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Conformational Studies of Peptide *Cyclo*-(D-Val-L-Pro-L-Val-D-Pro)$_3$, a Cation-Binding Analogue of Valinomycin†

Donald G. Davis,*,‡ B. F. Gisin, and D. C. Tosteson§

ABSTRACT: The solution conformation of *cyclo*-[D-Val-L-Pro-L-Val-D-Pro]$_3$ (PV) and its alkali-metal ion complexes was investigated by proton nuclear magnetic resonance spectroscopy. It is concluded that the cation complexes of PV have S_6 symmetry and are essentially isostructural with the K complex of valinomycin. In contrast to valinomycin, the Li- and Na-PV complexes are stable in methanol and have dissociation rate constants that are several orders of magnitude slower than the corresponding valinomycin complexes. Also in contrast to valinomycin, free PV exists in two different conformational states which interconvert at very slow rates ($\ll 1$ s^{-1}). One of these conformers has S_6 symmetry and is structurally similar to that of the cation complexes. The other species, which has lower symmetry than S_6, is the more stable conformer. Depending upon concentration and solvent polarity, the latter represents between 50 and 75% of the total mixture. It is proposed that PV may have a higher affinity for cations than valinomycin because of its higher potential energy in the uncomplexed state.

The facilitated and selective transport of cations across membranes by cyclic antibiotics such as valinomycin has been studied extensively (Läuger, 1972; Haydon and Hladky, 1972; Grell et al., 1972; Eisenman et al., 1973). Structural investigations show as well that the cation-carrier function of valinomycin is a consequence of its conformational properties. For example, the conformation of the K-valinomycin molecule provides for a polar, cation-binding site in which the ion is shielded from the low dielectric interior of the membrane by an exterior surface studded with hydrocarbon side chains (Ivanov et al., 1969; Pinkerton et al., 1969). This conformation, which is the basis for the ion-carrier activity of valinomycin, appears to be extremely sensitive to changes in the primary structure of the molecule (Merrifield et al., 1969; Ovchinnikov et al., 1972).

To investigate further the relationship between structure and function in these macrocyclic compounds and to test generalizations that have been proposed from studies on valinomycin, a cyclic dodecapeptide analogue of valinomycin was synthesized (Gisin and Merrifield, 1972). This compound, designated here as PV,[1] has the formula *cyclo*-[D-Val-L-Pro-L-Val-D-Pro]$_3$ and is related to valinomycin by the substitution of D- and L-prolines for D-hydroxyisovalerate and L-lactate residues, respectively. This substitution not only preserves the threefold periodicity of the sequence and the sequential order of optical isomers, but also restricts H-bond donors to the amide protons of the valines. In valinomycin, these latter constraints are thought to be important requirements for the formation and stabilization of the series of alternating β turns which characterize the bracelet-like secondary structure of the K ion complex (Ohnishi and Urry, 1971; Ovchinnikov et al., 1972).

Although the functional properties of PV resemble those of valinomycin qualitatively, PV is substantially different when compared to valinomycin in quantitative terms. Estimates of the affinities of PV for alkali metal ions in single and two phase bulk systems are at least 10^3 times larger than those for valinomycin (Gisin and Davis, 1973). On the other hand, when compared in terms of the concentrations required to produce a given membrane conductance in lipid-bilayer experiments, PV is less potent than valinomycin by a factor of 10^3 (Ting-Beal et al., 1974). The latter appears to be somewhat paradoxical in light of the higher cation affinities of PV and it points to the need for further experimental information about the structural properties of this peptide.

This paper deals with a proton nuclear magnetic resonance (^{1}H NMR) investigation into the solution conformations of PV and its complexes with alkali metal ions. Infor-

† From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710 (D.G.D., D.C.T., and B.F.G.), and the Rockefeller University, New York, N.Y. 10021 (B.F.G.). *Received July 22, 1975.* This work was supported by Research Grant HL12157 from the National Institutes of Health. The MPC 250 spectrometer facility (CMU) is supported by National Institutes of Health Grant RR-00292.
‡ National Institutes of Health Special Fellow FO 3GM52677-02.
* Author to whom to address correspondence. Present address: Department of Chemistry, Adelphi University, Garden City, New York 11530.
§ Present address: Division of Biological Sciences, Pritzker School of Medicine, University of Chicago, Chicago, Illinois 60637.

[1] Abbreviations used are: PV, *cyclo*-[D-Val-L-Pro-L-Val-D-Pro]$_3$; TNC, 2,4,6-trinitro-*m*-cresolate.

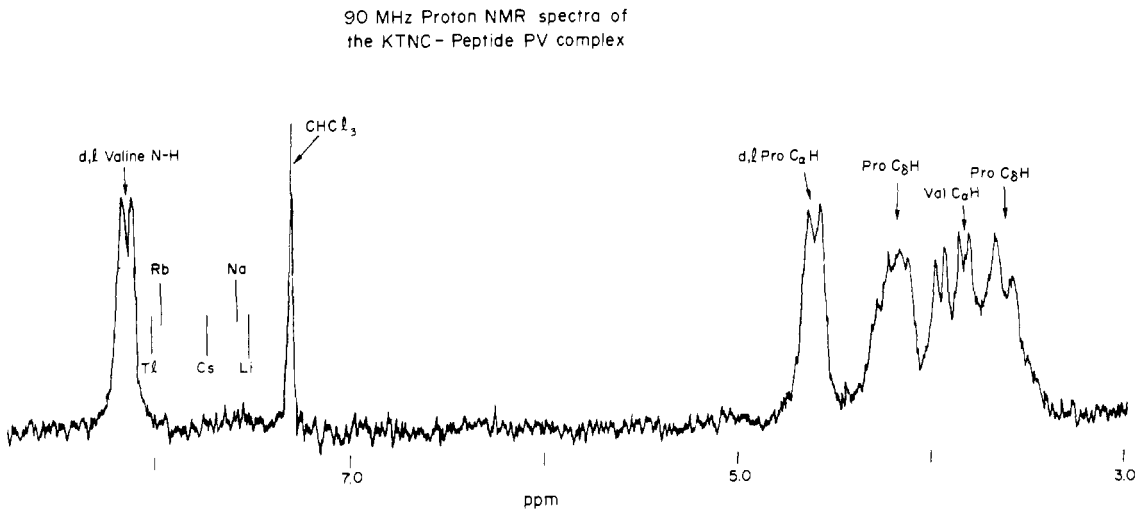


FIGURE 1: Low-field portion of the 90-MHz ^{1}H NMR spectrum of the potassium-PV complex in chloroform-d_1. The vertical lines indicate the position of the amide proton resonance for other monovalent cation complexes of PV. The chemical shifts are measured relative to Me_4Si.

mation about the conformation of PV and PV-cation complexes is given, and a structural basis for understanding some of the functional properties of this molecule is proposed.

Experimental Section

Materials. The synthesis of PV is described in a previous publication (Gisin and Merrifield, 1972).

TNC (2,4,6-trinitro-*m*-cresolate) salts were prepared by titration of the free acid (Eastman) to neutrality with the appropriate metal hydroxide in water. The solutions were reduced in volume until a precipitate formed. They were filtered and the residue was washed with hexane or $CHCl_3$ to remove any traces of the acid. After recrystallization from methanol, the TNC salts were dried and stored in closed vials.

Cation complexes of PV were formed by adding crystals of TNC salts to chloroform-d_1 (Merck) solutions of PV (~ 2–5×10^{-3} M). After an overnight incubation period the yellow-colored solutions were filtered through glass wool into NMR sample tubes (5 mm o.d.). Tetramethylsilane was added (1%, v/v) as an internal frequency reference and lock signal.

All other materials and solvents used in these experiments were obtained from commercial sources and were used without further purification. Acetone-d_6 (Merck) was stored over molecular sieves to remove traces of deuterated water.

Methods. Proton NMR spectra were recorded both in 90 MHz with a Bruker Scientific HFX spectrometer and at 250 MHz with the MPC-250 super-conducting NMR spectrometer (Dadok et al., 1970). Both spectrometers were operated in the field-frequency lock modes and signal-averaging techniques were used to improve the signal-to-noise quality of the spectra. At 90 MHz this involved accumulation of repeated slow passage traces in a Fabri-Tex 1024 digital storage scope. At 250 MHz, the signal-averaging procedure involved the use of correlation NMR spectroscopy. As described by Dadok and Sprecher (1975), slow passage NMR spectra are constructed by computer transformation of spectra recorded in linear fast passage. Correlation spectroscopy reduces substantially the accumulation time required to average out noise and yet obtain undistorted spectra.

Results

(A) Cation Complexes of PV in Chloroform. The 90-MHz proton NMR spectrum of the KTNC complex of PV in chloroform-d_1 is shown in Figure 1. This spectrum is also typical of the PV complexes formed from the TNC salts of the other alkali metal ions (Li, Na, Rb, Cs) and thallium. The line assignments for the valine N-H and C_α-H resonances are made on the basis of their chemical shifts and splitting patterns. The resonance of the proline ring protons are assigned by comparison with spectra of monomeric proline derivatives (Abraham and McLauchlan, 1962; D. G. Davis, unpublished). These assignments were also verified by double resonance experiments.[2] The chemical shifts of all the complexes examined are listed in Table I. Also listed in Table I are those spin–spin coupling constants which could be measured accurately and unambiguously. As these quantities do not vary significantly for the various complexes, a single set of coupling constants is tabulated. Because the lines are broad, the complex splitting patterns of the proline C_β and C_γ protons cannot be analyzed even in spectra recorded at 250 MHz; accordingly, their chemical shifts are given less precisely.

Only the chemical shifts of the valine N-H resonances differ substantially for the various cation complexes although trends are apparent in some of the other resonances. For ease of comparison, the N-H resonance positions for the other complexes are indicated in Figure 1. The temperature coefficients for the N-H chemical shifts are small and it is worth noting that $\Delta\delta/\Delta T$ (in ppm/°C) for the Na and Li complexes are zero or of opposite sign to those for K, Rb, and Cs complexes (see Table I).

(B) Cation Complexes of PV in Methanol. Some studies of the proton NMR spectra for the Li, Na, and K complexes of PV were made in methanol solutions. In order not to lose the amide protons to the solvent by exchange with deuterium atoms, the solutions were prepared with nondeu-

[2] Irradiation by a second radio-frequency field at the frequency of the proline C_β and C_γ proton resonances (not shown) collapses the line at 4.55 ppm (C_αH) to a singlet and the lines at 4.12 and 3.60 ppm (C_δH and C_δH′) into doublets, each split by ~10 Hz. Irradiation of the 4.55-ppm line (C_αH) does not affect the lines at 4.12 and 3.60 ppm; conversely, irradiation of either of these latter lines perturbs the other, but leaves the 4.55-ppm line unperturbed.

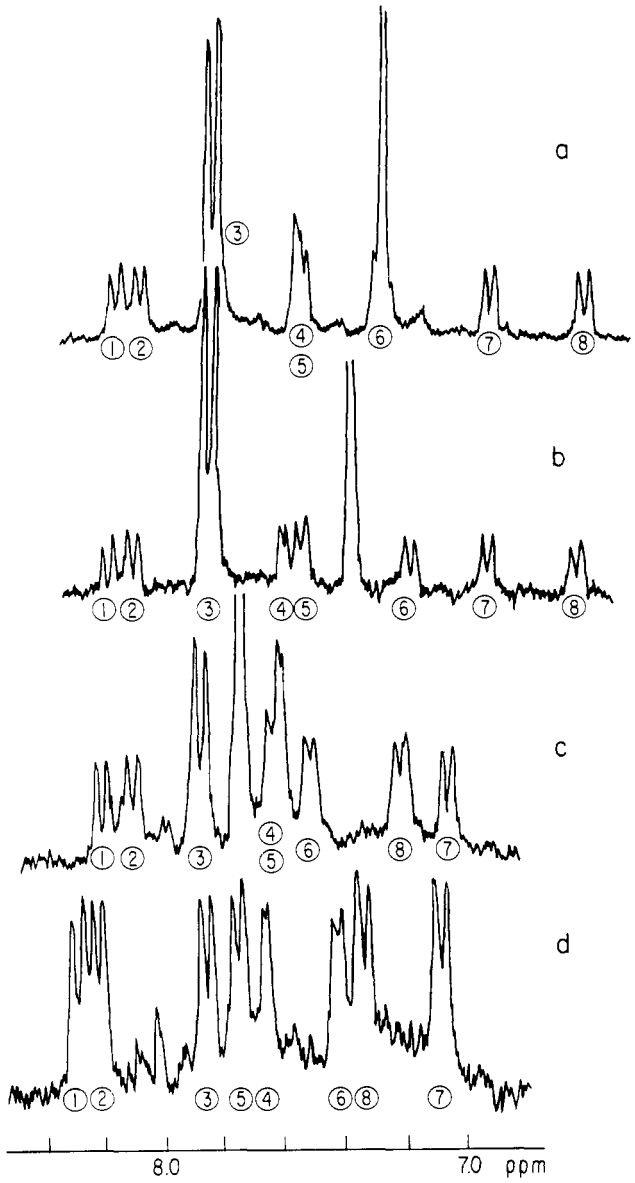


FIGURE 2: The 250-MHz ^1H NMR spectra of the amide protons of free PV in: (a) chloroform-d_1, [PV] ~4.2 × 10^{-3} M; (b) 89 mol % chloroform–11 mol % acetone; (c) 49 mol % chloroform–51 mol % acetone; (d) acetone-d_6, [PV] ~1.3 × 10^{-3} M. The numbering of the lines is explained in the text. The very intense, unnumbered line is from $CHCl_3$. The temperature of the samples was ~26 °C.

Table I: Proton Chemical Shifts and Coupling Constants for Monovalent Cation Complexes of PV.[a]

	Chemical Shift[b]						
	D,L-Valine				D,L-Proline		
Ion[d]	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H_3$	$C_\alpha H$	$C_\delta H_2$	$C_\beta H_2$-$C_\gamma H_2$
Li	7.50	3.87			4.73	4.20	
	(−0.5 × 10^{-3})[e]					3.59	
Na	7.56	3.88	~2.2	0.92	4.60	4.12	~2.0
	(0.8 × 10^{-3})			1.12		3.60	
K	8.10	3.84	~2.2	0.95	4.55	4.12	~2.0
	(3.0 × 10^{-3})			1.15		3.60	
Rb	7.96	3.81			4.53	4.12	
	(2.0 × 10^{-3})					3.58	
Cs	7.72	3.77			4.55	4.16	
	(2.0 × 10^{-3})					3.60	
Tl	8.00	3.82			4.61	4.16	
	(3.0 × 10^{-3})					3.61	

Coupling Constants[c,f]				
$J_{NHC_\alpha H}$	$J_{C_\alpha HC_\beta H}$	$J_{C_\beta HC_\gamma H}$	$\lvert J_{C_\alpha HC_\beta H'}\rvert$	$\lvert J_{HC_\delta H'}\rvert$
4.8	10.7	6.5	4.3	~10
			2.5	

[a] ca. 8 × 10^{-3} M in $CDCl_3$. [b] In ppm (±0.01) from Me_4Si. [c] In Hz (±0.2). [d] The anion is trinitro-*m*-cresolate (TNC). [e] Temperature coefficient (in ppm/°C). [f] Coupling constants are the same for all cations.

terated, spectroscopic grade methanol. Consequently, the extent to which the spectral properties could be investigated was limited by the resulting intense solvent resonances. Nevertheless, the amide proton lines are separated sufficiently from the solvent peaks to permit useful interpretation.

Judging from the position and splitting of the amide proton resonances,[3] the K and Na complexes of PV retain the same symmetry and conformation as they have in chloroform. This is also true of the Li–PV complex, although some process such as dissociation occurs fast enough (~5 s^{-1}) to average out the 5 Hz splitting which is observed in chloroform. Most of the PV molecules are complexed with Li for the spectrum is distinct from that of free PV (see below). The apparent stability of the Li- and Na–PV complexes is in marked contrast with what is known about valinomycin where Na and Li ions have little effect on the valinomycin spectra in most polar solvents (Ohnishi and Urry, 1969; Ivanov et al., 1971; Patel and Tonelli, 1973).

(C) Uncomplexed PV in Chloroform, Acetone, and Chloroform–Acetone Mixtures. The NMR spectra for the amide protons of the uncomplexed or free PV in deuterated chloroform, acetone, and chloroform–acetone mixtures are shown in Figure 2. They are clearly more complicated than the spectra of the cation complexes (Figure 1). Moreover, the positions and relative intensities of some of these amide resonances are quite sensitive to the polarity of the solvent, the temperature, and the concentration of free PV. The different resonance lines have been numbered 1, 2, . . . etc. as indicated in Figure 2. The same numbering applies to Figure 3 and Table II.

The spectra show eight amide doublets (Figure 2). This is seen most clearly in Figure 2b, where none of the lines are obscured by overlap with the solvent or other resonances. Since PV extracts sodium from the glassware, all spectra contain the signal for the Na–PV complex (line 4). The six lines labeled 1, 2, 5, 6, 7, and 8 make up ~50% of the total intensity. These lines all have equal intensity and this equality is maintained in all conditions of solvent polarity, temperature, and concentration. The remaining line, 3, is (except in pure acetone-d_6, Figure 2d) the most intense single line in the spectrum. In chloroform–acetone mixtures, the relative intensity of line 3 does not change significantly until the acetone concentration exceeds ~50 mol %. In pure acetone (Figure 2d) its intensity is comparable to the other six lines.

As can be seen most readily in Figure 3, the chemical shifts of lines 6 and 8 change substantially as the molar percentage of acetone in chloroform is increased. In a separate set of experiments, done in pure chloroform-*d*, it was found that the chemical shifts of these same two lines also have

[3] The amide-proton chemical shifts (and $J_{NHC_\alpha H}$ coupling constants) for the K-, Na-, and Li–PV complexes in methanol are: 8.19 ppm (4.1 Hz), 7.55 ppm (4.4 Hz), and 7.36 ppm (~5 Hz), respectively.

Table II: Proton Chemical Shifts of Free PV.[a]

Solvent	Proton Group	"S_6-Monomer" 3	"Low-Symmetry Form" 1	2	5	6	7	8
Chloroform-d_1[b]	Valine NH[c]	7.80[e]	8.15	8.06	7.58[f]	7.52	6.96	6.91[g]
	Valine $C_\alpha H$[d]	4.56	4.38	4.39	4.67	4.62	4.61	4.34
Acetone-d_6[h]	Valine NH[c]	7.86	8.30	8.22	7.76	7.42	7.08	7.34
Methanol[i]	Valine NH[c]	~7.9			~8.4			

[a] In ppm (±0.01) from Me_4Si; spectra were recorded at 250 MHz. [b] [PV] = 6.1×10^{-3} M. [c] $J_{NHC_\alpha H} \sim 10$–11 Hz. [d] $J_{C_\alpha HC_\beta H} \sim 10$ Hz. [e] Temperature coefficient $\sim 3 \times 10^{-3}$ ppm/°C. [f] Concentration dependent (see text), temperature coefficient, $\sim 1.6 \times 10^{-2}$ ppm/°C. [g] Concentration dependent (see text), temperature coefficient, $\sim 1.2 \times 10^{-2}$ ppm/°C. [h] [PV] = 1.3×10^{-3} M. [i] [PV] = $\sim 8 \times 10^{-4}$ M.

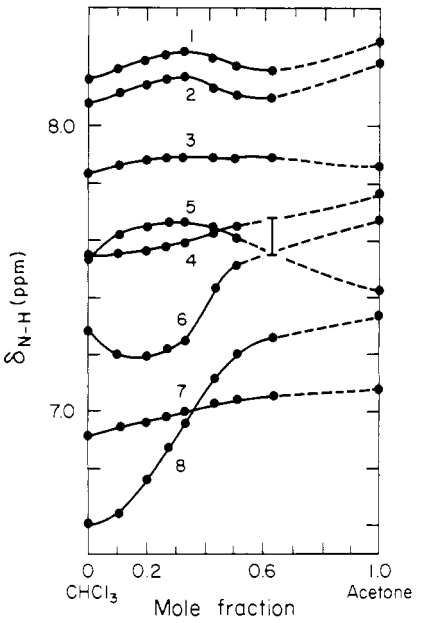


FIGURE 3: The chemical shifts of the amide protons of free PV as a function of the mole fraction of acetone-d_6 in chloroform-d_1. The solutions were prepared by adding successively (up to 62 mol %) measured amounts of acetone to a 4.2×10^{-3} M solution of PV in chloroform. The solution in pure acetone was prepared separately.

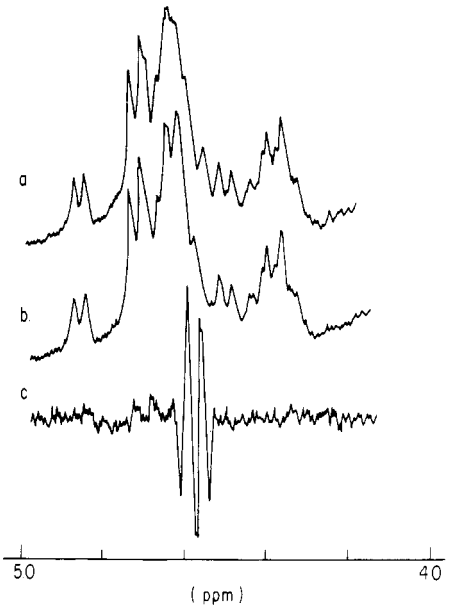


FIGURE 4: The 250-MHz proton NMR spectra of the valine and proline $C_\alpha H$ resonances in chloroform-d_1. (a) Normal one; (b) spectrum when the amide proton at 7.80 ppm (not shown) is irradiated with a second rf field. Spectrum (c) is the difference, (b) − (a), showing the normal $C_\alpha H$ quartet (which actually appears as a triplet) in the negative direction. Upon irradiation of the amide proton resonance the lines collapse to a doublet (positive direction).

temperature coefficients, $\Delta\delta/\Delta T$ (Table II) that are an order of magnitude greater than those of the other amide lines or for the amide protons of the cation–PV complexes (Table I). Moreover, the chemical shifts of lines 6 and 8 also move to higher field when the PV–chloroform solutions are diluted.

Two other observations are important to note. First, all the complicated multilined spectra for free PV can be converted completely to the simpler spectra of the cation complexes just by adding the appropriate salt. This implies that the multilined spectra do not represent chemically modified PV and, in particular, that the cyclic structure of the molecule is intact. Secondly, slight broadening of the amide lines was observed only above 50 °C (2 Hz vs. 1 Hz at 25 °C). This suggests that the molecular species represented by these spectra interconvert at a slow rate (<1 s^{-1} at 25 °C and ~ 0.5 s^{-1} at 50 °C; Pople et al., 1959).

Owing to the complexity of the spectrum, accurate chemical shifts and coupling constants can be measured directly only for the amide protons (Table II). Also listed in Table II, however, are the chemical shifts of the valine C_α protons which were measured indirectly by virtue of their coupling to the N–H protons. This approach involves the application of double-irradiation in a somewhat novel way and is described here briefly.[4]

In the particular example of PV, the region of the spectrum containing the C_α protons was scanned and stored in the computer while a second radio-frequency field irradiated one of the amide proton lines, thus "decoupling" this amide proton from its neighboring C_α proton (Figure 5b). The C_α proton region of the spectrum was again scanned and stored in the same computer storage locations but with the phase of the "lock-in" detector shifted by 180° and the frequency of the second irradiating field offset from the amide resonances by ~500 Hz. The phase shift inverts the spectrum, thus canceling out the unperturbed regions of the original (decoupled) spectrum. Residual images of the coupled and decoupled C_α–H transitions remain, however, as the difference (Figure 4c) with the one set of lines 180° out of phase from the other. As Figure 4c shows, these transitions can be identified clearly and accurately despite the fact that the total spectrum, either unperturbed (Figure 4a) or decoupled (Figure 4b), cannot be analyzed with any precision.

(D) Uncomplexed PV in Methanol. The spectrum of free

[4] Following the completion of these experiments Gibbons et al. (1975) reported a detailed description of this particular double resonance technique.

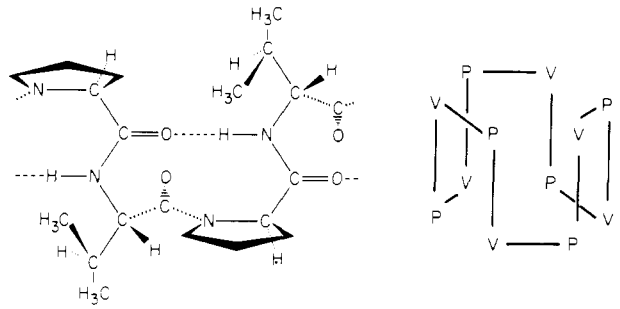


FIGURE 5: The proposed conformation of the peptide moiety for the PV–cation complexes and the S_6-symmetric monomer of free PV. The sketch on the left shows one L-Pro-L-Val-D-Pro-D-Val fragment. The estimated angles, ϕ and ψ (IUPAC-IUB Commission on Biochemical Nomenclature, 1970), for the various residues are as follows: L-Pro (−60°, 0°), L-Val (−60°, 120°), D-Pro (60°, 0°), D-Val (60, −120°). The figure on the right is a schematic representation of the backbone configuration showing the location of the amino acid α-carbon atoms.

PV in methanol shows two amide proton resonances: a broad line at ~8.5 ppm that accounts for 80% of the total intensity and another sharper line at 7.95 ppm. The positions of both lines are distinct from those of the complexes. Judging from the sharpness (~2 Hz) of the line at 7.95 ppm, this fraction of PV molecules does not interconvert rapidly with the broad-lined fraction. The line width for this latter fraction may be the consequence of slower molecular tumbling due to aggregation, broadening of the amide protons by exchange with solvent OH protons, or broadening by interconversion among different conformational states. At present, the low solubility of PV as well as technical limitations prevents the resolution of these possibilities.

Discussion

Cation Complexes of PV. A number of general structural features of the PV cation complexes are immediately apparent from their ^{1}H NMR spectra and the temperature coefficients of the amide proton chemical shifts. First, the spectra show that the protons of all six valine residues are in magnetically equivalent environments as are the protons of the proline residues. This implies that the PV complexes have (on the time average) S_6 ($C_3 \times i$) symmetry.[5] Any conformation of PV with less than S_6 symmetry and a lifetime that is long on the NMR time scale would most likely lead to magnetic nonequivalence thus increasing the number of resonance lines. It is interesting to note that the presence of the counterion, TNC^-, does not perturb this symmetry. Secondly, the magnitude of the coupling constant, $J_{C_\alpha HNH}$ (Table I), is consistent with a $C_\alpha HNH$ dihedral angle of either 60 or 120° (Bystrov et al., 1973). Likewise, the values for $J_{C_\alpha HC_\beta H}$ indicate a trans orientation[6] of the C_α–H and C_β–H bonds (Karplus, 1959). Thirdly, the amide proton shifts have small temperature coefficients and are rather insensitive to solvent polarity. This suggests that these protons are part of stable, solvent-shielded intramolecular hydrogen bonds (Kopple et al., 1969a,b; Ohnishi and Urry, 1969).

A more detailed specification of the solution conformation must be derived from an examination of molecular models. Such an analysis is facilitated by a comparison with what is known about valinomycin and its K complex. This is quite helpful for it includes both x-ray diffraction (Pinkerton et al., 1969; Daux et al., 1972; Neupert-Laves and Dobler, 1975) and ^{1}H NMR data (Ivanov et al., 1971; Patel and Tonelli, 1973). With molecular models only four conformers of PV can be constructed which possess all of the three features deduced solely from the spectra: (1) S_6 symmetry; (2) gauche orientations about the N–C_α valine bond; (3) intramolecular H-bonds to all six amide protons. Of these four conformers, two have what has been designated as 1–4 type H-bonds and two have the 1–3 type (Patel and Tonelli, 1973). The two conformations having the 1–3 H-bonds are unlikely for the following reasons. Generally, 1–3 type bonds are less stable than the 1–4 type (Tonelli, 1971) and when they are found, they have temperature coefficients on the order of 0.01 ppm/°C (Patel and Tonelli, 1973) which is in contrast to K–PV (0.003 ppm/°C). In addition, the 1–4 type is found in the K complex of valinomycin (Pinkerton et al., 1969). And lastly, PV models constructed with 1–3 type intramolecular H-bonds appear much more strained than those with the 1–4 type H-bonds. Hence, only the two conformers with the 1–4 type H-bonds will be considered further. The two remaining conformers may be distinguished by the orientations of the valine C_α–C_β bonds taken with respect to the threefold rotation axis. Borrowing from the nomenclature used to identify the conformational states of valinomycin (Ivanov et al., 1969), the A_1 confomer of PV has valine C_α–C_β bonds that are perpendicular (equatorial) to the C_3 axis, and in the A_2 conformer these bonds are approximately parallel (axial) to the C_3 axis.[7]

It is concluded that PV complexes, just like the K–valinomycin complex (Pinkerton et al., 1969; Neupert-Laves and Dobler, 1975) are in the A_2 conformation for the following reasons. First, the chemical shifts of the valine C_α protons are nearly identical with those in valinomycin complexes (Ivanov et al., 1971: Davis and Tosteson, 1975) of the same cation. Owing to bond magnetic anisotropy, the chemical shifts of protons adjacent to carbonyl groups are sensitive to the orientation of these groups (Pople et al., 1959). Since PV and valinomycin complexes show like chemical shifts for the valine C_α protons, the orientation of the carbonyl groups are most likely the same in both compounds. Secondly, the valine $C_\alpha H$–$C_\beta H$ pairs for PV complexes have a trans orientation. This too is consistent with the A_2 conformation for it has been shown experimentally and theoretically (Mayers and Urry, 1972; Urry and Kumar, 1974) that the preferred orientation of the protons about the C_α–C_β bond is trans for axially oriented isopropyl groups, but gauche for those with an equatorial orientation. Finally, examination of a CPK molecular model of PV reveals that in the A_1 conformation, the amide bonds of the proline ring nitrogens would be nonplanar and the proline ring and valine side chains would be eclipsed with obvious steric interactions. On the other hand, in the A_2 conformation, the proline amide bonds are planar and the valine residues do not interact sterically with the proline rings. Consequently, A_2 is the more likely conformation for PV complexes, the salient features of which are shown in Figure 5.

[5] A molecule with S_6 symmetry is one in which the atoms of a given unit (here any one of the dipeptide fragments) are spatially related to the other units in the molecule by either a 120° rotation, C_3, or a 60° rotation, C_6, and inversion (i) of the coordinates through the center of mass.

[6] The coupling constants for the PV complexes are not noticeably temperature dependent over the range from 20 to 50 °C, indicating that these particular orientations are relatively stable.

[7] In the A_2 conformation the $C_\alpha HNH$ dihedral angle is 120° and in A_1, 60°.

Uncomplexed PV. The NMR spectrum for some arbitrary conformer of PV can have at most six (one for each valine residue) distinct amide proton resonances. Since seven lines are observed, free PV must exist in at least two different conformational states, which, judging from the line widths, interconvert at a slow rate ($\ll 1\ s^{-1}$ at 25 °C). One of the conformers is represented by the large doublet, line 3, since its intensity varies independently of that for the six other amide proton lines. Conversely, the intensities of these latter lines are always equal suggesting that they represent the second and the only other conformational species of PV in these solutions. In fact, the second species is the more abundant one, making up between 50 and 75% of the total PV concentration.

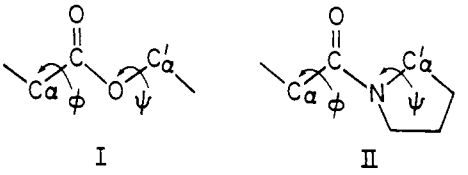
By the same kind of reasoning presented above for the cation complexes, the free PV molecules giving the large, single doublet must have S_6 symmetry. As suggested by the low temperature coefficient for the chemical shift of this line, these amide protons are intramolecularly H-bonded as well. Furthermore, the value of 10 Hz for the $C_\alpha H$-$C_\beta H$ vicinal coupling constant (Table II) is consistent with an axial orientation of the valine C_α-C_β bonds (Mayers and Urry, 1972). Again it can be concluded that A_2 is the only reasonable conformation having all three of these features.

The larger value (10 Hz) for the NH-C_αH coupling constant indicates, however, that the A_2 conformation of free PV differs somewhat from that of the cation complexes. According to the best empirical and theoretical estimates (Bystrov et al., 1973; Barfield and Gearhart, 1973), the NH-C_αH dihedral angle corresponding to a coupling constant of this magnitude is ~150° (±5°) or about 10–20° larger than that estimated for the cation complexes. As discussed in the next section, this increased angle might be due to distortions of the peptide backbone induced by repulsive interactions between the carbonyl groups.

With regard to the second conformer of free PV, a number of conclusions about its structure are clear from the NMR studies. First, the species has six nonequivalent amide and valine C_α protons. This means that it is distorted having less than S_6 symmetry. Secondly, two of the amide protons are unique in the sense that their chemical shifts are readily perturbed by temperature, dilution, and by polar solvents such as acetone. This suggests that these particular amide protons are only weakly, if at all, intramolecularly H-bonded or are readily exposed to the solvent and other PV molecules. The chemical shifts of the remaining four amide protons, however, are quite refractory to solvent and thermal perturbations and are most likely stabilized in intramolecular H-bonds. Thirdly, the distorted conformer is the more abundant species, and therefore, is more stable in this range of temperature, concentration, and solvent polarities. Finally, the rate of interconversion between the S_6 symmetric and distorted species is slow, possibly due to cis-trans isomerization about one or more Val-Pro bonds. Indeed such slow interconversions are commonly observed in proline-containing peptides. Alternatively, the breaking of several H-bonds could also slow down the rate of interconversion by adding significantly to the activation energy for this process.

Correlations between PV Structure and Function. The structural basis for the ability of PV to bind cations and facilitate their transport across bilayer membranes is clear. Like valinomycin, the PV-cation complex has a bracelet or doughnut-shaped structure with the cation held at the center via coordination to the inwardly directed valine carbonyl groups. The peptide moiety, by dissipating the cationic charge over a larger effective radius and by presenting a surface studded with hydrophobic valine side chains and proline rings, enhances the partition of the complex into the low dielectric interior of the membrane and lowers the energy barrier to ionic transport across the bilayer. Thus, the substitution of the amino acid proline for the hydroxy acids in valinomycin results in a compound which can form cation complexes that are isostructural with those of the prototype.

Despite these general similarities there are properties in which PV and valinomycin diverge markedly. Although PV has, for example, a larger affinity (10^3-fold) for cations (Gisin and Davis, 1973) it is 10^3 times less effective in lowering the ionic conductance of bilayer membranes (Ting-Beal et al., 1974). Such differences may be due to the different dipolar and electron-donating properties of the coordinating groups (amide vs. ester carbonyl groups) as well as the conformational energies of the ligand in the complexed states. However, structural differences in the uncomplexed forms may contribute as well to the divergent functional properties of the two compounds. For example, when there is no cation present to neutralize the strong repulsive interactions between inward-pointed carbonyl groups, the uncomplexed forms are less stable. For valinomycin the instability caused by these interactions can be lessened by *symmetry*-preserving rotations (ϕ and ψ) of the ester carbonyl groups about the C_α—C=O and —O—$C_{\alpha'}$ bonds (I). For free PV a similar rotation is not possible owing to the incorporation of the N-$C_{\alpha'}$ bond into the proline ring (II). The



I II

instability of the S_6-symmetric form of free PV is clearly demonstrated by the fact that the molecules exist predominantly in a distorted state of lower symmetry. Consequently, the larger affinity of PV for cations may be due in part to a less favorable free energy of the uncomplexed form (particularly an enthalpy term resulting from the carbonyl group repulsions) as compared to valinomycin.

With regard to the reduced effectiveness of PV in lowering the electrical resistance of bilayer membranes, an examination of the kinetics for complex formation and dissociation is informative. These reactions may be necessary steps in the carrier-mediated transport of ions across membrane interfaces. In CH_3OH, the rate-limiting steps for the dissociation of Na- and K-valinomycin complexes are $2 \times 10^6\ s^{-1}$ and $1.3 \times 10^3\ s^{-1}$, respectively (Grell et al., 1972). (Li apparently forms such a labile complex, that the kinetics of this system cannot be measured.) As estimated by the line widths in CH_3OH, the dissociation rate for Li-PV is $5\ s^{-1}$ and for Na- and K-PV, the rates are $\leq 1\ s^{-1}$. These values are several orders of magnitude slower than for the corresponding valinomycin complexes.

Although CH_3OH may only approximate the solvent conditions at the membrane-aqueous phase interface, the estimated rate for the K-valinomycin dissociation step at this membrane interface is $\sim 10^4\ s^{-1}$ (Läuger, 1972), i.e., only slightly faster than in CH_3OH. A similar extrapolation for PV leads to the conclusion that the dissociation of the complexes could be the rate-limiting factor in determining the electrical conductance of bilayer membranes exposed to PV.

The rates of PV–cation complex formation are not known. It can be pointed out, however, that regardless of which PV conformer (S_6-symmetric monomer or the distorted, asymmetric form) reacts fastest with cations, the slow conversion of an inactive to a more active species may limit the steady-state concentration of ion carriers at the interface. If membrane translocation of the loaded carrier is faster than the conversion rate between free PV conformers, a depletion in the number of carriers at the interface would arise and thereby reduce the conductance.

Acknowledgments

The authors thank the Chemistry Departments of Duke University and Carnegie-Mellon University for the use of their respective NMR facilities. The typing assistance of Miss J. Palumbo is also gratefully acknowledged.

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