Binding of dipeptides and tripeptides containing lysine or arginine by *p*-sulfonatocalixarenes in water: NMR and microcalorimetric studies †

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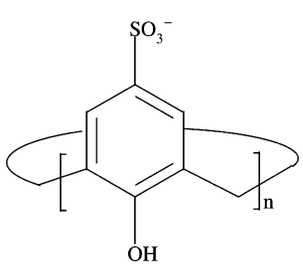
The water soluble *p*-sulfonatocalix[*n*]arenes (*n* = 4, 6 and 8) (**1**$_4$, **1**$_6$ and **1**$_8$) show glycosylaminoglycan (GAG) mimicry, being both active antithrombotics and modulators of lysyloxidase activity. In order to understand how these synthetic receptors interact with GAG receptor sequences, we have undertaken a study to thermodynamically characterize the binding of dipeptides and tripeptides bearing lysine or arginine residues by **1**$_4$, **1**$_6$ and **1**$_8$ in aqueous buffer at pH 8.0 and 298.15 K. The association constants, enthalpies and entropies of complexation have been determined using titration microcalorimetry. The structure of the complexes in solution has been studied by ^{1}H NMR spectroscopy. Combining the microcalorimetric data, the binding shifts and the 2D experiments, the nature of the complexation may be clarified.

Introduction

The interaction of glycosylaminoglycans with polypeptides plays a key regulatory role in many physiological processes.[1] For instance, the sulfated polysaccharide heparin binds serine proteinase inhibitors belonging to the serpin superfamily and increases the rate of inhibition of their target proteinases.[2] The heparin-promoted or glycosylaminoglycan (GAG) acceleration of thrombin inhibition by antithrombin provides the molecular basis for the anticoagulant effect of the sulfated glycosylaminoglycan.[3] Considering the importance of GAG–protein interactions, it would be highly interesting to have easily available synthetic GAG mimics.

The structure, charge density, charge distribution and size of the water-soluble *p*-sulfonatocalix[*n*]arenes (*n* = 4, 6 and 8), **1**$_4$, **1**$_6$ and **1**$_8$, (Scheme 1) make them excellent candidates as GAG mimics. Such GAG mimicry has been demonstrated by various groups and the compounds have been shown to be both active antithrombotics through a non-ATIII dependent mechanism[4] and modulators of lysyloxidase activity.[5] Probably *via* a non-heparinoid type mechanism **1**$_4$, **1**$_6$ and **1**$_8$ act as chloride ion channel blockers.[6] Lehn *et al.*[7] studied the affinity of **1**$_4$ and **1**$_6$ for acetylcholine and other quaternary ammonium cations. Other synthetic receptors based on a calixarene framework also showed affinity for peptides. For instance, a series of calix[4]arene based α-aminophosphonates exhibited remarkable selectivity as carriers for the membrane transport of the zwitterionic form of aromatic amino acids.[8] The affinities of four aminocalixarenes for nucleotides were determined by Shi and Schneider.[9] The synthesis of an antibody mimic based on calix[4]arene linked to four constrained peptide loops, used for recognition of protein surfaces, was also reported.[10] Recently, work on the immune responses to calixarenes has shown that they provoke essentially zero response.[11] Preliminary studies by the inhibition cells growth method show low cellular cytotoxicity for **1**$_4$, and for **1**$_6$ and **1**$_8$, essentially the nonexistent toxicity of glucose.[12]



1$_4$ (n=4), **1**$_6$ (n=6), **1**$_8$ (n=8)

Scheme 1 The *p*-sulfonatocalix[*n*]arenes.

Arginine and lysine play a key role in the peptide sequences present in GAG recognition sites.[13] In view of this, we have previously used ^{1}H NMR spectroscopy to study the possible mechanisms of recognition of these two amino acids by **1**$_4$, **1**$_6$ and **1**$_8$ in water: the formation of 1 : 1 complexes was observed between **1**$_4$ and the amino acids but with the higher calixarenes the effects were too weak to allow structural characterisation.[14] In contrast to the 1 : 1 situation in solution, a lysine–**1**$_4$ complex of 4 : 2 stoichiometry was observed in the solid state.[15]

In order to understand how synthetic receptors interact with peptides, it is necessary to thermodynamically characterize the binding process. Specific binding reflects the balance between favourable and unfavourable enthalpic and entropic components due to the modification of the species upon complexation. Several contributions may be involved due to multiple types of interaction (ionic, hydrophobic, π–π, cation–π,

† Electronic supplementary information (ESI) available: chemical shifts experienced by different protons of **KK**, **RR**, **KKK** or **RRR** in the presence of increasing amounts of **1**$_4$ or **1**$_6$; heat effects observed upon titration of **1**$_4$, **1**$_6$ or **1**$_8$ by **KK**, **RR**, **KKK** or **RRR**; COSY and ROESY 2D ^{1}H NMR spectra for **1**$_4$ complexed with **KK** or **RR**. See http://www.rsc.org/suppdata/p2/b1/b109553f/

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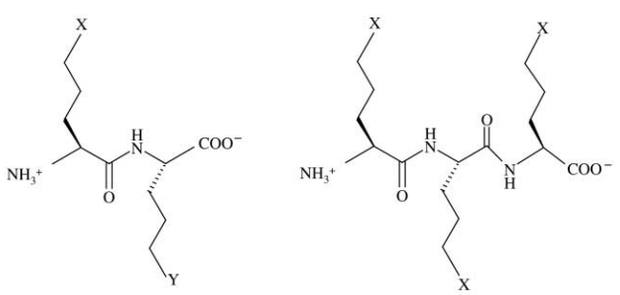
hydrogen bonding, *etc.*), which imply partial desolvation of the host and guest, and some modification of the translational and rotational degrees of freedom of the species. In most cases, it is not possible to evaluate separately the individual contributions but the sign of the major ones is known. A full thermodynamic characterization of the binding process is thus one of the key elements to understand how the units assemble and what are the stabilizing factors.[16]

Microcalorimetry is a powerful tool for measuring the thermodynamic parameters that characterize interacting molecules because it not only measures enthalpy changes but may also yield association constants, even in the case of weak interactions.[16,17] The highly sensitive modern instruments use reasonable amounts of material and are thus convenient tools to investigate the energetics of biomolecular recognition.[16,18] Such tools have been used, for instance, to study heparin–protein equilibria.[19] In continuation of our investigation of the interactions of *p*-sulfonatocalix[*n*]arenes with small peptides, we used microcalorimetry to determine the Gibbs energies, enthalpies and entropies of complexation of the basic amino acids lysine and arginine by $\mathbf{1}_4$, $\mathbf{1}_6$ and $\mathbf{1}_8$ in aqueous solution at pH 1.0 and pH 8.0,[20] thus reporting the first thermodynamic characterization of the binding of amino acids by water soluble calixarenes. A more recent report, which examined only the *K* values for the binding of calix[4]arene sulfonates with different amino acids, neglected to study lysine and arginine.[21]

In order to better identify the factors that control the binding process we have undertaken an extension of this study to include dipeptides and tripeptides bearing arginine and lysine residues. The patterns x**KK**x, x**RR**x, x**KR**x and x**RK**x are commonly found, along with x**K**x and x**R**x, in the known GAG binding peptide sequences in proteins, and tripeptide sequences are present in certain cases.[13] In the present work we study, using both ^{1}H NMR spectroscopy and microcalorimetry, the complexation of lysyl-lysine, **KK**, arginyl-arginine, **RR**, lysyl-arginine, **KR**, arginyl-lysine, **RK**, lysyl-lysyl-lysine, **KKK**, and arginyl-arginyl-arginine, **RRR**, (Scheme 2) by $\mathbf{1}_4$, $\mathbf{1}_6$ and $\mathbf{1}_8$ in



KK : X= Y = CH_2-NH_3^+

RR : X= Y= NHC(=NH_2^+)NH_2

KR : X= CH_2-NH_3^+ ; Y = NHC(=NH_2^+)NH_2

RK : X= NHC(=NH_2^+)NH_2 ; Y = CH_2-NH_3^+

KKK : X = CH_2-NH_3^+

RRR : X = NHC(=NH_2^+)NH_2

(In all cases, the sequence indicates L-amino acids)

Scheme 2 The dipeptides and tripeptides in aqueous solution at pH 8.0.

aqueous solution at pH 8.0. These dipeptides and tripeptides have been chosen because they can be directly and systematically compared with the amino acids, in continuation of our previous work.[20] These species, which are neither *N*-acylated nor *C*-amidated, apparently differ from a peptide sequence in a protein. However, it is interesting to note that the association constant and enthalpy change for the binding of $\mathbf{1}_4$ with *N*-acetyllysine amide[22] are similar to those observed with lysine,[20] suggesting that the thermodynamic properties for the association with polypeptides could be correlated with values obtained for non-protected small peptides. But this is beyond the scope of the present work.

Experimental

Materials

The three *p*-sulfonatocalixarenes were synthesized and purified using the method described by Arena *et al.*[23] For each of them, the final step before recrystallization was performed at pH 6. ^{1}H NMR (D_2O at 20 °C), atomic absorption spectroscopy (Na) and water analysis showed that the products have the following formulas at pH 6: $\mathbf{1}_4$, $C_{28}H_{19}O_{16}S_4Na_5$, 12% H_2O; $\mathbf{1}_6$, $C_{42}H_{28}O_{24}S_6Na_8$, 23 % H_2O; $\mathbf{1}_8$, $C_{56}H_{36}O_{32}S_8Na_{10}$, 20% H_2O. The dipeptides and tripeptides **KK**, **RR**, **RK**, **KR**, **RRR** and **KKK** (Bachem) were used as purchased; in all cases, the sequence indicates L-amino acids. Sodium dihydrogen phosphate dihydrate (Fluka, *pro analysi*) and disodium hydrogen phosphate anhydrous (Merck, BioChemika) were used without further purification. All the solutions were prepared by weight from triply distilled water. The pH of the calixarene and peptide solutions was set at 8.0 with a phosphate buffer prepared by mixing 96.6 mL of a 0.010 mol kg^{-1} Na_2HPO_4 solution and 3.4 mL of a 0.010 mol kg^{-1} NaH_2PO_4 solution. The pH was verified on a pH-meter (U-ISIS 20.000–1 SOLEA-Tacussel) calibrated with two different buffer solutions. The phosphate buffer was chosen because it has no apparent influence on complexation and can be used on a relatively large scale of pH (5 to 8) whereas other typical biological buffers we tested previously[24] interact with the calixarene sulfonates. The pK_a values[23,25] reported in the literature for the *p*-sulfonatocalix[*n*]arenes indicate that all the sulfonic acid groups and some of the hydroxy groups (one for the tetramer, two for the hexamer and two or three for the octamer) are dissociated at pH 8, yielding the following anions: (calix[4]arene sulfonate)$^{5-}$, (calix[6]arene sulfonate)$^{8-}$ and a mixture of (calix[8]arene sulfonate)$^{10-}$ and (calix[8]arene sulfonate)$^{11-}$.

Microcalorimetry

All measurements were performed at 298.15 K using a multichannel microcalorimeter (LKB-Thermometric 2277 Thermal Activity Monitor) equipped with a titration–perfusion vessel. Wadsö and co-workers have thoroughly described this twin thermopile heat-conduction calorimeter and analyzed its performance.[26] The 1 mL stainless steel titration vessel was charged with 0.8 mL of calixarene buffered solution and 14 μL of peptide buffered solution was injected in each step using a Lund syringe pump (Thermometric) equipped with a 250 μL Hamilton syringe fitted with a stainless steel cannula. 15 injections were made for each titration experiment. The solution molalities were, prior to titration, in the range 0.005–0.01 mol kg^{-1} for calixarenes and 0.05–0.3 mol. kg^{-1} for peptides. Static and dynamic calibrations were used; the power values observed upon titration ranged from 30 to 300 μW. Separate dilution experiments were performed. Since the heats of dilution of calixarenes were found to be negligible, the heat effects observed upon titration were simply corrected for the heats of dilution of the peptides. Each experiment was repeated three times to verify reproducibility. Values for the apparent association constant K' and apparent standard enthalpy of reaction $\Delta_r H'^{\ominus}$ in the buffer were calculated by use of the Digitam 4.1 minimization program (Thermometric), the three series of data obtained for each system being treated simultaneously in the regression analysis.

NMR Spectroscopy

The ^{1}H NMR spectra of the *p*-sulfonatocalixarene–peptide solutions in 90% H_2O–10% D_2O buffered at pH 8.0 were collected at 298.15 K on a Varian Unity 500 MHz spectrometer.

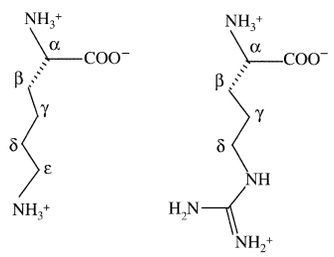
The chemical shifts were referenced to deuterated TSP [2,2,3,3-d_4-3-(trimethylsilyl)propionic acid sodium salt]. The solution molalities were, prior to titration, in the range 0.01–0.05 mol kg^{-1} for calixarenes and 0.002–0.005 mol kg^{-1} for peptides. The titration was carried out by introducing 700 μL of peptide buffered solution into the NMR tube and by adding increasing amounts of calixarene buffered solution. An NMR spectrum was recorded after each injection. 2D COSY and ROESY experiments were carried out at a ratio of [$\mathbf{1}_4$] to [peptide] sufficiently high as to ensure full complexation ([$\mathbf{1}_4$]/[peptide] = 2).

Results and discussion

The spectroscopic and microcalorimetric data that are not shown in the Tables and in the Figures can be found in the electronic supplementary information.

Spectroscopic results for complexes with $\mathbf{1}_4$

The assignment of the protons of the lysine and arginine residues (identified by lower case Greek letters as shown in Scheme 3) was made from the tabulated ^{1}H chemical shifts



K : L-lysine **R** : L-arginine

Scheme 3 Identification of the protons of the lysine and arginine residues.

for twenty common amino acid residues in 'random coil' polypeptides.[27] The ^{1}H NMR spectra show that the proton signals of both lysine and arginine residues are shifted upfield upon complexation with the *p*-sulfonatocalix[4]arene. The values of the apparent association constants and limiting chemical shifts deduced from the fit of the data using a 1 : 1 binding model are reported in Table 1.

The values for the amino acids **K** and **R** have been redetermined in the present study. As expected, lysine and arginine are more strongly complexed than the other amino acids (the association constants reported by Arena *et al.*[21] for valine, leucine, phenylalanine, histidine and tryptophan are between 16 and 63). Arginine differs from lysine simply by the replacement of a CH_2–NH_3^+ group by a guanidinium group but it exhibits a K' value that is twice as large.

Fig. 1 shows the upfield shifts experienced by the lysine ε protons of **KK** in the presence of increasing amounts of $\mathbf{1}_4$.

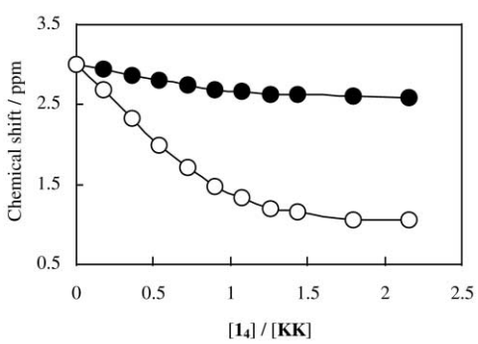


Fig. 1 Chemical shifts experienced by the lysine ε_1 (○) and ε_2 (●) protons of **KK** in the presence of increasing amounts of $\mathbf{1}_4$.

Table 1 Apparent stability constants K' and limiting ^{1}H NMR upfield shifts $\Delta\delta_{lim}$ calculated for the complexes formed between the *p*-sulfonatocalix[4]arene and some amino acids, dipeptides and tripeptides in water at pH 8 (phosphate buffer) and 298.15 K[a]

	$10^{-3}K'$ [b]	$\Delta\delta_{lim}$ [c]
Lysine-containing peptides		
$\mathbf{1}_4$-**K** [d]	0.8 ± 0.1	2.40 ± 0.09
$\mathbf{1}_4$-**KK**	3.4 ± 0.4	2.06 ± 0.02
$\mathbf{1}_4$-**KKK**	30 ± 7	1.43 ± 0.02
Arginine-containing peptides		
$\mathbf{1}_4$-**R** [d]	1.5 ± 0.6	2.7 ± 0.1
$\mathbf{1}_4$-**RR**	7 ± 3	2.06 ± 0.05
$\mathbf{1}_4$-**RRR**	33 ± 6	1.51 ± 0.01
Mixed dipeptides		
$\mathbf{1}_4$-**KR**	3.7 ± 0.5	1.76 ± 0.02
$\mathbf{1}_4$-**RK**	3.9 ± 0.7	2.19 ± 0.03

[a] K' and $\Delta\delta_{lim}$ deduced from the non-linear least-squares fit of the data using a 1 : 1 binding model. [b] Molar scale. [c] Shifts of the lysine ε protons for **K**, **KK**, **KR**, **RK**, **KKK** and shifts of the arginine δ protons for **R**, **RR**, **RRR**, given in ppm at 500 MHz. [d] The NMR spectra of the amino acids have been redetermined in the present study.

The peaks of both lysine residues, which are chemically equivalent in the absence of ligands, are differentially displaced in the presence of $\mathbf{1}_4$. To simplify, the residues are designated as $\mathbf{K}_1$ and $\mathbf{K}_2$, the lower script 1 being used for the residue that shows the most important shifts. The ε protons of residue $\mathbf{K}_1$ (ε_1) are strongly perturbed ($\Delta\delta$ = 1.95) whereas those of $\mathbf{K}_2$ (ε_2) are much less affected ($\Delta\delta$ = 0.4). The δ protons show similar behaviour whereas the γ protons display much less important effects. Although the β protons are not significantly perturbed, the α_1 proton experiences a tiny upfield shift effects. If upon complexation either $\mathbf{K}_1$ or $\mathbf{K}_2$ is included in the ligand cavity with the same probability then one would expect the peaks of both lysine residues to be shifted in a similar fashion. The data in Fig. 1 indicate that $\mathbf{K}_1$ is preferentially included within the cavity of $\mathbf{1}_4$ whereas $\mathbf{K}_2$ stays mainly outside. The 2D COSY experiments allow assignment of side chain 1 as the N-terminal and side chain 2 as the C-terminal. Similar spectra have been obtained with **RR** and, again, COSY has allowed assignment of side chain 1 as the N-terminal and side chain 2 as the C terminal.

It can be seen that the signals for $\mathbf{R}_1$ and $\mathbf{K}_1$ are less perturbed than those observed upon complexation of the amino acids themselves: the difference between the $\Delta\delta_{lim}$ of **RR** and that of **R** is equal to −0.64 and the difference between the $\Delta\delta_{lim}$ of **KK** and that of **K** is equal to −0.34. Two hypotheses can be advanced: firstly that $\mathbf{K}_1$ is not as deeply embedded in the cavity and secondly that the environment is modified by the presence and alignment of the second side chain. This probably indicates that the second side chain of the dipeptide, because it keeps part of its freedom, pulls on the first side chain, which, as a result, is less deeply embedded than the corresponding amino acid. The solid state complex of $\mathbf{1}_4$ with lysine shows that a particular conformation of the hydrophobic lysine side chain exists with a folded conformation inserted into the cavity.[15] The 2D ROESY experiments for **KK** complexed to $\mathbf{1}_4$ in water show through space correlations between CONH and α_1, β_1, β_2 and γ_2 which are consistent with a compact folded conformation. The **RR** chain is not capable of forming a folded structure, as shown by the lack of through space correlation in the 2D ROESY experiments.

For the two mixed dipeptides **KR** and **RK**, the ε protons of the **K** residue are more strongly shifted than the δ protons of the **R** residue (Fig. 2). In the case of the mixed dipeptide **KR**, the N-terminal **K** residue bears two favourable charged sites (the NH_3^+ group in position ε and the NH_3^+ in position α)

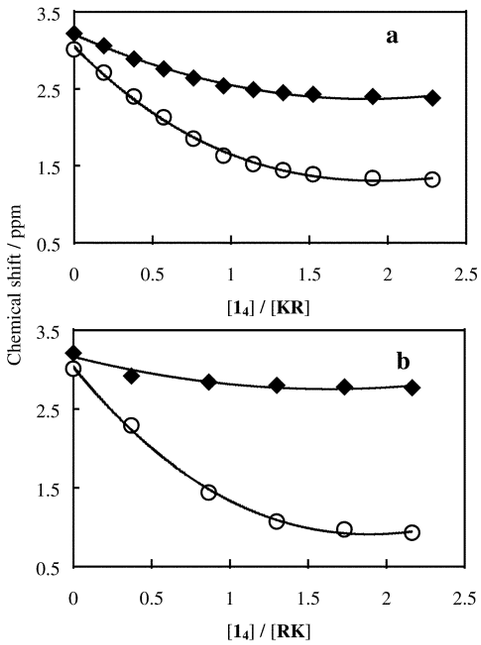


Fig. 2 Chemical shifts experienced by the lysine ε protons (○) and the arginine δ protons (◆) of **KR** (a) and of **RK** (b) in the presence of increasing amounts of $\mathbf{1}_4$.

whereas the C-terminal **R** residue bears one favourable charged site (the guanidinium group) and one unfavourable charged site (the COO^- group). For the mixed dipeptide **RK** the situation is reversed. The $\Delta\delta_{lim}$ values show that the ε protons of the **K** residue of **RK** are more strongly perturbed than those of **KR**. The association constants of **RK** and **KR** are, within the uncertainty limits, equal to that observed for **KK** but only half that observed for **RR**. It can thus be stated that for the lysine-containing dipeptides, it is the lysine residue that is preferentially complexed in the ligand cavity. For **KK**, this is the N-terminal, and also for **KR**, however, for **RK** it becomes the C-terminal and hence apparently the less electrostatically favourable residue. CPK models show that in the three cases, the amide NH is in a position favourable to bonding with the sulfonate groups.

Fig. 3 shows the upfield shifts experienced by the lysine ε

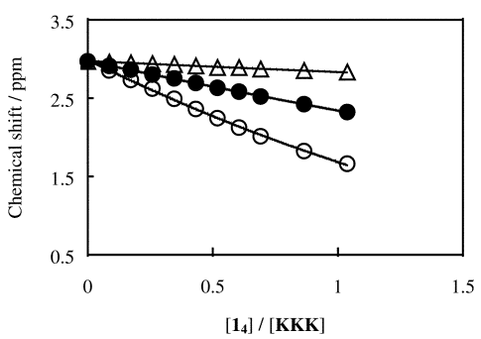


Fig. 3 Chemical shifts experienced by the lysine ε_1 (○), ε_2 (●) and ε_3 (△) protons of **KKK** in the presence of increasing amounts of $\mathbf{1}_4$.

protons of **KKK** in the presence of increasing amounts of $\mathbf{1}_4$: the ε protons of $\mathbf{K}_1$ (ε_1) are strongly perturbed, those of $\mathbf{K}_2$ (ε_2) are moderately affected whereas those of $\mathbf{K}_3$ (ε_3) are almost unchanged. The δ protons show shifts that are very similar to those experienced by the ε protons whereas the γ protons are much less affected. Similar spectra have been obtained with **RRR**. These data indicate that while one of the chains of **KKK** or **RRR** is strongly bound, the second chain experiences a moderate variation in its environment whereas the third one is

Table 2 Thermodynamic parameters characterizing the complexation of some amino acids, dipeptides and tripeptides by the *p*-sulfonatocalix[4]arene in water at pH 8 (phosphate buffer) and 298.15 K [a,b,c]

	$10^{-3}K'$	$\Delta_rG'^{\circ}$	$\Delta_rH'^{\circ}$	$T\Delta_rS'^{\circ}$
Lysine-containing peptides				
$\mathbf{1}_4$-**K** [d]	0.74 ± 0.01	−16.4 ± 0.1	−14.4 ± 0.1	2.0 ± 0.2
$\mathbf{1}_4$-**KK**	3.8 ± 0.3	−20.4 ± 0.2	−29.0 ± 0.3	−8.6 ± 0.5
$\mathbf{1}_4$-**KKK**	30 ± 4	−25.5 ± 0.4	−27.3 ± 0.3	−1.8 ± 0.7
Arginine-containing peptides				
$\mathbf{1}_4$-**R** [d]	1.52 ± 0.09	−18.2 ± 0.1	−20.3 ± 0.3	−2.1 ± 0.4
$\mathbf{1}_4$-**RR**	7.7 ± 0.6	−22.2 ± 0.2	−26.1 ± 0.2	−3.9 ± 0.4
$\mathbf{1}_4$-**RRR**	35 ± 9	−25.9 ± 0.6	−31.2 ± 0.5	−5.3 ± 1.1
Mixed dipeptides				
$\mathbf{1}_4$-**KR**	3.7 ± 0.1	−20.4 ± 0.1	−29.4 ± 0.1	−9.0 ± 0.2
$\mathbf{1}_4$-**RK**	4.3 ± 0.2	−20.8 ± 0.1	−31.0 ± 0.2	−9.7 ± 0.3

[a] K' and $\Delta_rH'^{\circ}$ deduced from the non-linear least-squares fit of the data using a 1 : 1 binding model. [b] Molar scale. [c] $\Delta_rG'^{\circ}$, $\Delta_rH'^{\circ}$ and $T\Delta_rS'^{\circ}$ in kJ mol^{-1}. [d] Ref. 20.

in an environment essentially unperturbed by $\mathbf{1}_4$. N-Terminal or central residue binding is consistent with the data. As was observed for the complexation of the amino acids,[14] the changes in the chemical shifts are greater for the arginine-containing species than for the lysine-containing species. It can be noticed that the $\Delta\delta_{lim}$ values vary almost linearly in the order **R** > **RR** > **RRR** and **K** > **KK** > **KKK**. By analogy with the dipeptides, we may assign $\mathbf{R}_1$ or $\mathbf{K}_1$ to the N-terminal residue. We may also consider that the COO^-–SO_3^- repulsion is reduced to its minimum when the C-terminal chain keeps its freedom, that is, if it corresponds to $\mathbf{R}_3$ or $\mathbf{K}_3$.

Microcalorimetric results for complexes with $\mathbf{1}_4$

Fig. 4 shows the results of the microcalorimetric titrations of $\mathbf{1}_4$

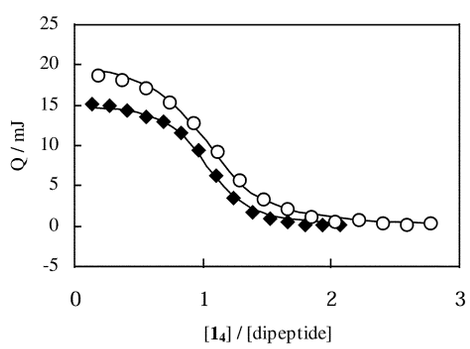


Fig. 4 Heat effects observed upon titration of $\mathbf{1}_4$ by **KK** (○) and by **RR** (◆).

with **RR** and **KK** in aqueous solution at pH 8.0. Similar plots were obtained with **RRR** and **KKK**. The fit of the data with a 1 : 1 binding model yields the thermodynamic properties listed in Table 2. For comparison, the values previously obtained[20] for the complexation of the amino acids **R** and **K** are also given. Within the uncertainty limits, the apparent association constants obtained by microcalorimetry appear to be in good agreement with those deduced from NMR spectroscopy (Table 1). It should be underlined, however, that microcalorimetry is particularly well adapted to the study of weak complexes, the uncertainty in K' for such species being smaller than the uncertainty associated with the NMR determinations.

Our previous study of the complexation of **R** and **K** at pH 8 and pH 1 has shown[20] that the binding of these amino acids by

$\mathbf{1_4}$ is controlled by the enthalpy: at both pH values, the enthalpy is more favourable for the binding of arginine than lysine whereas it is the opposite for the entropy. The recent determination[28] of the enthalpies and entropies for phosphate complexation by ammonium and guanidinium groups also showed more favourable enthalpies and less favourable entropies for the guanidinium hosts. Our results[20] are consistent with the possible existence of π–π interactions between the guanidinium group of arginine and the phenyl units of the calixarene. For both **R** and **K**, the enthalpy of binding becomes more favourable at pH 1 whereas the entropy of binding becomes more unfavourable. When the medium becomes acidic, the carboxy group of the amino acid is no longer ionized and the repulsion that existed with the sulfonate groups then disappears. As a result, the nonpolar chain of the amino acid can penetrate more deeply into the host, which results in a favourable enthalpic contribution and an unfavourable entropic contribution. This is consistent with what was observed by NMR and with the X-ray structure of the lysine complex.[15]

Although it is not possible to estimate what is the exact part of each contribution (ionic, hydrophobic, van der Waals, *etc.*) to the binding of $\mathbf{1_4}$ with **R** or **K**, one can try to identify the major effects by looking at analogous but simpler species. Thus, let us compare the binding of *n*-propylammonium ion and *n*-propanol by $\mathbf{1_4}$ in water at 298.15 K. It has been shown by using microcalorimetry that the binding of C_3H_7–NH_3^+ yields[29] $\Delta_r G'^\circ = -23.5$ kJ mol^{-1}, $\Delta_r H'^\circ = -16.9$ kJ mol^{-1} and $T\Delta_r S'^\circ = 6.6$ kJ mol^{-1} and that the binding of C_3H_7–OH yields[30] $\Delta_r G'^\circ = -7.6$ kJ mol^{-1}, $\Delta_r H'^\circ = -16.6$ kJ mol^{-1} and $T\Delta_r S'^\circ = -9.0$ kJ mol^{-1}. A comparable Gibbs energy change was reported for *n*-propanol from ^{1}H NMR data.[31] The thermodynamic behaviour of *n*-propanol ($\Delta_r H'^\circ \ll 0$ and $T\Delta_r S'^\circ < 0$) is typical[32] of what is observed upon tight binding of an apolar solute into the hydrophobic cavity of a ligand through van der Waals interactions, the structural changes giving rise to negative enthalpic and entropic contributions that largely outweigh the positive contributions due to the desolvation of the species. As underlined by Diederich and co-workers,[32] who thoroughly examined this type of binding, this thermodynamic behaviour is in sharp contrast with what is observed for processes driven by the classical hydrophobic interaction ($\Delta_r H'^\circ \approx 0$ and $T\Delta_r S'^\circ > 0$). The thermodynamic behaviour of *n*-propylammonium ion ($\Delta_r H'^\circ \ll 0$ and $T\Delta_r S'^\circ > 0$) shows that the addition of an amino group on the propyl chain does not significantly modify the enthalpy of binding but gives a positive contribution to the entropy of binding. It is known that purely ionic bindings are endothermic and strongly entropy-driven,[33] due essentially to the positive enthalpy and entropy changes resulting from the partial desolvation of the charged species upon interaction. This suggests that the binding of $\mathbf{1_4}$ with C_3H_7–NH_3^+, which is exothermic and strongly enthalpy-driven, is controlled by the favourable enthalpic term resulting from the tight inclusion of the hydrocarbon chain into the ligand cavity. It is, however, the desolvation of the NH_3^+ and SO_3^- groups upon their ionic interaction which governs the entropy of binding and which explains why the affinity of $\mathbf{1_4}$ for *n*-propylammonium ($K' = 13100$) is higher than that for *n*-propanol ($K' = 22$).

The binding of the amino acids can be interpreted in the same way: lysine shows indeed thermodynamic behaviour ($\Delta_r H'^\circ = -14.4$ kJ mol^{-1} and $T\Delta_r S'^\circ = 2.0$ kJ mol^{-1}) quite similar to that of C_3H_7–NH_3^+ ($\Delta_r H'^\circ = -16.9$ kJ mol^{-1} and $T\Delta_r S'^\circ = 6.6$ kJ mol^{-1}); arginine shows additional negative enthalpic and entropic contributions ($\Delta_r H'^\circ = -20.3$ kJ mol^{-1} and $T\Delta_r S'^\circ = -2.1$ kJ mol^{-1}) possibly because of π–π interactions. One expects the situation to be more complicated with the dipeptides and the tripeptides principally because part of the guest remains in the solvent upon binding and thus keeps some mobility. Moreover, polypeptides bear NH groups that can be involved in hydrogen bonding. The situation is also complicated by the fact that the α-amino function of the polypeptide is probably not fully protonated at pH 8. There could then be, upon binding, transprotonation from the phosphate buffer to the α-amino group of the peptide; fortunately, if this happens, it cannot significantly perturb our heat effects because the enthalpy for the acid dissociation (second ionisation) of phosphate is endothermic and very small.[34]

The values reported in Table 2 indicate that, upon binding with $\mathbf{1_4}$, the thermodynamic behaviours of **KK**, **KR** and **RK** are similar. The NMR data have shown that, in the three cases, it is the lysine residue which inserts into the cavity and that it adopts a compact conformation. CPK models show that this constricted structure puts the amide NH in a position favourable for hydrogen bonding with an SO_3^- group of the host. The fact that the thermodynamic behaviour of **RK** is similar to that of **KR** and **KK** seems to indicate that this hydrogen bond plays, for the three guests, a more important role than the interaction with the α-NH_3^+ group. This conclusion is strongly supported by the values reported in Table 2 which show that the substitution of **KK** (or **KR**, or **RK**) for **K** yields a negative enthalpic contribution of −15 kJ mol^{-1} and a negative entropic contribution of −10 kJ mol^{-1}, which is consistent with the formation of a hydrogen bond. The complexation of **RR** yields an entropy loss much smaller than the complexation of the lysine-containing dipeptides, in agreement with the NMR data which do not support a folded structure for the arginine residue.

The values reported in Table 2 show that the enthalpy change is more favourable for **RRR** than for **KKK** but this is partly compensated by an entropy change that is more unfavourable for **RRR** than for **KKK**: as a result, the affinity of $\mathbf{1_4}$ for **RRR** is only slightly more important than for **KKK**. $\Delta_r H'^\circ$ and $T\Delta_r S'^\circ$ vary almost linearly within the series **R** ⟶ **RR** ⟶ **RRR**, which is consistent with the fact that the dipeptide can form one hydrogen bond whereas the tripeptide can form, as shown by CPK models, two hydrogen bonds through the amide NH. Replacing **K** by **KKK** shows trends that are similar but less pronounced than those observed when replacing **K** by **KK**. Obviously, the addition of a third chain, which keeps most of its degrees of freedom, perturbs the very ordered arrangement adopted by **KK** and slightly pulls out the included lysine residue.

Spectroscopic and microcalorimetric results for complexes with $\mathbf{1_6}$

The NMR spectra show that the peaks of the lysine residues of **KK** and of the arginine residues of **RR** are much less displaced in the presence of $\mathbf{1_6}$ than in the presence of $\mathbf{1_4}$. The upfield shifts experienced by both residues of the dipeptide do not differ as much as upon complexation by $\mathbf{1_4}$. Also, the heat effects observed upon microcalorimetric titrations of $\mathbf{1_6}$ with **RR** and **KK** are smaller than those observed upon titration of $\mathbf{1_4}$ (Fig. 5). Within the uncertainty limits of the experiments, the spectroscopic and microcalorimetric data for **KR** are indistinguishable from those for **RK**.

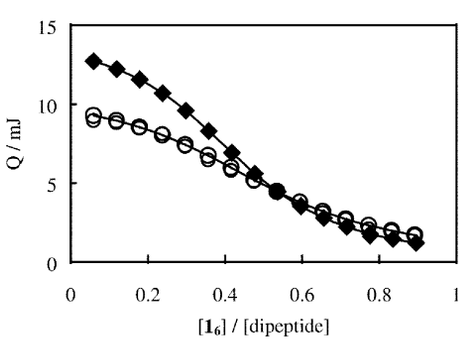


Fig. 5 Heat effects observed upon titration of $\mathbf{1_6}$ by **KK** (○) and by **RR** (◆).

Table 3 Thermodynamic parameters characterizing the complexation of some dipeptides and tripeptides by the *p*-sulfonatocalix[6]arene in water at pH 8 (phosphate buffer) and 298.15 K[a,b,c]

	$10^{-2}K'$	$\Delta_rG'^{\circ}$	$\Delta_rH'^{\circ}$	$T\Delta_rS'^{\circ}$
Lysine-containing peptides				
1_6-K[d]	0.94 ± 0.04	−11.3 ± 0.1	−21.8 ± 0.3	−10.5 ± 0.2
1_6-KK	23 ± 7	−19.2 ± 0.9	−26 ± 2	−7 ± 3
1_6-(KK)$_2$	7.0 ± 0.8	−16.2 ± 0.3	−15 ± 3	1 ± 3
1_6-KKK	31 ± 7	−20.0 ± 0.4	−34 ± 2	−14 ± 2
1_6-(KKK)$_2$	34 ± 4	−20.2 ± 0.2	−19 ± 3	1 ± 3
Arginine-containing peptides				
1_6-R[d]	1.86 ± 0.07	−13.0 ± 0.1	−41.2 ± 0.5	−28.2 ± 0.6
1_6-RR	12 ± 2	−17.5 ± 0.4	−26.1 ± 0.8	−9 ± 1
1_6-(RR)$_2$	12 ± 1	−17.5 ± 0.3	−31 ± 2	−14 ± 1
1_6-RRR	16 ± 8	−18 ± 1	−54 ± 11	−36 ± 12
1_6-(RRR)$_2$	113 ± 41	−23.1 ± 0.8	−15 ± 12	8 ± 13
Mixed dipeptides				
1_6-RK or **1_6-KR**	16 ± 4	−18.3 ± 0.5	−35 ± 2	−17 ± 3
1_6-(RK)$_2$ or **1_6-(KR)$_2$**	7.3 ± 0.9	−16.3 ± 0.3	−23 ± 4	−7 ± 4

[a] K' and $\Delta_rH'^{\circ}$ deduced from the non-linear least-squares fit of the data using a 1 : 2 binding model. [b] Molar scale. [c] $\Delta_rG'^{\circ}$, $\Delta_rH'^{\circ}$ and $T\Delta_rS'^{\circ}$ in kJ mol^{-1}. [d] Ref. 20.

In all cases, a 1 : 2 binding model involving the following stepwise equilibria

$$\mathbf{1_6} + \text{guest} = \mathbf{1_6}\text{–guest}$$
(characterized by K'_1 and $\Delta_rH'^{\circ}_1$)

$$\mathbf{1_6}\text{–guest} + \text{guest} = \mathbf{1_6}\text{–(guest)}_2$$
(characterized by K'_2 and $\Delta_rH'^{\circ}_2$)

must be considered in order to fit the microcalorimetric data. It is known that the *p*-sulfonatocalix[6]arene adopts a 1,2,3-alternate conformation (double partial cone) in the solid state, showing on each half of the molecule three adjacent sulfonate groups and one phenolate anion involved in intramolecular hydrogen bonding with the neighbouring undissociated hydroxy groups.[25c] The crystal structures of calix[6]arenes crystallized from hydrogen-bonding solvents also show a 1,2,3-alternate conformation, with co-crystallized solvent molecules symmetrically located on opposite sides of the calixarene.[35] This is consistent with the stepwise formation of a 1 : 2 complex and suggests that $\mathbf{1_6}$ adopts the same conformation in aqueous solution. The fit of the microcalorimetric data yields the stepwise apparent association constants and apparent standard enthalpy changes reported in Table 3. In all cases, the binding process is enthalpy-driven. As shown previously,[20] $\Delta_rH'^{\circ}$ and $T\Delta_rS'^{\circ}$ are much more negative for the binding of **R** than for the binding of **K**. These pronounced enthalpic and entropic effects can possibly be ascribed to the fact that the π–π interactions between the guanidinium group of **R** and the phenyl groups of $\mathbf{1_6}$ are particularly favoured in a 1,2,3-alternate conformation. The thermodynamic properties for the complexation of the dipeptides strengthen this assumption. The fact that the ratio K'_2/K'_1 (0.30) differs only slightly from the statistical ratio (0.25)[36] shows that $\mathbf{1_6}$ binds two **KK** in a non-cooperative manner and this is consistent with the 1,2,3-alternate conformation. Binding the first **KK** results in an important loss of enthalpy that is partly compensated by an unfavourable entropic contribution. The binding of the second guest also results in a favourable enthalpic contribution but of much less importance. Surprisingly, this is accompanied by a favourable entropy gain which probably indicates that the first guest is displaced or partly ejected and that, on the whole, the system gains degrees of freedom. The behaviour of **KR** (or **RK**) follows that observed with **KK**: the values of K_1', K_2', $(\Delta_rH'^{\circ}_2 - \Delta_rH'^{\circ}_1)$ and $(T\Delta_rS'^{\circ}_2 - T\Delta_rS'^{\circ}_1)$ are, within the uncertainty limits, the same for the mixed dipeptides and lysyl-lysine. This is consistent with a preferential inclusion of the lysine residue. The fact that the enthalpy and entropy changes are more negative for the binding of **KR** (or **RK**) than for the binding of **KK** is possibly due to π–π stacking in the former case: CPK models show indeed that the arginine residue, which remains outside the double partial cone, is of the appropriate length for perfect stacking of its guanidinium group on a phenyl group of the host. In the case of **RR**, the constants characterizing the formation of the 1 : 1 and 1 : 2 complexes are equal. Binding the first **RR** results in a loss of enthalpy and a loss of entropy that are quite similar to those observed upon binding the first **KK**. However, the situation differs when the second **RR** is added. In fact, the second guest interacts more strongly with the host and seems to be more immobilized than the first guest, π–π stacking being possibly responsible for this.

The NMR spectra show that the peaks of **KKK** and **RRR** are less displaced in the presence of $\mathbf{1_6}$ than in the presence of $\mathbf{1_4}$ but, whereas the three residues of the tripeptide experienced different upfield shifts in the presence of increasing amounts of the tetramer, it appears that in the presence of the hexamer two of the residues show the same behaviour. The dissymmetry of the NMR spectra suggests that only one of the side chains is turned towards the interior of the cavity and that it is less strongly perturbed than the other two chains.

The heat effects observed upon microcalorimetric titrations of $\mathbf{1_6}$ with **RRR** and **KKK** are also smaller than those observed upon titration of $\mathbf{1_4}$. The only way to fit the microcalorimetric data is to apply the 1 : 2 binding model used with the dipeptides: the results are reported in Table 3. Here again, the binding process is enthalpy-driven. The complexation of **RRR** is noteworthy with enthalpy and entropy changes upon binding the first guest that are remarkably negative: $\Delta_rH'^{\circ}_1 = -54$ kJ mol^{-1} and $T\Delta_rS'^{\circ}_1 = -36$ kJ mol^{-1}. The enthalpy change upon binding the second guest is much less pronounced whereas the entropy change is markedly positive. **KKK** shows similar behaviour but to a much lesser extent. CPK models show that the first **RRR** fits particularly well the ligand in the 1,2,3-alternate conformation: if the shorter chain is turned towards the interior of the cavity then the guanidinium groups of the two longer side chains can almost exactly and symmetrically reach two phenyl groups of the partial cone from the outside, possibly giving rise to important π–π interactions. This is consistent with the NMR spectra and suggests that it is the central residue which is turned towards the interior of the cavity. The addition of the second **RRR** probably disrupts this nice

arrangement, in particular because of the repulsion between the two included residues and, as a result, the whole system gains degrees of freedom. It may be noticed that the complexation of the first **RRR** is a remarkable case of tight binding for which the important favourable enthalpy change is largely compensated by the unfavourable entropy change. This yields an affinity for **RRR** that is weaker than for the loosely bound **KKK**, which is fully consistent with the idea put forward earlier[37,38] that loose association is required to achieve strong binding.

Spectroscopic and microcalorimetric results for complexes with $\mathbf{1_8}$

The ^{1}H NMR titration spectra obtained on addition of increasing amounts of $\mathbf{1_8}$ to the dipeptides and tripeptides show very broad bands precluding determination of the association constant. Furthermore, it is not been possible to fit the heat effects observed upon microcalorimetric titrations of $\mathbf{1_8}$ with **KK**, **RR**, **KKK** and **RRR** on the basis of classical 1 : 1 or 1 : 2 binding models. Obviously, higher order complexes are formed in solution leading to aggregation but it is not possible to thermodynamically characterize them in a reliable way.

Correlation between the enthalpies and entropies of binding

It has been shown that the enthalpies and entropies for complexation of different guests (cations, neutral or charged molecules) by various cyclic (crown ethers, cryptands, cyclodextrins, cyclophanes, calixarenes, *etc.*) or acyclic flexible (glymes, podands, enzymes, antibiotics, *etc.*) hosts are linearly correlated, whatever the solvent.[19d,32,39–41] These correlations have been thoroughly analyzed in terms of the degree of conformational change of the host and/or guest and the extent of host and guest desolvation upon complexation.[32,39–42]

In Fig. 6, we have plotted $T\Delta_rS'^{\circ}$ *versus* $\Delta_rH'^{\circ}$ for the complexation of the amino acids **K** and **R**, the dipeptides **KK**, **RR**, **KR** and **RK**, and the tripeptides **KKK** and **RRR** by $\mathbf{1_4}$ and $\mathbf{1_6}$, respectively, in water at 298.15 K and pH 8 (from the values reported in Tables 2 and 3). The $T\Delta_rS'^{\circ}$ and $\Delta_rH'^{\circ}$ values for both steps of the 1 : 2 binding processes have been plotted. A straight line is observed but, since the thermodynamic properties for the complexation of several other guests by *p*-sulfonatocalix[*n*]arenes in water are now available, it is interesting to check if a more general correlation exists. We have thus also plotted in Fig. 6 $T\Delta_rS'^{\circ}$ *versus* $\Delta_rH'^{\circ}$ for the complexation of $\mathbf{1_4}$ with *n*-alkylammoniums ions [H–$(CH_2)_n$–NH_3^+, n = 1–7],[29,33] tetraalkylammonium ions [$(H–(CH_2)_n)_4N^+$, n = 1–4],[33] alkaline-earth-metal cations (Mg^{2+} and Ca^{2+}),[33] lanthanide cations (La^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Gd^{3+}, Dy^{3+} and Yb^{3+}),[33] and *n*-propanol[30] (it should be noted that the values were obtained at pH 8 for *n*-propanol, at pH 7 for the series of ions going from *n*-propylammonium to *n*-heptylammonium, and at pH 2 for the other species). To this, we have added the values for the complexation of $\mathbf{1_4}$, $\mathbf{1_6}$ and $\mathbf{1_8}$ with *N*,*N*-dimethylindoaniline,[41] *N*,*N*,*N*-trimethylanilinium ion[43] and 1-adamantyltrimethylammonium ion[43] (including, for $\mathbf{1_8}$, the values for both steps of the 1 : 2 binding process). The values given by Tao and Barra[41] for the binding of *N*,*N*-dimethylindoaniline by methyl and hexyl ether derivatives of $\mathbf{1_6}$ have also been included, as well as the values given by Arena *et al.*[44] for the binding of *N*,*N*,*N*-trimethylanilinium, benzyltrimethylammonium and *p*-nitrobenzyltrimethylammonium cations by a derivative of $\mathbf{1_4}$ bearing four O–CH_2–COO^- groups on the lower rim. We have also added our values[20] for the complexation of lysine and arginine by $\mathbf{1_4}$, $\mathbf{1_6}$ and $\mathbf{1_8}$ at pH 1 and by $\mathbf{1_8}$ at pH 8 (including the values for both steps of the 1 : 2 binding processes). Altogether, 70 points have been plotted. Interestingly, in spite of the diversity of guests, a unique linear relationship is observed (r = 0.96) with a slope α = 1.10 and an intercept $T\Delta_rS'^{\circ}_o$ = 20.3 kJ mol^{-1}. These values can be compared with those reported for complexation by various host molecules: antibiotics (0.95 and 23.4 kJ mol^{-1}),[39a] biological receptors (1.07 and 42.6 kJ mol^{-1}),[40] crown ethers (0.76 and 10.0 kJ mol^{-1}),[39a] cryptands (0.51 and 16.7 kJ mol^{-1}),[39a] cyclodextrins (0.90 and 13.0 kJ mol^{-1}),[39c] modified cyclodextrins (1.07 and 20.9 kJ mol^{-1}),[39d] and cyclophanes (0.72 and 12.7 kJ mol^{-1}).[32]

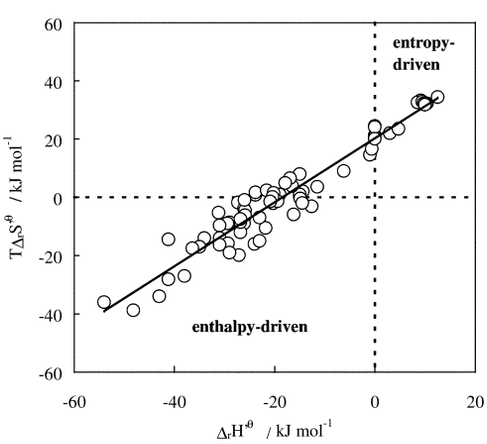


Fig. 6 Variation of $T\Delta_rS'^{\circ}$ with $\Delta_rH'^{\circ}$ for the complexation of 35 different guests by *p*-sulfonatocalix[*n*]arenes in water at 298.15 K and different pH (see text for a full description of the guests and hosts considered here).

The points that fall in the upper right quadrant of Fig. 6 correspond to entropy-driven bindings whereas those that fall in the lower left quadrant correspond to enthalpy-driven bindings. According to a classification commonly used for biological ligands, guests of the agonist type are characterized by their ability to elicit a conformational response in the receptor whereas those of the antagonist type act by blocking the receptor site, yielding entropy-driven bindings for the former and mostly enthalpy-driven bindings for the latter.[40] The only guests that fall in the upper right quadrant are the alkaline-earth-metal and lanthanide cations. The interactions between these metal ions and the host are purely ionic and controlled by the entropy of dehydration: one can thus consider that the metal ions, which essentially block the sulfonate groups of the host as clearly evidenced by the recent molecular dynamics simulation of the La^{3+}–$\mathbf{1_4}$ complex in water,[45] act as antagonists, although this type of classification is usually restricted to biomolecules. The ions bearing an ammonium group and a non-polar chain (*n*-alkylammonium, tetraalkylammonium, trimethylanilinium, 1-adamantyltrimethylammonium) fall in the upper left quadrant. The major contribution to their favourable enthalpy of complexation comes from the van der Waals interactions associated with the inclusion of the alkyl chain but it is the partial desolvation of the charged groups upon ionic interaction that is responsible for their small favourable entropy of complexation. This is consistent with the recent molecular dynamics simulations of the R_4N^+–$\mathbf{1_4}$ complexes in water.[45] The points that fall in the lowest part of the lower left quadrant correspond to the binding of arginine with $\mathbf{1_6}$ and $\mathbf{1_8}$ and to the binding of arginyl-arginine and arginyl-arginyl-arginine with $\mathbf{1_6}$ and $\mathbf{1_8}$. The points for the binding of lysyl-lysine and lysyl-lysyl-lysine are slightly above. These dipeptides and tripeptides seem to act as agonists towards $\mathbf{1_6}$ and $\mathbf{1_8}$ by eliciting a conformational response of the host which decreases both the entropy and the enthalpy. This is in fact induced by a combination of different effects more or less opposed: the ionic interactions between the ammonium groups of the guest and the sulfonate groups of the host, the tight inclusion of the non-polar chain into the hydrophobic cavity of the host through van der Waals interactions, and the hydrogen bonding between the amide NH of the guest and the sulfonate groups of the host. The effect is amplified with the arginine-containing guests because the conformational response of the host favors π–π stacking. As a consequence of this large enthalpy–entropy compensation, the

affinity for the very tightly bound species shown on the lowest end of the correlation is rather small. More generally, molecular engineering has to take into account the fact that the strengthening of host–guest interactions is inefficient if it results in an important reduction of the conformational freedom of the system.

Conclusion

The complexation of a series of lysine-containing and arginine-containing dipeptides and tripeptides by the *p*-sulfonatocalix[*n*]-arenes in water at pH 8 has been studied by both ^{1}H NMR and microcalorimetry. For calix[4]arene sulfonate, only a 1 : 1 stoichiometry is observed. The binding process is controlled by the favourable enthalpy resulting mainly from the tight inclusion of the apolar part of the guest into the hydrophobic cavity of the host through van der Waals interactions, but the favourable entropy accompanying the desolvation of the charged groups upon ionic interaction also plays an important role. The thermodynamic properties of association and the NMR data show that lysyl-lysine adopts a very compact folded structure upon binding by the tetrameric host. The mixed dipeptides that bear a lysine residue behave like lysyl-lysine, the lysine residue being preferentially complexed in the ligand cavity. Addition of a third chain perturbs this very nice arrangement and, because the entropy of binding then becomes more favourable, the affinity for the tripeptide is more important. Arginyl-arginine, which cannot adopt such a folded structure, shows different behaviour. With calix[6]arene sulfonate both 1 : 1 and 1 : 2 complexes are observed. The hexameric host binds two lysyl-lysine guests in a non-cooperative manner, which suggests that its adopts in solution a conformation of the 1,2,3-alternate type, as observed in the solid state. Its binding to the first arginyl-arginyl-arginine molecule is noteworthy with enthalpy and entropy changes remarkably negative. The thermodynamic data and the dissymmetry of the NMR spectra suggest that only the central side chain of the tripeptide is turned towards the interior of the partial cone. A very rigid structure is then obtained by stacking of the three guanidinium groups of the guest over the phenyl units of the hexameric host, which possibly gives rise to important π–π contributions. The complexation of arginyl-arginyl-arginine by the calix[6]arene sulfonate appears in fact to be a remarkable case of tight binding for which the important favourable enthalpy change is almost compensated by an unfavourable entropy change, yielding an affinity that is weaker than for the loosely bound lysyl-lysyl-lysine. Complex behaviour, associated with higher order stoichiometry and probably aggregation, occurs in the case of the calix[8]arene sulfonate.

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