248. Alkali Cation Complexation and Transport Properties of Synthetic Bicyclic Decapeptides: Structural, Thermodynamic, and Kinetic Analysis[1])

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Summary

Alkali cation complexation and bilayer transport by the bicyclic decapeptides *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-glycyl-glycyl-L-prolyl (**1**), *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-glycyl-glycyl-D-prolyl (**2**), *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-sarcosyl-sarcosyl-L-prolyl (**3**), and *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-sarcosyl-sarcosyl-D-prolyl (**4**) were analyzed according to structural, thermodynamic and kinetic criteria; valinomycin was used as a reference ionophoretic system.

Structural analysis of peptide **3** with spectroscopic methods showed different conformational arrangements in the bicyclic system depending on its state of complexation. Circular dichroism indicated the presence of a multitude of conformations with differing helicities around the disulfide bridge in both free and complexed states.

Thermodynamic analysis by microcalorimetry demonstrated a far lower cation selectivity among the synthetic peptides than displayed by valinomycin. Peptide **3** shows cation affinities of about two orders of magnitude higher than peptide **4**, but still much lower than found for the complexes of valinomycin with K^+ and Rb^+. In contrast to the latter case, the complexation reactions of peptides **3** and **4** are driven by both enthalpy and entropy contributions. Neither peptide **1** and **2** nor the cyclic partial structures of all four peptides displayed significant cation complexation.

A kinetic analysis of the K^+-complexation by peptide **3** based on the microcalorimetry experiments showed far lower rates of cation exchange for the synthetic peptide than those reported for valinomycin. Transport studies with peptide **3** using artificial lipid bilayer membranes gave negative results. The apparent lack of ionophoretic properties of these synthetic peptides despite their considerable ability to form complexes with cations is discussed in terms of structural parameters.

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Introduction. – The ability of many antibiotics of depsipeptide, polyester, or polyether structure (valinomycin, nonactin, nigericin, *etc.*) to specifically complex alkali metal cations and facilitate their transport across hydrophobic membrane structures [1] [2] raises the question whether it might be possible to construct similar 'ion cages' using exclusively amino acids as building blocks. Such systems might then serve as models for ionophoretic substructures in membraneous transport proteins.

A simple approach to conceive suitable carbonyl cages around an alkali cation with spherical charge distribution resides in the cooperative action of two small cyclic peptides which can not themselves be penetrated by the cations in question. To enhance the probability of complex formation, the two peptide rings might be covalently linked at one or more sites. Reversible formation of these links, *e.g.* with disulfide bonds, might form the base of a naive model for active, energy driven ion transport [3] [4].

Following this concept, the bicyclic decapeptide *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-glycyl-glycyl-L-prolyl (**1**) was synthesized as the first model system [4]. To imitate more closely the natural ionophoretic antibiotics, the L-prolyl residue in **1** was replaced by D-prolyl to obtain *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-glycyl-glycyl-D-prolyl (**2**). In a further attempt to increase the surface hydrophobicity of the complex structure, two of the three glycyl residues were replaced by sarcosyl in *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-sarcosyl-sarcosyl-L-prolyl (**3**) and *S,S'*-bis-*cyclo*-glycyl-L-hemicystyl-sarcosyl-sarcosyl-D-prolyl (**4**) [5].

Conformational analysis of the single peptide rings forming these bicyclic structures has been carried out by NMR. spectroscopy and semiempirical energy calculations [6–9]. Several deuteriated derivatives of the ring structures of peptides **1** and **2** were synthesized [5] [10] in order to clear the last remaining ambiguities concerning hydrogen bonding in the peptide rings [11].

In this communication we describe the structural, thermodynamic, and kinetic analysis of alkali cation complexation of peptides **1** to **4** by spectroscopic methods and microcalorimetry as well as their ion transport properties in an artificial lipid bilayer system. The antibiotic valinomycin is used as a reference compound regarding cation affinities and selectivities. Preliminary reports on the characterization of the valinomycin-K^+ complex by microcalorimetry have been presented [12] [13].

Experimental Part

General. 'Purissimum' grades of NaI, KI, RbI, ethanol, methanol, and dimethylsulfoxide (DMSO) were purchased from *Fluka Ltd.* Valinomycin (A-grade) was a product of *Calbiochem* (Lucerne). Metal iodide solutions were kept from light during storage.

IR. spectra were recorded in DMSO using a *Beckman* IR-33 Infrared Spectrophotometer. ^{1}H-NMR. spectra were obtained in (D_6)DMSO with a *Varian* HR-220-NMR.-Spectrometer. UV. spectra were recorded in absolute ethanol on a *Beckman* Acta V UV.-Visible-Spectrophotometer. Lipid bilayer studies were performed using oxidized cholesterol/decane membranes [14]. The apparatus and techniques have been described [15].

Microcalorimetry. Enthalpy changes were determined in a LKB 10700 batch microcalorimeter equipped with a pair of gold cells *(LKB-Produkter AB).* The temperature of the calorimeter unit was maintained at 25.0±0.01°. 2.0 ml of a 0.1 to 0.4mM solution of peptide or valinomycin were introduced into the smaller compartment of the reaction cell, and 4.0 ml of solvent containing 0.1 to 100 g-atoms of metal iodide per mol of ligand into the larger compartment. To compensate for the

small heat of dilution of the salt during mixing, the reference cell was filled with 2.0 ml of pure solvent and 4.0 ml of the same salt solution used in the reaction cell. Thermal equilibration was indicated by the absence of a significant base-line drift and was usually reached 1 to 2 h after introduction of the thermostatted solutions. The reaction was initiated by rotation of the calorimeter unit, and the heat of reaction was recorded until thermal equilibrium was reestablished. A second rotation cycle after completion of the reaction gave the heat of friction due to mixing, which was subtracted from the observed heat of reaction. The heat of dilution of the ligand determined separately was negligible compared to the measured heats of reaction. After each experiment the gold cells were cleaned by passage of pure solvent, with rotation of the calorimeter unit, and dried by a gentle stream of N_2. Absolute values for the observed heats of reaction were determined by electrical simulation of the heat effect after each experiment. Observed reaction heats ranged between 0.5 and 5.0 mcal.

Experiments with peptides **1** and **2** were done in methanol/water (1:1, *v/v*) or DMSO, the measurements with peptides **3** and **4** and with valinomycin in absolute ethanol. For the strong valinomycin-K^+ and -Rb^+ complexes the standard reaction enthalpy, ΔH^0, the complex stoichiometry, $\bar{n}$, and the equilibrium constant, K_a, were calculated as described in [12]; in all other cases an iterative graphical procedure of curve fitting was employed to determine ΔH^0 and K_a, assuming a complex stoichiometry of $\bar{n}=1$ for the metal/peptide complexes. The free reaction enthalpy, ΔG^0, and the reaction entropy, ΔS^0, were determined as $\Delta G^0 = -R \cdot T \cdot \ln k_a$, and $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$, respectively.

Results. – *Structural information from spectroscopic studies.* IR. spectra of the bicyclic peptide **3** show a relative shift of the *sec.* amide bands upon cation complexation from the position of free amide (1705 cm^{-1}) towards the associated state (1655 cm^{-1}). In parallel, a shift of the N,H-stretch bands from the associated (3420 cm^{-1}) towards the free state (3275 cm^{-1}) is observed. These results conform well with the notion of a disruption of one or both of the ring hydrogen bonds [9] [11] upon metal complexation which probably involves more than two carbonyl groups per peptide ring.

^{1}H-NMR. spectra are virtually identical in the range of the amide protons for the bicyclic structure **3** and its component cyclopeptides, giving no indication of any interaction between the two ring structures in the uncomplexed state. Some changes are, on the other hand, observed upon metal complexation by **3**. A detailed conformational analysis of the cyclic and bicyclic peptides by NMR. spectroscopy is contained in [9] [11].

UV. spectra of **3** in its free and complexed state show no sign of the disulfide absorption band expected between 220 and 300 nm, the exact location of which is indicative for the dihedral angle of the disulfide bond [16]. The corresponding *Cotton* effect in the CD. spectrum [17] is, however, well discernable. As shown in *Figure 1*, the free peptide displays a main positive band at 232 nm with a molar ellipticity of $\theta = +9900$ and a second maximum at 292 nm ($\theta = +1800$), the latter representing the long-wavelength disulfide transition, the former probably the superposition of the short-wavelength disulfide transition and the perturbed peptide (n,π^*)-transition [18]. If the minimum at 265 nm represents a superimposed negative *Cotton* effect, it cannot be unequivocally assessed from the spectrum. The spectrum of the Na^+-complex of **3** shows a comparatively large decrease in the rotational strength for both bands, red-shifted for the short-wavelength transition (247 nm; $\theta = +3700$), unchanged for the long-wavelength band (292 nm; $\theta = +330$). Independent of the state of complexation, rotational strengths found for **3** are all much lower than reported for comparable cyclic peptide systems containing a single, rigid disulfide bridge, like the decapeptide [2,7-cystine]-gramicidin S

($\theta = -7730$ for the long-wavelength band [19]), corresponding much more closely to values associated generally with open chain disulfide bonds.

Thermodynamic analysis of cation complexation. Enthalpy changes, ΔH^0, obtained by microcalorimetry upon titration of valinomycin with Na^+, K^+, and Rb^+ are shown in *Figure 2.* As reported before [20a, b], high selectivity of the ligand for K^+ and Rb^+ over Na^+ is observed, with clear indication for complex stoichiometries

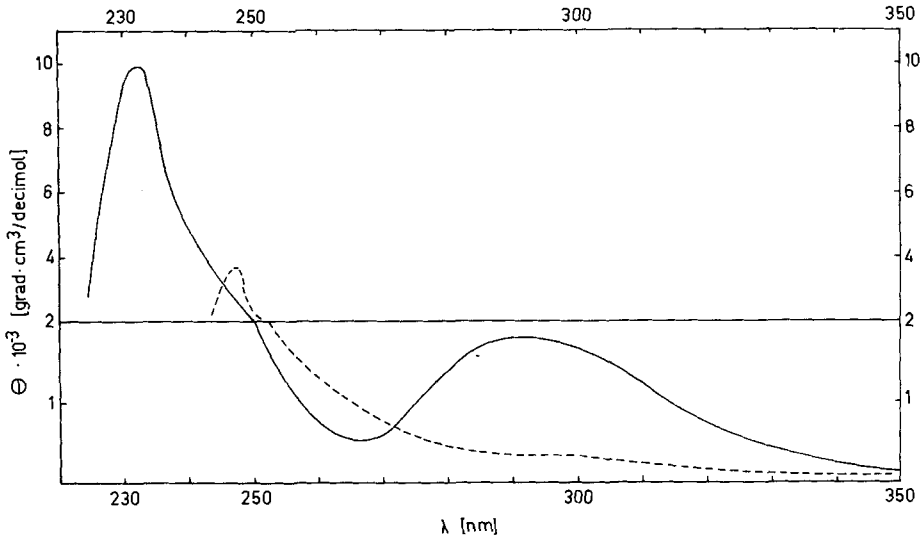

Fig. 1. *CD. spectra of peptide* **3** *in its uncomplexed* (———) *and* Na^+*-complexed* (– – – –) *state* (counterion: ClO_4^-)

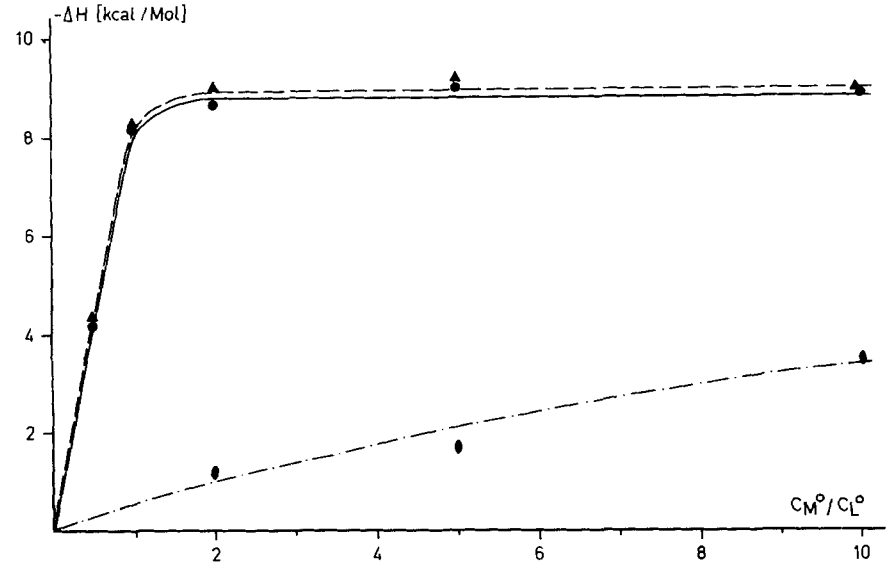

Fig. 2. *Enthalpy titration of valinomycin with* Na^+ (◖), K^+ (●), *and* Rb^+ (▲)

Table 1. *Thermodynamic parameters for alkali cation complexation in ethanol by peptides* **3** *and* **4** *and by valinomycin* (all parameters calculated assuming complex stoichiometries $\bar{n}=1$)

Ligand	Cation	K_a [l/mol]	ΔG^0 [kcal/mol]	ΔH^0 [kcal/mol]	ΔS^0 [e.u.]
Peptide **3**	Na^+	$5.5 \cdot 10^3$	−4.9	−2.9	+6.7
	K^+	$1.3 \cdot 10^4$	−5.4	−3.8	+5.4
	Rb^+	$6.4 \cdot 10^4$	−6.3	−3.7	+8.9
Peptide **4**[a])	Na^+	$9.0 \cdot 10^1$	−2.6	−2.1	+1.7
	K^+	$2.0 \cdot 10^2$	−3.0	−2.0	+3.4
	Rb^+	$3.0 \cdot 10^2$	−3.2	−2.1	+3.7
Valinomycin	K^+	$2.5 \cdot 10^6$	−8.4	−8.9	−1.7
	Rb^+	$2.2 \cdot 10^6$	−8.3	−9.0	−2.3

[a]) Parameters for peptide **4** are only rough estimates *(cf. Fig. 4)*.

of $\bar{n}=1$ for both the K^+- and Rb^+-complex. Thermodynamic parameters calculated from the titration curves are listed in *Table 1*. The equilibrium constant, K_a, obtained for the K^+-complex is in good agreement with published values [20a, b]. Na^+-complexation is too weak to lend itself to the calculation of reaction parameters from the microcalorimetry results.

Analogous enthalpy titration curves for the bicyclic peptides **3** and **4** are shown in *Figures 3* and *4*, respectively. For solubility reasons, saturation could be reached only with peptide **3**. Thermodynamic parameters, obtained by curve fitting, assuming complex stoichiometries of $\bar{n}=1$, are listed in *Table 1;* in the case of peptide **4** they represent only rough approximations.

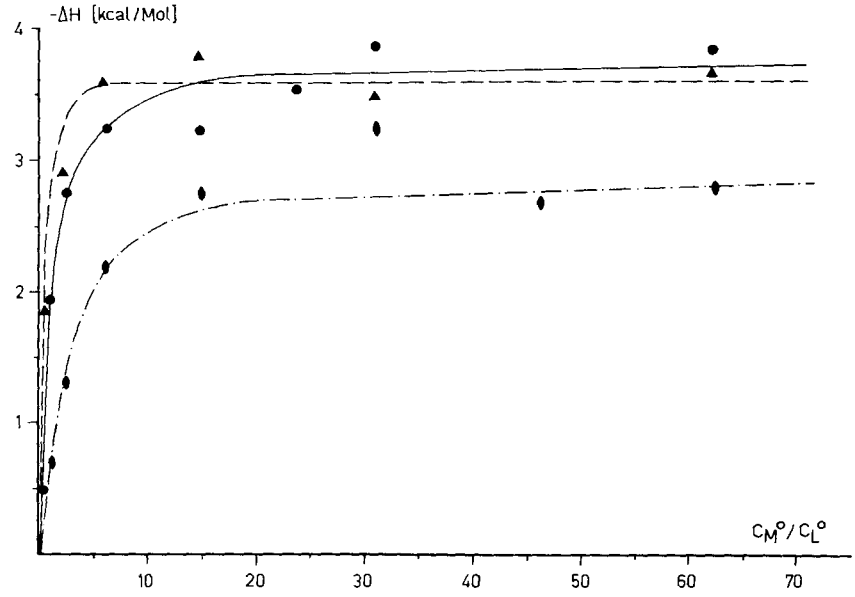

Fig. 3. *Enthalpy titration of peptide* **3** *with* Na^+ (◖), K^+ (●), *and* Rb^+ (▲)

The comparative values listed in *Table 1* show clearly that both synthetic peptides **3** and **4** exhibit far lower selectivity among alkali metal cations than the natural compound valinomycin. This finding conforms well with the higher flexibility of the bicyclic structure which tends to render the peptide far more adaptable to different cation radii. A restricted preference which parallels increasing ion radius can, however, still be observed for both peptides **3** and **4**. The absolute affinities displayed by valinomycin for K^+ and Rb^+ were attained with neither of the two synthetic structures, peptide **3** showing affinities up to two orders of magnitude lower than the antibiotic. The simple exchange of the L-prolyl residue in the pentapeptide ring of **3** by D-prolyl causes a further drop in the cation affinities of **4** by another two orders of magnitude *(Table 1)*.

Contrary to the case of valinomycin, where complex formation causes a net decrease in the entropy of the system, both peptides **3** and **4** show positive entropy changes accompanying cation complexation *(Table 1)*. This finding again points to the important differences in the structural concepts associated with the two molecular systems. In the case of the flexible synthetic peptides, the gain in structural order in the ligand system upon cation complexation is evidently not sufficiently high to compensate for the entropy increase due to the concomitant liberation of water from the hydration sphere of the cation. Furthermore, the fact that reaction entropies, ΔS^0, do not decrease monotonically with increasing ion radius is indicative of conformational differences between the different cation complexes.

The single cyclopeptide components of **3** and **4** show no significant enthalpy changes upon metal titration, either with a diphenylmethyl group protecting the cysteyl side chain or after catalytic hydration of the L-cysteyl residue to L-alanyl

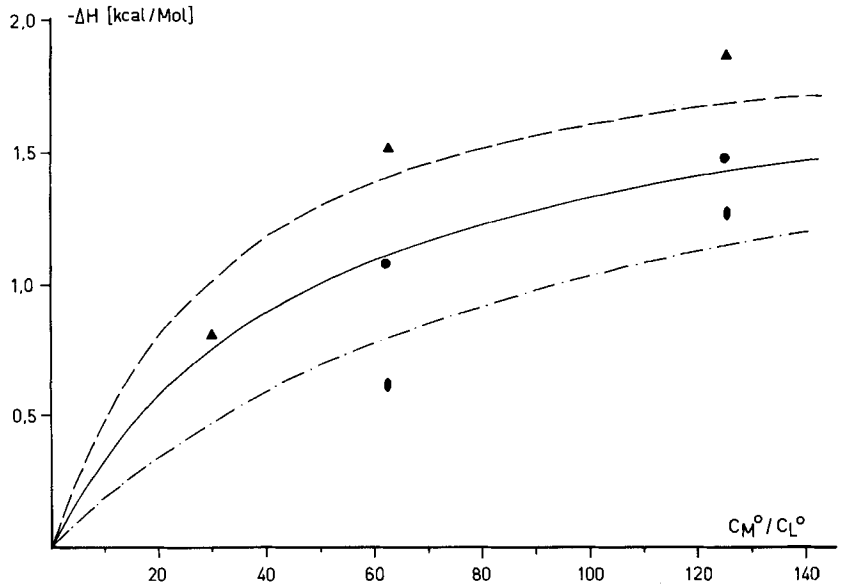

Fig. 4. *Enthalpy titration of peptide* **4** *with* Na^+ (⧫), K^+ (●), *and* Rb^+ (▲)

[10]. This demonstrates that one pentacycle alone is not suitable for cation complexation, and that a link between two rings is a prerequisite for the formation of a stable sandwich-like complex. The bicyclic structures **1** and **2**, as well as their cyclopeptide components, display no measurable enthalpy changes upon metal titration. This is likely to be due to their insufficient cation affinities as observed for peptide **1** in preliminary experiments using different techniques [4].

Kinetic analysis of cation complexation. The kinetics of K^+-complexation by the bicyclic peptide **3** is slow enough to allow analysis using the time-dependence of the calorimeter response. From the time constant of the reaction

$$K^+ + P \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \{K, P\}^+$$

under pseudo first-order conditions ($[P_o] \ll [K_o^+]$), and the thermodynamic equilibrium constant $K_a = k_+/k_-$ determined separately, the two rate constants k_+ and k_- could be determined. A detailed description of this kinetic analysis based on batch microcalorimetry data is given in a separate communication [21].

Values obtained for peptide **3** are listed in *Table 2*, together with the results reported by *Grell et al.* [22] for the K^+-valinomycin complex in methanol. Despite the different solvents used in the two cases, it is evident that both complexation and decomplexation rate constants are much lower for the synthetic peptide. This slow cation complexation may well represent a limiting factor for the transport properties of these bicyclic peptides.

Cation transport properties of peptide **3**. The capability of peptide **3** to transport Na^+, K^+, and Rb^+ across artificial lipid bilayer membranes [14] [15] was studied by conductivity and diffusion potential experiments. In contrast to control experiments with valinomycin no significant increase in membrane permeability for up to 0.2 M Na^+, K^+, or Rb^+ was observed in the presence of 0.1 to 10 mM aqueous solutions of peptide **3**. Despite its definite ion complexing capabilities this molecule thus seems to lack the ability to transport alkali cations across lipid barriers.

In experiments with peptide added to one side of the membrane only, small potentials of sign opposite to that expected for carrier transport were sometimes detected, possibly indicating a certain adsorption of the peptide-cation complex to the lipid-water interface. Capacitance relaxation studies [23] displayed a significant increase in the two shortest relaxation times in the presence of the peptide. This indicates a restriction in the mobility of the membrane dipoles, which conforms well with the notion of an adsorption of the peptide complex to the membrane/water interface.

A further possible cause of the lack of transport capability despite cation complexation was found in the unfavorable decane/water partition coefficient of

Table 2. *Kinetic parameters for K^+-complexation by peptide* **3** *and valinomycin*

Ligand	k_+ [$M^{-1} \cdot s^{-1}$]	k_- [s^{-1}]
Peptide **3** (in ethanol)	$(2.0 \pm 0.5) \cdot 10^1$	$(1.5 \pm 0.4) \cdot 10^{-3}$
Valinomycin (in methanol; [22])	$3.5 \cdot 10^7$	$1.2 \cdot 10^3$

around 1/1000 found for peptide **3**, which may well prevent the complex from entering the lipid phase.

Discussion. – The structural analysis of cation complexation by peptide **3** with IR. and ^{1}H-NMR. spectroscopy demonstrates the presence of different conformational arrangements of the bicyclic system in its free and complexed state. Moreover, the low rotational strengths observed in the CD. spectra clearly show that even in the complexed state the peptide does not exist in a single conformational arrangement around the disulfide bridge, but rather in an ensemble of conformations with opposite helicities of the disulfide bond. The weak positive shoulder of the long-wavelength *Cotton* effect found for the K^+-complex of peptide **3** *(Fig. 1)* could indicate an ensemble of conformations with a predominant dihedral angle of the disulfide bond of 50–55° with positive helicity or, alternatively, an angle of 125–130° with left-handed helical arrangement. The former possibility is represented in *Figure 5* with ring conformations deduced from NMR. data [9] [11]. Similar 'sandwich' complexes have been described for (2:1) ligand/cation complexes of certain macrocyclic polyethers where the cation is too large to fit into the ligand ring structure [24], as well as for enniatin B at high concentrations of K^+ [20a, b]. The antibiotics X-537A and A23187 also seem to form (2:1) complexes with bivalent cations [25] [26].

The thermodynamic analysis by microcalorimetry shows definite alkali cation complexation in ethanol by the bicyclic peptide **3**. This demonstrates the viability of the approach to build 'ion cages' by joining two small peptide rings to one single unit. The fact that the two unbridged rings do not display any significant ion complexation corroborates the structural prediction that a cyclic pentapeptide is too small and inflexible to sufficiently cover the coordination sphere of an alkali cation. It furthermore lends support to the postulated ionophoretic model for such bicyclic

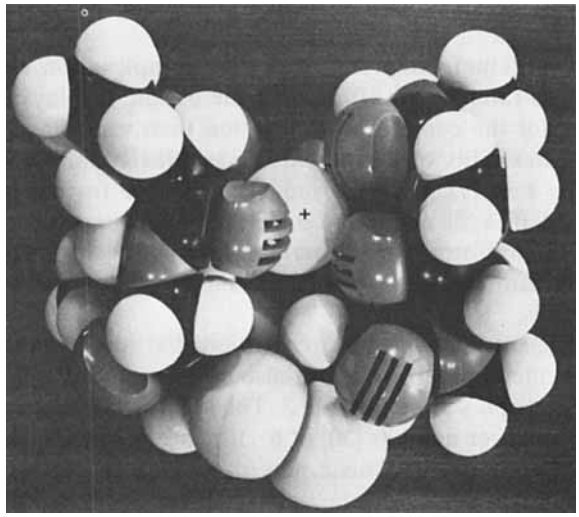

Fig. 5. *Space-filling model of a possible complex structure of peptide* **3**, *representing a cation* (+) *of the size of* Rb^+

systems based on a reversible linkage between two peptide rings [3] [4]. The low cation selectivity found for peptide **3** conforms well with the results of the spectroscopic studies, demonstrating a high flexibility of the bicyclic ligand system even in the complexed state. Furthermore, the two sarcosyl residues unable to form hydrogen bonds at their methylated nitrogen atoms render the single peptide ring rather well adaptable to changes in cation radii. Analogous behaviour has been shown for enniatin B, which contains only methylated amide bonds and displays a highly flexible ring structure of relatively low cation selectivity, as compared to the highly selective valinomycin *(Fig. 2),* which contains six free amide protons [20a] [27].

The rather drastic loss of cation affinity upon change in the configuration of one single amino acid residue of peptide **3** from L-prolyl to D-prolyl to obtain peptide **4** is comparable to analogous and even more pronounced variations found with valinomycin [20a] [27] and demonstrates that even such a structurally flexible 'ion cage' composed of two conformationally quite independent partial structures is strikingly sensitive to minor structural modifications. The positive reaction entropies observed for complex formation by both peptides **3** and **4** – in contrast to valinomycin, the analogous process of which is entirely enthalpy driven – places these peptide structures in a class with certain low molecular weight metal binding proteins like skeletal muscle troponin-C and parvalbumin for which positive values of ΔS^0 have been found for Ca^{2+}- and Mg^{2+}-complexation [28] [29]. The complete loss of cation complexation upon replacement of two sarcosyl residues by glycyl (peptides **1** and **2**) can hardly be explained solely by solubility problems. The main structural difference lies in the introduction of two new amide protons capable of forming hydrogen bonds, what is tantamount to competition for cation complexation. The most crucial structual prerequisite for ionophoretic systems thus appears to be the presence of the exact number of hydrogen bond formers needed to sustain a stable ligand cage structure without overly competing with the cation complexation reaction.

The results of the kinetic analysis of the K^+-complexation reaction show that peptide **3**, despite its rather high affinity for the cation, displays far less favorable kinetic parameters for the complexation reaction than valinomycin. This might be due to insufficient flexibility of the peptide rings. NMR. double resonance experiments with peptide **1** in DMSO show indeed a lifetime for the predominant conformation of about 0.02 s [6], compared to a value of around 10^{-7} s for valinomycin [22]. Since the main conformational energy barrier of each of the peptides **1**–**4** is the *cis/trans* isomerization of the X-pro bond, similar lifetimes may well be assumed in all four compounds.

The goal of efficient ion transport across lipid barriers is hindered not only by these unfavorable kinetic parameters, but also by the equally unfavorable oil/water partition coefficient displayed by peptide **3**. The kinetic parameters given in *Table 2* yield a maximum turnover number [30] of $6 \cdot 10^{-4}\ s^{-1}$ per molecule. Combined with the partition coefficient of the uncomplexed peptide **3**, this gives a maximum expected carrier current density of about 10^{-14} A/cm^2 or about 10^{-16} A for typical bilayer diameters. As the basic noise level is greater than 10^{-12} A, it is not surprising that no ionophoric activity was found.

Improved model compounds might be prepared by rendering their ring structure more flexible through further elimination of hydrogen bonding amide protons and, perhaps, by increasing the ring size. Furthermore, amino acids with more lipophilic side chains should be introduced in order to increase the surface hydrophobicity of the transport species, thereby increasing its solubility in lipid systems.

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