Conformational Studies of Cyclo-(-Pro-Gly-)$_3$ and Its Complexes with Cations by Vibrational Circular Dichroism

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Abstract: We report vibrational circular dichroism (VCD) and infrared absorption spectra of a cyclic hexapeptide, cyclo-(-Pro-Gly-)$_3$ in aqueous and nonaqueous media, and in the presence of a number of mono- and divalent metal cations. Cyclo-(-Pro-Gly-)$_3$ was studied previously by CD, Raman, and NMR spectroscopies, and its ionophoric properties were recognized. VCD in the amide I spectral region monitors the relative orientation of amide linkages of the peptide with respect to each other. We find that VCD is an enormously sensitive technique to monitor the conformation of small peptides, and can distinguish more than a dozen different molecular shapes, depending on the ionic strength of the solvent media.

Introduction

Infrared or vibrational circular dichroism (VCD) is a relatively new spectroscopic technique[1] which bears several advantages over conventional methods used for the determination of peptide solution conformation. Compared with electronic CD, it provides the possibility to monitor the optical activity of a number of specific functional groups, such as the amide linkage, which exhibits a C=O stretching vibration between 1600 and1700 cm^{-1}. These amide vibrations undergo dipolar coupling to produce the observed VCD. Since vibrational transition moments are smaller than those encountered in electronic CD (ECD) observed in the UV spectral region, the coupling in VCD extends over shorter distances than in ECD.[2] Therefore, VCD is sensitive to short range structural order. This advantage, coupled with the selectivity of vibrational spectroscopy toward different chemical environments, and the high resolution and narrow band widths achievable in vibrational spectroscopy, make VCD a nearly ideal tool to monitor conformational changes of biological molecules. Due to its chiroptical origins, it adds an enormous amount of information to that available from standard vibrational techniques.

Even compared to other structural techniques used previously to study biomolecules, such as X-ray crystallography and NMR and Raman spectroscopies, VCD offers significant advantages. Obviously, it is a solution conformational technique and is therefore free from crystal effects which may distort the shape of molecules. Furthermore, it circumvents the difficulties associated with crystal growth. Compared to NMR spectroscopy, VCD offers the advantages of permitting a qualitative interpretation of spectral changes in terms of structural changes without requiring elaborate calculations, although quantitative information is available from VCD as well. In addition, there are a number of arguments involving the vibrational *vs* NMR time scale, which are favorable for VCD.

However, one problem persists with the understanding of VCD of biomolecules: it is presently impossible to perform detailed VCD intensity calculations on molecules of the size discussed here. Empirical VCD calculations, to be discussed later, have been carried out for some biological molecules, but they yield the best results for molecular systems where the interacting groups exhibit only dipolar and no mechanical coupling. In peptides, however, both dipolar and mechanical coupling, as well as other factors, contribute to the overall VCD intensity.[3] Therefore, we restricted ourselves in this paper to a qualitative interpretation of the VCD results, using mostly geometric parameters to assess whether or not dipolar coupling occurs and contributes to the observed VCD intensities.

Primary Structural Considerations

Cyclo-(-Pro-Gly-)$_3$, to be abbreviated CPG3 henceforth, is a model for ion carrier peptides. Its conformations were studied systematically by Blout's group in the 1970s, mostly by NMR and CD spectroscopies.[4,5] Raman[6] and X-ray data[7,8] are available as well. Results from these techniques will be reviewed briefly in the next section.

CPG3 is composed of the two amino acids proline and glycine. Proline has a $-(CH_2)_3-$ side chain linked to the amide nitrogen atom, which makes it a tertiary amide and restricts the conformational freedom about the N−C$_\alpha$ bond. Thus, incorporation of a proline residue into a peptide sequence reduces the number of possible rotamers. Proline also plays an important

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role in peptide turns which reverse the direction of the peptide chain. Turns are particularly common when proline is followed by glycine in a peptide. Glycine, which has a H as the side group, can fit into structures where amino acids with large side groups would be excluded.

Proline and glycine exhibit amide linkages with quite different amide I vibrational frequencies: a Pro-Gly peptide bond contains N as a secondary amide, whereas in a Gly-Pro group, the nitrogen is a tertiary amide. The carbonyl stretching vibration of a secondary amide linkage, such as the $C{=}O_{(Pro)}$ in Pro-Gly, is observed at 1670 cm^{-1}, whereas the corresponding vibration in a tertiary amide such as Gly-Pro is observed at 1640 cm^{-1}. These frequencies and assignments have been established through studies of model peptides such as Pro-Gly and Gly-Pro by Asher and co-workers.[5] The *cis* and *trans* forms of a tertiary (Gly-Pro) amide linkage exhibit nearly the same potential energy, and the corresponding vibrations can be distinguished by the slightly higher amide I frequency of the *cis* form (about 1650 cm^{-1}). The ability to form *cis* linkages increases the conformational flexibility of CPG3, for which *cis* peptide bonds have been found to occur in polar solvents.[4]

CPG3 has six carbonyl but only three NH groups. Even when intramolecular hydrogen bonds are formed, three carbonyl groups can form hydrogen bonds with the solvent, or interact with metal ions. These factors contribute to the conformational variety of CPG3, which is determined primarily by the solvent environment. In most complexes with metal ions, it appears that the three $C{=}O_{(Pro)}$ groups point toward one side of the molecular ring whereas the three $C{=}O_{(Gly)}$ groups point toward the other side.

Results from Other Techniques

The conformations of CPG3 were studied previously by NMR,[4] electronic CD,[4] and Raman spectroscopies,[6] as well as by X-ray crystallography.[7,8] The 3-fold repetition in the primary sequence of CPG3 allows this molecule to form a conformation with 3-fold symmetry under certain conditions. In nonpolar solvents, for example, CPG3 exhibits three γ-turns, which are stabilized by intramolecular 1−3 H-bonds between the glycine residues. These are chemically equivalent as indicated by the fact that only one N−H proton signal is observed.[4] This symmetric structure is called conformation **S**. In aqueous or polar solvents, on the other hand, some or all intramolecular hydrogen bonds may break, and the tertiary amide linkage of the prolyl groups may assume the *cis* conformation. Results from crystallography confirm that in crystals grown from polar solvents (methanol:water = 2:1), CGP3 exhibits one *cis* and five *trans* peptide linkages. The occurrence of one *cis* linkage causes a loss of symmetry, and the resulting asymmetric structure is referred to as conformation **A**. This conformation, for which NMR spectroscopy detects three different N−H resonances, also exists in solution.

Because of the alternating Pro-Gly sequence, one can visualize all prolyl and all glycyl carbonyl groups to be located above and below the peptide ring, respectively. Metal ions can therefore bind to two faces, or binding sites, composed of either prolyl or glycyl carbonyl groups. The complexation with cations can move either set of carbonyl groups closer together toward the center of the ring. Several conformations of cation complexes are possible, depending on the solvent medium and the nature of the cation. These can be classified into 1:1 complexes, peptide sandwiches (peptide/metal ion/peptide), or salt sandwiches (metal ion/peptide/metal ion).

X-ray results show[7] that CPG3 can form a peptide sandwich with Ca^{2+} ions. Here, calcium binds with only the glycyl carbonyls of two separate peptide molecules. The Ca−O distance is 2.26 Å, which is about the same as the length observed between a calcium and the carbonyl oxygen in proteins. CPG3 also can form longer sandwich structures in sodium/calcium-containing crystals. Here, one finds solvent/Na^+/peptide/Ca^{2+}/peptide/ Na^+/solvent structures in which the sodium ion binds to the three prolyl carbonyls which are on the face of the peptide ring not occupied by the calcium ions. With magnesium ions, one finds a 1:1 complex, with the Mg^{2+} ion coordinated to the three glycyl carbonyls as in the case with calcium ions. In addition, three water molecules are found complexed with the magnesium ion. The carbonyl Mg−O distance was found to be 2.03 Å, and the Mg−O distance with the water molecule is 2.11 Å. This complex exhibits 3-fold symmetry.

In solution, NMR results suggest two kinds of complexes formed between CPG3 and cations. One of them, called conformation **S1***, is formed by Ca^{2+}, Na^+, and K^+ at 1:1 stoichiometry. In these complexes, the prolyl carbonyl groups are rotated by 80° as compared to the **S** conformation, and form an open cup on one face of the molecule. The rotation of the prolyl carbonyl groups also brings the glycyl carbonyls together to form a similar cup on the other side of the molecule.[3] The other conformation, **S2***, is a salt sandwich formed with magnesium ions only. Thus, the NMR results on complexes formed in solution are quite different from the X-ray results discussed above.

Raman spectroscopy can distinguish the tertiary amide I band of $C{=}O_{(Gly)}$ in Gly-Pro at 1651 cm^{-1} and the secondary amide I band of $C{=}O_{(Pro)}$ in Pro-Gly at 1677 cm^{-1}. Two bands observed in crystalline CPG3 at 1634 and 1671 cm^{-1} were assigned accordingly, although the shift of the glycyl carbonyl frequency from 1651 cm^{-1} in Gly-Pro to 1634 cm^{-1} in CPG3 was not explained satisfactorily. In solution in chloroform, the observed frequencies were 1674 and 1641 cm^{-1}, with the latter number very uncertain because proper band analysis was not carried out by the authors.[6] In fact, we believe that the Raman data for the uncomplexed peptide, as well as for the peptide/ion complexes, are so strongly influenced by crystal effects that the interpretation presented is questionable.

The Raman data of the complexes were interpreted in terms of the Na^+ and K^+ ions binding to the prolyl carbonyls, because a band attributed to the $C{=}O_{(Pro)}$ amide I vibration shifted from 1674 to 1706 cm^{-1}. This band is strong and sharp, with three more Raman bands observed at 1672, 1653, and 1643 cm^{-1}. We contend that the highest frequency peak at 1706 cm^{-1} is due to crystal effects or overtones of the anion vibrations (SCN^-). This is based on a comparison of its intensity with those of other peptide Raman bands, and bands marked with (an unexplained) letter S in Figure 2 of ref 6.

Calcium binding was observed for the deuteriated peptide in the solid phase. The amide I′ vibrations were observed at 1690, 1656, and 1619 cm^{-1}. The vibrations of the anion used, perchlorate, are very strong and again might contribute overtone and combination bands. On the basis of the Raman frequency shifts observed for the carbonyl groups, Asher and co-workers[6] concluded that Na^+ and K^+ bind with prolyl carbonyls, and Ca^{2+} binds with glycyl carbonyls. We do not observe in solution phase FT-IR spectra any comparable frequency shifts, and believe that the Raman data are dominated by solid phase artifacts.

Experimental Aspects

Experimental details of infrared VCD and absorption data acquisition are discussed in the literature.[9] Absorption data were verified at higher resolution using a MIDAC, Inc., M-Series FT-IR spectrometer equipped

with a HCT detector. CPG3 was purchased from Bachem Biochemicals, Inc., and used without further purification. However, the peptide was lyophilized from D_2O to exchange the three labile protons to deuterons. The reason for doing so is that VCD spectra obtained from solution in D_2O are naturally those of the deuteriated peptide linkage. Thus, in order to avoid problems of comparing deuteriated peptide linkages in D_2O and protonated linkages in other solvents, only deuteriated peptide samples were used. Consequently, our reported frequencies are those of the amide I′ vibration, which is about 10−15 cm^{-1} lower than the corresponding amide I frequencies reported in the Raman data.

The solvents DMSO, $CDBr_3$, D_2O, and ethanol-d_1 were obtained from Sigma Chemical Co., and were used after distillation. All salts utilized were the chlorides, and were obtained from Sigma Chemical Co. as well.

The visualization of molecular shapes, and the determination of the distances and the geometry of the prolyl and glycyl carbonyl groups, was carried out using the program HyperChem (SciVision, Inc., Lexington, MA) operating on a desktop computer equipped with a 90 MHz Pentium processor.

Results

In the following section, the observed VCD spectra of CPG3 and its complexes with metal cations will be presented. These spectra will be interpreted qualitatively in terms of the dipolar coupling mechanism used before by us[1] and others[3] to describe the origin of VCD features. Previous comparisons of observed and computed VCD intensities[10,11] have demonstrated that dipolar coupling provides a significant portion of the observed VCD intensity, but that other mechanisms may contribute as well. However, to fully exploit the conformational information contained in VCD, intensity calculations at the *ab initio* level need to be carried out.[12]

Although it is anticipated that such calculations will become practical for molecules of the size of CPG3 in the future, it is presently impossible to carry out the numerous calculations necessary to interpret the observed spectral data, considering the large number of different structures and environments studied. We feel that empirical VCD calculations, on the level of the nondegenerate extended coupled oscillator (NECO) model,[13] may be helpful in the interpretation of the observed VCD data.

However, in this paper, we shall concentrate on the experimental results and a qualitative interpretation of the VCD results. This interpretation will use distance arguments alone to determine whether or not coupling between carbonyl transitions is possible. Since structural data on a number of different conformations are available, we are able to assess the validity of our approach. A favorable coupling geometry is given, for example, for the S conformation in low polarity solvents, where all three glycyl carbonyl groups point toward the inside of the peptide to form three γ-turns. Here, the glycyl carbonyl groups are very close (3.2 Å) whereas the prolyl groups are about 8 Å apart. Under these circumstances, the coupling is nearly exclusively due to the glycyl groups. Similarly, in mixed

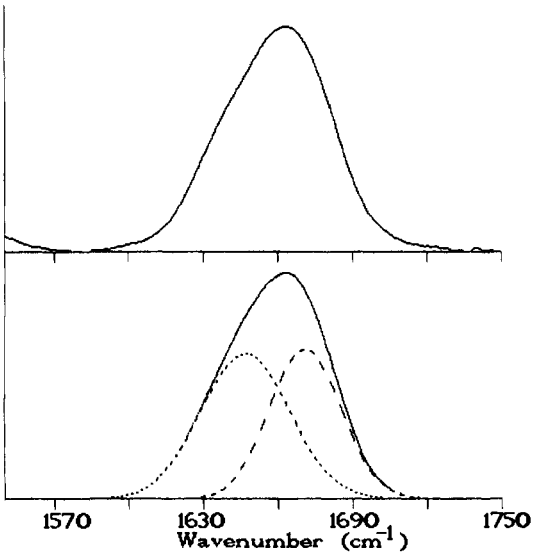

Figure 1. Observed (top) and band-fitted (bottom) infrared absorption spectra of cyclo-(Pro-Gly-)$_3$ in $CDBr_3$: fitting parameters, Gaussian envelopes; frequencies, 1642, 1671 cm^{-1}; FWHH, 42, 34 cm^{-1}; intensities, 0.0709, 0.0731 OD units.

solvents of somewhat higher polarity, the S conformation is destroyed, and a structure appears which may be described as having a binding cavity, or "cup", on both faces of the molecule.[4] In this conformation, both the glycyl carbonyl as well as the prolyl C=O groups can undergo dipolar coupling *(vide infra)*. Consequently, very different VCD spectra result. The solution structural arguments based on changes in VCD spectra, and absorption frequency shifts, do not necessarily agree in detail with the crystallographic data of the ion/CPG3 complexes. Previously available Raman spectroscopic results of crystalline CPG3 probably cannot be used to make any statements about the solution structure of these complexes.

1. Conformational Studies of CPG3 in Solvents with Various Polarities. The conformation of CPG3 is determined in a sensitive way by the solvent environment. In a low polarity solvent such as bromoform, CPG3 shows an infrared absorption spectrum shown in Figure 1. The amide I′ band has its absorption maximum at 1663 cm^{-1} which is a relatively high frequency for an amide I′ vibration. This high value is determined, in part, by the absence of hydrogen bonding with the solvent. The absorption peak, which is asymmetric with a low frequency shoulder, was resolved into two components at *ca.* 1636 and 1668 cm^{-1} *via* second-derivative spectroscopy. Subsequent band deconvolution into Gaussian envelopes resulted in two peaks at 1642 and 1671 cm^{-1} which we assign to the $C{=}O_{(Gly)}$ and $C{=}O_{(Pro)}$ vibrations, respectively, in agreement with the previous Raman data. Alternatively, one could argue that the difference in frequencies is mostly determined by the presence or absence of hydrogen bonding in the glycyl and prolyl carbonyl groups, respectively, in the S conformation. However, the relatively small shifts discussed below with different solvents, or in the presence of metal ions, make us favor the former interpretation.

The VCD spectrum shows a strong positive couplet (implying positive VCD intensity at lower wavenumber) with a zero crossing point of about 1642 cm^{-1} (*cf.* Figure 2 and Table 1), near the center of the glycyl carbonyl vibrations. Thus, we conclude that the VCD couplet is due to the three $C{=}O_{(Gly)}$ groups, held close to each other (3.2 Å, measured from the center of the C=O bonds) in this 3-fold symmetric structure by the three 1−3 H-bonds of the γ-turns, and that these C=O groups undergo dipolar coupling which is responsible for the appearance

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Table 1. Observed Frequencies (cm^{-1}) and VCD Intensities of CPG3 and Its Metal Ion Complexes

	absorption maximum (cm^{-1})	wavenumber of VCD maxima (M) and zero (Z) crossing (cm^{-1})	VCD intensity in units of $\Delta\epsilon$ (L/(mol cm))
CPG3 in$CDBr_3$	1663 (1642/1671)[a]	(M) 1630/1657 (Z) 1642	−0.029/−0.060
CPG3 in $CDBr_3$/ethanol (1:2 (v/v))	1654 (1642/1665)[a]	(M) 1633/1654/1680 (Z) 1645/1667	−0.026/0.032/−0.032
CPG3 in DMSO	1656	(M) 1679	−0.066
CPG3 in D_2O	1638 (1632/1657)[a]	(M) 1619/1667 (Z) 1642	0.018/−0.012
CPG3/Ca^{2+} 1:1in D_2O	1643 (1636/1659)[a]	(M) 1621/1641/1665 (Z) 1626/1652	−0.009/0.032/ −0.031
CPG3/Na^+ 1:1 in $CDBr_3$/Ethanol (1:2 (v/v))	1664 (1649/1669)[a]	(M) 1636/1661/1689 (Z) 1648/1676	−0.042/0.054/−0.023
CPG3/K^+ 1:1 in $CDBr_3$/Ethanol (1:2 (v/v))	1664	(M) 1635/1662/1692 (Z) 1647/1681	−0.046/0.070/−0.016
CPG3/Ca^{2+}/Na^+ 1:0.5:1 in D_2O	1643	(M) 1641/1665 (Z) 1652	0.027/−0.025
CPG3/Mg^{2+} 2:1in $CDBr_3$/Ethanol (1:2 (v/v))	(1638/1666)[a]	(M) 1624/1656/1685 (Z) 1636/1677	−0.018/0.074/−0.010
CPG3/Mg^{2+} 1:1 in $CDBr_3$/Ethanol (1:2 (v/v))	1642/1661 (1637/1670)[a]	(M) 1621/1652/1683 (Z) 1631/1670	−0.016/0.088/−0.044
CPG3/Mg^{2+} 1:2 in $CDBr_3$/Ethanol (1:2 (v/v))	1653	(M) 1624/1656/1684 (Z) 1636/1677	−0.018/0.075/−0.018

[a] These frequencies were obtained from second-derivative spectra to approximately locate the composite bands, followed by a band decomposition into mixed Gaussian/Lorentzian band shapes.

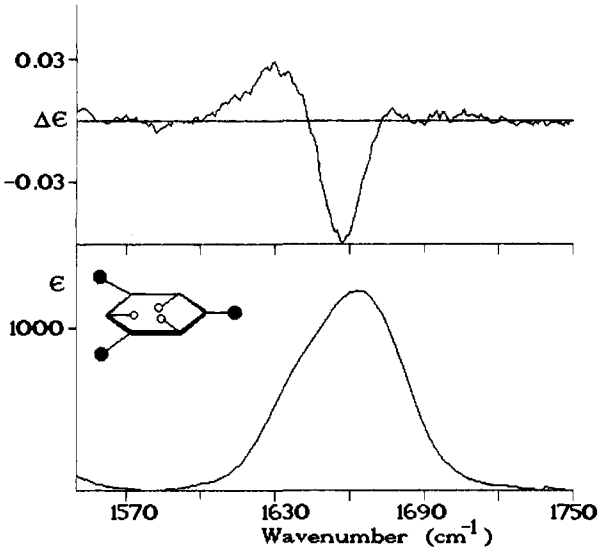

Figure 2. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in $CDBr_3$. The inset presents a schematic structure of the peptide: black circles, prolyl carbonyls; white circles: glycyl carbonyls.

of the observed VCD spectrum. The three $C{=}O_{(Pro)}$ groups, on the other hand, are far away from each other (8 Å), and do not contribute significantly to the observed VCD. Here, we used structural data available from other techniques to interpret the observed VCD features.

1,4-Dioxane is a nonpolar solvent, but a strong H-bond acceptor. In 1,4-dioxane, CPG3 shows VCD spectra identical to those in bromoform (data not shown). This result implies that the polarity, rather than the H-bond acceptor properties, determines the conformation of CPG3. In more polar solvents, however, the symmetric conformation observed in bromoform or dioxane will be perturbed. In a 1:2 mixture of bromoform/ethanol, CPG3 exists in another conformation which exhibits spectral features different from those in bromoform. The absorption band is symmetric and shifted to lower frequency (1654 cm^{-1}). A band deconvolution revealed the prolyl and glycyl carbonyl frequencies at 1665 and 1642 cm^{-1}, respectively. For this and subsequent interpretations, we assume that differences in H-bonding do not change the order of glycyl and prolyl carbonyl stretching frequencies, *i.e.*, that the prolyl carbonyl frequencies are always larger than those of the glycyl groups. This assumption is well justified, since band deconvolution in all cases studied here does reveal a component shift of about 10 cm^{-1}.

A negative/positive/negative VCD spectrum is observed for CPG3 in this solvent which is interpreted to be due to the superposition of a negative couplet of the glycyl carbonyl groups, with a zero crossing at 1645, and a positive couplet of the prolyl carbonyl groups, with a zero crossing at 1667 cm^{-1}. The zero crossing points are remarkably close to the calculated band maxima of the curve-fitted absorption peak (*cf.* Table 1). The fact that there are couplets under both prolyl and glycyl absorptions makes us believe that coupling among the prolyl and among the glycyl carbonyl groups does occur. In order for such coupling to be possible, the distances between the glycyl and the distances between the prolyl carbonyls should be below 4 Å, and should be approximately equal. Inspection of the structures formed by CPG3 and metal ions *(vide infra)* reveals that the distances between glycyl carbonyl groups may vary from 3.1 to 3.7 Å and those between prolyl carbonyl groups between 3.1 and 3.9 Å in these complexes. Thus, we conclude that the structure of CPG3 in bromoform/ethanol exhibits a geometry where the distances between the three prolyl and the three glycyl carbonyl groups are approximately equal. We represent this conformation schematically by the inset in Figure 3.

DMSO is a more polar solvent system, but a weak H-bond acceptor. In DMSO, the frequency of the amide I′ mode is shifted to 1656 cm^{-1}, and shows increased intensity and a high frequency shoulder (Figure 4). The VCD is negative, and has its largest intensity under the high frequency shoulder (1673 cm^{-1}). The frequency of the VCD feature suggests that it is due to the prolyl carbonyls, which must have turned even closer to allow interactions. The strong VCD signal implies a well-defined conformation with some intramolecular H-bonds still existing. Previous NMR studies have shown that in more polar solvents, an asymmetric structure called conformation **A** exists which appears to contain one *cis* peptide linkage *(vide supra)*.[4]

D_2O is still more polar, and a good H-bond acceptor and donor. It can form intermolecular H-bonds with both the C=O

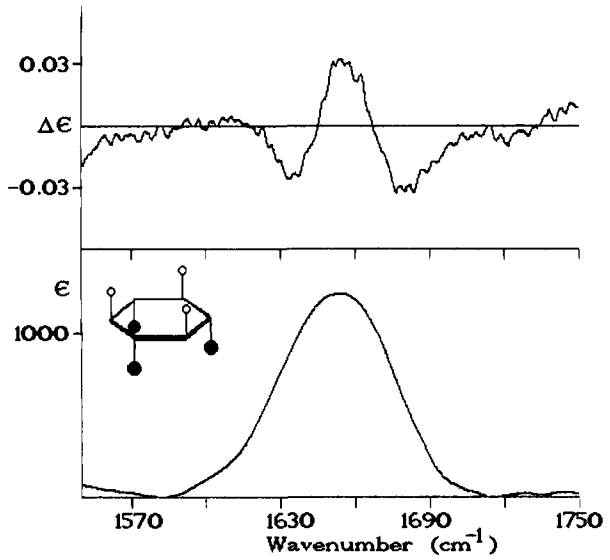

Figure 3. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in $CDBr_3$/ethanol (1:2 by volume). The inset presents a schematic structure of the peptide: black circles, prolyl carbonyls; white circles, glycyl carbonyls.

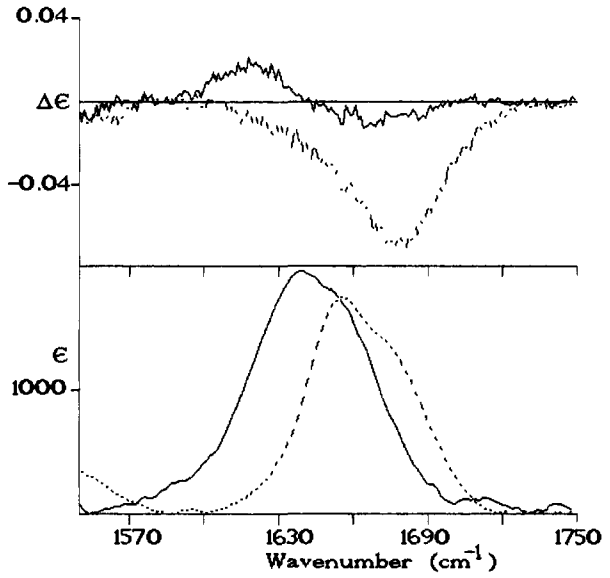

Figure 4. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in D_2O (solid traces) and DMSO (dashed traces).

and ND groups, which may release the constraint of the peptide even further. The absorption maximum occurs at 1638 cm^{-1} with both the prolyl and glycyl frequencies shifted to lower wavenumber, to 1632 and 1657 cm^{-1}, respectively. We attribute this shift to solvent exposure, and hydrogen bonding, of all carbonyl groups. The VCD spectrum exhibits a weak positive couplet. Since the zero crossing point of the VCD couplet (1645 cm^{-1}) is located near the center of the IR peak and not under the individual prolyl and glycyl components, it appears that the VCD is due to small, residual VCD of uncoupled prolyl and glycyl groups. The weak VCD intensities point toward a poorly defined structure, or an equilibrium of many structures, with water molecules bridging the carbonyl groups.

The VCD and infrared absorption spectra of CPG3 in D_2O and DMSO are quite different, indicating different solution structures for the peptide in these solvents. Interestingly, these differences were not obvious in other spectroscopic experiments.[4] We believe that the fast time scale and high spectral resolution of VCD allow these different conformations to be detected, whereas NMR and CD spectroscopic methods cannot do so.

Table 2. Properties of Metal Ions Used in This Study

metal ion	ionic radius[a] at coordination number 6 (Å)	binding constant[b] for protein binding	comments
Ca^{2+}	1.02	$<10^6$	prefers oxygen as electron donor; donor–ion distance 2.2–2.6 Å; coordination character; ionic
Mg^{2+}	0.72	10^3	higher affinity for N than Ca^{2+}; donor–ion distance 2.0–2.1 Å; coordination character: more covalent
Na^+	1.02	$<10^3$	
K^+	1.38	$<10^3$	

[a] From Einspahr, H.; Bugg, C. Crystal Structure of Calcium Complexes and Implications for Biological Systems. In *Metal Ions in Biological Systems*; Sigel, H., Ed., Marcel Dekker: New York, 1984; Vol. 17, pp 51–97. [b] Frausto da Silva, J. J. R.; Williams, R. J. P. *The Biological Chemistry of Elements: The Inorganic Chemistry of Life*; Clarendon Press: Oxford, U.K., 1991; p 104.

An inspection of Table 1 reveals frequency shifts of the absorption maxima from 1663 (in bromoform) to 1638 cm^{-1} (in D_2O). However, the shifts in the band maxima of the component bands is somewhat smaller, between 1632 and 1642 cm^{-1} for the glycyl and between 1657 and 1671 cm^{-1} for the prolyl carbonyl groups. The observed shifts in the band maxima are actually due to relatively small shifts of the component bands, accompanied by intensity variations in the components.

2. Conformational Studies of CPG3 with Various Metal Ions. An interesting feature of CPG3 is its ability to act as an ionophore, that is, to bind to metal ions while undergoing a conformational change. Peptides with such properties are thought to fulfill important biological tasks *in vivo*, such as the transport of metal ions through membranes. The cations studied here change the geometry of the C=O groups, mostly by twisting either the glycyl or the prolyl carbonyl groups toward the center of the molecule. The formation of such complexes depends on the charge and size of the cation as well as the solvent used.

Some properties of the metal ions of interest are listed in Table 2, along with peptide binding constants of the cations. Calcium plays a very important role in biological systems, especially its interactions with proteins. Calcium is a large, divalent cation, which favors coordinate numbers of 8, 7, 6, and 9 (in order of preference). Thus, calcium has the flexibility to bind with ligands which have binding pockets of different sizes.

Figure 5 shows VCD and absorption spectra of CPG3 in the presence of Ca^{2+} ions in aqueous solution at a ratio CPG3: Ca^{2+} = 1:1. The absorption maximum shifts up to 1643 cm^{-1} from 1639 cm^{-1} in water. The band decomposition results in two peaks at about 1636 and 1659 cm^{-1}, as compared to 1632 and 1657 cm^{-1} found in D_2O. The small changes in the absorption maxima, as well as the positions of the component bands, suggest that the order of prolyl and glycyl frequencies is unchanged. The changes in the solution phase infrared spectra are very small and inconclusive. In light of these results, the enormous changes observed in the Raman spectra of the solid CPG3/Ca^{2+} complex are most likely due to solid state effects.

The VCD exhibits a (weak negative)/positive/negative pattern with a zero crossing at 1653 cm^{-1}. Since the main VCD couplet is formed in the high frequency range of the amide I′ peak, we conclude that the calcium ion binds to the peptide in such a fashion that the prolyl carbonyl groups are able to undergo dipolar coupling. We believe that this implies that the calcium

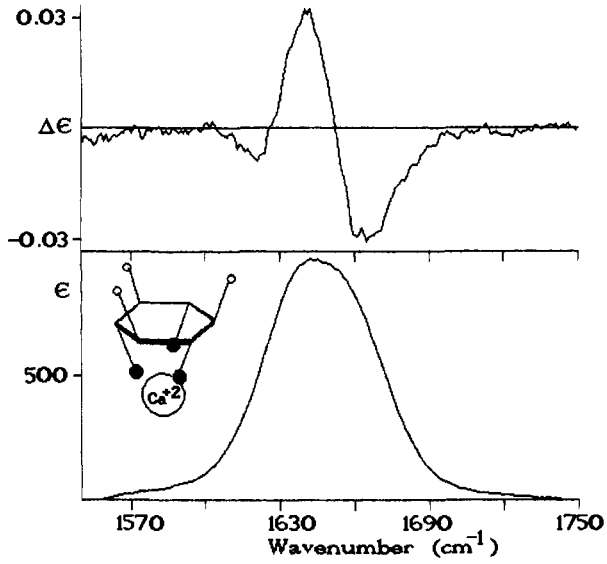

Figure 5. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in D_2O in the presence of equimolar Ca^{2+}. The inset presents a schematic structure of the peptide: black circles, prolyl carbonyls; white circles, glycyl carbonyls.

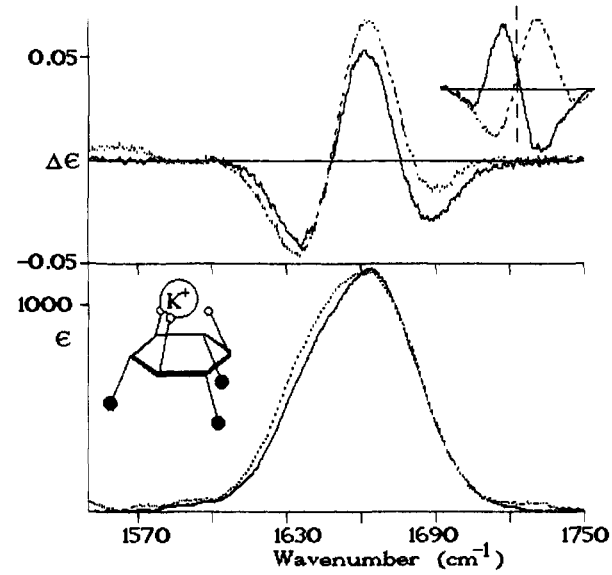

Figure 6. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in $CDBr_3$/ethanol (1:2 by volume) in the presence of equimolar Na^+ (solid traces) and K^+ ions (dashed traces). The inset at the upper right corner compares the Ca^{2+} and the K^+ spectra. The lower left inset presents a schematic structure of the peptide: black circles, prolyl carbonyls; white circles, glycyl carbonyls.

ion actually binds *via* the prolyl carbonyl groups and distorts the peptide as shown in the inset in Figure 5. However, the possibility exists that the calcium ion actually binds to the glycyl carbonyl groups, and that the spectral change observed in the prolyl region is due to a better coupling geometry of the prolyl residues once the calcium ion is in the glycyl pocket. However, further results with other ions make us favor the first interpretation.

Upon increasing the CPG3:Ca^{2+} ratio to 1:2 or even 1:10, no further spectral changes are observed, except for a slight shift toward lower wavenumbers of the zero crossing point of the VCD trace. We believe that this indicates that no Ca^{2+}/peptide/Ca^{2+} complex is formed, and that Ca^{2+} binds selectively to prolyl carbonyls. This finding is in contrast to results from the solid phase, and the different conclusions drawn in this study will be elaborated upon in the discussion section below.

We found that, in aqueous solution, other cations such as Li^+, Na^+, K^+, and Mg^{2+} cannot induce similar conformational changes in CPG3 due to their lower binding constants. Although they may cause a frequency shift in the IR absorption spectrum, due to the weak interaction between the ions and the carbonyl oxygen atoms, they cannot compete with the solvent and produce a complex with a discernible conformation. Thus, the solvent polarity is the dominant factor controlling not only the conformation of CPG3 but also its complexes with metal ions.

However, in moderately polar environments, the carbonyls of CPG3 exhibit weaker interaction with the solvent medium, and therefore allow some cations to bind with CPG3. Figure 6 shows the VCD and absorption spectra of Na^+ and K^+ with CPG3 in bromoform/ethanol (1:2). Na^+ and K^+ have relatively large sizes (*cf.* Table 2) and a single positive charge. Addition of these cations induces a shift of the absorption maximum to 1664 cm^{-1} from 1654 cm^{-1} in bromoform/ethanol, with the glycyl and prolyl components appearing at 1649 and 1669 cm^{-1}. This shift toward higher wavenumber, as compared with the pure bromoform/ethanol solvent, is due to the metal ion binding; however, when comparing the prolyl and glycyl components in the Na^+/peptide complex with those of the Ca^{2+}/peptide complex (*cf.* Table 1), one has to remember that the calcium data were collected in aqueous solution, which always exhibits lower frequencies. Furthermore, the symmetric band shape observed in bromoform/ethanol in the absence of metal ions (*cf.* Figure 3) is lost, and the absorption spectra in the presence of Na^+ and K^+ ions show distinct low frequency shoulders.

The observed VCD spectra are more intense than that shown in Figure 3, and this increase occurs mostly in the glycyl carbonyl couplet at the expense of the prolyl couplet. Since the spectral changes between the complexed and uncomplexed peptides in bromoform/ethanol occur in the low frequency shoulder (the glycyl carbonyl groups) of the amide I′ band, we conclude that both Na^+ and K^+ bind with the glycyl carbonyl groups, and that a similar distortion of the carbonyl groups occurs that was discussed for the Ca^{2+} ion in D_2O before. It is interesting to note that the shape of the VCD spectrum of the CPG3/K^+ complex is related to that of the calcium complex in D_2O by a reflection by a vertical plane, indicated by the dashed line in the small (upper) inset in Figure 5. We conclude that the binding cups formed on both faces of the molecule are similarly related by a mirror image. Available structural data[7,8] have allowed us to assess the distances between the carbonyl groups. These distances vary between 3.1 and about 3.9 Å, which is well within the range at which we have observed carbonyl coupling to occur.[11]

A further increase in the sodium or potassium ion concentration does not change the observed spectral features, and the formation of an ion/peptide/ion sandwich can be excluded. This can be understood in terms of the two binding sites of CPG3 being sufficiently different that they cannot easily interact with a second sodium or potassium ion (*vide infra*).

The previously presented data suggest that calcium and sodium (or potassium) ions bind to different faces of CPG3. Therefore, it should be possible to form ion/peptide/ion sandwich complexes with a mixture of these ions. In Figure 7, we present the VCD data of a CPG3/ Ca^{2+}/ Na^+ complex at a ratio 1:0.5:1 in D_2O. Sodium ions at any concentration cannot induce a conformational change of CPG3 in aqueous solution since sodium shows a weak interaction with the glycyl carbonyl groups; consequently, complexation of sodium ions was observed in nonaqueous solutions only.

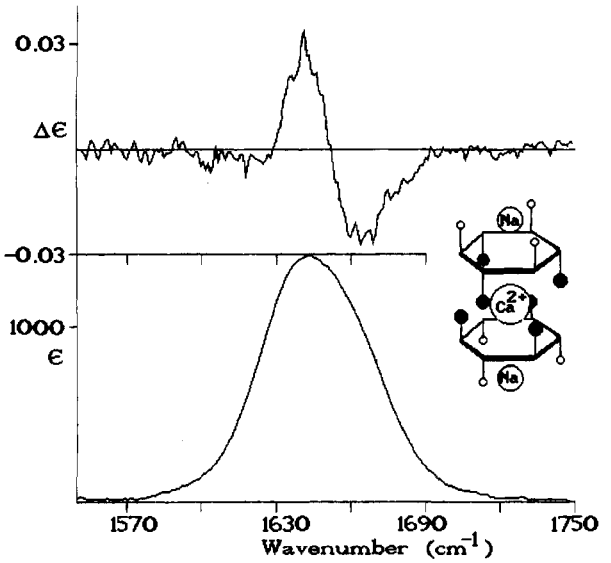

Figure 7. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in D_2O in the presence of Ca^{2+} and Na^+ at a ratio of CPG3: Ca^{2+}:Na^+ = 1:0.5:1. The inset presents a schematic structure of the peptide/ion complex: black circles, prolyl carbonyls; white circles, glycyl carbonyls.

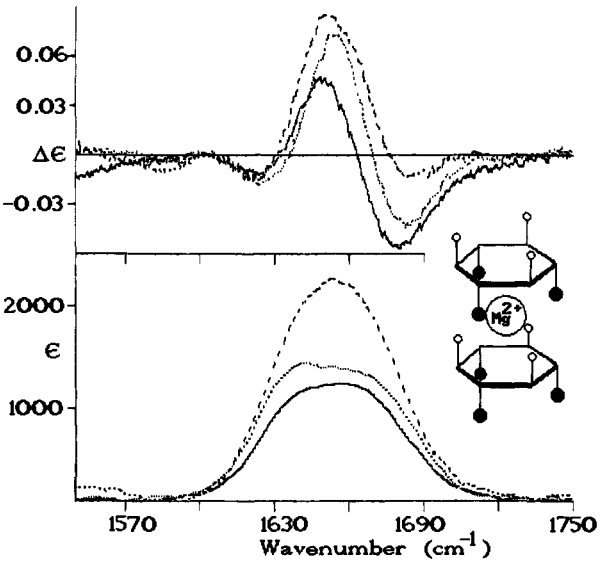

Figure 8. Infrared VCD (top) and absorption (bottom) spectra of cyclo-(-Pro-Gly-)$_3$ in $CDBr_3$/ethanol (1:2 by volume) in the presence of Mg^{2+} ions: solid trace, CPG3:Mg^{2+} = 1:1; dotted trace: CPG3:Mg^{2+} = 1:2; dashed trace, CPG3:Mg^{2+} = 2:1. The inset presents a schematic structure of the peptide/ion complex: black circles, prolyl carbonyls; white circles, glycyl carbonyls.

However, at a ratio of CPG3: Ca^{2+}:Na^+ of 1:0.5:1, the observed VCD spectrum is the same as that of CPG3 in the presence of a 1:1 ratio of Ca^{2+} ions. Thus, we conclude that the binding of sodium and calcium occurs cooperatively: since one cation binding to one side of the peptide can produce a conformational change which facilitates the binding of the other cation, binding proceeds preferentially in the presence of both ions.

VCD data do not allow us to determine the exact nature of the complex formed in the presence of both sodium and calcium ions. However, since X-ray crystallography has suggested the structure Na^+/peptide/Ca^{2+}/peptide/Na^+ (*vide supra*), which has the same stoichiometry as the solution used in the VCD study, we are inclined to suggest that a similar complex might be formed in solution, with the calcium ion being sandwiched by two peptide molecules, and one sodium ion binding to each of the peptide rings *via* the glycyl groups.

Different binding behavior was observed for Mg^{2+} in bromoform/ethanol. Mg^{2+} is chemically quite different from the other cations. Its small size, combined with its high charge, gives the interaction of magnesium ions with the carbonyl groups more covalent character. Consequently, it exhibits totally different bioactivity from the other cations. In bromoform/ethanol, Mg^{2+} can bind with CPG3 in at least two distinct ways, depending on the molar ratio of peptide to metal ion. In Figure 8, the VCD and absorption spectra of 0.5:1 , 1:1, and 1:0.5 ratios of CPG3/Mg^{2+} are shown.

At a ratio of CPG3:Mg^{2+} of 1:1, one observes that the absorption peak is broader and nearly resolved into two components, which are found by band deconvolution to occur at 1638 and 1666 cm^{-1}. The VCD spectrum exhibits a strong couplet in the C=O$_{(Pro)}$ region with a much weaker couplet in the C=O$_{(Gly)}$ band. The observed band shape is very similar to that observed for the Ca^{2+} ion complex, but with larger splitting of the components. Thus, we conclude that the preferential binding site of the magnesium ion is the prolyl pocket as well.

The VCD and absorption data observed for a 1:2 ratio of CPG3/Mg^{2+} are similar to those observed for the 1:1 ratio, except that, at higher cation concentration, the prolyl absorption and VCD shift to somewhat higher wavenumber. We interpret the similarity between the 1:1 and 1:2 complexes in terms of the absence of an ion/peptide/ion complex, in which metal binding would have to occur at both prolyl and glycyl carbonyl groups. In this case, we would expect that their VCD couplets be equal. This is clearly not the case.

At a 2:1 ratio of CPG3/Mg^{2+}, however, both IR and VCD spectra become symmetric with respect to the absorption center at about 1655 cm^{-1}. The strong VCD spectrum, with a dominant positive center and two negative peaks on each side, shows two zero crossing points at 1636 and 1677 cm^{-1}. We have interpreted this spectrum in terms of a peptide/ion/peptide sandwich in which both faces of the peptides are involved. The magnesium ion binds with C=O$_{(Gly)}$ from one CPG3 and C=O$_{(Pro)}$ from another. This hypothesis is based on a comparison of the observed spectrum of the 2:1 complex (Figure 8) with a coaddition of the VCD spectra of a glycyl/metal interaction (such as the 1:1 Na^+/CPG3 complex) and a prolyl/metal interaction (such as the 1:1 Mg^{2+} complex). The resulting coadded spectrum has a shape similar to that of the experimental data of the 2:1 complex. We believe that, at this ratio, the complex formed is a peptide/Mg^{2+}/peptide sandwich where the metal ion binds to both the C=O$_{(Gly)}$ and C=O$_{(Pro)}$ of different CPG3 molecules.

Discussion

The solution conformation of CPG3 in low polarity solvents is well established from CD and NMR spectroscopies.[4] This solution structure serves as the starting point of the interpretation of the VCD results. The VCD peak of CPG3 in bromoform has the same sign pattern as the VCD observed in other molecules containing a γ-turn;[14] however, band frequencies and relative intensities are quite different. We attribute the distinct shape of the VCD peak in CPG3 in bromoform to the fact that there are three highly coupled, identical γ-turns in very close vicinity, whereas previously studied γ-turns either were isolated species[14] or occurred next to a type II β-turn.[15]

(14) Xie, P.; Zhou, Q.; Diem, M. Infrared CD of Turns in Small Peptides. *Proc. Faraday Soc.*, submitted for publication.

In solvents of increasing polarity, such as bromoform/ethanol, a conformational change occurs in CPG3 which turns both the glycyl and prolyl carbonyl groups to form binding pockets, or cups, which are accessible to metal ions.[4] It is interesting to note that the spectrum of CPG3 in bromoform/ethanol is nearly symmetrical (Figure 3), indicating nearly equally good coupling geometry for both the prolyl and the glycyl carbonyl groups. This spectrum is quite similar to the spectra of CPG3 with certain metal ions. Thus, we may conclude that the largest conformational change occurs between very weakly polar and more polar solvents, and that metal ions produce subsequent and smaller conformational changes. We believe that the best representation of the solution conformation in bromoform/ethanol is given by the inset in Figure 3.

The interaction of calcium ions with CPG3 produces a number of interesting features. In aqueous solution, in the absence of metal ions, the structure of CPG3 is ill-defined. The presence of Ca^{2+} ions reestablishes a structure which is most likely highly symmetric, and which shows strong VCD features. Compared with the symmetric structure observed in pure $CDBr_3$, it is apparent that the main VCD couplet occurs in the prolyl, rather than the glycyl carbonyl groups. Thus, we conclude that the prolyl carbonyl groups are pulled toward each other by the calcium ion, and undergo dipolar coupling. The glycyl carbonyl groups, on the other hand, are solvent exposed and are expected to be hydrogen bonded to solvent molecules. In crystalline CPG3/Ca^{2+} complexes, water molecules actually were found bound to the glycyl carbonyl groups. These solvent molecules, along with the unfavorable geometry of the glycyl pocket, will not permit a second calcium ion to bind at the glycyl face.

However, even in the CPG3/Ca^{2+} complex in aqueous solution, there is evidence for some coupling of the glycyl groups, which we believe is manifested by the small negative couplet at low frequency. A schematic structure proposed for the Ca^{2+} complex with CPG3 is shown in the inset of Figure 5; the glycyl carbonyl groups will point away from each other, but will still be in reasonably good coupling geometry. In a less polar solvent, sodium and potassium ions bind in a very similar manner, but on the face opposite to the calcium ion binding site.

At this point, it is appropriate to comment once more on the different conclusions derived from the Raman studies on the solid complexes. We present here not only the VCD data but also absorption spectra which were collected independently on the VCD spectrometer, and on a commercial FT-IR instrument. Thus, we believe that an error in our frequency measurement is unlikely. The IR absorption frequencies do show some variation with metal ions, but not nearly as much as the Raman data reported. Furthermore, band deconvolutions reveal that the component bands stay within a 12 cm^{-1} range for the glycyl and a 14 cm^{-1} range for the prolyl carbonyl groups. Although band resolution algorithms have some inherent uncertainties, the calculated band positions do agree very well with the zero crossing points of the VCD spectra. Thus, we are confident that our results represent an accurate picture of the vibrational frequencies as a function of solvent polarity or the presence of metal ions. We believe that using shifts of band maxima may be very misleading, since in a composite band, intensity variations may contribute to the apparent shifts of a band.

The VCD data, much more so than the infrared absorption spectra, identify the binding site (*i.e.*, prolyl *vs* glycyl pockets) occupied by the cation in a complex in solution. We believe that these changes in VCD patterns are much more reliable than changes in frequency and intensity observed in standard vibrational spectroscopy, especially in the solid phase. Furthermore, we have ensured that all anions used in this study are the same (chloride), and are therefore not concerned about vibrational bands and/or overtones contributed by the anions. It is certainly possible that the solid phase data reported earlier are correct, but we claim that they do not represent the structure of CPG3 complexes with calcium, sodium, or potassium ions in solution phases.

Next, we shall attempt to present a rationale for the different binding of CPG3 to calcium and magnesium ions. Calcium has a less rigidly defined coordination number than magnesium. It prefers to interact with neutral oxygen donors such as carbonyl groups and alcohols, and prefers to form a loose complex. Magnesium, on the other hand, prefers sites with high charge density and small cavity size. It forms tight complexes with a coordination number of 6. Thus, the binding of magnesium to many ligands is much more restricted than, for example, the binding of calcium to the same ligands.

In general, the binding of CPG3 with metal ions will depend on four factors: the polarity and size of the peptide cavity, and the charge and size of the cation. The cavity size of CPG3 depends on the geometry of the carbonyl groups, which, in turn, depends on the polarity of the solvent as discussed before. The data of CPG3 in solvents of various polarity show how the alignment of the carbonyl groups varies; since the $C{=}O_{(Pro)}$ and $C{=}O_{(Gly)}$ groups are linked by the peptide backbone, the rotation of any carbonyl group is not arbitrary, but is correlated to the rotation of other carbonyl groups in the ring. On the basis of this consideration, we may postulate a first binding step that causes the carbonyl groups on one side of the ring to complex the metal ion, whereas the carbonyl groups on the other side form a binding pocket that may induce another ion to bind.

In water, for example, the conformation of CPG3 is such that both C=O and NH groups can form hydrogen bonds with the solvent. The cavity size is relatively large, and a calcium ion can fit in it. In a less polar medium, it has a shape with two well defined, and much smaller, binding pockets, or cups. In this situation Mg^{2+} forms a tight complex with a coordination number of 6 with either two peptide molecules or one peptide molecule and anions or solvent molecules.

Mg^{2+}/peptide/Mg^{2+} or Ca^{2+}/peptide/Ca^{2+} sandwiches were not found in either case because the binding of one ion leaves the binding pocket on the other side of the molecules so distorted that a second ion does not fit. Only in the case of Ca^{2+} and Na^{+} did we find evidence for such a sandwich, since the sodium and the calcium naturally prefer different faces of the peptide, which have totally different binding properties. The glycyl site favors monovalent cations, whereas the prolyl site prefers divalent cations.

At this point, we need to discuss the unusual structure of the peptide/Mg^{2+}/peptide complex, which we believe is formed *via* the prolyl carbonyl groups on one of the CPG3 molecules, and *via* the glycyl carbonyl groups of the other. If both CPG3 molecules were binding *via* the prolyl groups, the VCD spectrum of the magnesium complex would not change as the peptide concentration is raised. This behavior was observed in the case of calcium ions. Thus, we offer the following explanation for the formation of the peptide/Mg^{2+}/peptide complex. After the first magnesium ion binds to the three prolyl carbonyl groups from one CPG3 molecule (we call this the first binding step), this magnesium ion loses some of its polarity and behaves more like a monovalent cation. Therefore, it will no longer interact and complex with prolyl carbonyls from a second CPG3, but

(15) Wyssbrod, H. Diem, M. Infrared (Vibrational) CD of Peptide β-turns: A Theoretical and Experimental Study of *cyclo*-(-Gly-Pro-Gly-D-Ala-Pro-). *Biopolymers* **1992**, *31*, 1237–1242.

rather with its glycyl carbonyl groups. Larger sandwich structures, such as $\cdots Mg^{2+}{}_{(Gly)}CPG3_{(Pro)}Mg^{2+}{}_{(Gly)}CPG3_{(Pro)}Mg^{2+}\cdots$, have 1:1 stoichiometry and should exhibit the VCD spectrum shown in Figure 8 for the 1:1 ratio. Thus, we believe that the 2:1 ratio complex contains peptide molecules binding with different faces to the magnesium ion.

Conclusion

We find that VCD is an enormously sensitive tool for monitoring conformation changes in peptides. Its ability to identify different kinds of C=O groups allows for a qualitative interpretation, and its sensitivity to detect local dipolar coupling interactions allows one to determine which carbonyl groups are nearby in a given conformation. Therefore, VCD is an extremely powerful solution conformational probe for small peptides.

CPG3 is an excellent model to study the interaction of various metal ions with an ion-binding peptide. The results of uncomplexed CPG3, several 1:1 complexes of CPG3, and two peptide sandwiches lead to the following conclusions: (1) The solvent is the most important factor determining the conformation of the peptide. (2) The size of the ion binding cavity, formed by carbonyls, and the size and charge of the metal ions play important, but secondary roles to the solvent polarity.

For metal ions, the real size depends on the coordination of the ion prior to peptide complexation. Solvent molecules attached to the cavity may determine the shape of the binding cup. The differences in binding geometries of various cations with CPG3 confirm why these cations have different properties in biological systems.

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