Metal-ion-binding properties of synthetic conantokin-G

Tamas BLANDL, Jaroslav ZAJICEK, Mary PROROK and Francis J. CASTELLINO¹

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, U.S.A.

The secondary structure of the synthetic 17-residue peptide, conantokin-G (con-G), a γ -carboxyglutamate-containing marine cone snail neuroactive protein, is altered from a random conformation to one containing a very high level (> 70%) of α -helix on binding of multivalent cations. The proportion of α -helix formed correlated well with the size of the cation and ranged from a low of approx. 7% with large cations, such as Ba²⁺, to more than 70% with smaller cations, such as Mn²⁺, Mg²⁺ and Zn²⁺. The valency of the multivalent cation was not as important, since trivalent lanthanides (Eu³⁺, Gd³⁺ and Tb³⁺) of ionic radius 106–109 pm induced similar levels (50–60%) of helix to those induced by Ca²⁺ and Cd²⁺ (ionic radii 109 and 114 pm respectively). Although the correlation was not as tight, smaller cations of the same valency allowed the helical transition to occur at lower concentrations than the larger cations. The spectroscopic and spectrometric properties of some of these cations permitted a more detailed analysis of the molecular nature of the cation–con-G binding. EPR-based titrations with Mn²⁺ provided a binding isotherm that was deconvoluted to a single class of 2–3 Mn²⁺ sites of average K_d 3.9 μ M. This number of sites was similar to that for Ca²⁺ [Prorok, Warder, Blandl and Castellino (1996) *Biochemistry* 35, 16528–16534], but a much lower K_d was displayed with Mn²⁺. Determinations by ¹H NMR of the longitudinal relaxation rates of the water protons in

Mn²⁺/con-G solutions at different magnetic field strengths corresponding to the proton Langmuir frequencies of 24, 300 and 500 MHz permitted calculation of the hydration number of Mn²⁺ in the complex, which was found to be 1.0. This indicates that five of the six co-ordination sites of Mn²⁺ are occupied by peptide atoms, probably oxygens. Titrations of the changes in Tb³⁺ fluorescence as a result of its binding to con-G gave an EC₅₀ of 58 μ M, a value nearly identical with that obtained by titration of the change in helicity of the peptide as a function of Tb³⁺ concentration. This shows that the macroscopic binding of Tb³⁺ to con-G is directly responsible for the alteration in secondary structure of the peptide. Finally, Cd²⁺ was found to be an extremely suitable cation for an NMR-based investigation of the amino acid residues of apo-con-G that are perturbed by cation binding. In a limited example of the results of this study, it was discovered that originally equivalent CH₂^δ protons of Arg¹³ became distinctly magnetically non-equivalent in the Cd²⁺-bound helical form of con-G. This indicates that Arg¹³ is situated in the helix in such a way that the mobility of its side chain is highly restricted. In conclusion, the data show that a variety of multivalent cations with measurable spectroscopic and spectrometric properties interact similarly with con-G and generate extensive α -helical conformation in this peptide.

INTRODUCTION

Venomous predator snails of the *Conus* family employ a variety of neuroactive peptides to paralyse their prey. These agents selectively block ion channels by acting on various types of neuro- and/or skeletal-muscular receptors, thereby elaborating their biological effects. Among the large number of peptides present in these venoms are conantokin-G (con-G) and conantokin-T (con-T), found in *Conus geographus* and *Conus tulipa* respectively [1,2]. These polypeptides produce a sleep-like state in young mice and a hyperactive response in older mice [3,4]. The molecular basis of these effects resides in the ability of the peptides to act on ligand-gated Ca²⁺ channels, specifically as antagonists of glutamate/glycine receptors of the *N*-methyl-D-aspartate subclass [2,5,6]. This antagonist function has been described on a molecular level to result from the non-competitive inhibition by the peptides of the polyamine potentiation of agonist binding at the *N*-methyl-D-aspartate receptors [7].

Con-G and con-T contain 17 and 21 amino acids respectively. Neither of these peptides possess disulphide bonds, in contrast with a number of conotoxins isolated from the venoms of some members of this marine snail family. In addition, a relatively high abundance of γ -carboxyglutamic acid (Gla) residues is present in both peptides, namely four residues/molecule in con-T and five residues/molecule in con-G [1,2]. This finding suggests that these conantokins have an affinity for multivalent cations. Indeed, it

has been found that Ca²⁺ interacts with both con-G and con-T thereby stabilizing their α -helical forms [4]. Other metal ions, such as Mg²⁺ [4] as well as Zn²⁺ and Cu²⁺ [8], also stabilize the helical forms of these peptides. This is especially observable for con-G, which lacks significant amounts of α -helix in the absence of metal cations [4,8]. It has therefore been proposed that binding of cations such as Ca²⁺ and Mg²⁺ results in and stabilizes peptide conformations necessary for their interactions with the receptor sites of con-G and con-T [4].

To understand more completely the specificity of the cation-binding site(s) of these neuroactive peptides, the binding properties of several multivalent cations that possess a variety of spectroscopic and spectrometric properties are the subject of the current investigation. Initial focus is centred on cation binding to con-G because this peptide displays a more dramatic conformational alteration in the presence of multivalent cations.

EXPERIMENTAL

Peptide synthesis, purification and characterization

Peptides were synthesized and characterized as previously reported [4] using an Applied Biosystems (Foster City, CA, U.S.A.) 433A peptide synthesizer and standard Fmoc protecting group strategy. The following side-chain-protecting groups were used:

Abbreviations used: con-G, conantokin-G; con-T, conantokin-T; Gla, γ -carboxyglutamic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; TOF-MALDI-DE-MS, time-of-flight matrix-assisted laser-desorption-ionization-delayed-extraction MS.

¹ To whom correspondence should be addressed.

O-tBu for Glu, Ser, Tyr; trityl for Asn, Gln; 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg; Boc for Lys. The peptides were cleaved from the support resin with trifluoroacetic acid in the presence of the free-radical scavengers ethanedithiol and thioanisole. The resulting peptides were purified on a DEAE-20 column (1.5 cm × 20 cm), connected to an FPLC system (Pharmacia, Piscataway, NJ, U.S.A.). A 600 ml linear gradient of 100–600 mM NaCl was applied in a buffer composed of 10 mM sodium borate, pH 8.0. The peptides were characterized by reverse-phase HPLC, time-of-flight matrix-assisted laser-desorption-ionization delayed-extraction MS (TOF-MALDI-DE-MS) and alkaline amino acid hydrolysis (for Glu content), as previously reported [4].

Ca²⁺ binding

Ca²⁺-binding isotherms were determined potentiometrically at 25 °C using a Ca²⁺-selective electrode (Orion Research, Boston, MA, U.S.A.) with a single-junction Ag⁺/AgCl reference electrode. The peptide was dissolved in 3 ml of 10 mM Mes (sodium salt)/100 mM NaCl, pH 6.5, to a final concentration of 2 mM. The samples were then treated with Chelex-100 resin before titration with the appropriate concentration range of CaCl₂. Electrode calibrations were performed immediately before and after titration of the sample. The electrode was used to measure the free Ca²⁺ concentrations in peptide/Ca²⁺ mixtures, and the bound Ca²⁺ concentrations were determined by subtraction of the free Ca²⁺ from the total. Treatment of the data to determine the equilibrium binding constants (*n* and *K_d*) was carried out as previously described [9].

EPR

Mn²⁺-binding studies were conducted in 10 mM Mes/100 mM NaCl, pH 6.5, at room temperature. The concentration of free Mn²⁺ in the presence of peptide was determined by the intensity of the EPR spectrum using a Bruker ER 100E X-band EPR spectrometer. Before the experiments, the buffer and peptide solutions were treated with Chelex-100 resin. EPR measurements were made in quartz capillaries (1 mm internal diameter) on 50 µl samples containing different concentrations of Mn²⁺ at a constant peptide concentration, or at various concentrations of peptide at a constant Mn²⁺ concentration. Binding isotherms were generated and analysed by non-linear multiparameter least-squares fitting, with models involving one or two classes of binding site.

Proton relaxation rate

The NMR studies were carried out in 10 mM Mes/100 mM NaCl, pH 6.5, at 25 °C. The longitudinal nuclear magnetic relaxation time of water protons was measured by the 180°–*τ*–90° inversion recovery method [10] using a Seimco pulsed NMR spectrometer operating at 24.3 MHz. Measurements were made on 50 µl samples at 50 µM total Mn²⁺ and increasing (2–100 µM) peptide concentrations. The binary enhancement values (*e_b*) were calculated from the observed enhancement of the relaxation rates, *R₁* (*R₁* ≡ 1/*T₁*), and independent measurements of the free Mn²⁺ concentration by EPR, as previously described [11].

The hydration number (*q*) of Mn²⁺ bound to con-G was calculated using the Solomon–Bloembergen equation [12,13], describing the longitudinal relaxation rate of water protons bound to paramagnetic ions, in the following form [14]:

$$\frac{1}{pT_{1M}} = \left(\frac{812}{r^6} \right) q \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2 \tau_c^2} \right)$$

where (1/*pT_{1M}*) is the normalized measured relaxation rate, *p* is the ratio of bound Mn²⁺ to the concentration of the ligand (water in this case), *r* is the Mn²⁺–water nucleus distance in the hydrated complex (2.86 Å), *τ_c* is the overall correlation time, and *ω_I* and *ω_S* are the Larmor frequencies for the proton and electron respectively. Separate values of *q* were obtained at different *p* and *T₁* values using the above equation and averaged for all points [14].

To determine the overall correlation time, *τ_c*, of the Mn²⁺–con-G complex, the above Solomon–Bloembergen equation was used once again. For this calculation, the 1/*T₁* values for water protons were measured at magnetic field strengths of 7.1 T (300 MHz) and 11.7 T (500 MHz) at several Mn²⁺ concentrations on two Varian NMR spectrometers, employing the inversion recovery pulse sequence [10]. For the experiment, a solution of 0.5 mM con-G (final concentration) was dissolved in 10 mM Mes/100 mM NaCl, pH 6.5, without inclusion of ²H₂O. The Mn²⁺ concentrations were 0, 0.25, 0.5, 0.75 and 1.0 mM.

CD

CD spectra of con-G as a function of metal ion concentration were monitored at 222 nm on an AVIV model 62DS spectrometer using a 1 cm path length cell. The peptide concentration was 35 µM in 10 mM sodium borate/100 mM NaCl, pH 8.0. Alternatively, experiments were performed in the same buffer, at pH 6.5. Mean residue ellipticities were calculated by using a mean residue molecular mass of 133 Da for con-G. The α-helical content was determined from the mean residue ellipticities at 222 nm along with the empirical relationship *f_α* = (–[Θ]₂₂₂ – 2340)/30 300 [15]. The EC₅₀ values for each metal ion were determined by non-linear least-squares fitting of the data using a Michaelis–Menten-type equation. It should be noted that *n*[peptide]/2 imposes a lower limit on EC₅₀ values obtainable with any technique, regardless of the true *K_d*.

Tb³⁺ fluorescence

Tb³⁺ was excited at 241 nm and emission was monitored at 546 nm using an SLM 8100C spectrofluorimeter. Samples (1 ml) of 50 µM peptide solutions in 10 mM Mes/100 mM NaCl, pH 6.5, at 25 °C, were titrated with TbCl₃ with continuous stirring. In the absence of con-G, the fluorescence of Tb³⁺ at any given concentration over the titration range was less than 2% of that in the presence of con-G. Nonetheless, the appropriate fluorescence of Tb³⁺ in the absence of peptide was subtracted from that present in con-G/Tb³⁺ mixtures at the same Tb³⁺ concentration. The EC₅₀ value was determined by the analysis described for the CD titrations.

Tb³⁺ emission scans were also collected in the absence and presence of con-G. In these cases, the TbCl₃ concentration was 1 mM and the con-G concentration 0 or 100 µM. Tb³⁺ was excited at 241 nm and the resulting emission scanned from 400 to 700 nm.

¹H-NMR

¹H-NMR spectra were collected on a 600 MHz Varian (Palo Alto, CA, U.S.A.) UnityPlus spectrometer, using a Nalorac 3 mm dual ¹H–¹³C probe. The peptide was dissolved in 10 mM sodium borate/100 mM NaCl, pH 6.5, in ²H₂O at 5 °C, at a final concentration of 8 mM, and treated with Chelex-100 resin. A Cd²⁺ titration was performed on the same sample in the NMR tube by small-volume additions of 100 mM CdCl₂ dissolved in the same deuterated buffer. For each sample, 512 transients were acquired in 32K complex data points over a 7400 Hz spectral

window. Zero-filling to 128K and a Lorentzian weighting function were applied before Fourier transformation.

Resonance assignments were achieved by performing two-dimensional TOCSY [16], double-quantum-filtered COSY [17] and NOESY [18] experiments. Spin systems were identified using TOCSY and double-quantum-filtered COSY followed by sequential assignments through NOESY connectivities [19]. The ^1H two-dimensional experiments were accomplished over 7400 Hz, and 40 transients were sampled in 2K complex data points for 400 t_1 increments. A 1.5 s relaxation delay and transmitter presaturation of water were used. TOCSY experiments were collected with mixing times of 30 and 85 ms. A mixing time of 200 ms was used for the NOESY experiments. All two-dimensional spectra were obtained using the hypercomplex phase-sensitive method [20], and processed as $4\text{K} \times 2\text{K}$ complex data sets with baseline correction and Gaussian weighting functions in both dimensions. ^1H chemical shifts are reported in p.p.m. using 4,4-dimethyl-4-silapentane-1-sulphonate as an external standard. Spectra were processed and analysed using VNMR (Varian) and FELIX (Hare Research) software packages.

RESULTS

Complete chemical synthesis of con-G, namely $\text{NH}_2\text{-Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn-CONH}_2$, was accomplished and the resulting material was purified and characterized as detailed in a previous publication [4]. As revealed through analytical reverse-phase-HPLC, TOF-MALDI-DE-MS and Gla analysis, highly purified con-G was obtained with the requisite chemical integrity. Ca^{2+} -binding profiles, as assessed by Ca^{2+} -specific electrode titrations, were as reported previously [4].

As CD analysis proved to be highly revealing of the conformational changes that accompanied Ca^{2+} binding to con-G [4], this approach was used to evaluate the relative abilities of other multivalent cations to induce these types of change. Examples of the data obtained with Cd^{2+} , Mn^{2+} and Eu^{3+} are illustrated in Figure 1. Clearly, binding of all three cations induced large conformational alterations in apo-con-G, with Mn^{2+} inducing the most profound change. It is seen that apo-con-G has essentially no α -helical structure, whereas in the presence of these cations, a large population of con-G (60–70%) exists in the α -

Figure 1 Effect of metal ions on the CD properties of con-G at 25 °C

The buffer used was 10 mM sodium borate/100 mM NaCl, pH 8.0. The CD spectra of con-G (35 μM) in the absence of multivalent cations (—) and in the presence of 10 mM Ca^{2+} (+ + + +), 500 μM Mn^{2+} (....), 500 μM Cd^{2+} (— · — ·) and 200 μM Eu^{3+} (— — —) are illustrated. The CD spectrum of con-G/ Ca^{2+} recorded at pH 6.5 is also shown as indicated by the solid line.

Table 1 Binding properties of metal cations to con-G as revealed by CD analysis

α -Helix content was determined from the computed saturation value of the binding curves.

Metal ion	Ion radius (pm)	α -Helix (%)	EC_{50} (mM)
Ba^{2+}	149	7	11.5
Sr^{2+}	132	20	9.1
Ca^{2+}	114	50	3.9
Cd^{2+}	109	60	0.11
Eu^{3+}	109	54	0.04
Gd^{3+}	108	61	0.21
Tb^{3+}	106	51	0.04
Mn^{2+}	97	67	0.04
Co^{2+}	89	58	0.02
Zn^{2+}	88	68	0.03
Cu^{2+}	87	43	0.12
Mg^{2+}	86	73	0.12

Figure 2 Effect of Mg^{2+} on the α -helical content of con-G

The titration with MgCl_2 was conducted at 222 nm at room temperature. The buffer used was 10 mM sodium borate/100 mM NaCl, pH 8.0, at a constant con-G concentration of 35 μM . The EC_{50} for Mg^{2+} determined from this plot was 118 μM .

helical conformation. However, these values should only be viewed as estimates of α -helical content, and are mainly used here to compare different cations. During the course of these studies, it was found to be of critical importance to rigorously remove bivalent cations from the con-G with Chelex-100 resin before the experiments, otherwise misleading information on the α -helical content of apo-con-G is obtained, as has been the case previously [21,22]. Proper removal of residual cations from con-G provides an apo-con-G CD spectrum as illustrated in Figure 1, with no observable α -helix content.

The α -helix-inducing capabilities of several other bi- and trivalent cations are listed in Table 1, along with the concentrations of the cations necessary to induce 50% of the final conformational change in con-G (EC_{50}). These latter values were determined from titrations of the CD changes with metals, and a sample of one of these experiments with Mg^{2+} is shown in Figure 2. In terms of the relative levels of α -helical conformation adopted in con-G, Mg^{2+} and Mn^{2+} induce the largest amount, and Ba^{2+} and Sr^{2+} result in much smaller conformational changes in apo-con-G. Although Ca^{2+} has been used as the inducing agent, more effective cations exist. For example, as compared with Ca^{2+} , an approximately 200-fold lower concentration of

Figure 3 Binding isotherm of Ca^{2+} to con-G using Ca^{2+} -specific electrode titrations

The peptide concentration was 2 mM. Titrations were performed in 10 mM Mes/100 mM NaCl, pH 6.5, at room temperature. The fit shown is for a single class of binding sites. $K_d = 2.4$ mM, $n = 2.9$, $h = 0.94$.

Co^{2+} is required to produce 50% of the α -helical change in con-G, and the EC_{50} values are 100- and 30-fold less with Mn^{2+} and Mg^{2+} respectively. The extent of α -helix induced by these cations, as revealed by the final molar ellipticity values, was independent of peptide concentration, up to at least 2 mM con-G. Except for the case of Cd^{2+} , which is considered separately below, this peptide concentration is the highest used in any of the experiments reported here. This, along with previous CD and NMR data [4,23], strongly suggests that secondary events accompanying cation binding, such as peptide aggregation, probably do not occur.

Tervalent cations are also very effective at inducing α -helix formation in apo-con-G. All of the metal ions tested from this group produced a similar extent of conformational alteration in con-G, at approx. 15–100-fold lower concentrations than required for Ca^{2+} (Table 1). Eu^{3+} and Tb^{3+} were equally efficient in this regard, and Gd^{3+} was approximately 10-fold less so.

The CD spectra were obtained at pH 8.0. These conditions are useful for internal comparisons under the same conditions for relative effectiveness of cations in elaborating a conformational change in the peptide, and in providing comparisons with our previous work with Ca^{2+} -con-G and Ca^{2+} -con-T complexes [4]. However, since some of these other bivalent and trivalent cations were difficult to work with at pH 8.0 using other biophysical techniques, the remaining studies were carried out at pH 6.5. No differences were found in the extent of conformational change (Figure 1) or in the EC_{50} value for Ca^{2+} of con-G at pH 6.5, compared with pH 8.0, but we needed to know whether the Ca^{2+} -binding isotherms were similar at pH 8.0 [4] and 6.5. The Ca^{2+} -binding isotherm obtained for con-G at pH 6.5 is illustrated in Figure 3. Deconvolution of this curve yielded approx. 2–3 equivalents of Ca^{3+} sites per mol of con-G, with an average K_d of 2.4 mM. These parameters were nearly identical with the values obtained previously for Ca^{2+} at pH 8.0 [4]; thus this change in pH was acceptable.

Other methods were used to examine the binding of cations to con-G. For Mn^{2+} , EPR measurements of free Mn^{2+} in Mn^{2+} /con-G mixtures allowed assessment of the stoichiometry and affinity constant for this metal ion. The binding isotherm of Mn^{2+} to con-G is provided in Figure 4. The data were best-fit to a single class of binding sites with $n = 2.2$ and $K_d = 3.9$ μM . This K_d value

Figure 4 Binding isotherm of Mn^{2+} to con-G obtained by EPR measurements

The titrations were performed at room temperature in 10 mM Mes/100 mM NaCl, pH 6.5, at a constant concentration of 50 μM Mn^{2+} with increasing concentrations of con-G. The fit illustrated is for a single class of binding sites, with $n = 2.2$, $K_d = 3.9$ μM , $h = 1.01$.

is approximately 10-fold lower than the EC_{50} obtained for Mn^{2+} from the CD titration. However, the EPR titration gave a true K_d , whereas the CD measurements under the conditions of Table 1 cannot provide EC_{50} values lower than the peptide concentration used (35 μM). Assuming tight binding (such that essentially all added cations are bound) with a two-site model, the $\text{EC}_{50} \approx [\text{peptide}]$. Thus the EC_{50} measurements of Table 1 are intended only to provide comparisons of the relative abilities of different metal cations to cause conformational change under the same conditions, and, given the characteristics of the binding isotherms defined here, the peptide concentration dictates the lower EC_{50} limit.

Paramagnetic metal cations such as Mn^{2+} also have distance-dependent effects on relaxation rates of protons. Thus ^1H -NMR

Figure 5 Binary enhancement (ϵ_b) of water proton relaxation rates by con-G

Measurements were performed on 50 μl samples at 50 μM Mn^{2+} with increasing (2–100 μM) peptide concentrations. The observed relaxation rate of water protons in the buffer was identical with the rate in a 100 μM con-G solution with no Mn^{2+} present. Binary enhancement values were calculated from the observed enhancement by con-G in the presence of Mn^{2+} over the enhancement by Mn^{2+} alone. The concentration of free Mn^{2+} was determined by EPR on the same solutions as were used for proton relaxation rate measurements. Binary enhancement values are plotted as a function of binding-site occupancy. The buffer was 10 mM Mes/100 mM NaCl, pH 6.5, at room temperature.

Figure 6 Effect of con-G on the fluorescence emission spectrum of Tb^{3+}

The fluorescence emission spectra of 1 mM TbCl_3 before (····) and after (—) the addition of con-G (100 μM) are shown. The buffer was 10 mM Mes/100 mM NaCl, pH 6.5, at 25 °C. The emission spectrum was scanned from 400 to 700 nm. The excitation wavelength was 241 nm.

Figure 7 Fluorescence analysis of the interaction of Tb^{3+} with con-G

Binding of Tb^{3+} to con-G was monitored by measuring the fluorescence emission of this cation at 546 nm (excitation at 241 nm). Titrations were performed at 25 °C in 10 mM Mes/100 mM NaCl, pH 6.5, at 25 °C. The concentration of con-G used in the titrations was 50 μM . The EC_{50} determined from this plot was 58 μM .

methods can be used to examine the effects on specific proton relaxation of Mn^{2+} binding to peptides and proteins. In the present study, pulsed NMR techniques were used to measure the effects of the Mn^{2+} –peptide complexes on the observed enhancement of the relaxation rates of solvent water protons. These binary enhancement values (ϵ_b) as a function of binding-site occupancy are illustrated in Figure 5, and are nearly constant, at approx. 3.5, across the Mn^{2+} –con-G binding range measured. The ϵ_b was also used to measure the hydration number of Mn^{2+} in the binary complex, with separate determinations of the overall correlation time, τ_c , of the Mn^{2+} –con-G complex. The value for τ_c , as determined from the dependency of T_1 values of water protons at two separate magnetic field strengths, 7.1 and 11.7 T (300 and 500 MHz), was 0.34 ns. Using these experimental parameters in the Solomon–Bloembergen equation provided a value for q of 1.0 ± 0.4 .

The fluorescence emission spectrum of Tb^{3+} after excitation at 241 nm is shown in Figure 6 in the absence and presence of con-G. The large increase in fluorescence intensity observed at 546 nm was used to monitor Tb^{3+} –con-G binding, and a relevant titration curve characteristic of this interaction is shown in Figure 7. A typical saturation curve was observed with an EC_{50} of 58 μM .

This value is similar to that obtained from titrations of the effect of Tb^{3+} on the molar ellipticity values (at 222 nm) of con-G.

Finally, the effects of Cd^{2+} on the ^1H -NMR spectrum of con-G were analysed. Figure 8 illustrates the effect of Cd^{2+} on a downfield region of the ^1H -NMR spectrum of con-G that includes many of the α -protons of con-G. Examples of resonances that undergo gradual chemical-shift changes are Asn⁸-H $^\alpha$ at 4.60 p.p.m., Glu³-H $^\alpha$ at 4.37 p.p.m., Ile¹²-H $^\alpha$ at 3.73 p.p.m., and Arg¹³-H $^\delta$ and H $^\epsilon$ at 3.31 and 3.14 p.p.m. respectively. The fact that there is little line broadening in many residues in this spectrum demonstrates that Cd^{2+} does not induce aggregation of con-G at the peptide concentration used (8 mM), which is the highest peptide concentration used in any of the techniques in this study.

DISCUSSION

The conantokins, con-G and con-T, are excellent vehicles for the investigation of structure–function relationships of biologically active macromolecules. In general, they exist in their natural states as low-molecular-mass materials, and thus are readily chemically synthesized in large quantities, and they are complete and separate gene products. In essence, these polypeptides provide concentrated forms of genetically derived protein functional sites. In addition, their primary structures are not complicated by the presence of disulphide bonds and their conformations in the presence of metal cations are fully helical [4,8,23]. Further, for our particular interests, these peptides serve as low-molecular-mass models for receptor binding, with easily manipulated primary structures for assessment of structure–receptor binding relationships, and possess a high relative abundance of Glu residues. Because of this latter property, these peptides interact with multivalent cations, and also undergo large and defined conformational alterations as a result of metal ion binding [4]. In this way, these peptides also allow us to model the cation-binding properties of Glu domains of blood coagulation proteins using intact gene products in place of protein fragments.

The characteristics of the binding isotherm of Ca^{2+} to both con-G and con-T have previously been elucidated, and have identified the types of conformational changes that these peptides undergo as a result of binding to this cation [4]. Conformational analyses are particularly noteworthy for con-G, for which an alteration of a non-helical structure to a highly α -helical conformation is observed to accompany Ca^{2+} binding. It thus became relevant to probe the nature of the binding and conformational transitions that occur in these polypeptides as a result of their interaction with other metal cations. Con-G was chosen because of the more dramatic conformational changes that resulted on metal binding. For initial considerations, a helical wheel projection of the peptide sequence (Figure 9) reveals that all five of the Glu side chains are located on one side of the helical barrel. Moreover, four Glu residues, at positions 3, 7, 10 and 14, are the only residues that fall in an area corresponding to a one-quarter turn segment of the helix. In the absence of charge neutralization, the eight negative charges that these side chains possess would provide major destabilizing forces throughout the entire length of the peptide. With multivalent metal ions bridging the Glu side chain carboxylates of these residues, the interactions between them would favour α -helix formation.

A number of bivalent and trivalent cations induce α -helical conformational transition in con-G (Figures 1 and 2 and Table 1). Some, however, *e.g.* Ba^{2+} and Sr^{2+} , do not allow the transition to proceed to completion. This could be due to the properties of the ions, differences in the numbers of cation sites on the peptides, and/or differences in the nature of the co-ordination of

Figure 8 Cd^{2+} titration of the ^1H -NMR spectrum of con-G at 5 °C

A downfield segment of the aliphatic region is shown. The peptide concentration is 8 mM, in 10 mM sodium borate/100 mM NaCl, pH 6.5, in $^2\text{H}_2\text{O}$. The Cd^{2+} concentrations are (top to bottom): 9.6, 7.2, 5.6, 4.0, 2.4, 0.8 and 0 mM. Examples of resonances that undergo gradual chemical-shift changes are highlighted above the top spectrum.

Gly-1
Glu-2
Gln-3
Arg-4
Leu-5
Ile-6
Gln-7
Asn-8
Gla-9
Gln-10
Leu-11
Glu-12
Gla-13
Asn-14
Gln-15
Lys-16
Gla-17

Figure 9 Amino acid sequence and helical-wheel projection of con-G

the metal ion-peptide complexes. The strongest correlation appears to reside in the relationships between the ionic radii of the cations and their relative abilities to induce helices in the peptides. Except for Co^{2+} and Cu^{2+} , cations that prefer nitrogen ligands over oxygen, there is a reasonable correlation between the radius of the cation and the extent of helix formation (Table 1). Smaller cations, such as Mn^{2+} , Mg^{2+} and Zn^{2+} , appear to induce relatively greater amounts of helix in con-G than larger cations, such as Ba^{2+} and Sr^{2+} . Ions with radii between these

groups of cations, such as Ca^{2+} , Cd^{2+} and the lanthanides, induce correspondingly intermediate helical contents in the peptide. The overall correlation, however, between cationic radius and extent of helical formation in the peptide induced by cation binding is reasonable, since it might be expected that smaller cations would be able to serve better as helical nucleators. Larger cations might restrict this small polypeptide from folding appropriately. No obvious fine correlation is observed between the effects of the charge and/or radius of the cation and the EC_{50} values. In support of this, it should be noted that an approximately 7-fold difference in EC_{50} values exists between Eu^{3+} and Gd^{3+} , despite their similar charges and ionic radii. A similar 3-fold disparity in EC_{50} values is seen between Mg^{2+} and Zn^{2+} . However, it is also clear from the data that the cations with the largest ionic radii, namely Ba^{2+} , Sr^{2+} and Ca^{2+} , exhibit the distinctly highest EC_{50} values for induction of α -helix in con-G, further indicating the difficulties that are encountered by the peptide in folding into a helical conformation in the presence of large cations. However, for the reasons cited above for the lower limitation of the EC_{50} values, a stronger correlation in this regard might exist if K_d values were obtained for the metal ion-con-G binding rather than EC_{50} values.

To address the question of whether these cations interacted differently with con-G, the thermodynamics of binding of Mn^{2+} to this polypeptide were measured, using EPR to monitor free Mn^{2+} concentrations in mixtures of Mn^{2+} /con-G samples. A value for n of 2.2 indicates that at least two sites are present for Mn^{2+} on con-G, similar to the two to three binding sites revealed by Ca^{2+} titrations using a Ca^{2+} -selective electrode to measure free

Ca^{2+} concentrations [4]. The average K_d for Mn^{2+} was found to be $4\ \mu\text{M}$. Thus it would appear that Mn^{2+} and Ca^{2+} interact similarly with con-G, the tighter binding of Mn^{2+} being entirely predictable from a consideration of the well-known strength of its binding to ligands such as citrate and malonate relative to that of Ca^{2+} .

Studies with Mn^{2+} were extended in order to obtain the hydration number (q) for Mn^{2+} when it is present in the Mn^{2+} –con-G complex. From the average binary enhancement value (ϵ_p) of Mn^{2+} (Figure 5) in complex with con-G, of 3.5, along with the overall correlation time (τ_c) of the Mn^{2+} –con-G complex (0.34 ns), the value of q was calculated to be approx. 1.0. From this, it is concluded that five of the six ligands to Mn^{2+} in the complex originate from the peptide, and are probably oxygen atoms. Thus Mn^{2+} is highly co-ordinated by peptide residues, perhaps explaining its relatively tight binding constant.

Another example of a useful spectroscopic probe in these systems is the fluorescence enhancement shown by Tb^{3+} when this cation is bound to con-G (Figures 6 and 7). This fluorescence change is readily titrated, thereby allowing an important comparison to be made between EC_{50} values for this cation as obtained by evaluation of its effects on the peptide and the effects of the peptide on the properties of the metal cation. The fact that the EC_{50} values obtained are virtually identical suggests that the macroscopic binding of this cation is directly responsible for the conformational change in the peptide.

Finally, Cd^{2+} was employed as an NMR probe of areas of the peptide that are perturbed by cation binding. Such studies were unsuitable with paramagnetic cations such as Mn^{2+} because they cause line broadening of nearly all the protons. The ^1H -NMR spectra of con-G obtained in the presence of a series of concentrations of Cd^{2+} (Figure 8) display little generalized line broadening, and the extent to which broadening occurs appears to reflect perturbations of specific resonances in con-G. Many of the resonances shown in Figure 8 correspond to backbone α -protons. Their resonance signal shifts to higher field on Cd^{2+} binding are readily observable in several cases, *e.g.* Asn^8 and Ile^{12} . These changes in chemical shift are attributable to the transition from random coil to α -helix. The resonances of the α -protons are usually shifted 0.1–0.6 p.p.m. upfield for a given amino acid when present in α -helix rather than a random-coil conformation [24].

Of the easily observable changes in Figure 8, integration of the multiplet at 3.23 p.p.m. yielding two protons is particularly dramatic, and this has been assigned by extensive two-dimensional homonuclear and heteronuclear NMR experiments (T. Blandl, unpublished work) to the two δ -protons of Arg^{13} . The fact that this two-proton multiplet is split into two one-proton resonance signals after additions of Cd^{2+} demonstrates that these δ protons of Arg^{13} become magnetically non-equivalent (one proton each at chemical shifts of 3.31 and 3.14 p.p.m. in 9.6 mM Cd^{2+}) in the Cd^{2+} –con-G complex. This is probably due to more restricted rotation of the side chain of Arg^{13} in the presence of Cd^{2+} , which is slow on the NMR time scale. Examination of the three-dimensional structure of the cation–con-G complex suggests that the explanation for the reduced mobility of Arg^{13} resides in the likelihood of interactions of its side chain with side chains of Gla^{10} and Gla^{14} in the helix (S. E. Warder, unpublished work). Thus this residue is important to the integrity of the helix and a particularly sensitive monitor of the Cd^{2+} -induced helical conformation of con-G. Interestingly, the resonances of the γ -protons of the Gla residues appear at the same region of chemical shifts (3.16–3.24 p.p.m.) as the H^δ protons of Arg^{13} , when the

spectrum of apo-con-G is obtained in water. The fact that these protons are not visible in the $^2\text{H}_2\text{O}$ -containing buffer of Figure 8 shows that these relatively acidic protons are exchangeable with solvent protons at pH 6.5. Correspondingly, in ^1H – ^{13}C hetero multi-quantum correlation experiments in $^2\text{H}_2\text{O}$, no H^γ – C^γ cross-peaks were observed for any of the Gla residues (T. Blandl, unpublished work), suggesting that a C– ^2H bond exists at each of these locations.

In conclusion, it is clear that a variety of multivalent cations interact with con-G in similar fashions and induce extensive helical conformation in this peptide. The fact that a variety of cations with measurable spectroscopic and spectrometric properties are able to provide such valuable information on the characteristics of this cation–peptide interaction and concomitant conformation alteration suggests that a battery of methodological approaches with rationally designed synthetic variants of this peptide will define even more rigorously the molecular details of these important events.

We gratefully acknowledge the assistance of Professor Thomas Nowak with the proton relaxation rate experiments and Dr. Graham Lappin for discussions on the properties of the different metal cations. These studies were supported by grant HL-19982 from the National Institutes of Health, the Kleiderer-Pezold Family endowed professorship (to F.J.C.) and a postdoctoral fellowship from the American Heart Association, Indiana Affiliate (to M.P.).

REFERENCES

- McIntosh, J., Olivera, B. M., Cruz, L. and Gray, W. (1984) *J. Biol. Chem.* **259**, 14343–14346
- Haack, J. A., Rivier, J., Parks, T. N., Mena, E. E., Cruz, L. J. and Olivera, B. M. (1990) *J. Biol. Chem.* **265**, 6025–6029
- Rivier, J., Galyean, R., Simon, L., Cruz, L. J., Olivera, B. M. and Gray, W. R. (1987) *Biochemistry* **28**, 8508–8512
- Prorok, M., Warder, S. E., Blandl, T. and Castellino, F. J. (1996) *Biochemistry* **35**, 16528–16534
- Mena, E. E., Gullak, M. F., Pagnozzi, M. J., Richter, K. E., Rivier, J., Cruz, L. J. and Olivera, B. M. (1990) *Neurosci. Lett.* **118**, 241–244
- Skolnick, P., Zhou, L.-M., Chandler, P., Nashed, N. T., Pennington, M. and Maccaccheni, M.-L. (1994) in *Pharmacology and Toxicology: Basic and Clinical Aspects: Direct and Allosteric Control of Glutamate Receptors* (Palfreyman, M. G., Reynolds, I. J. and Skolnick, P., eds.), pp. 155–165, CRC Press, Boca Raton, FL
- Skolnick, P., Boje, K., Miller, R., Pennington, M. and Maccaccheni, M.-L. (1992) *J. Neurochem.* **59**, 1516–1521
- Skjærbaek, N., Nielsen, K. J., Lewis, R. J., Alewood, P. and Craik, D. J. (1997) *J. Biol. Chem.* **272**, 2291–2299
- Colpitts, T. L. and Castellino, F. J. (1994) *Biochemistry* **33**, 3501–3508
- Carr, H. Y. and Purcell, E. M. (1954) *Phys. Rev.* **94**, 630–639
- Bajaj, S. P., Nowak, T. and Castellino, F. J. (1976) *J. Biol. Chem.* **251**, 6294–6299
- Solomon, I. (1995) *Phys. Rev.* **99**, 559–565
- Bloembergen, N. (1975) *J. Chem. Phys.* **27**, 572–573
- Lee, M. E. and Nowak, T. (1992) *Biochemistry* **31**, 2172–2180
- Chen, Y.-H., Yang, J. T. and Martinez, H. M. (1972) *Biochemistry* **11**, 4120–4131
- Davis, D. G. and Bax, A. (1985) *J. Am. Chem. Soc.* **107**, 2820–2821
- Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R. and Wuthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **117**, 479–485
- Kumar, A., Ernst, R. R. and Wuthrich, K. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1–6
- Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York
- States, D. J., Haberkorn, R. A. and Ruben, D. J. (1982) *J. Magn. Reson.* **48**, 286–293
- Chandler, P., Pennington, M., Maccaccheni, M. L., Nashed, N. T. and Skolnick, P. (1993) *J. Biol. Chem.* **268**, 17173–17178
- Zhou, L.-M., Szendrei, G. I., Fossom, L. H., Maccaccheni, M.-L., Skolnick, P. and Otvos, L. (1996) *J. Neurochem.* **66**, 620–628
- Warder, S. E., Chen, Z., Zhu, Y., Prorok, M., Castellino, F. J. and Ni, F. (1997) *FEBS Lett.* **411**, 19–26
- Wishart, D. S., Sykes, B. D. and Richards, F. M. (1992) *Biochemistry* **31**, 1647–1651