The binding isotherms of the divalent metal cations, Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\), to the synthetic \(\gamma\)-carboxyglutamic acid-containing neuroactive peptides, conantokin-G (con-G) and conantokin-T (con-T), have been determined by isothermal titration calorimetry (ITC) at 25 °C and pH 6.5. We have previously shown by potentiometric measurements that con-G contains 2–3 equivalent Ca\(^{2+}\) sites with an average \(K_d\) value of 2800 \(\mu\)M. With Mg\(^{2+}\) as the ligand, two separate exothermic sites are obtained by ITC, one of \(K_d = 46\) \(\mu\)M and another of \(K_d = 311\) \(\mu\)M. Much tighter binding of Zn\(^{2+}\) is observed for these latter two sites (\(K_d = 0.2\) \(\mu\)M and 1.1 \(\mu\)M), and a third considerably weaker binding site is observed, characterized by a \(K_d\) value of 286 \(\mu\)M and an endothermic enthalpy of binding. con-T possesses a single exothermic tight binding site for Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\), with \(K_d\) values of 428 \(\mu\)M, 10.2 \(\mu\)M, and 0.5 \(\mu\)M, respectively. Again, in the case of con-T, a weak (\(K_d = 410\) \(\mu\)M) endothermic binding site is observed for Zn\(^{2+}\). The binding of these cations to con-G and con-T result in an increase in the \(\alpha\)-helical content of the peptides. However, this helix is somewhat destabilized in both cases by binding of Zn\(^{2+}\) to its weakest site.

Since the differences observed in binding affinities of these three cations to the peptides are substantially greater than their comparable \(K_d\) values to malonate, we conclude that the structure of the peptide and, most likely, the steric and geometric properties imposed on the cation site as a result of peptide folding greatly influence the strength of the interaction of cations with con-G and con-T. Further, since the Zn\(^{2+}\) concentrations released in the synaptic cleft during excitatory synaptic activity are sufficiently high relative to the \(K_d\) of Zn\(^{2+}\) for con-G and con-T, this cation along with Mg\(^{2+}\), are most likely the most significant metal ion ligands of these peptides in neuronal cells.

The \(N\)-methyl-\(d\)-aspartate (NMDA)\(^1\) subtype of glutamate receptor is a ligand-gated ion channel that displays high permeability for Ca\(^{2+}\). The marked excitotoxicity of glutamate is generally regarded as ascribable to its persistent interaction with the NMDA receptor (NMDAR), resulting in the establishment of neurodegenerative glutamatergic loops defined by uncontrolled elevations of intracellular Ca\(^{2+}\), followed by cell lysis and death. Because the Ca\(^{2+}\)-mediated neuronal cell death, which is attendant to both acute (e.g. ischemia) and chronic (e.g. epilepsy, Parkinson’s disease) neurodegenerative disorders, can be ameliorated by antagonists specific for the NMDAR (1–5), extensive biochemical characterization of drug-receptor interactions focused on this receptor is a widely studied topic.

Isolated from the venom ducts of predatory snails of the genus Conus, the conantokins-G (con-G) and -T (con-T) are potent and selective inhibitors of NMDAR function (6–8) and are the only peptide antagonists of this receptor subtype described to date. More specifically, this antagonism derives from a noncompetitive inhibitory effect of the polyamine agonist site of the receptor (6). The physiological responses elicited following intracranial injections in mice include a sleep-like state in neonatal mice and a hyperactive response in older animals (9).

An unusual feature of con-G and con-T is their high abundance of \(\gamma\)-carboxyglutamic acid (Gla) (10, 11). Prior to its discovery in the conantokins, the presence of this amino acid in polypeptides and proteins had been noted only in certain bone proteins and in various blood coagulation factors. In these contexts, the ability of Gla to bind to Ca\(^{2+}\) plays an integral role in the adoption of functional protein conformers (12). The interaction of Gla with Ca\(^{2+}\) has been established for the conantokins, in which cases it has been demonstrated that both con-G and con-T adopt a significant degree of \(\alpha\)-helicity in the presence of this cation (13). This \(\alpha\)-helical induction is particularly profound in the case of con-G, which is essentially structureless in its apo form but assumes a full end-to-end \(\alpha\)-helical conformation when Ca\(^{2+}\) or Mg\(^{2+}\) is fully bound to the peptide (13–15). In contrast, con-T manifests appreciable \(\alpha\)-helicity in the apo form which increases slightly in the divalent cation-bound state (13, 16, 17). Because the smaller population of conformers associated with the more structurally rigid metal-bound forms of these peptides would be expected to afford more discriminate binding to the NMDAR site, metal complexation to these peptides may be necessary for full bioactivity. Of the many Conus peptides currently characterized, all of which appear to target neuronal or muscle cell receptors, only con-G and con-T lack intramolecular disulfide bridges. This observation lends support to the idea that conformational rigidity, either covalently or noncovalently imposed, is an important element of receptor recognition among members of the Conus-derived peptide family. In the case of the conantokins, metal ions other than Ca\(^{2+}\) have also been shown to effect \(\alpha\)-helix induction. Of these, Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) are the most physiologically relevant metal cations in brain cells (14). Through CD-monitored titrations of con-G, we have found that the affinity of these divalent metal ions for the peptide is significantly greater for

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1 The abbreviations used are: NMDA, \(N\)-methyl-\(d\)-aspartate; NMDAR, NMDA receptor; con-G, conantokin-G; con-T, conantokin-T; Gla, \(\gamma\)carboxyglutamic acid; ITC, isothermal titration calorimetry; Mes, 4-morpholineethanesulfonic acid.
Peptide samples ranging in concentrations from 0.23–1.0 mM in a total buffer used was 10 mM Mes, 100 mM NaCl, pH 6.5. The partial specific volume of con-T (0.72 ml/g) was calculated from its amino acid composition by assigning the Gla residue the value of glutamate (0.66 ml/g). The density of the buffer was determined to be 1.005 g/ml. Sedimentation data were analyzed using the single ideal species model included in the Beckman XL-I software. Baseline offset values were constrained to zero for all data sets. Calculated molecular weights represent the average of the results from three separate scans.

RESULTS

Calorimetric titrations of con-G, con-T, and malonate with various divalent metal cations were performed at 25 °C, pH 6.5. Examples of the heat changes accompanying the binding of incremental additions of Zn\(^{2+}\) to con-G and con-T are shown in the upper panels of Fig. 1. The binding isotherm corresponding to a plot of integrated heats as a function of the molar ratio of Zn\(^{2+}\)/peptide is displayed in the lower panels. These Zn\(^{2+}\) tritinations represent the more complicated isotherms of the Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) data sets with 3 and 2 enthalpic transitions resulting from the complexation of Zn\(^{2+}\) with con-G and con-T, respectively. The deconvolution of the data for con-G was achieved by fixing a multiple site model to an n = 3, thereby allowing only K\(_d\) and ΔH values to float during the iterative nonlinear least squares minimization. For con-T, n\(_1\) was con-

\[ K_{d1} = \frac{[\text{Peptide}][\text{Metal}]}{[\text{Peptide} \cdot \text{Metal}]} \]

Mg\(^{2+}\) and Zn\(^{2+}\) than for Ca\(^{2+}\) (14). Although CD-monitored titrations represent a convenient approach for assessing relative metal ion affinities, their corresponding K\(_d\) values cannot be accurately determined from this method since the estimated K\(_d\) values fall in the range of working peptide concentrations. This situation also complicates determination of the stoichiometry of metal binding. To gain a comprehensive quantitative assessment of these parameters, we have employed isothermal titration calorimetry (ITC) for monitoring the heat changes that accompany metal binding to the peptides. In addition to K\(_d\) and stoichiometry, values for ΔH and ΔS can also be extracted from an ITC profile. With these data in hand, the nature of metal ion binding to both con-G and con-T can be more rigorously analyzed. The purpose of the current communication is to elaborate the thermodynamic properties of metal ion–conantokin binding.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The materials, synthetic protocols, and purification procedures for obtaining con-G (G-E-γ-Y-L-Q-γ-N-Q-γ-L-R-γ-K-S-N-CONH\(_2\), γ = Gla) and con-T (G-E-γ-Y-Q-K-M-L-γ-N-L-R-γ-A-E-V-K-K-N-A-CONH\(_2\), γ = Gla) have been described earlier (13). Concentrations of peptide stock solutions were determined by quantitative amino acid analysis.

Isothermal Calorimetry—The binding isotherms of Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) to the conantokins and malonate were determined by ITC measurements of the heat changes accompanying titration of the metal ions into solutions of the relevant sample. The titrations were performed with an OMEGA titration calorimeter (Microcal, Inc., Northampton, MA) at 25 °C in a buffer containing 10 mM Mes, 100 mM NaCl, pH 6.5. Peptide samples ranging in concentrations from 0.23–1.0 mM in a total volume of 1.4 ml were placed in the reaction cell. After equilibration, an appropriate concentration of CaCl\(_2\), MgCl\(_2\), or Zn(OAc)\(_2\) (typically 30–50× higher in concentration than the peptide solution) in matching buffer was delivered at discrete intervals. The observed heat was measured after each injection. The total observed heat effects were corrected for the heat of dilution of ligand by performing control titrations in the absence of peptide. The resulting titration curves were deconvoluted for the best-fit model using the ORIGIN for ITC software package supplied by Microcal.

Circular Dichroism—CD titrations of con-G and con-T as a function of metal ion concentration were performed on an AVIV model 62DS spectrometer at 222 nm using a 0.1-cm path length cell thermostatted at 25 °C. The peptides were dissolved in 10 mM Mes, 100 mM NaCl, pH 6.5, to a final concentration of 0.5 mM. Mean residue ellipticities were calculated by using a mean residue molecular mass of 133 Da for con-G and 128 Da for con-T. The fractional α-helical content was determined from mean residue ellipticities at 222 nm using the empirical relationship

\[ \phi_\text{helix} = -\frac{\theta_{222}}{2340} \times 2000 \]

where θ\(_{222}\) is the observed ellipticity at 222 nm. The fractional α-helical content was calculated from mean residue ellipticities at 222 nm using the empirical relationship

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TABLE I

| Metal Peptide | Metal | Kd | AΔH | AΔG | TΔS |
|---------------|-------|----|-----|-----|-----|
|               |       | μM | kcal/mol | kcal/mol | kcal/mol |
| Ca²⁺ | con-G | 2–3 | 2800 |     |     |
| Mg²⁺ | con-G | 0.93 | 46 | -3.0 | -5.9 | 2.9 |
| Zn²⁺ | con-G | 1.0 | 311 | -5.7 | -4.8 | -0.9 |
| Ca²⁺ | con-T | 1.0 | 92.2 | +4.2 | -9.0 | 4.8 |
| Mg²⁺ | con-T | 1.0 | 1.1 | -5.6 | -8.1 | 2.5 |
| Zn²⁺ | con-T | 1.0 | 286 | +4.8 | -4.8 | 9.6 |
| Ca²⁺ | Malonate | 0.83 | 428 | -2.7 | -4.6 | 1.9 |
| Mg²⁺ | Malonate | 0.84 | 10.2 | -4.4 | -6.8 | 2.4 |
| Zn²⁺ | Malonate | 0.90 | 0.5 | -5.7 | -8.6 | 2.9 |
| Ca²⁺ | Malonate | 0.90 | 410 | +8.2 | -4.6 | 12.8 |
| Mg²⁺ | Malonate | 0.86 | 9900 | +2.0 | -2.7 | 4.7 |
| Zn²⁺ | Malonate | 0.62 | 3850 | +2.9 | -3.3 | 6.2 |

*Potentiometric data from Ref. 13. The binding of Ca²⁺ to con-G is too weak to lend itself to ITC methodology since prohibitively high concentrations of peptide would be necessary for probing such an interaction.

binding to malonate are positive. The stoichiometry of approximately 0.6 determined for the association of Zn²⁺ with malonate suggests that some degree of dimerization (or possibly higher order aggregation) may accompany binding of this particular metal ion to malonate.

In an attempt to address the phenomena underlying the endothermic transitions observed for con-G and con-T, CD-monitored titrations of these peptides with Zn²⁺ were performed under conditions that paralleled those implemented in the ITC experiments. As can be seen for con-G (Fig. 2A), an essentially linear increase in α-helicity attends the addition of Zn²⁺, up to an apparent plateau occurring at a metal/peptide ratio of 2:1 (m:m). The linear phase of this titration, with a midpoint of 1:1 (m:m), reflects extremely tight binding up to 2 eq of Zn²⁺, such that virtually all added metal ion exists in the peptide-bound form. The inset of Fig. 2A reveals that above a Zn²⁺/peptide molar ratio of 2, a small but defined decrease in α-helicity is induced. This phenomenon was not noted at similar metal/peptide ratios of Ca²⁺ and Mg²⁺ (data not shown).

The CD-monitored titration of con-T with Zn²⁺ is illustrated in Fig. 2B. High affinity binding of Zn²⁺, as with con-G, is seen in the early portion of the profile, while a significant decrease in α-helicity accompanies occupation of the second Zn²⁺ site. Again, this decrease in α-helical content was not observed in similarly conducted Ca²⁺ and Mg²⁺ titrations. Because the CD-titratable diminutions in α-helical content correlate closely with the endothermic phases of the ITC profiles, it appears that occupancy of the weakest Zn²⁺ site of both con-G and con-T effects a partial unwinding of the α-helix, which is considerably more pronounced for the latter peptide.

As indicated above, calorimetric analysis of the Zn²⁺-titanium profile of con-T was suggestive of a more complex model of metal binding involving peptide association. Zn²⁺-induced peptide dimerization (or higher order aggregation) could also account for the decrease in con-T and con-G α-helicity observed at higher Zn²⁺/peptide molar ratios. To address this issue, sedimentation equilibrium analysis of con-T was performed in both the apo state and at various Zn²⁺ concentrations. As shown in Fig. 3, the apparent molecular weight of con-T increases from 3160 in its uncomplexed state to 3390 at a Zn²⁺/peptide ratio of 1:1 (m:m), and 3650 at a cation/peptide ratio of 5:6:1 (m:m), an ~10% increase in the early portion of the profile, while a significant decrease in α-helicity accompanies occupation of the second Zn²⁺ site. Also to be noted from Table I are the positive entropies of approximately 1, all parameters (n, Kd, ΔH) were allowed to float during the minimization process. Excellent fits were obtained for stoichiometries corresponding to integer or near-integer values. This strongly suggests that neither metal-induced aggregation of peptide monomer nor metal-induced dissociation of an apo aggregate occurred under the conditions of the calorimetric titrations. An exception to this general observation occurred in the case of the Zn²⁺ titration for con-T. For these data, a second acceptable model was generated corresponding to Kd = 60 nM, n = 0.45; Kd = 760 nM, n = 0.53; Kd = 440 μM, n = 0.95. Insofar as these parameters might indicate Zn²⁺-induced peptide aggregation, further exploration of this possibility was pursued, as described below.

In addition to the additional peptide binding sites that can be occupied by Zn²⁺, the thermodynamic signature of Zn²⁺ binding to the conantokins was also unique compared with Mg²⁺ and Ca²⁺: that a late endothermic transition occurs in the titration of both peptides. This is significantly more pronounced for the latter peptide. Also to be noted from Table I are the positive entropies attending occupation of each titratable site, with the exception of the entropy associated with the weaker of the two Mg²⁺ sites in con-G.

Comparison of the Kd values for Ca²⁺, Mg²⁺, and Zn²⁺ for con-G and con-T shows a dramatic increase in affinity for Zn²⁺ when compared with Mg²⁺ which, in turn, binds to the peptides much tighter than Ca²⁺. For con-G, the tightest of the 3 Zn²⁺ sites (Kd = 0.2 μM) displays 200-fold more avid binding than the tightest of the 2 Mg²⁺ sites (Kd = 46 μM) and a 12,000-fold increase in affinity relative to the 2–3 eq Ca²⁺ sites. For con-T, this discrimination is somewhat less pronounced, with the strength of the interaction for the tight Zn²⁺ site being approximately 40- and 1000-fold greater than that associated with the Mg²⁺ and Ca²⁺ sites, respectively. In contrast, malonate manifests only a very modest selectivity for Zn²⁺ as compared with Mg²⁺ and Ca²⁺ (2.3- and 6.5-fold, respectively). In addition, the absolute Kd values that characterize the binding of these cations to malonate range from 1 to 4 orders of magnitude higher than the same Kd values associated with their complexation to the conantokins (Table I). Both ΔH and ΔS values for metal ion

**Discussion**

The multiple metal cation sites detected for con-G (Table I) are consistent with the multiple cation sites proposed from modeling by a genetic algorithm/molecular dynamics simulation method using the coordinates provided from the NMR-derived structures of the Ca²⁺- and Mg²⁺-bound peptides (15, 19). The NMR structure of the Mg²⁺-complexed form is notable for the spatial proximity of the side chain carboxylates of Glu10 and Glu14, which are optimally positioned for metal ion coordination. This proximity would be unlikely in the absence of a stabilizing carboxyl group. A cluster comprised of Glu3, Glu6, and Glu8 represents a second potential coordination site. The viability of these side chains as metal binding loci was supported by the simulated docking of Mg²⁺ to the Mg²⁺-loaded NMR-derived structure using the genetic algorithm/molecular dynamics simulation method (15). This approach also identified Glu7 as another likely site for Mg²⁺ in con-G. ITC experiments failed to detect this putative third locale which may bind.
Mg\(^{2+}\) too weakly to be amenable to this calorimetric approach. However, a third, albeit relatively weak site, was detected during the Zn\(^{2+}\) titration of con-G and may reflect binding at this proposed Gla\(^7\) site. Synthetic peptide variants containing individual Gla to Ala substitutions strongly support a model wherein the tight Mg\(^{2+}\) site is maintained by Gla\(^{10}\) and Gla\(^{14}\), with the weaker site being comprised of Gla\(^3\), Gla\(^4\), and Gla\(^7\) (15). The orientation of Gla\(^{10}\) and Gla\(^{14}\) as determined from the Ca\(^{2+}\)-bound NMR solution structure of con-T is strikingly similar to that found in con-G and, by analogy, can be considered a likely binding locus for metal ions (16). Docking of Ca\(^{2+}\) into this structure using the aforementioned genetic algorithm/molecular dynamics simulation yielded a lowest energy structure showing the carboxylates of Gla\(^{10}\) and Gla\(^{14}\) as coordination site donors. A second site appears maintained by the two carboxylates of Gla\(^3\) and the side chain amide carbonyl of Gln\(^6\). A second binding site was not experimentally detected for Ca\(^{2+}\) and Mg\(^{2+}\), but was observed with Zn\(^{2+}\), a more avid ligand.

Previous studies have demonstrated that no detectable higher order species are present upon Ca\(^{2+}\) loading of either con-G or con-T (13). In the presence of saturating concentrations of Mg\(^{2+}\), the monomeric molecularity of both peptides has also been established.\(^3\) However, with Zn\(^{2+}\), the calorimetric titration of con-T yielded data for which a fit consistent with peptide dimerization could be generated. In addition, from the CD titration data, one interpretation of the unusual trend of decreasing con-T helicity coinciding with increasing Zn\(^{2+}\) concentration could include Zn\(^{2+}\)-induced peptide aggregation upon weak-site occupancy, which leads to helix unwinding. To test the possibility of Zn\(^{2+}\)-induced con-T aggregation under the conditions employed in both the calorimetry and CD experiments, we opted for sedimentation equilibrium analysis of the apo and Zn\(^{2+}\)-chelated peptide states (Fig. 3). Inspection of the residuals of the fits to the single ideal species model for uncomplexed and Zn\(^{2+}\)-chelated con-T reveals the random distribution of points diagnostic of ideal solute behavior.

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\(^{2}\) S. E. Warder, unpublished data.  
\(^{3}\) M. Prorok, unpublished data.
additional carboxylate in Gla should effectively lower the ν associated with this residue by virtue of its increased hydrophilicity and capacity for hydration. In addition, in the presence of Zn2+ the intimate and preferential interaction of the metal ion with the Gla side chains may profoundly perturb the ν of Gla. When considering the relatively high weight percentage of Gla in con-T, its contribution to the ν value of the peptide is significant. The observation that an increase in molecular weight does not attend increased peptide concentration at high Zn2+ concentrations is additional compelling evidence in support of the nonassociative behavior of con-T.

The exotherms associated with peptide-metal binding correlate in the expected fashion with the degree of conformational change effected by metal binding. In the case of Mg2+ complexation to con-G, wherein the peptide undergoes a dramatic change in α-helicity upon Mg2+ binding (14), the total change in the ΔH for binding of 2 eq of ligand is −8.7 kcal/mol. The enthalpic change for con-T, which undergoes a distinctly smaller increase in α-helicity upon saturation of its metal ion sites with Mg2+, is −4.4 kcal/mol. Clearly, bond formation occurs to a greater extent in the con-G metal-induced transition than in the con-T system. It seems unlikely that electrostatic components contribute to these favorable ΔH values since metal ion binding to malonate, which occurs strictly through electrostatic interactions, possesses an unfavorable ΔH (Table I), leaving entropic forces to drive the binding event. The effective neutralization of clustered, destabilizing negative charge that occurs upon chelation of metal ions to the Gla head groups of the peptide may simply allow favorable intrapeptide hydrogen bonds to prevail.

The positive, albeit small, entropies that accompany the exothermic transitions are somewhat surprising considering the significant degree of order that metal ion binding imposes on these peptides. Factors with favorable entropies, such as release of the metal ion from its coordination sphere and release of structured water surrounding polar and non-polar residues, are likely contributing in the peptide systems. From the positive TΔS values for metal complexation to malonate, it is probable that such contributions take entropic precedence, despite the absence of some elements of rotational bond freedom.

The positive ΔH values observed for occupancy by Zn2+ of the weak sites of both con-G and con-T correlate with a small degree of α-helix unwinding as implied from parallel CD-monitored titrations (Fig. 2). The relatively large positive entropy values linked to weak site Zn2+ binding for both peptides is also consistent with the notion of increased structural disorder. The nature of this destabilization in con-G is difficult to state with certainty because the results of simulated Mg2+ docking to con-G (15) point toward Gla7 as a third binding locus. Because all five Gla residues of con-G exist on the same face of the helix
in the metal-loaded structure (15), inclusion of an additional divalent cation would be expected to impart increased stabilization to the peptide. We expect that ITC titrations with Zn^{2+} employing various Glu to Ala variants of con-G will address the nature of this third site and these studies are currently in progress. For con-T, we contend that the greater degree of \( \alpha \)-helix unwinding that occurs upon Zn^{2+} loading can be explained in terms of the capping potential of Glu. The NMR solution structures of both apo- and Ca^{2+}-loaded con-T indicate that the side chain of Glu is an apparent capping residue in both structures. This contention is supported by CD data on the [Glu]Ala]con-T variant which displays compromised \( \alpha \)-helical content in both its apo- and Ca^{2+}-loaded forms (17). In the presence of high Zn^{2+} concentrations, we suggest that Glu is recruited from its side-chain to main-chain interaction role to share with Glu in the maintenance of a weak metal-binding site, thus destabilizing the \( \alpha \)-helix in the N-terminal vicinity.

Finally, the current study raises important issues concerning the identity of the metal ion that predominate in the bioactive conformation of these peptides. It has been established in numerous studies with Gla-containing bone and blood proteins that Ca^{2+} mediates their functional aspects. In the case of prothrombin, factor IX, and protein C, which contain 10, 12, and 9 Gla residues, respectively, in their N-terminal domains, no major preference for Mg^{2+} versus Ca^{2+} has been noted from intrinsic fluorescence titration experiments (20–23). Only a slight (2.5-fold) preference is observed for Mg^{2+} over Ca^{2+} in terms of their \( K_d \) values for malonate (Table I). Despite the smaller ionic radius for Mg^{2+} (86 pm) as compared with Ca^{2+} (114 pm), this disparate charge to radius ratio fails to impart appreciable selectivity differences in these cases. However, for con-G, the Mg^{2+}-tight site and weak site affinities in comparison with Ca^{2+} are 175- and 9-fold, greater, respectively. For con-T, Mg^{2+} displays a 42-fold increase in binding strength compared with Ca^{2+}. Both con-G and con-T display distinctly lower \( K_d \) values for Zn^{2+} than for either Ca^{2+} or Mg^{2+}. A pronounced (ca. 10,000-fold) greater affinity for Zn^{2+} over Ca^{2+} has recently been reported for a Gla-containing de novo designed peptide, which undergoes a metal-induced helical transition (24). These investigators maintain that the greater covalent character of electrostatic interactions involving Zn^{2+}, ascribable to its filled 3d orbitals as well as higher charge density for this cation, are the major contributors to this preferential binding. However, the primacy of these factors is not borne out by our malonate data, which indicate that Zn^{2+} manifests only a modest (6.5-fold) increase in affinity for malonate compared with Ca^{2+}. This would suggest that the binding sites of those peptides, which avidly bind Zn^{2+}, are particularly amenable to this smaller metal ion simply because their induced binding site cation pockets are intrinsically small. Hence, the strength of metal-ion binding in these molecules may rely more on proper steric and geometric fit than on purely electrostatic considerations.

The consideration that Zn^{2+} is the dominant metal ion effector of conantokin function is supported by studies that indicate that transiently high local concentrations of Zn^{2+} (ca. 300 \( \mu \)M) can be attained in the synaptic cleft during excitatory synaptic activity (25, 26). Intracerebral injections of the conantokinins into pre-2-week-old mice have been shown to induce a prolonged sleep-like state in these subjects at an estimated final brain concentration of 15 \( \mu \)M (13). Assuming a free Zn^{2+} concentration of 300 \( \mu \)M, this would result in essentially complete occupancy of the two tight Zn^{2+} sites in con-G and approximately 50% occupancy of the weak site. In the case of con-T, saturation of the high affinity Zn^{2+} site and 40% occupancy of the low affinity Zn^{2+} site would be attained at these peptide and ligand concentrations. At a cerebrospinal fluid Mg^{2+} concentration of 1.5 mM, tight and weak site loading of con-G would be 97% and 80%, respectively. For con-T, essentially 100% of the peptide molecules would be complexed with Mg^{2+}. At a cerebrospinal fluid Ca^{2+} concentration of 1.5 mM, Ca^{2+} loading would be considerably less than Mg^{2+} and Zn^{2+}, with 30% and 77% occupancy of the metal sites in con-G and con-T, respectively. We therefore propose that Mg^{2+}, as well as Zn^{2+}, are more plausible candidates for the role of in vivo metal ions for the conantokins. However, it should be noted that the high coordination numbers and irregular coordination geometry displayed by Ca^{2+} confer upon Ca^{2+}-bound proteins the ability to bridge phospholipid membranes and/or other proteins. This particular feature of Ca^{2+}-complexed biomolecules may mediate the functionality of the conantokins, despite the compromised binding capacity displayed by Ca^{2+} compared with Mg^{2+} and Zn^{2+}. Dissecting the roles of these metal ions in the conantokins with respect to the NMDAR is complicated by their involvement in receptor function, namely the Ca^{2+} permeability associated with the receptor (see above), voltage-dependent Mg^{2+} blockage of the receptor (27, 28), and allosteric voltage-insensitive and -sensitive blocks by Zn^{2+} (29, 30). Elucidating the precise role of these metal ions in terms of conantokin antagonism of the receptor is essential for a full pharmacological profile of the these peptides and represents one of the major investigative challenges associated with future work in this area.

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