamma\gamma$ VAKMAA γ LAR γ NIAKGCKVNCYP

Table 2Data collection and refinement statistics for the Ca^{2+} /con-G[Q6A] complex.

Ca^{2+} /con-G[Q6A]	
Data collection	
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)	23.5, 40.1, 26.9
α, β, γ (°)	90.0, 104.1, 90.0
Resolution (Å)	1.4(1.45-1.40)
R_{merge}	7.7(12.2)
$I / \sigma I$	42.68(5.44)
Completeness (%)	95.2(74.6)
Refinement	
Resolution (Å)	24-1.3
No. measured reflections	502,416
No. unique reflections	9584
$R_{\text{work}} / R_{\text{free}}$	14.54/18.26
No. atoms	
Protein	889
Ca^{2+}	6
Water	75
Mean B -factors (Å ²)	17.3
R.m.s deviations	
Bond lengths (Å)	0.01
Bond angles(°)	1.26

* Highest resolution shell is shown in parentheses

Table 3

thermodynamic parameters for metal binding to con-G[Q6A]

Metal sites	Ca			Cd		
	Kd μM	ΔH kCalmol ⁻¹	T ΔS kCalmol ⁻¹	Kd μM	ΔH kCalmol ⁻¹	T ΔS kCalmol ⁻¹
M1	590	0.77	5.1	20	-2	4.4
M2	720	-0.44	3.8	20	-6	0.4
M3				55	-0.8	5.0