Cyclic Peptides. 19. Cation Binding of a Cyclic Dodecapeptide *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ in an Aprotic Medium[1]

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Abstract: The synthesis of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ is reported. This homodetic cyclic dodecapeptide contains only naturally occurring amino acids and is a model of an ion carrier related to valinomycin. Titration curves obtained from circular dichroism data in acetonitrile solutions reveal three kinds of cyclic dodecapeptide (P)–cation (C) complexes: PC (1:1 complex), P_2C (peptide–sandwich complex), and PC_2 (ion–sandwich complex). Binding constants of several cations and molar ellipticities of the bound species were determined using a new computer program. The stabilities of the 1:1 complexes are found to be correlated with the diameter of the divalent cation in the series $Mg^{2+} \ll Ca^{2+} \ll Ba^{2+}$. The charge of the cation is also an important factor: Ca^{2+} is more strongly bound than K^+, which has a diameter close to that of Ba^{2+}. Nevertheless, the stability of the peptide–K^+ complex is comparable to that of valinomycin–K^+. Another important finding is that P_2C complexes are stable; indeed, these are the major species for small cations, e.g., Li^+, Mg^{2+}, and Na^+.

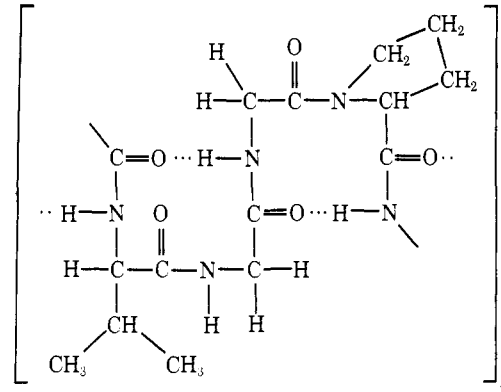
Current interest in the functions of biological membranes, particularly ion transport, has led to several studies of ion binding and transport with antibiotics[4] and other natural peptides[5] and with synthetic ionophores.[6,7] Extrapolation of the findings of such studies should help in understanding the physical-chemical basis for the functions of membrane proteins. In an effort to facilitate the extension of analyses of antibiotic ionophores to protein systems, the present study uses a cyclic peptide designed to retain the functional properties of a well-known antibiotic ionophore and to contain only amino acids normally found in proteins. The ion-binding properties of this synthetic cyclic peptide are described in this paper.

Valinomycin is generally considered to be a good molecule for binding ions and transporting them through lipid membranes, both natural and synthetic. Its sequence confers on it the capability of adopting a C_3 symmetric conformation with a polar cavity, lined with carbonyls, and a hydrophobic exterior (formed by side chains on the hydroxy and amino acid residues) (Figure 1a). The molecular backbone in the ion-binding conformation is made up of alternating type II (-L-Val-D-HyIv-) and II′ (-D-Val-L-Lac-) β turns.[8,9]

Although valinomycin is an appropriate subject for study as an ion carrier, the occurrence of ester linkages and of D residues in its sequence makes valinomycin a poor model for structures that might exist in eukaryotic membrane proteins. With a goal of finding a more appropriate, yet functional, model for such proteins, it was decided to design a *homo*detic cyclic peptide, analogous to valinomycin so that ion-binding properties would be expected, but also lacking D residues. The use of Gly residues in place of the D-HyIv's and D-Val's leaves open the same regions of conformational space that were available to the D residues (and, of course, those regions available to L residues, as well).[10] Furthermore, the use of Pro's in place of the Lac residues replaces the ester function without introducing an additional NH.

Precedent for the analogies in the conformation described above was established by Gisin and Merrifield.[11] These workers synthesized *cyclo*-(L-Val-D-Pro-D-Val-L-Pro)$_3$ by solid-phase methods and found that this cyclic dodecapeptide did indeed bind potassium cations analogously to valinomycin. The cyclic dodecapeptide described herein incorporates Gly's in the positions in the sequence where Gisin and Merrifield utilized D residues, i.e., *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$.

cyclo-(L-Val-Gly-Gly-L-Pro)$_3$ is potentially able to retain two features of the valinomycin–K^+ complex: (i) a conformation commonly referred to as the "bracelet" structure[8,9] and (ii) a hydrophobic exterior around a hydrophilic cavity. The sequence, -L-Val-Gly-Gly-L-Pro-, allows only one "bracelet" conformation due to the lack of an amide hydrogen on the proline:



In the above conformation the three Val side chains are on one face, and the three Pro rings are on the other. A Corey-Pauling-Koltun (CPK) model of the bracelet conformation of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ is shown in Figure 1b.

The use of the (L-Val-Gly-Gly-L-Pro) repeating sequence introduces several specific conformational features *not* present in valinomycin. First, the Gly residues are less restricted in conformational space than their counterparts in the valinomycin sequence. As a result, the present cyclic dodecapeptide could assume *different* conformations from valinomycin and would be expected to have more conformational freedom. Second, the decreased number of bulky side chains leads to a more "open" structure, and to greater accessibility of a complexed ion to solvent, or to interactions with other peptide molecules (cf. Figures 1a and 1b).

We have studied the binding of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ in acetonitrile solutions to alkali metal and alkaline earth cations. In order to compare the effects of size and charge, cations of related diameter were chosen for study: K^+ and Ba^{2+}, Na^+ and Ca^{2+}, and Li^+ and Mg^{2+}. Problems of NH-anion and cation-anion interactions have been reduced by using perchlorate rather than halogen salts.

Acetonitrile has been chosen as a solvent over protic solvents such as water or methanol because of the potentially better resolution of the different kinds of cation complexes which occur in this solvent.[6,12]

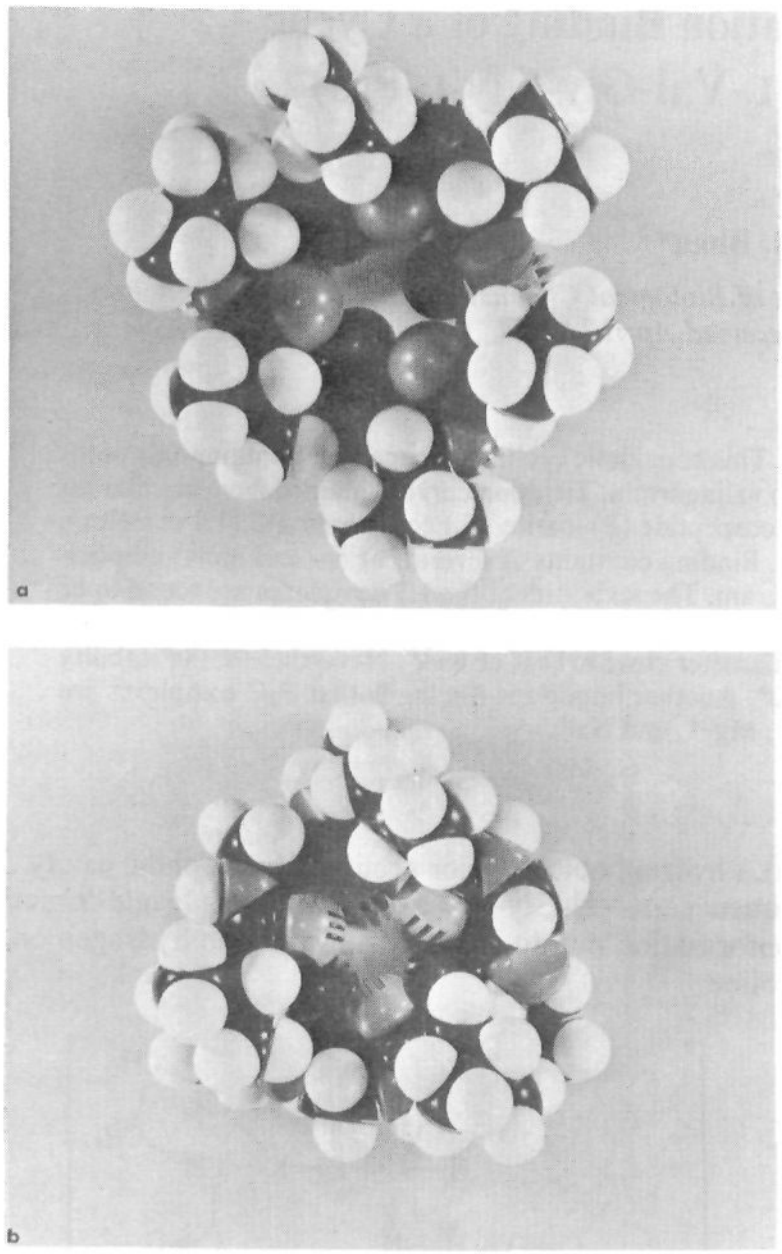


Figure 1. Photographs of CPK models: (a) valinomycin in bracelet conformation; (b) *cyclo*-(L-Val-Gly-Gly-Pro)$_3$.

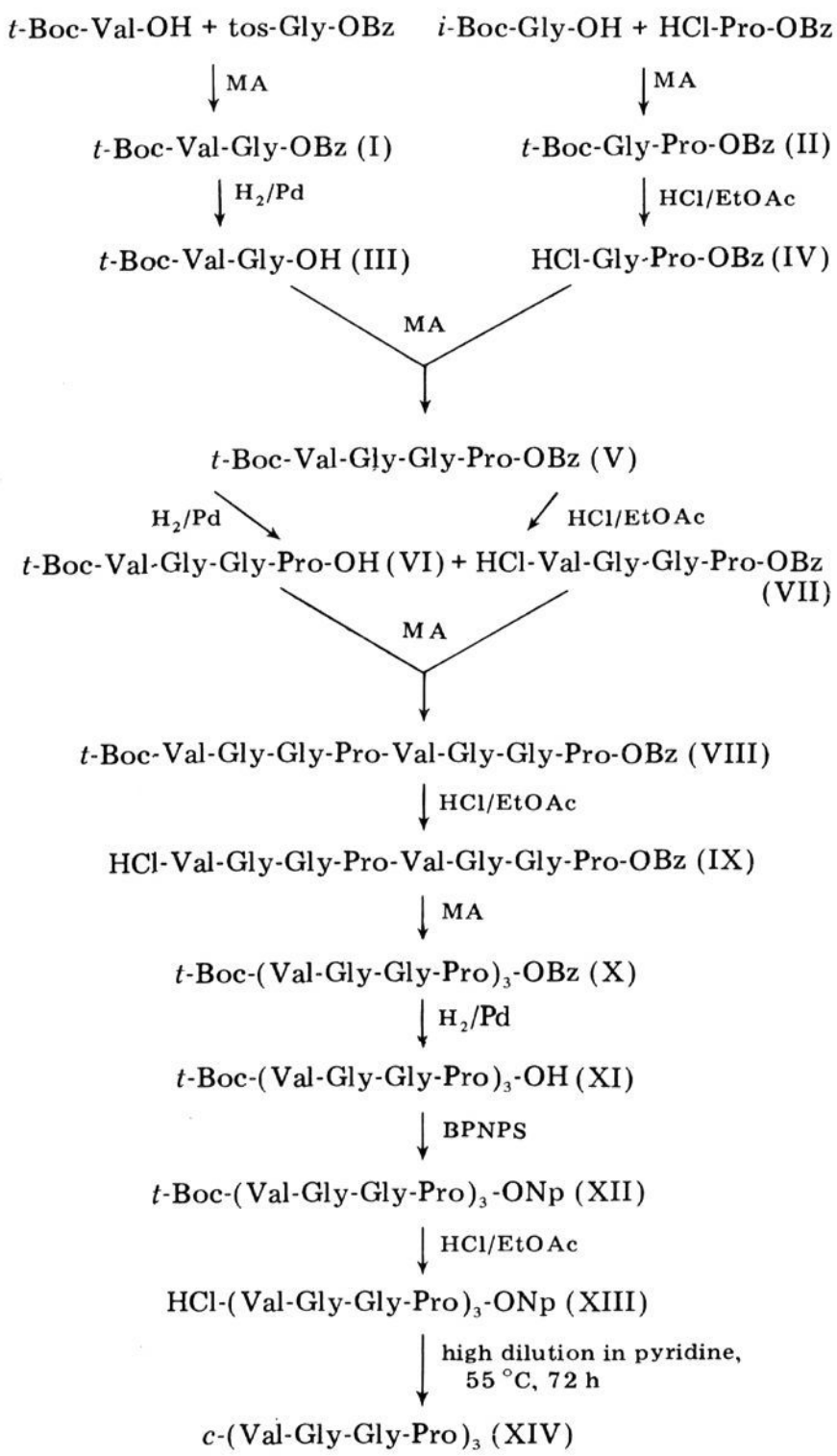
Titration curves were obtained from circular dichroism spectra in the amide region (below 250 nm), which includes both n→π* and π→π* transitions. Circular dichroism is particularly appropriate for such studies because it is sensitive to peptide conformation,[13] and conformational changes are expected to occur upon complex formation.[6,8,12,14-16] Ideally, distinct conformations of particular complexes can be detected and analyzed in terms of the binding interactions. In the present work the emphasis was on determination of binding constants and molar ellipticities, especially when several complexed species are in equilibrium. In such cases, data at several wavelengths were analyzed, generally with a computer program.

Experimental Section

Synthesis. *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ was synthesized via cyclization of the *p*-nitrophenyl ester of the linear dodecapeptide, HCl-(Val-Gly-Gly-Pro)$_3$-ONp. Scheme I is a summary of the overall synthetic scheme. The identity and purity of all intermediate compounds were checked by thin-layer chromatography (TLC), infrared spectroscopy, and nuclear magnetic resonance. All compounds used in succeeding steps showed appropriate spectral properties and a single spot on TLC. Details of synthetic procedures are given below.

***t*-Boc-Val-Gly-OBz (I).** A solution of *t*-Boc-Val-OH (43.4 g, 200 mmol) in $CHCl_3$ (ca. 300 mL) was cooled to −20 °C in dry ice/CCl_4 and treated with 22.3 mL (200 mmol) of *N*-methylmorpholine and 28.8 mL (220 mmol) of isobutyl chloroformate. After the reaction mixture was stirred for 15 min at −20 °C, 67.2 g of Tos-Gly-OBz (200 mmol) and an additional equivalent (22.3 mL) of *N*-methylmorpholine were added, and the reaction mixture was allowed to warm slowly to room temperature and stirring was continued overnight. The product mixture was purified by successive extractions with 225-mL portions of 0.2 N HCl (twice) and 5% $NaHCO_3$, drying the $CHCl_3$ layer over $MgSO_4$, and evaporation to yield a pale orange syrup. The crude product was dissolved in ether and crystallized overnight to give I in 85% yield (61.9 g) as white hygroscopic crystals which tended to collapse on storage: mp 63–69 °C.

Scheme I. Cyclic Dodecapeptide Synthetic Scheme[a]



[a] Abbreviations used are: ONp, *p*-nitrophenyl ester; *t*-Boc, *tert*-butoxycarbonyl; tos, *p*-tosylate; BPNPS, bis(*p*-nitrophenyl) sulfite; EtOAc, ethyl acetate; MA, mixed anhydride; *c*-, *cyclo*.

***t*-Boc-Gly-Pro-OBz (II).** Preparation was as previously reported.[17]

***t*-Boc-Val-Gly-OH (III).** I (21.0 g, 58 mmol) was dissolved in 250 mL of *tert*-butyl alcohol (required warming) and was treated with 10% Pd/C. Hydrogenation of 20 psi for 16 h at room temperature, followed by removal of the catalyst by filtration through Celite, evaporation of solvent, and pumping in vacuo yielded III as a solid foam (90% yield, 14.2 g) which was used directly in the next step.

HCl-Gly-Pro-OBz (IV). A solution of *t*-Boc-Gly-Pro-OBz (II) (50.7 g, 140 mmol) in ethyl acetate (500 mL) was cooled to 0 °C and treated with HCl gas for 45 min. The solvent was evaporated, and the resulting white foam was triturated with ether and dried; yield, 43.3 g (100%).

***t*-Boc-Val-Gly-Gly-Pro-OBz (V).** The tetrapeptide (V) was prepared from the reaction of a mixed anhydride of III (formed by adding 12.9 mL (115 mmol) of *N*-methylmorpholine and 14.6 mL (127 mmol) of isobutyl chloroformate to a solution of 31.5 g (115 mmol) of III in 300 mL of $CHCl_3$ at 15 °C) with 34.4 g (115 mmol) of IV, added after 20 min with an additional equivalent of *N*-methylmorpholine. The reaction mixture was allowed to warm to room temperature slowly, and stirring was continued overnight. The product was obtained by

successive extractions with 250 mL of 0.2 N HCl (twice) and 5% $NaHCO_3$. Evaporation of the $CHCl_3$ layer (dried over $MgSO_4$) yielded a yellow syrup which crystallized from ether/hexane to give 43.0 g (72% of theoretical) of V: mp 67–70 °C.

***t*-Boc-Val-Gly-Gly-Pro-OH (VI).** Hydrogenation of 29.7 g (57.3 mmol) of V under 30 psi of H_2 for 16 h (in *tert*-butyl alcohol with 10% Pd/C as catalyst) yielded, upon filtration through Celite and evaporation of the solvent, VI as an amorphous solid which was not crystallized; yield, 22.8 g (93%).

HCl-Val-Gly-Gly-Pro-OBz (VII). A solution of V (13.0 g, 25 mmol) in ethyl acetate (250 mL) was treated at 0 °C with HCl gas for 45 min. When the solvent was evaporated, a yellow residue, which solidified on trituration with ether, remained. The off-white amorphous solid was dried in vacuo to yield 11.0 g (96.5%) of VII.

***t*-Boc-(Val-Gly-Gly-Pro)$_2$-OBz (VIII).** A solution of the tetrapeptide acid VI (10.36 g, 24.2 mmol) in $CHCl_3$ (100 mL) was cooled to −15 °C and treated with 2.7 mL (24.2 mmol) of *N*-methylmorpholine and 3.5 mL (26.6 mmol) of isobutyl chloroformate. After 10 min at −15 °C, a solution of VII (11.0 g, 24.2 mmol) in 30 mL of $CHCl_3$ was added dropwise to the reaction mixture, followed by an additional equivalent of *N*-methylmorpholine. The reaction was allowed to warm slowly to room temperature and to stir an additional 12 h. Work-up by extractions with 250-mL portions of distilled water, 5% $NaHCO_3$, and saturated NaCl solutions, followed by drying of the $CHCl_3$ layer, yielded on evaporation of the solvent a yellow foam. On dissolving in hot acetone and cooling, the octapeptide VIII crystallized as a microcrystalline, hygroscopic solid which was isolated under nitrogen; yield, 15.5 g (78%).

HCl-(Val-Gly-Gly-Pro)$_2$-OBz (IX). A suspension of the octapeptide VIII (15.0 g, 18.1 mmol) in 200 mL of ethyl acetate was cooled to 0 °C and bubbled with HCl gas for 2 h (time necessary for all of starting material to dissolve). Evaporation of the solvent left the octapeptide IX as a white solid, which was triturated with ether and dried in vacuo; yield, 13.3 g (93%).

***t*-Boc-(Val-Gly-Gly-Pro)$_3$-OBz (X).** A solution of the tetrapeptide acid VI (3.10 g, 7.23 mmol) in $CHCl_3$ (50 mL) was cooled to −15 °C and treated with 0.81 mL (7.2 mmol) of *N*-methylmorpholine and 1.04 mL (7.9 mmol) of isobutyl chloroformate. After the reaction mixture stirred for 10 min at −15 °C, 5.52 g (7.4 mmol) of the octapeptide hydrochloride IX and an additional equivalent of *N*-methylmorpholine were added. The reaction mixture was allowed to warm slowly and to continue stirring for a further 10 h. Extraction with water (300 mL), 5% $NaHCO_3$ (200 mL), and saturated NaCl (200 mL), followed by drying and evaporation of the $CHCl_3$ layer, yielded the dodecapeptide X as an off-white foam which crystallized from methanol as a microcrystalline solid: mp 177–182 °C; yield, 6.0 g (73%).

***t*-Boc-(Val-Gly-Gly-Pro)$_3$-OH (XI).** A trifluoroethanol solution of X (6.0 g, 5.3 mmol, in 100 mL) was treated with a catalytic amount of 10% Pd/C and hydrogenated at 30 psi for 20 h. The catalyst was removed by filtration through a pad of Celite, and the solvent was evaporated, leaving an off-white foam which came out of methanol as a microcrystalline solid. Trituration with ethyl acetate and drying in vacuo yielded 4.2 g (76%) of XI.

***t*-Boc-(Val-Gly-Gly-Pro)$_3$-ONp (XII).** The dodecapeptide acid XI (4.2 g, 4.0 mmol) was dissolved in 100 mL of dry, distilled pyridine, and the reaction vessel was kept in the dark. Bis(*p*-nitrophenyl) sulfite (BPNPS) (1.56 g; 4.8 mmol) was added and stirring was continued. Monitoring the reaction mixture by TLC showed a significant amount of unreacted starting material, so that 0.6 g more of BPNPS was added after 2 h and an additional 0.5 g after 12 h. The product was obtained by evaporation of the solvent and trituration of the residue with ether. The resulting off-white solid was dissolved in $CHCl_3$ and extracted two times with 30-mL portions of water. The $CHCl_3$ layer was dried with Na_2SO_4 and treated with ether to the cloud point to yield the dodecapeptide active ester XII as a microcrystalline solid; yield, 2.81 g (60%).

HCl-(Val-Gly-Gly-Pro)$_3$-ONp (XIII). Treatment of XII (2.74 g, 2.3 mmol) in 60 mL of ethyl acetate at 0 °C with HCl gas for 20 min yielded the dodecapeptide active ester hydrochloride XIII, which was isolated by evaporation of the solvent; yield, 2.45 g (95%).

***cyclo*-(Val-Gly-Gly-Pro)$_3$ (XIV).** A 100-mL solution of 1.23 g (1.1 mmol) of XIII in dimethylformamide (dried over Na_2SO_4) was added dropwise over a 7-h period with efficient stirring to 1.5 L of spectrophotometric grade pyridine at 55 °C. The reaction was allowed to proceed at 55 °C for 72 h, during which time the reaction mixture became bright yellow. The mixture was cooled, and the solvents were evaporated. The resulting brown residue was triturated with ether, taken up in 70 mL of 60:40 (v/v) ethanol–water, and stirred for 1.5 h with Rexyn I-300 ion exchange resin at room temperature. Removal of the resin by filtration and the solvents by evaporation yielded a white solid (0.98 g). The crude product was dissolved in methanol, and a semicrystalline gel formed and was isolated (presumably polymeric material). The mother liquor was evaporated and redissolved in acetone. Crystalline *cyclo*-(Val-Gly-Gly-Pro)$_3$ was then isolated in 59% yield: 614 mg (combined first and second crops), mp 240 °C dec.

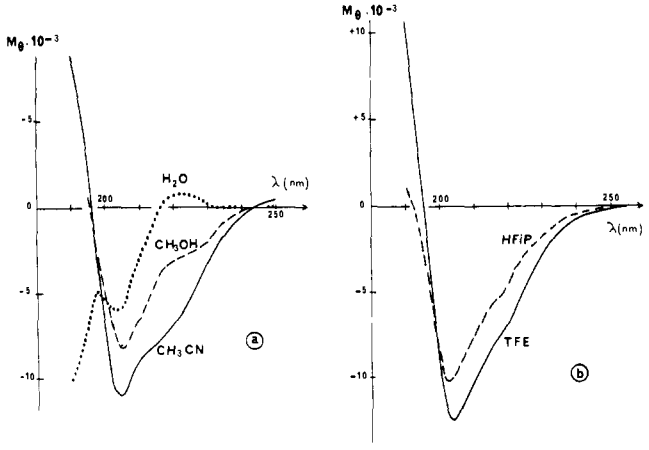


Figure 2. Mean residue ellipticity of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ in various solvents; [peptide] ≃ 10^{-4} M: (a) CH_3CN (—), CH_3OH (- - -), H_2O (···); (b) Trifluoroethanol (—), hexafluoro-2-propanol (- - -).

The identity of the cyclic dodecapeptide was confirmed by ^{13}C nuclear magnetic resonance (NMR) and by infrared spectroscopy. Both of these methods indicated that the molecule was cyclic; ^{13}C NMR spectra revealed only one resonance for each carbon of the tetrapeptide repeat unit, in contrast to spectra of the linear precursors which were complex with multiple resonances.

Anal. Calcd for $C_{42}H_{66}N_{12}O_{12}\cdot H_2O$: C, 53.16; H, 7.17; N, 17.72. Found: C, 53.52; H, 7.15; N, 17.74.

Circular Dichroism Measurements. Spectrophotometric grade acetonitrile (Eastman Kodak) was dried over molecular sieves before use, and perchlorate salts were dried in vacuo at 120 °C.

Solutions used in circular dichroism titrations were prepared by mixing varying amounts of two stock peptide solutions, one which contained no salt, and the other containing 1 M perchlorate salt (except in the case of K^+, where the maximum concentration limit was 10^{-2} M). The concentrations of the resulting solutions containing salt and peptide were calculated from the weights of the solutions combined, using measured densities. Negligible excess volume of mixing was assumed.

Circular dichroism (CD) spectra were recorded on a Cary 60 spectropolarimeter with a Model 6001 CD attachment, using, generally, a 0.1-cm path length and 0.04° full range and peptide concentrations of 10^{-4} M. A blank run of solvent and cell was subtracted from the measured spectrum.

Results

Circular Dichroism Spectra. Free Peptide. Circular dichroism spectra of the dodecapeptide in several organic solvents (trifluoroethanol, hexafluoro-2-propanol, and methanol) do not differ markedly from that in acetonitrile (see Figure 2). All show a minimum at ca. 205 nm with a shoulder near 225 nm. There is no obvious correlation with CD spectra of valinomycin.[15] The spectrum of *cyclo*-(Val-Gly-Gly-Pro)$_3$ in water is quite different from that in less polar solvents (Figure 2a) and suggests that a different conformation(s) occur(s) in aqueous solution. Molar ellipticities of the cyclic peptide in CH_3CN do not depend on concentration in the range 10^{-6} to 10^{-4} M.

Small Amounts of K^+, Ca^{2+}, or Ba^{2+}. Solutions which are ca. 10^{-3} M in any of these three salts yield similar spectra (Figure 3a). The spectra are characterized by the disappear-

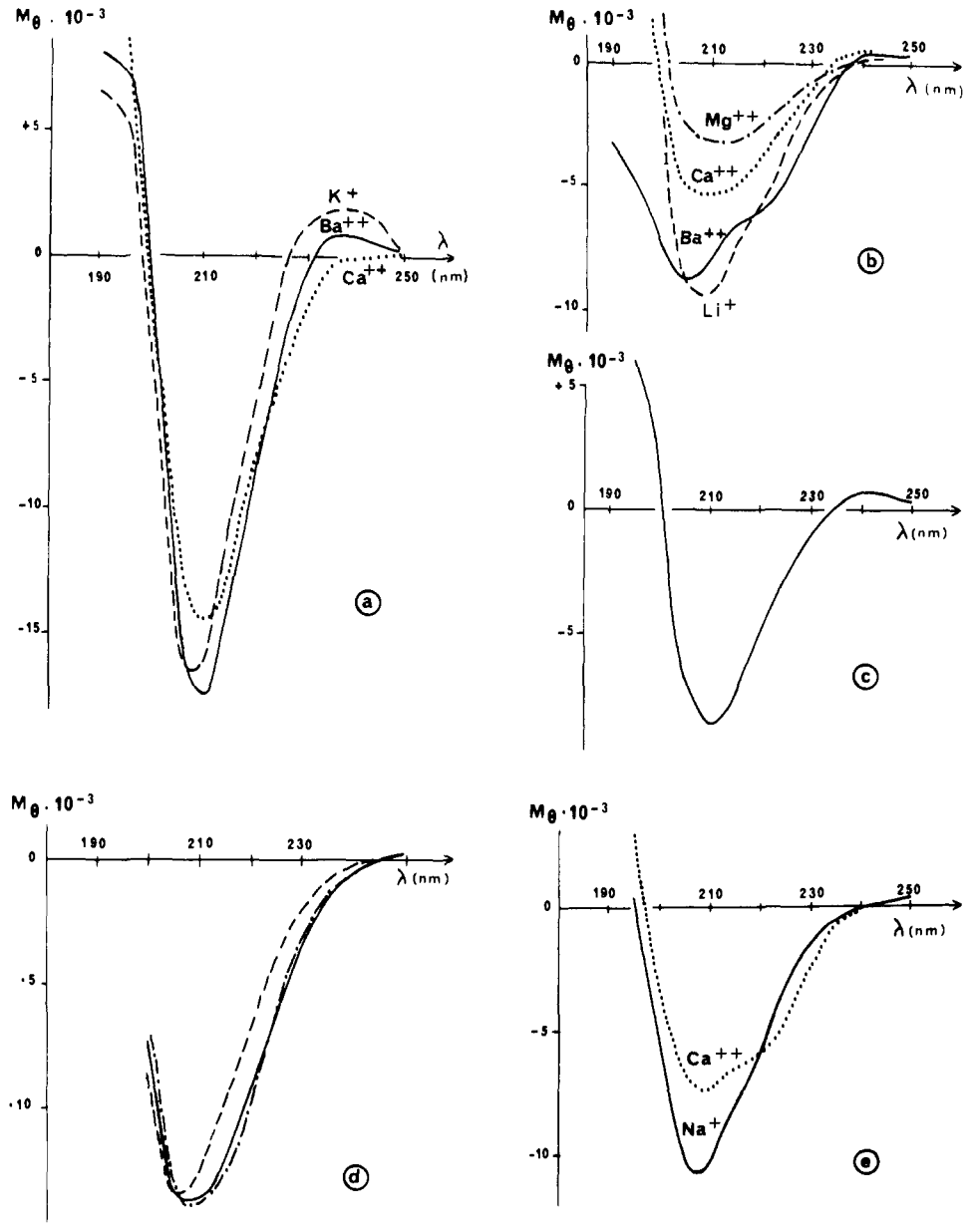


Figure 3. Mean residue ellipticity of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ in CH_3CN: (a) [peptide] $\simeq 10^{-4}$ M; [K^+] = 7.4×10^{-3} M (- - -), [Ca^{2+}] = 2.6×10^{-4} M (···), [Ba^{2+}] = 3.5×10^{-4} M (—); (b) 1 M solution of Li^+ (- - -), Mg^{2+} (···), Ba^{2+} (—), and 0.19 M Ca^{2+} (···); (c) 2×10^{-3} M Na^+, [peptide] = 10^{-4} M; (d) [peptide] $\simeq 10^{-5}$ M; [Li^+] = 1.1×10^{-3} M (- - -), [Na^+] = 0.66 M (—), [Mg^{2+}] = 1.7×10^{-3} M (···); (e) [peptide] $\simeq 10^{-4}$ M; [Na^+] = 0.6 M (—), [Ca^{2+}] = 1 M (···).

ance of the 225-nm shoulder and a strong enhancement of the main negative band. The extremum is shifted to 210 nm (from 205 nm for the free peptide). These spectra are designated type I.

Molar Solutions of Li^+, Mg^{2+}, and Ba^{2+}. In these cases there is a sharp decrease of the negative ellipticity, which becomes even less negative than in the free peptide (Figure 3b). While not strictly similar, these spectra are referred to as type II.

Small Amounts of Na^+. Concentrations of Na^+ of 10^{-3} M in 10^{-4} M peptide solution result in a disappearance of the 225-nm shoulder of the free peptide spectrum and a shift of the extremum to 210 nm but no increase of the negative ellipticity (Figure 3c). Significant changes take place at shorter wavelengths.

Other Cases. Small amounts of Li^+ or Mg^{2+} lead to an approximate type I spectrum, but the enhancement of the negative ellipticity is less marked (Figure 3d). At high concentrations of Ca^{2+} two different spectra can be observed: with less than 0.2 M the spectrum is type II (Figure 3b), while higher concentrations display an increased negative ellipticity of the main band (Figure 3e). Sodium ion concentrations greater than 0.1 M lead to two different spectra depending on the concentration of *peptide*: at low peptide concentrations (10^{-5} M) a spectrum similar to the type I of Li^+ or Mg^{2+} is observed (Figure 3d), but at higher concentration of peptide, less negative ellipticity is observed (Figure 3e).

The above results suggest that several different complexes are being detected, and that most of them are encountered in various cation/peptide systems. The titration curves give a complementary view of these results.

Titration Curves. Titration curves are generated by plotting $\Delta M^{\lambda}_{\theta}$ (the difference between the molar ellipticity, M_θ, at wavelength λ (in nanometers) of peptide plus ion and that of free peptide) as a function of the log of cation concentration. Clearly, data from several wavelengths can be plotted; the discussion below focuses on those curves which show the largest deviations: 210 nm for Ba^{2+}, Ca^{2+}, K^+, and Mg^{2+} and 200 nm for Li^+ and Na^+.

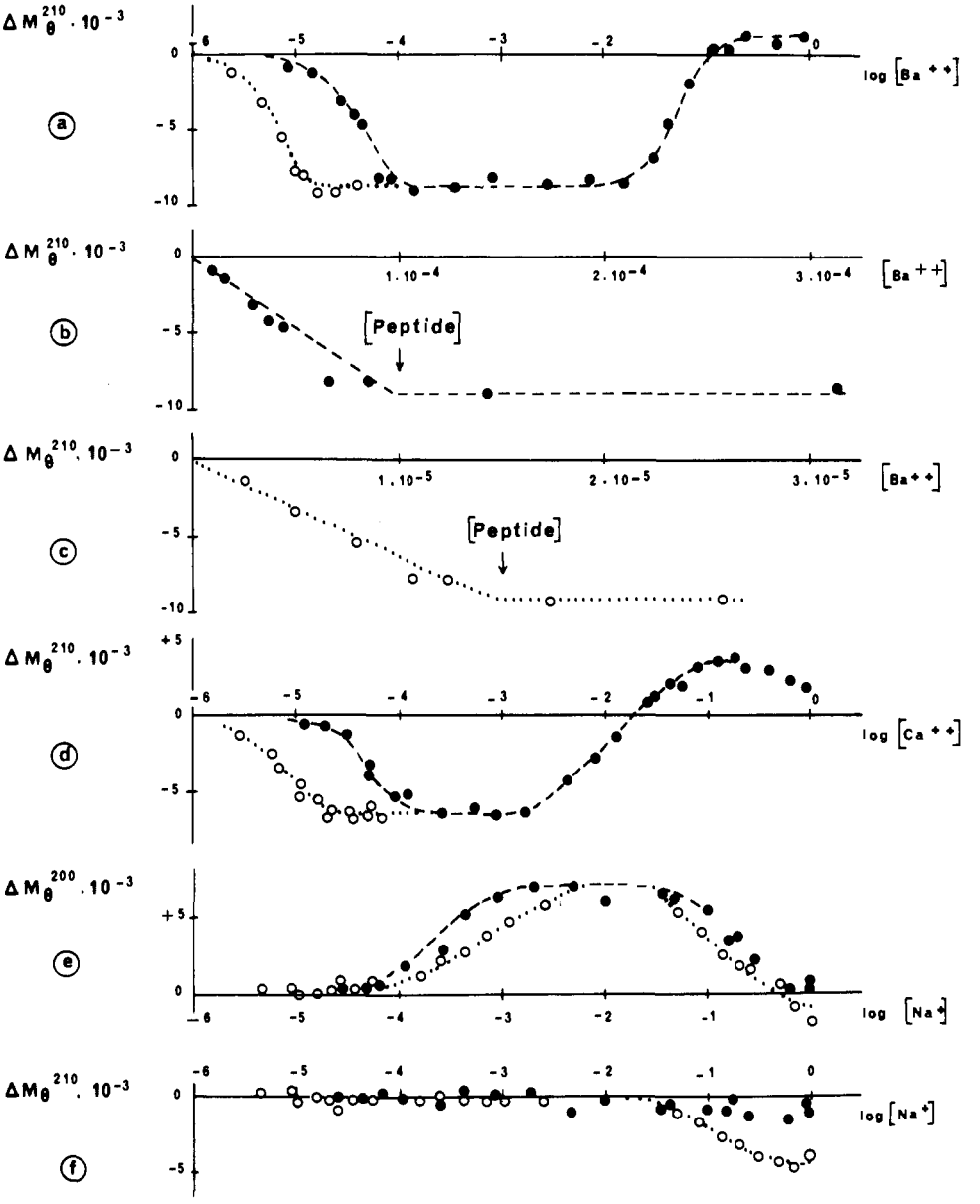


Figure 4. Titration curves ($\Delta M^{\lambda}{}_{\theta} = M^{\lambda}{}_{\theta}$ − $M^{\lambda}{}_{\theta}$ free peptide) of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ in CH_3CN; [peptide] ≃ 10^{-4} M (●) or 10^{-5} M (○); dashed or dotted lines are calculated curves: (a) $\Delta M^{210}{}_{\theta}$ vs. log [Ba^{2+}]; (b) $\Delta M^{210}{}_{\theta}$ vs. [Ba^{2+}]; (c) $\Delta M^{210}{}_{\theta}$ vs. [Ba^{2+}]; (d) $\Delta M^{210}{}_{\theta}$ vs. log [Ca^{2+}]; (e) $\Delta M^{200}{}_{\theta}$ vs. log [Na^+]; (f) $\Delta M^{210}{}_{\theta}$ vs. log [Na^+].

Three representative titration curves are described.

Ba^{2+} Titration Curves (Figure 4a). Two plateaus corresponding to type I and type II spectra are observed. The curve for 10^{-5} M peptide displays larger ΔM_{θ} than that obtained with 10^{-4} M peptide for the same concentration of salt, because a greater proportion of the peptide is complexed. Note that the plots of ΔM_{θ} vs. [Ba^{2+}] (Figure 4b,c) indicate that the first step of complexation is the formation of a 1:1 peptide-cation species.

Ca^{2+} Titration Curves (Figure 4d). The second plateau in this system appears as a maximum because of the formation of another species at high Ca^{2+} concentration, as cited above. As in the case of Ba^{2+}, the first plateau can be associated with the formation of a 1:1 complex. However, the data show that more peptide is complexed than added salt in the early parts of the titration. This finding can be explained by assuming that complexes of stoichiometry P_nC ($n > 1$) form first, then, upon addition of more cation, are transformed into PC complexes.

Na^+ Titration Curves (Figure 4e,f). Two complexation steps, *both* strongly influenced by the peptide concentration, are observed. For a given [Na^+] the amount of complexed peptide *increases* with peptide concentration (as seen in the 200-nm data) in the first step. This finding is a clear proof of P_nC complex formation. Moreover, the corresponding spectrum for the solution composition of the first plateau is distinct from the more usual type I spectrum (Figure 3a,c). At higher cation concentration two species are detected; their ratios, [Na^+]/[peptide], are different and are greater than in the P_nC complex of the first step.

Other Cases. The titration curves of Li^+ and Mg^{2+} are similar to those of Ba^{2+} with two differences: smaller deviations in the first step, particularly for Li^+, and some evidence of P_nC complexes in the first step for Mg^{2+} (as seen for Ca^{2+}). Finally, the second step of complexation has not been observed with K^+, presumably because of the low solubility of $KClO_4$ in CH_3CN; otherwise, the titration curve for K^+ resembles those of Ca^{2+} or Ba^{2+} up to 10^{-2} M salt.

Calculated Binding Constants. Identification of Complexed Species. PC complexes are concluded to be responsible for the type I spectra, based on data on the Ba^{2+} and Ca^{2+} systems. With other ions similar (K^+) or related (Li^+, Mg^{2+}, and even

Table I. Binding Constants[a] and Molar Ellipticities of the Complexes of *cyclo*-(L-Val-Gly-Gly-L-Pro)$_3$ with Perchlorate Salts in Acetonitrile

Cation	Diameter, Å	Binding constants, M^{-1}			ΔM_θ[b] (deg) × 10^{-3}		
		K_1 (P + C ⇌ PC)	$K_{1/2}$ (PC + P ⇌ P_2C)	K_2 PC + C ⇌ PC_2)	PC	P_2C	PC_2
Li^+	1.20	1.1×10^3	5.5×10^6	2.4×10^2	−6.50	−2.90	−0.73
					(−10.46	−1.40	+4.40)[c]
Na^+[d]	1.90	5.8×10^1	6.9×10^5		−6.27	−0.17	
					(−5.20	+9.07)[c]	
K^+	2.66	1.7×10^5	2.3×10^5		−7.64	−4.17	
Mg^{2+}	1.30	2.4×10^3	1.0×10^6	1.5×10^2	−4.73	−6.90	+6.33
Ca^{2+}[e]	1.98	3×10^8	8×10^5	5.5×10^1	−6.13	−3.93	+4.97
Ba^{2+}	2.70	2×10^{10}	2×10^6	1.5×10^1	−8.53	−4.17	+3.00

[a] Temperature = 25 °C. [b] Mean difference *per residue* at λ 210 nm unless otherwise noted. [c] λ 200 nm. [d] $[Na^+] < 0.1$ M for 10^{-4} M peptide solutions. [e] $[Ca^{2+}] < 0.2$ M.

Na^+) spectra are obtained; hence, it is assumed that PC complexes are occurring in all systems under varying conditions.

Type II spectra are observed with Li^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} only. As they always occur at a higher cation concentration that PC complexes do, type II spectra are interpreted to correspond to PC_n ($n > 1$). By comparison with previous ion binding studies of cyclic peptides,[6,12,18] we assume these complexes to be PC_2.

The existence of other complexes is required to explain the results completely, particularly a P_nC complex with Na^+, Mg^{2+}, and Ca^{2+}. A P_2C ("peptide sandwich") complex has been postulated in related cation–macrocyclic systems[6,12,18,19] and seems to occur generally. Moreover, in the Li^+, K^+, and Ba^{2+} systems preliminary examination of the data does not exclude the possibility of P_nC complexes. For these latter cases results obtained assuming the presence of P_2C species are compared with those obtained using no P_2C species. Two phenomena were not analyzed quantitatively because of ambiguity in interpreting the observations: the complex at high Ca^{2+} concentration—data with $[Ca^{2+}] > 0.2$ M were not used; and the complex formed at high concentrations of Na^+ in 10^{-4} M peptide solutions—only data at 10^{-5} M peptide were taken into account when $[Na^+]$ exceeded 0.1 M.

Binding Constants. The detailed procedures used for the calculation of the binding constants and molar ellipticities of the complex species are given in the Appendix. Data at three or four wavelengths were used for each system.

The results of the binding constant calculations confirm the identity of the complexed species (vide supra). The *F* statistical tests (see Appendix) allow us to assign the second step of complexation (in all cases except Na^+) to the formation of a PC_2 complex rather than a PC complex. This is especially significant in the Ca^{2+} system where the existence of three steps of complexation might have suggested that the second step corresponded to PC formation. Furthermore, the assumption that only PC species are involved in the second step of the Na^+ titration curve is confirmed, providing that data at low peptide concentration are utilized in the calculation.

Inclusion of P_2C complexes in the calculation generally yields better results (as judged by the *F* test) even in the cases of Li^+, K^+, and Ba^{2+}. However, it is found that P_2C species are not necessary to fit correctly the titration curve with Ba^{2+} at 210 nm.

Binding constants and molar ellipticities of the complexes are given in Table I. The accuracies of the binding constant values are estimated to be: between 10 and 20% for K_2 (PC + C ↔ PC_2), between 20 and 30% for $K_{1/2}$ (PC + P ↔ P_2C) and for K_1 (P + C ↔ PC) (except for Ca^{2+} and Ba^{2+}), and better than an order of magnitude for $K_{1/2}$ and K_1 (Ca^{2+} and Ba^{2+} systems only).[20]

Discussion

As expected from the design of the cyclic dodecapeptide, *cyclo*-(Val-Gly-Gly-Pro)$_3$, binding in 1:1 complexes is *stronger* the *larger* the cation for ions of the same charge. (No cations larger than 2.8 Å in diameter have been examined because of the insolubility of their perchlorate salts in acetonitrile.) In addition, it appears that *cyclo*-(Val-Gly-Gly-Pro)$_3$ is approximately as good a complexing agent as valinomycin ($K \approx 3 \times 10^5$ M^{-1} in CH_3CN).[21]

The charge of the cation has also been shown to have a significant effect on the strength of binding in *cyclo*-(Val-Gly-Gly-Pro)$_3$ complexes. For example, even though the diameters of the cations are close (2.66 Å vs. 2.70 Å), the ratio of the binding constants, $K_{1(Ba^{2+})}$:$K_{1(K^+)}$, is 10^5. Moreover, the binding constant (K_1) of Ca^{2+} is three orders of magnitude higher than that of K^+. Thus, despite the fact that the diameter of this divalent cation appears to be smaller than the size of the *cyclo*-(Val-Gly-Gly-Pro)$_3$ cavity (measured from a CPK model of the bracelet conformation), the 2+ charge is sufficient to stabilize the 1:1 complex.

In contrast to the above trends, the binding of Na^+ is much lower than that of the other monovalent ions, even the smaller cation, Li^+. The Na^+ results suggest that there may be two different structures for the PC complex: one in which the cation resides in the center of the cavity and interacts with six carbonyl oxygens—requiring a large diameter cation or high charge density and an intermediate diameter—and the other, in which the cation utilizes fewer carbonyl oxygens for binding but approaches more closely to them (either inside or outside the cavity), seemingly the better binding arrangement for small cations with high charge density, such as Li^+ and Mg^{2+}.

In either of these binding modes Na^+ would not be expected to bind very well, as it is too small to interact effectively with six carbonyls but does not have a large enough charge density to form a stable complex with any fewer carbonyl oxygens. Hence, it should show, as it does, the lowest binding constant of the PC complexes.

Other aspects of our results, such as the occurrence of PC_2 and P_2C complexes, support the existence of several complexation sites on the peptide. It is likely that the P_2C species are true "peptide sandwich" complexes (as opposed to dimeric peptides binding one cation), similar to those found for valinomycin,[19,22] the enniatins,[22,23] and antamanide.[19,22] Model building suggests that the formation of peptide-sandwich complexes should be more favorable with large cations, as the two peptides can approach the cation closely enough for a stable interaction without steric hindrance from the peptide side chains. The expected affinities of cation and peptide in a 1:1 complex with the cation in the cavity and in a peptide sandwich with the cation in a cavity formed between the two

peptides should, therefore, follow the same trends. However, the data show remarkable constancy among the $K_{1/2}$ values. This result may be due to an opposing effect. Namely, the formation of a peptide sandwich may require an open PC complex (as postulated above for the small cations), and the proportion of such complexes present at any time follows a reverse trend from the tendency to yield a stable cavity complex. The consequence of the two effects could be a relatively constant value of $K_{1/2}$, as is observed.

In some cases (Li^+, Na^+, and Mg^{2+}), $K_{1/2}$ is larger than K_1. These cations are the smallest of those studied and are likely to form open PC complexes. The association constants suggest that the binding arrangement in the peptide sandwich complex is more favorable than in the PC complex. In these cases, the small size of the cation probably restricts the capability of the PC complex to optimize binding interactions. The sandwich complexes formed by two cyclic peptide molecules do not suffer from the same limitation. In fact, P_2C complexes are the major species for Li^+, Na^+, and Mg^{2+} in the range of low cation concentration. PC species are formed only at high $[Na^+]$; with Li^+ or Mg^{2+} neither predominates because of the formation of PC_2 complexes.

The opposing trends of K_2 and K_1 values can be explained using the same model for the binding. The formation of PC_2 is not expected to be favorable for divalent ions (due to repulsive interactions) or for large cations because of the inaccessibility of the necessary two sites. Furthermore, open PC complexes would be required in order to form PC_2; for those cations which form stable PC cavity complexes, higher cation concentrations would be required in order to produce PC_2.[24]

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of the free dodecapeptide and of the 1:1 complexes in CH_3CN show only three amide proton and four carbonyl resonances.[29] C_3 symmetry is, therefore, present at least on the NMR time scale, as required by the bracelet structures. Similar results have been reported by Gisin et al. for their analogous cyclic dodecapeptide.[30] Further spectroscopic studies[29] will aid in the identification of the conformations of all the binding species.

Conclusions

cyclo-(Val-Gly-Gly-Pro)$_3$ forms several complexes in acetonitrile solutions with alkali metal and alkaline earth cations, including two types of "sandwich" complexes. Data obtained from circular dichroism spectra and titration curves have been interpreted in terms of PC, P_2C, and PC_2 species. Two different 1:1 complexes are postulated in order to explain the quantitative binding constant data. One of these is analogous to the valinomycin-K^+ complex while the other appears to occur in order to accommodate a cation which is too small for the cavity of the peptide.

Both the size and the charge of the cation affect the stabilities of complexes: K^+, Ca^{2+}, and Ba^{2+} form the most stable PC complexes, and, of these, the doubly charged ions bind better by a large factor. A proposal has been put forward to explain the trends in the stabilities of both peptide- and ion-sandwich complexes.

Acknowledgments. This work has been supported, in part, by U.S. Public Health Service Grants AM07300 and AM10794. Acknowledgment is made also to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. Finally, we wish to express thanks to Ms. Paula Olsiewski for her enthusiastic and able technical assistance in the synthesis of the cyclic dodecapeptide.

Appendix

Method of Calculation of the Binding Constants and Molar Ellipticities of the Complexes. When several equilibria occur in the same step of a titration curve, it is necessary to calculate binding constants by using a computer program which minimizes the deviations between experimental and calculated data. Moreover, it is useful to take into account different sets of data (in our case, M_θ at several wavelengths).

Assuming that there is no bulk ionic effect on the ellipticities (assumption justified by the plateaus obtained at high concentration of salt in the Mg^{2+} and Ba^{2+} systems), one has:

$$\Delta M_\theta^\lambda = \alpha[\Delta M^\lambda_{\theta,\,P_2C}] + \beta[\Delta M^\lambda_{\theta,\,PC}] + \gamma[\Delta M^\lambda_{\theta,\,PC_2}] \quad (1)$$

where $\alpha = 2[P_2C]/P_0$, $\beta = [PC]/P_0$, and $\gamma = [PC_2]/P_0$ (P_0 being the formal concentration of the peptide and $[\Delta M^\lambda_{\theta,i}]$ the difference between the molar ellipticity of the i species and that of the monomer.)

α, β, and γ are obtained from the values of the binding constants:

$$P + C \rightleftharpoons PC \qquad K_1 = \frac{[PC]}{[P][C]}$$

$$PC + P \rightleftharpoons P_2C \qquad K_{1/2} = \frac{[P_2C]}{[PC][P]}$$

$$PC + C \rightleftharpoons PC_2 \qquad K_2 = \frac{[PC_2]}{[PC][C]}$$

and the relationships:

$$P_0 = [P] + K_1[P][C] + 2K_1K_{1/2}[P]^2[C] + K_1K_2[P][C]^2 \quad (2)$$

$$C_0 = [C] + K_1[P][C] + K_1K_{1/2}[P]^2[C] + 2K_1K_2[P][C]^2 \quad (3)$$

As [P] and [C] are involved in a nonlinear way, an iterative process must be used to get [P], [C], and α, β, and γ for a given set of binding constants and for each solution. The procedure in this process is as follows. An upper limit of [C] is taken as $C_1 = C_0 = C_{max}$; [P] is calculated from eq 2, and $C_{0,max}$ from eq 3. A lower limit of [C] is calculated from the relationships:

$$\left[\frac{d[C]}{dC_0}\right]_{C_0=0} = \frac{1}{1 + K_1P_0 + K_1K_{1/2}P_0^2}$$

and

$$C_2 = \frac{C_0}{1 + K_1P_0 + K_1K_{1/2}P_0^2} = C_{min}$$

[P] and $C_{0,min}$ are calculated from eq 2 and 3. A weighted value of [C] is then determined: $C' = \{[(C_{0,max} - C_0)C_{min}C_0]/C_{0,min} + [(C_0 - C_{0,min})C_{max}C_0]/C_{0,max}\}/(C_{0,max} - C_{0,min})$. The corresponding value, C_0', is compared to C_0 and exchanged with $C_{0,min}$ or $C_{0,max}$; a new C' is determined as previously, and so on.

The process is repeated until a convergence is reached for C_0' and C_0. Then, α, β, and γ are calculated for this solution.

When α, β, and γ have been obtained for each solution, a least-squares method is applied to eq 1, independently at each wavelength, leading to the values of $[\Delta M^\lambda_{\theta,i}]$. Finally, the total sum of square deviations:

$$S = \sum_\lambda \sum_j [\Delta M^\lambda_{\theta,j(exp)} - \Delta M^\lambda_{\theta,j(calcd)}]^2$$

is found for the initial set of binding constants.

Values of the binding constants are automatically varied in order to find the minimum value of S. Generally, the first trial value of K_1 is taken from the midpoint of the first step of complexation (on the titration curve) and $K_{1/2}$ is put equal to

K_1; the trial value of K_2 comes from the inflection point of the second step of complexation. The accuracy of K_2 is generally good enough to keep it constant in the first stage of the process, when K_1 and $K_{1/2}$ are varied until a local minimum of S is reached; in the second stage variations of the three constants are used until a final minimum is obtained, giving an accuracy better than 5% on the binding constants. Convergence is rapid, and no subminimum occurs.

Following Sillen,[31] the allowed values of the parameters are given from:

$$S_{\text{allowed}} \leq S_{\min}\left(1 + \frac{1}{n-p}\right) = S_{\max}$$

where n is the number of independent data points and p the number of unknowns (binding constants and molar ellipticities of the complexes). Keeping two Ks constant, the third is varied until $S > S_{\max}$; both limits of the binding constants are calculated in this way.

Finally, to discuss the validity of a model assuming three complexes (q unknowns) with respect to an alternative model involving only two complexes (p unknowns), the F statistical test has been used, wherein:

$$F_{(q-p,n-q)} = \frac{S_p - S_q}{S_q} \cdot \frac{n-q}{q-p}$$

The three complexes are considered to be justified when a confidence level of at least 90% is obtained.[32]

References and Notes

(1) For the preceding paper in this series, see C.-H. Niu, L. Q. Pease and E. R. Blout, *Biopolymers*, in press.
(2) Address correspondence to this author at Harvard Medical School.
(3) B. F. Gisin and D. G. Davis, *Biophys. J.*, **15**, 288a (1973).
(4) (a) C. Moore and B. C. Pressman, *Biochem. Biophys. Res. Commun.*, **15**, 562 (1964); (b) P. Mueller and D. O. Rudin, *Ibid.*, **26**, 398 (1967).
(5) T. Wieland in "Chemistry and Biology of Peptides", J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 377.
(6) V. Madison, M. Atreyi, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 6725 (1974).
(7) G. Eisenman, C. M. Deber, and E. R. Blout, *Biophys. Soc. Abstr.*, **16**, 81a (1976).
(8) (a) M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, V. K. Antonov, A. M. Shkrob, I. I. Mikhaleva, A. V. Evstratov, and G. G. Malenkov, *Biochem. Biophys. Res. Commun.*, **29**, 834 (1967); (b) M. Ohnishi and D. W. Urry, *ibid.*, **36**, 194 (1969); (c) V. T. Ivanov, I. A. Laine, N. D. Abdullaev, L. B. Senyavina, E. M. Popov, Yu. A. Ovchinnikov, and M. M. Shemyakin, *ibid.*, **34**, 803 (1969); (d) D. F. Mayers and D. W. Urry, *J. Am. Chem. Soc.*, **94**, 77 (1972).
(9) (a) M. Pinkerton, L. K. Steinrauf, and P. Dawkins, *Biochem. Biophys. Res. Commun.*, **35**, 512 (1969); (b) K. Neupert-Laves and M. Dobler, *Helv. Chim. Acta*, **58**, 432 (1975).
(10) G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.*, **23**, 284 (1968).
(11) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 6165 (1972).
(12) V. Madison, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **99**, 4788 (1977).
(13) J. Schellman and C. Schellman, *Proteins*, **2**, 1 (1964).
(14) M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, V. K. Antonov, E. I. Vinogradova, A. M. Shkrob, G. G. Malenkov, A. V. Evstratov, I. A. Laine, E. I. Melnik, and I. D. Ryabova, *J. Membr. Biol.*, **1**, 402 (1969).
(15) D. J. Patel and A. Tonelli, *Biochemistry*, **12**, 486 (1973).
(16) Yu. A. Ovchinnikov, V. T. Ivanov, V. F. Bystrov, N. D. Abdullaev, and A. I. Miroshnikov, *Pept. Proc. Eur. Pept. Symp., 11th, 1971*, 403 (1973).
(17) C. M. Deber and E. R. Blout, *Isr. J. Chem.*, **12**, 15 (1974).
(18) C.-H. Niu, L. G. Pease, and E. R. Blout, manuscript in preparation.
(19) (a) V. T. Ivanov, L. A. Fonina, N. N. Uvarova, S. A. Koz'min, T. B. Karapatnitskaya, N. M. Checkhlayeva, T. A. Balashova, and Yu. A. Ovchinnikov, *Pept.: Chem. Struct. Biol. Proc. Am. Pept. Symp. 4th, 1975*, 195 (1975); (b) Yu. A. Ovchinnikov and V. T. Ivanov, *Tetrahedron*, **31**, 2177 (1975).
(20) The method used for estimating accuracy is described in the Appendix. The relatively large errors in the Ca^{2+} and Ba^{2+} constants are a result of their strong binding which leads to a very low (and, hence, inaccurately known) free cation concentration.
(21) M. C. Rose and R. W. Henkens, *Biochim. Biophys. Acta*, **372**, 426 (1974).
(22) V. T. Ivanov, *Ann. N.Y. Acad. Sci.*, **264**, 221 (1975).
(23) V. T. Ivanov, A. V. Estratov, L. V. Sumskaya, E. I. Melnik, T. S. Chumburidze, S. L. Portnova, T. A. Balashova, and Yu. A. Ovchinnikov, *FEBS Lett.*, **36**, 65 (1973).
(24) An alternative interpretation of the type II spectra is that the species responsible is a peptide–cation–anion (PCA) complex as with valinomycin–Na^+–A^- complex in $CDCl_3$ and CCl_4[25] (since in the last step of complexation, [PC] is negligible compared to salt concentration, it is not possible to distinguish theoretically between PC + C $\rightleftharpoons$ PC_2 and PC + A $\rightleftharpoons$ PCA). However, ion-pair formation is low in acetonitrile ($K_A \simeq 10$ for alkaline perchlorates[25]) and will be reduced if the cation is bound to the peptide; comparatively K_2 is high: from 15 with Ba^{2+} to 240 with Li^+. Another possibility is to consider some interaction between NH and ClO_4^- leading to a destabilization of the PC complex structure; but here, also, the constant seems to be less than 10.[26] Finally, PC_2 complexes have been found with *cyclo*-(Pro-Gly)$_3$, with Mg^{2+} in CH_2Cl_2 (as demonstrated by extraction procedure),[6] and with quaternary ammonium of L-valinylmethyl ester hydrochloride in $CDCl_3$ (through ^{13}C NMR relaxation measurements).[27] Taking into account the large values of K_2 in CH_3CN, ion–sandwich complexes (PC_2) seem more probable in our systems than PCA complexes.
(25) D. G. Davis and D. C. Tosteson, *Biochemistry*, **14**, 3962 (1975).
(26) "Physical Chemistry of Organic Solvent Systems", A. K. Covington and T. Dickinson, Ed., Plenum Press, London, 1973, p 598.
(27) M. H. Baron and C. de Loze, *J. Chim. Phys. Phys. Chim. Biol.*, **69**, 1084 (1972).
(28) C. M. Deber, B. Bartman, and E. R. Blout, *Pept.: Chem. Struct. Biol., Proc. Am. Pept. Symp. 4th, 1975*, 203 (1975).
(29) K. R. K. Easwaran, L. G. Pease, and E. R. Blout, unpublished results.
(30) D. G. Davis, B. F. Gisin, and D. C. Tosteson, *Biochemistry*, **15**, 768 (1976).
(31) L. G. Sillen, *Acta Chem. Scand.*, **16**, 159 (1962).
(32) See C. J. Brookes, I. G. Betteley, and S. M. Loxton, in "Mathematics and Statistics", Wiley, New York, N.Y., 1966, for tables of F values at 90% confidence level.