Speciation of Cyclo(Pro-Gly)$_3$ and Its Divalent Metal-Ion Complexes by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESI-MS) was used to study the binding of selected group II and divalent transition-metal ions by cyclo(Pro-Gly)$_3$ (CPG3), a model ion carrier peptide. Metal salts ($CatX_n$) were combined with the peptide (M) at a molar ratio of 1:10 M/Cat in aqueous solvents containing 50% vol/vol acetonitrile or methanol and 1 or 10 mM ammonium acetate (NH_4Ac). Species detected include $[M + H]^+$, $[M + Cat - H]^+$, $[M_2 + Cat]^{2+}$, $[M + Cat + Ac]^+$, and $[M + Cat + X]^+$. The relative stabilities of complexes formed with different cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) were determined from the abundance of 1:1 and 2:1 M/Cat species relative to that of the unbound peptide. The largest metal ions (Ca^{2+}, Sr^{2+}, and Mn^{2+}) formed the most stable complexes. Reducing the buffer concentration increased the overall extent of metal binding. Results show that the binding specificity of CPG3 depends upon the size of the metal ion and its propensity for electrostatic interaction with oxygen atoms. Product ion tandem mass spectrometry of $[M + H]^+$ and $[M + Cu - H]^+$ confirmed the cyclic structure of the peptide, although the initial site(s) of metal attachment could not be determined. (J Am Soc Mass Spectrom 2005, 16, 1536–1544) © 2005 American Society for Mass Spectrometry

The biogeochemistry of a particular metal ion is strongly influenced by its ability to form stable complexes with dissolved organic compounds under ambient conditions [1–3]. Identification and characterization of these complexes is critical to understanding and predicting the bioactivity and distribution of metals in biological systems and the environment. Peptides constitute one of the most important classes of compounds involved in the binding and transportation of metal ions [4, 5]. Cysteine-containing peptides such as phytochelatins (PCs) and metallothioneins (MTs) have long been implicated in the binding, detoxification, and sequestration of heavy metals [5]. These compounds have been found in bacteria, plants, and animals, suggesting a universal role in metal detoxification and homeostasis. Alteration of MT expression and PC biosynthesis has been investigated as a means of increasing metal tolerance and uptake in plants, with the intention of using them for remediation of metal-contaminated sites [5–8]. However, this approach currently is limited by the low specificity with which metals such as cadmium, copper, and zinc bind to and induce expression of these peptides. Moreover, interactions between these metals and MTs or PCs in vivo remain poorly understood, while apparent localization of MT–metal complexes in roots limits uptake, translocation, and recovery of metals from aerial tissues [5, 6].

Biosynthesis and release of peptides and related compounds is another mechanism by which microorganisms such as soil bacteria and marine phytoplankton are able to regulate uptake of essential and nonessential metals from their surroundings [9, 10]. Such compounds include cysteine-containing tripeptides similar to glutathione and PCs [11] and cyclic peptides such as rhodotorulic acid, a bacterial siderophore [12]. Many cyclic peptides have antibiotic or antifungal properties that arise from the ability to bind and transport metals across biological membranes [4, 13, 14]. These peptides show a high degree of specificity, forming stable complexes with metal ions of a particular charge and ionic radius. Metal binding specificity is related to the size and conformation of the peptide and the type and orientation of groups involved in metal binding [14]. It also is affected by solvent–metal and solvent–ligand interactions [4, 14]. Biosynthesis of cyclic peptides in plants could, in principle, be used to promote uptake of specific metals, thereby enhancing the phytoremediative properties of metal (hyper)accumulators or the

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nutritional value of crop species, provided that suitable peptides can be designed and expressed *in planta*.

Cyclo(Pro-Gly)$_3$ (CPG3) is a synthetic cyclic peptide composed of alternating proline and glycine residues linked in a six-membered ring [14]. Proline residues form peptide bonds containing tertiary amino groups, which restrict rotation about the N—Cα bond. Glycine residues are small enough to accommodate such constraints and often are paired with proline residues to enable turns and reversals in polypeptide chains. These characteristics give CPG3 a well-defined yet flexible structure [15], making it an ideal model for studying interactions between metal ions and natural cyclic peptides. Furthermore, the metal binding properties of CPG3 match closely those of antamanide and the enniatins, cyclic peptides, which are known to transport metal ions across biological membranes [15]. The three-dimensional structure of CPG3 and the conformational changes that occur on complexation of selected metal ions (Na^+, K^+, Mg^{2+}, and Ca^{2+}) have been studied extensively using a variety of techniques, including theoretical and computer modeling, X-ray crystallography, electronic circular dichroism, vibrational circular dichroism (VCD), and Raman and NMR spectroscopy [14–18]. Of these techniques, VCD and NMR spectroscopy provide the most reliable information concerning the formation of CPG3 metal-ion complexes in solution. For example, NMR studies indicate that Ca^{2+} ions form 1:1 complexes with CPG3 [15], whereas VCD suggests that Ca^{2+} does this by interacting with the carbonyl oxygen atoms of the three proline residues, drawing them toward each other [14]. This distorts slightly the so-called S1* conformation of *apo*-CPG3, in which the three proline and three glycine carbonyl groups lie on opposite sides of the ring. Interaction between calcium and CPG3 apparently is favored by the ionic radius of Ca^{2+} (1.0 Å for sixfold coordination) and the distance between proline carbonyl groups (3.1-3.9 Å in the S1* conformation). Strong interactions between CPG3 and aqueous solvent appear to inhibit binding of metal ions of different size or charge, including Na^+, K^+, and Mg^{2+} (ionic radius, 0.7 Å). However, these interactions are disrupted in less polar solvents, allowing metals other than Ca^{2+} to bind to CPG3. Under these conditions, VCD analysis suggests that Mg^{2+} binds simultaneously to the proline carbonyl groups of one CPG3 molecule and the glycine carbonyl groups of another to form a "peptide sandwich" complex. In contrast, "salt sandwich" complexes containing one CPG3 molecule and two Ca^{2+} or Mg^{2+} ions are not observed. This is because binding of a metal ion to the carbonyl groups on one side of the cyclic peptide increases the distance between the carbonyl groups on the other side, preventing a second metal ion from binding. Hence, complexation of a particular metal ion by CPG3 depends on the size and charge of the metal ion and the conformation of the peptide, which in turn depends on solvent composition and the resulting orientation of proline and glycine carbonyl groups [14].

The purpose of this study was to evaluate electrospray ionization mass spectrometry (ESI-MS) as a tool for speciation of cyclic peptides and their metal-ion complexes in solution. The use of ESI-MS for dissolved metal speciation has been shown using a variety of model synthetic and natural ligands, including glutathione and tannic acid [19, 20]. Structural analysis of cyclic peptides using single stage (MS/MS) and multistep (MS^n) tandem mass spectrometry also has been documented, and an annotation system was developed for the resulting product ions [21, 22]. Recently, the use of metal-ion adduction to promote fragmentation and sequencing of cyclic peptides by ESI-MS^n with an ion-trap mass spectrometer has been successfully established [23]. Here, we use ESI-MS to determine the distribution of dissolved CPG3 species formed in the presence of selected divalent metal ions and to determine the relative stabilities of CPG3 complexes under different solution conditions. Results are compared with those obtained using other analytical techniques and are interpreted with reference to the physicochemical properties of the cyclic peptide. Fragmentation of CPG3 and selected complexes by low-energy collision-induced dissociation (CID) also was investigated, and the use of ESI-MS and MS/MS for characterizing cyclic peptides and their metal-ion complexes is discussed.

Materials and Methods

All reagents were obtained from Sigma-Aldrich (Milwaukee, WI) and used without further purification. Acetonitrile (ACN) and methanol (MeOH) were high-performance liquid chromatography grade and obtained from EM Science (Gibbstown, NJ). De-ionized water was prepared in-house using the Milli-Q Element system from Millipore (Bedford, MA). Samples were analyzed by flow-injection ESI-MS, as previously described [19], using a Quattro LC quadrupole tandem mass spectrometer (Micromass, Manchester, UK) connected to an HP1100 solvent delivery system and autosampler (Agilent, Palo Alto, CA). Ten microliters of each sample was injected into a carrier stream consisting of the sample solvent flowing at 25 μL/min. The mass spectrometer was operated in the positive ion mode, and ESI conditions were adjusted to optimize sensitivity while preserving solution-phase interactions. Typical operating conditions were capillary, 3.3 kV; cone, 42 V; source temperature, 100 °C; desolvation gas temperature, 120 °C; desolvation gas flow, 266 L/h; and nebulizer gas flow, 90 L/h. A 100-μM solution of CPG3 in 50:50 ACN/H_2O containing 1 mM of ammonium acetate (NH_4Ac) was first analyzed by ESI-MS and product ion MS/MS to characterize the molecular ion. Speciation experiments were then initiated by combining CPG3 (M) with divalent metal salts ($CatX_n$) of selected group II and transition metals (Table 1) at a M/Cat mole ratio of 1:10 in different solvents and performing full scan ESI-MS analysis on each sample. These concentrations were necessary to ensure detec-

Table 1. Selected properties of the divalent metal ion salts used in this study

Metal cation (Cat)	Ionic radius (pm)[a]	Lightest isotope	Fractional abundance (*A*)	Salt anion (X)	Lightest isotope(s)	Fractional abundance (*B*)
Mg^{2+}	72	^{24}Mg	0.79	SO_4^{2-}	^{32}S, ^{16}O	0.95
Ca^{2+}	100	^{40}Ca	0.97	Cl^-	^{35}Cl	0.76
Sr^{2+}	118	^{88}Sr	0.83	NO_3^-	^{14}N, ^{16}O	1.00
Mn^{2+}	83	^{55}Mn	1.00	NO_3^-	^{14}N, ^{16}O	1.00
Co^{2+}	65	^{59}Co	1.00	Cl^-	^{35}Cl	0.76
Ni^{2+}	69	^{58}Ni	0.68	Cl^-	^{35}Cl	0.76
Cu^{2+}	73	^{63}Cu	0.69	SO_4^{2-}	^{32}S, ^{16}O	0.95
Zn^{2+}	74	^{64}Zn	0.49	Br^-	^{79}Br	0.51

[a]Ref. [26].

tion of all significant CPG3 species, the distribution of which has not been found to change significantly between M/Cat mole ratios of 1:1 and 1:10 using other techniques [14].

As in previous studies [19, 23], the peptide species detected in solution included $[M + Cat - H]^+$, $[M + Cat + Ac]^+$, and $[M + Cat + X]^+$, where X is the anion present in the metal salt. All three may be regarded as different forms of the 1:1 M/Cat complex, assuming that the interaction between the peptide and metal ion is stronger than interactions between the metal and other solutes [19]. This was verified by the low relative abundance of $[Cat + Ac]^+$, $[Cat + X]^+$, and $[Cat(solvent)_i]^{n+}$ peaks in full scan spectra, with the notable exception of copper in aqueous ACN (*vide infra*). For the purposes of this study, it was necessary to combine the responses for $[M + Cat - H]^+$, $[M + Cat + Ac]^+$, and $[M + Cat + X]^+$ to compare directly the relative stabilities of 1:1 M/Cat complexes formed by different metals. In each case, the monoisotopic peak generally was the peak of greatest intensity and, hence, gave the most accurate measurement of relative abundance. After determining the *m/z* ratios for each of these peaks, accurate speciation measurements were performed by analyzing the samples again, in triplicate, using the same experimental conditions. An extracted ion chromatogram (EIC) was generated for each species [10], using a window of 0.75 u about the centroid mass of the monoisotopic peak. The relative abundance of $[M + H]^+$ and metal species were then determined by integration and comparison of the corresponding EIC peaks.

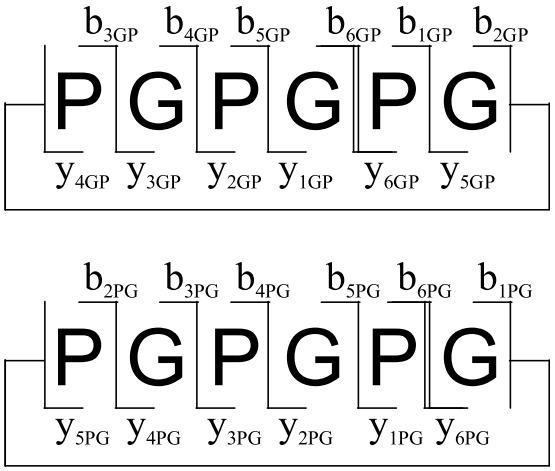
In cases where the metal has more than one isotope it was necessary to apply a correction factor of $1/A$, where A is the fractional abundance of the lightest isotope (Table 1), to the peak areas for $[M + Cat - H]^+$ and $[M + Cat + Ac]^+$. The same correction factor was applied to $[M_2 + Cat]^{2+}$ ions, which usually were observed between $[M + H]^+$ and 1:1 M/Cat species. For $[M + Cat + X]^+$ a correction factor of $1/A.B$, where B is the fractional abundance of the lightest isotope(s) of X, was applied to the monoisotopic peak area. Corrected peak areas for $[M + Cat - H]^+$, $[M + Cat + Ac]^+$, and $[M + Cat + X]^+$ were then combined to give a total value (ΣMCat), and the metal binding properties of CPG3 were investigated by determining ΣMCat/M and M_2Cat/M peak area ratios under different conditions. Structural analysis of $[M + H]^+$ and selected $[M + Cat - H]^+$ ions by CID and product ion MS/MS also was performed using a collision energy (E_{LAB}) of 29 eV, a collision gas (Ar) pressure of 1×10^{-3} mbar, and ESI conditions similar to those used for the mass spectrometry experiments.

Flow-injection ESI-MS analysis of 100 μM of CPG3 in 50:50 MeCN/H_2O containing 1 mM of NH_4Ac identified $[M + H]^+$ (*m/z* 463) as the predominant molecular ion, along with small amounts of $[M + NH_4]^+$ (*m/z* 480), $[M + Na]^+$ (*m/z* 485), and $[M + K]^+$ (*m/z* 501). The relative abundance of $[M + NH_4]^+$ increased slightly in

Table 2. Product ions for CPG3

m/z	Peak assignment[a]	Relative abundance
463	$[M + H]^+$	25.2
445	$[M + H - H_2O]^+$	100.0
435	$a_6^+{}_{PG}/a_6^+{}_{GP}$	0.9
406	$b_5^+{}_{GP}$	0.3
378	$a_5^+{}_{GP}$	0.2
366	$b_5^+{}_{PG}$	12.9
348	$b_5^o{}_{PG}$	1.8
338	$a_5^+{}_{PG}$	0.9
321	$a_5{}^*{}_{PG}$	4.7
309	$b_4^+{}_{PG}/b_4^+{}_{GP}$	16.2
291	$b_4^o{}_{PG}/b_4^o{}_{GP}$	0.4
281	$a_4^+{}_{PG}/a_4^+{}_{GP}$	1.7
269	$c_3^+{}_{GP}$	1.3
264	$a_4{}^*{}_{PG}/a_{4*GP}$	1.4
252	$b_3^+{}_{GP}$	2.0
234	$b_3^o{}_{GP}$	0.2
224	$a_3^+{}_{GP}$	0.6
212	$b_3^+{}_{PG}$	5.0
194	$b_3^o{}_{PG}$	1.2
184	$a_3^+{}_{PG}$	0.5
167	$a_3{}^*{}_{PG}$	2.3
155	$b_2^+{}_{PG}/b_2^+{}_{GP}$	5.3
137	$b_2^o{}_{PG}/b_2^o{}_{GP}$	0.9
127	$a_2^+{}_{PG}/a_2^+{}_{GP}$	0.9
115	$c_1^+{}_{GP}$	1.8
70	i_p^+	3.4

[a]For annotation, see text.



Scheme 1. Product ions formed by initial cleavage of protonated or metal-cationized CPG3 at a G—P or P—G bond, as indicated by the position of the double vertical lines.

10 mM of NH_4Ac (not shown). Low-intensity peaks corresponding to $[M_2 + H]^+$ (*m/z* 925), $[M_2 + NH_4]^+$ (*m/z* 941), and $[M_2 + Na]^+$ (*m/z* 947) also were observed; however, no multiply charged ions were detected.

Product ion MS/MS analysis of $[M + H]^+$ showed facile loss of water (*m/z* 445) and sequential loss of intact proline (97 Da) and glycine (57 Da) residues on CID (Table 2). By convention [22], fragments generated after initial cleavage of a P—G bond (i.e., a bond C-terminal to proline and N-terminal to glycine) are designated $y_{n\ PG}^+$, $b_{n\ PG}^+$, etc., and vice versa. Alternation of P and G residues in CPG3 creates a situation in which $y_{i\ PG}^+$ coincides with $b_{i\ GP}^+$ for all values of *i*, and in which $b_{i\ PG}^+$ is isobaric with $b_{i\ GP}^+$, and $b_{i\ PG}^+$ is isobaric when *i* is an even number. Furthermore y, b, and a ions are all subject to loss of water (to give y^o, b^o, and a^o) or ammonia (to give y*, b*, and a*) on low-energy CID, and therefore can not be assigned as N- or C-terminal ions on the basis of these neutral losses. However, close inspection of the product ion MS/MS spectrum for $[M + H]^+$ reveals that most of the ions corresponding to sequential loss of P and G have satellite ions at −28 u, and therefore can be assigned as b ions (Table 2).

The intensities of $b_{3\ PG}^+$ and $b_{5\ PG}^+$ ions relative to $b_{3\ GP}^+$ and $b_{5\ GP}^+$ further suggest that initial ring cleavage occurs predominantly at a P—G bond (Scheme 1). This differs from the high bias toward N-terminal fragmentation observed for proline in doubly charged, linear tryptic peptides [24]. For cyclic peptides, this bias usually results in the formation of b ions with a cationized proline residue at the N-terminus and an aziridinone group at the C-terminus [25]. However, glycine is second only to proline in its tendency toward N-terminal fragmentation [24], and the flexibility inherent in the structure of CPG3 [14] apparently serves to promote initial fragmentation at a P—G bond. Nevertheless, fragments corresponding to $a_{3\ GP}^+$, $a_{5\ GP}^+$, $c_{1\ GP}^+$, and $c_{3\ GP}^+$ confirm that initial ring cleavage also can occur at a G—P bond. These initial observations establish the identity and fragmentation behavior of $[M + H]^+$, and because multiple charging of CPG3 was negligible in all solvents, ΣMCat/M ratios were determined using M equal to the $[M + H]^+$ peak area for each sample.

Results and Discussion

Speciation of 1:1 M/Cat Complexes

Speciation experiments were performed using solvents of near-neutral pH to promote complex formation and better approximate normal physiological and environmental conditions under which peptide–metal interactions might naturally occur. Results obtained using 50:50 vol/vol ACN/H_2O containing 1 or 10 mM of NH_4Ac are summarized in Figure 1. The highest ΣMCat/M ratio was obtained for the Ca^{2+} complex (Figure 2a), suggesting that this metal ion forms the most stable 1:1 complex with CPG3. This is in good agreement with results obtained using VCD and NMR spectroscopy [14, 15]. These studies also show that Mg^{2+} and other metal ions can form complexes with

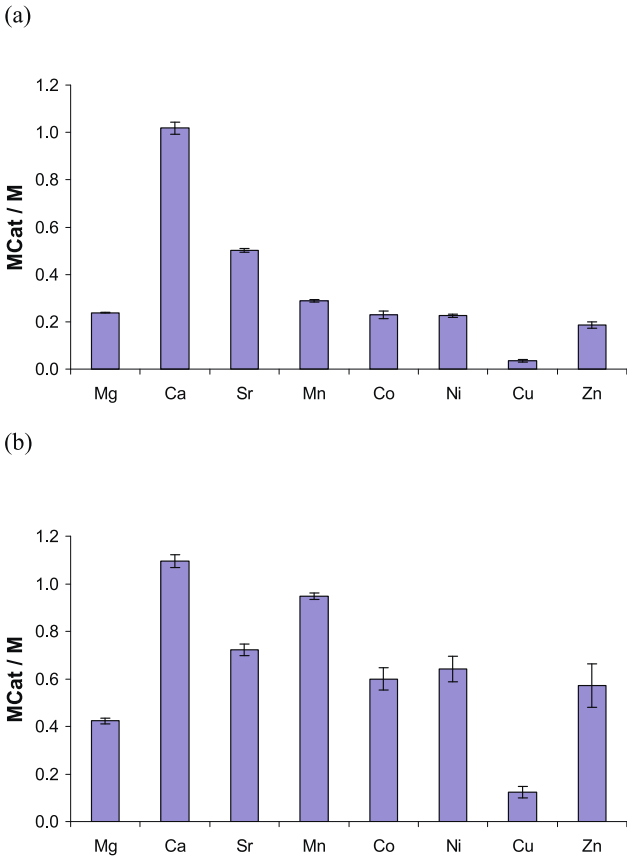


Figure 1. Relative stabilities of 1:1 complexes formed by selected group II and divalent transition-metal ions with CPG3 in 50:50 vol/vol ACN/water containing (**a**) 10 mM (pH 7.6) and (**b**) 1 mM (pH 6.7) of ammonium acetate buffer, as determined from the ratio (MCat/M) of the sum of normalized ESI-MS monoisotopic peak intensities for 1:1 M/Cat species relative to that of the unbound peptide (see text).

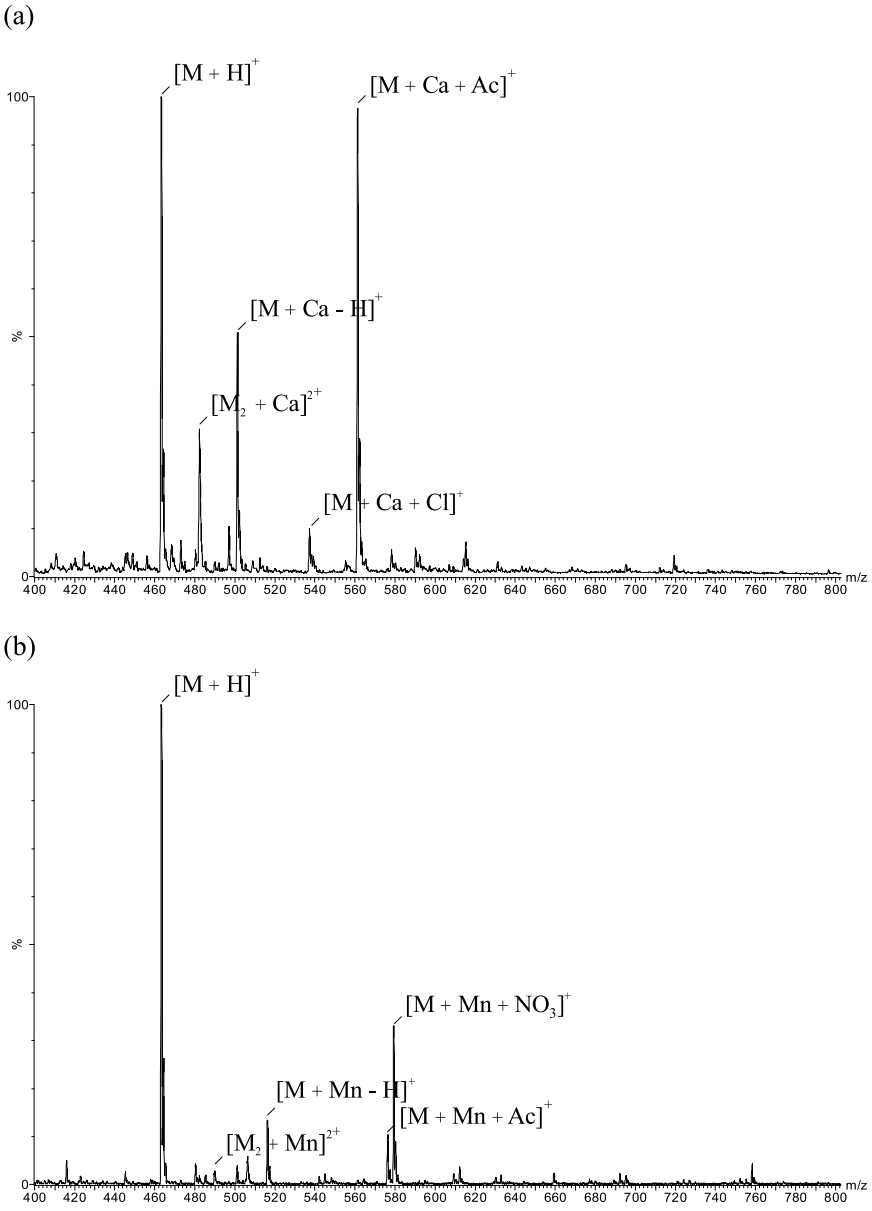


Figure 2. ESI-MS spectra showing species formed in 50:50 vol/vol ACN/water by CPG3 with (**a**) Ca^{2+} and 10 mM of ammonium acetate and (**b**) Mn^{2+} and 1 mM of ammonium acetate at a molar ratio of 1:10 M/Cat.

CPG3 in solvents less polar than pure water. Addition of an organic cosolvent such as ACN or MeOH to facilitate electrospray of aqueous samples apparently reduces the polarity of the solvent sufficiently to permit formation of such complexes [19].

Of the group II metals studied, Mg^{2+} appears to form the least stable 1:1 complex with CPG3 and the Sr^{2+} complex is second in order of stability. This can be rationalized by considering the ionic radii of the metal ions (Table 1), Sr^{2+} (118 pm) being closer in size to Ca^{2+} (100 pm) than Mg^{2+} (72 pm). Hence, Sr^{2+} is able to fit more closely than Mg^{2+} into the pocket formed by carbonyl groups on one side of the peptide molecule. In 10 mM of acetate (Figure 1a), ESI-MS results indicate that the Sr^{2+} complex also is more stable that those of the divalent transition-metal ions, which have ionic radii similar to Mg^{2+}. These values assume sixfold coordination [14, 26], with three sites occupied by carbonyl groups and three available to interact with other ligands, including X, Ac, and M.

When the buffer concentration is reduced to 1 mM of acetate (Figure 1b), the 1:1 complex formed by Mn^{2+} (Figure 2b) becomes more stable than that of Sr^{2+}, and complexes of Co^{2+}, Ni^{2+}, and Zn^{2+} are of comparable stability. This is consistent with the ionic radius of Mn^{2+} (at 83 pm the largest of the five transition-metal ions) and an approximately spherical (d^5) electron distribution that promotes electrostatic interactions with oxygen and other atoms that are not easily polarized [3]. Results suggest that these electrostatic interactions are

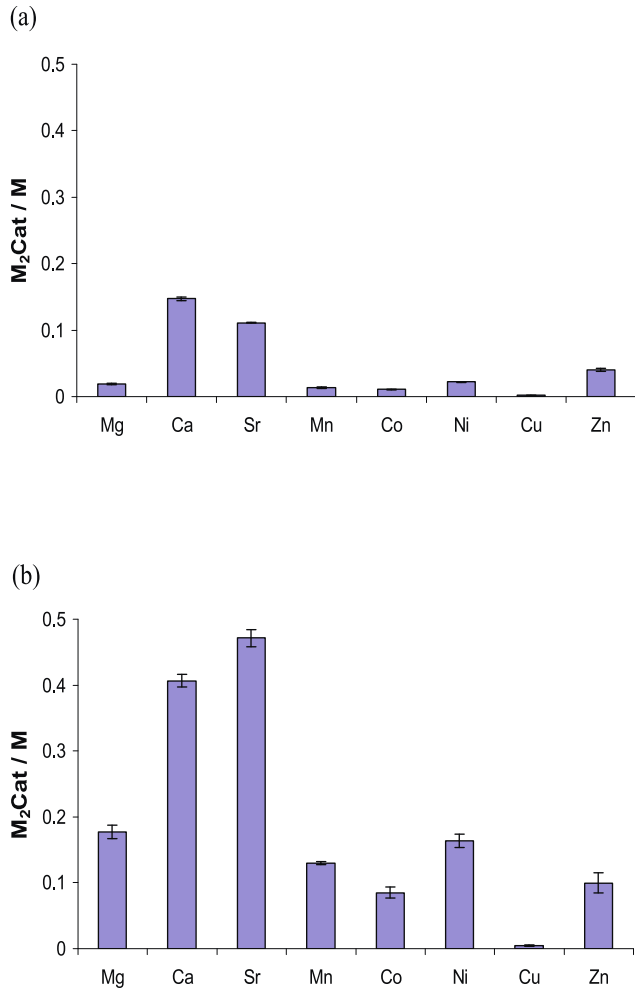


Figure 3. Relative stabilities of 2:1 M/Cat complexes formed by selected group II and divalent transition-metal ions with CPG3 in 50:50 vol/vol ACN/water containing (**a**) 10 mM and (**b**) 1 mM of ammonium acetate buffer, as determined from the ratio (M_2Cat/M) of the normalized ESI-MS monoisotopic peak intensity for the 2:1 complex relative to that of the unbound peptide (see text).

suppressed at higher buffer concentrations but become more evident as ionic strength is reduced. This has important implications for biological and environmental systems, in which ionic strength can change significantly across cell membranes and other interfaces. For example, complexes formed by cyclic peptides with certain metals could be destabilized on transfer from freshwater to saline systems, such as seawater, cytoplasm, and xylem sap, while other complexes continue to transport selected metal ions in these systems, based on steric effects. This scenario could form the basis for selective uptake and sequestration of metals from contaminated soils and (interstitial) groundwater, using plants adapted to release and/or take up cyclic peptides via their roots.

The anomalous results obtained for Cu^{2+} in aqueous ACN are likely because of reductive complexation of copper by ACN [3], a situation that often occurs when interactions between Cu^{2+} and the added organic ligand (in this case, CPG3) are relatively weak [19]. This is confirmed by the appearance of strong $[Cu^{I}(ACN)_i]^+$ ions in the full scan ESI-MS spectrum (not shown). When ACN is replaced with MeOH the ΣMCat/M ratio for Cu^{2+} increases to 0.45, a value comparable with those for the other transition metals in 1 mM of acetate (Figure 4b), although ESI-MS sensitivity is significantly reduced [19].

Speciation of 2:1 M/Cat Complexes

Previous studies suggest that formation of 1:2 salt sandwich M/Cat complexes by CPG3 is precluded by allosteric effects that result in the distortion of a second potential binding site on initial formation of the 1:1 complex [14]. That no such complexes were detected by ESI-MS supports this hypothesis. In contrast, Mg^{2+} is known to form peptide sandwich complexes in solution by binding to the opposite faces of two CPG3 molecules [14], while similar complexes have been observed for Ca^{2+} using X-ray crystallography and crystals grown in aqueous MeOH [15]. Peaks corresponding to 2:1 M/Cat complexes were observed in ESI-MS spectra for all of the group II and transition-metal ions included in this study. Such complexes were detected as doubly charged $[M_2 + Cat]^{2+}$ ions, which have *m/z* ratios in the same range as the singly charged 1:1 complexes and free peptide ions. Hence, M_2Cat/M ratios determined using $[M_2 + Cat]^{2+}$ and $[M + H]^+$ peak areas provide a good indication of the propensity with which different metal ions form 2:1 M/Cat complexes (Figure 3).

In 10-mM acetate the order of relative stabilities for M_2Cat complexes is similar to that for 1:1 species in the same solvent, with Ca^{2+} forming the most stable complex (Figure 3a). Reducing the buffer concentration to 1 mM again promotes complexation and enhances the stabilities of other complexes relative to Ca^{2+}, to the extent that Sr^{2+} forms the most stable M_2Cat complex in this solvent (Figure 3b). M_2Cat accounts for 20-30% of total complexed metal for the group II cations in 1 mM of acetate but only 10-20% for the transition metals, based on the measured peak areas for $[M_2 + Cat]^{2+}$ and the 1:1 M/Cat species. In 10 mM of acetate the distribution shifts even further toward the 1:1 complexes for both group II and transition metals, and $[M + Cu - H]^+$ accounts for over 95% of complexed copper in all solvents. It is interesting to note that, of the three species represented by ΣMCat, it is $[M + Cat + X]^+$ that predominates for transition metals in 10 mM of acetate, whereas $[M + Cat + Ac]^+$ is the most abundant complex for group II metals, which are known to interact strongly with oxygen [3]. $[M + Cat + X]^+$ also predominates for transition metals in 1 mM of acetate, whereas $[M + Mg + Ac]^+$, $[M + Ca - H]^+$, and $[M + Sr + NO_3]^+$ are the more abundant species in this solvent. Hence, ESI-MS provides information regarding the specificity with which CPG3 and other ligands interact

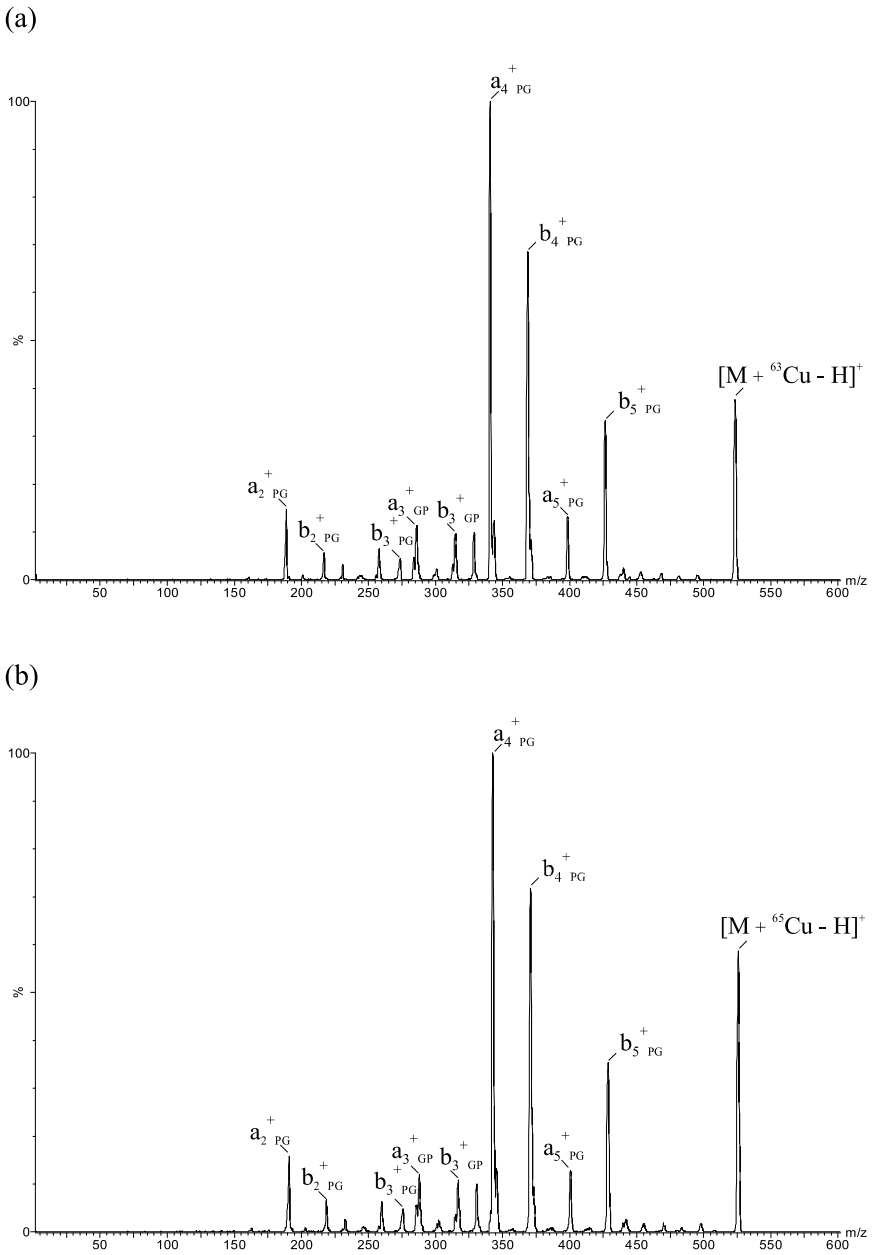
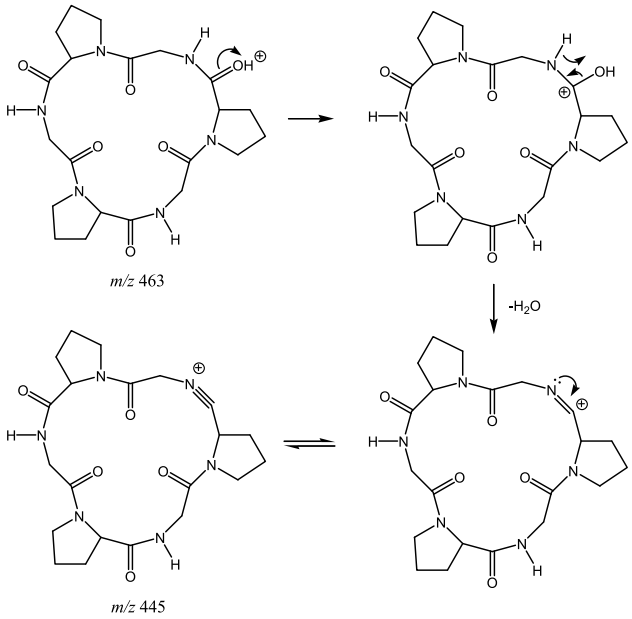


Figure 4. Product ion MS/MS spectra containing N-terminal fragments formed by CID of the 1:1 copper complex ions (**a**) $[M + {}^{63}Cu - H]^+$ (*m/z* 524) and (**b**) $[M + {}^{63}Cu - H]^+$ (*m/z* 526), showing retention of copper by all fragments, suppression of water loss, and enhancement of a ions relative to the product ion spectrum for $[M + H]^+$ (Table 2).

with these metal ions, as well as the relative stabilities of 1:1 and 2:1 M/Cat complexes formed by CPG3.

Characterization of M/Cat Complexes

Product ion MS/MS experiments were performed on selected $[M + Cat - H]^+$ ions in an attempt to obtain structural information for complexes formed by CPG3. MS/MS characterization of cyclic peptides is complicated by the lack of well-defined termini and the potential for ring cleavage at several positions on the peptide backbone (*vide supra*). However, recent studies indicate that structurally informative fragmentation of cyclic peptides can be obtained by attachment of particular metal ions, notably Na^+ and Pb^{2+} for depsipeptides and Ni^{2+}, Sr^{2+}, Ag^+, and Li^+ for certain other cyclic peptides [23]. Because the structure of CPG3 is already known, it was hoped that MS/MS analysis would provide additional information from which the location and geometry of the metal binding site(s) could be inferred. In practice, however, the symmetry of the CPG3 molecule, combined with the nature of the product ion MS/MS spectra, provided limited information about the mode of metal-ion binding.



Scheme 2. Proposed mechanism for loss of water from protonated CPG3 on low-energy CID.

This is exemplified by MS/MS spectra obtained for the $[M + Cat - H]^+$ complexes of $^{63}Cu^{2+}$ and $^{65}Cu^{2+}$ (Figure 4). These show sequential loss of proline and glycine to form a series of a and b ions, as observed for the protonated ligand (Table 2). However, loss of water from $[M + Cu - H]^+$ is not observed, suggesting that protonation is necessary for this process to occur. ^{18}O-labeling experiments have shown that singly protonated peptides can lose water from the C-terminal carboxylic acid, the side-chain hydroxyl groups of serine or threonine residues or the amide carbonyl of a peptide bond [27]. In the case of CPG3, the options are limited to loss of water from a proline or glycine carbonyl group. Furthermore, initial loss of water appears to be competitive with sequential loss of intact amino acid residues, because *m/z* 445 is the base peak in the product ion spectrum for $[M + H]^+$ and the b^o ions are of relatively low abundance (Table 2). The apparent stability of the *m/z* 445 ion suggests that it is probably cyclic, while the absence of an equivalent product ion for $[M + Cu - H]^+$ further suggests that protonation of a prolyl carbonyl group is required for elimination of water from CPG3. This likely proceeds via transfer of an electron pair from the C=O double bond to the protonated oxygen (Scheme **2**), followed by transfer of the glycyl amide hydrogen to oxygen and the remaining electron pair from the C—O bond to the N—Cα bond. This concerted reaction, which proceeds via a four-membered cyclic intermediate, releases H_2O to form a stable cation in which the integrity of the CPG3 ring is maintained.

Each product ion in the MS/MS spectrum of $[M + {}^{65}Cu - H]^+$ (Figure 4b) is offset by +2 u from the corresponding peak in the $[M + {}^{63}Cu - H]^+$ spectrum (Figure 4a), indicating that copper is associated with every fragment. Furthermore, the relative intensity of the $a_{4\,PG}^+$ ion peak is much greater for $[M + Cu - H]^+$ than for $[M + H]^+$, suggesting that attachment of copper promotes loss of CO from $b_{4\,PG}^+$, which is the most stable $[M + H]^+$ fragment after loss of water (Table 2). The fact that other a ions also are enhanced makes the product ion spectrum for $[M + Cu - H]^+$ much easier to interpret than that of the protonated molecular ion, illustrating the use of metal cationization for structural elucidation of cyclic peptides [23]. Copper is known to interact strongly with basic residues and, in the case of an N-terminal arginine, to remain immobilized while sequential loss of amino acids from the C-terminus produces a series of a and b ions containing copper [28]. However, previous studies also suggest that proline does not possess a strong affinity for copper (at least, for Cu^+) [28]. This would explain the apparent retention of copper by an N-terminal glycine, followed by sequential loss of alternating proline and glycine residues from the opposite end of the linearized peptide. It also suggests that Cu^{2+} initially is bound to at least one glycine residue, whereas Ca^{2+} and Mg^{2+} tend to interact preferentially with proline carbonyl groups in 1:1 complexes with CPG3 [14]. Another possible interpretation is that Cu^{2+} initially is bound to more than one site on the peptide, an observation consistent with binding of the metal ion by multiple carbonyl groups (*vide supra*). Unfortunately, the tendency of metal cationized cyclic peptides to undergo "charge remote" fragmentation after ring cleavage [25] means that the initial site(s) of metal attachment can not be confirmed using CID and MS/MS, even though the resulting product ions may be structurally informative.

Conclusions

ESI-MS is a powerful method for studying interactions between metal ions and cyclic peptides in solution. Results for Mg^{2+} and Ca^{2+} are in good agreement with those obtained using VCD, NMR, and other methods, indicating the speciation and relative stabilities of complexes formed by CPG3 in aqueous solvents. ESI-MS also provides this information for divalent transition-metal ions, some of which are paramagnetic and therefore less amenable to NMR spectroscopic analysis. Results are consistent with the ionic radius of the metal ion and its propensity for electrostatic interaction with neutral oxygen donors such as CPG3 carbonyl groups. Product ion MS/MS analysis confirmed the cyclic structure of the peptide, although the location and geometry of the metal binding site(s) could not be determined using this technique.

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