A comparative study of the determination of the stability constants of inclusion complexes of *p*-sulfonatocalix[4]arene with amino acids by RP-HPLC and ^{1}H NMR

Olga I. Kalchenko,[a] Florent Perret,[b,c] Nicole Morel-Desrosiers[c] and Anthony W. Coleman*[b]

[a] *Institute of Organic Chemistry, National Academy of Sciences of Ukraine, Murmanskaya, 5, 02094, Kiev-94, Ukraine*
[b] *IBCP, CNRS UMR 5086, 7, Passage du Vercors, 69367 Lyon Cedex 07, France. E-mail: aw.coleman@ibcp.fr*
[c] *Laboratoire de Thermodynamique des Solutions et des Polymères, UMR CNRS 6003, Université Blaise Pascal (Clermont-Ferrand II), 24 avenue des Landais, 63177 Aubière cedex, France*

Received (in Cambridge, UK) 10th July 2000, Accepted 19th December 2000
First published as an Advance Article on the web 7th February 2001

Reversed-phase high-performance liquid chromatography (Separon SGX C 18, UV detection at 254 nm and acetonitrile–water–trifluoroacetic acid (70 : 30 : 0.5, v/v), and methanol–water–trifluoroacetic acid (3 : 97 : 0.5, v/v) as mobile phases) was applied to the study of the host–guest complexation of *p*-sulfonatocalix[4]arene (**SC[4]A**) with amino acids as guests in the mobile phase. It was established that the formation of the inclusion complexes results in changes in the retention times of the amino acids. Stability constants of the complexes were determined. The variations in stability constants may be explained in terms of the various interactions (ion-pairing, hydrophobic, aromatic–aromatic and electrostatic interactions) that may occur between a given amino acid and **SC[4]A**. For the amino acids aspartic acid and proline the association constants were also derived from ^{1}H NMR experiments.

Introduction

Calix[4]arenes, composed of four phenolic units linked *via* methylene groups, have been intensively studied as cavity-shaped host molecules able to recognize a wide range of guest molecules.[1] The binding properties of these compounds have been examined in solution,[2,3] the gaseous phase,[4] and in the crystalline state.[5] In order to increase the binding properties of calixarenes in aqueous solutions, water soluble *p*-sulfonatocalix[*n*]arenes (**SC[4]A**) were synthesized.[6,7] In view of the activity of **SC[4]A** towards different proteins, including chloride-ion channels,[7] lysyl oxidase[8] and its anti-thrombotic activity,[9] knowledge of the nature and strength of the interactions of **SC[4]A** with amino-acids should provide important information on the mechanism of the binding of **SC[4]A** to complex bio-macromolecules.

Previous work on the complexation of the amino acids Lys and Arg with **SC[4]A** by NMR,[10] and microcalorimetry[11] has shown that **SC[4]A** forms 1 : 1 complexes with these amino acids in water. Their stability constants determined respectively by NMR and microcalorimetry are 760 and 735 M^{-1} for Lys, and 1490 and 1520 M^{-1} for Arg. The solid-state crystal structure of the complex of Lys with *p*-sulfonatocalix[4]arene has been determined.[12] The system is complex with a stoichiometry of 4 Lys:2 **SC[4]A**, although two of the Lys molecules are present in positions that will occur only in the solid state.

For the Lys directly complexed into **SC[4]A** the alkyl side chain is embedded in the calixarene cavity in an unusual folded conformation. Another series of complexation studies of amino acids with **SC[4]A** has been published by Arena *et al.*[13]

Whilst the study of amino acid complexes with **SC[4]A** is possible by NMR and microcalorimetry, for peptides having a chain length above 10 amino acids problems of solubility have been encountered, making K_A measurements impossible. For this reason the greater sensitivity of HPLC may allow the study of the complexation of the sulfonated calix[4]arenes with bioactive peptides, and consequently it is of interest to compare K_A values obtained by different analytical methods.

In this work we have investigated host–guest interactions of **SC[4]A** with 10 amino acids (Fig. 1) under reversed-phase high-performance (RP-HPLC) conditions. The stability constants of the host–guest inclusion complexes were determined. For two further amino acids, Pro and Asp, K_A values were also obtained from ^{1}H NMR at pH 2 and 8.

Experimental

Reagents

Acetonitrile, trifluoroacetic acid, sodium dihydrogen phosphate dihydrate and anhydrous disodium hydrogen phosphate were analytical grade and were used without purification. **SC[4]A** was synthesised by the published method.[6] Amino acids were purchased from Sigma.

Apparatus

The conditions of RP-HPLC analysis were as follows. The LC system consisted of a high-pressure pump HPP 4001 (Laboratorni Pristroje, Prague, Czech Republic) connected to a Rheodyne Model sample 7120 injector (20 μL, Rheodyne Inc., Berkeley, CA) and an ultraviolet–visible (UV–vis) detector LCD 2563 (Laboratorni Pristroje, Prague, Czech Republic). The column (150 × 3 mm id) was packed with Separon SGX C 18 (5 μm) (Lachema, Czech Republic).

For ^{1}H NMR titrations, we used a 500 MHz Varian instrument.

^{1}H NMR analysis

The ^{1}H NMR titrations were carried out in 95% H_2O–5% D_2O

 DOI: 10.1039/b005497f

Table 1 Capacity factors of the guests k'_0, k', $(\Delta k') = k' - k'_0$ and the host–guest stability constants K_A in mobile phase A (CH_3CN–H_2O–CF_3COOH with **SC[4]A** additive)

k'_0 (No additive)	$10^3 k'$ $(\Delta k')/M^{-1}$				K_A/M^{-1} (RSD, %)
	0.16	0.32	0.64	1.3	
Asp 0.51	0.50 (0.01)	0.48 (0.03)	0.46 (0.05)	0.41 (0.10)	171 (24.0)
Gly 0.35	0.34 (0.01)	0.33 (0.02)	0.31 (0.04)	0.29 (0.06)	180 (24.8)
Trp 0.60	0.57 (0.03)	0.55 (0.05)	0.51 (0.09)	0.44 (0.16)	282 (12.8)
Pro 0.46	0.44 (0.02)	0.41 (0.05)	0.39 (0.07)	0.34 (0.12)	285 (12.6)
Tyr 0.51	0.48 (0.03)	0.45 (0.06)	0.39 (0.12)	0.32 (0.19)	433 (32.5)
Phe 0.60	0.56 (0.04)	0.52 (0.08)	0.46 (0.14)	0.39 (0.21)	448 (18.4)
His 1.05	0.96 (0.09)	0.89 (0.16)	0.81 (0.24)	0.61 (0.44)	541 (15.2)
Ala 0.40	0.36 (0.04)	0.33 (0.07)	0.29 (0.11)	0.22 (18)	648 (8.3)
Arg 0.41	0.34 (0.07)	0.30 (0.11)	0.24 (0.17)	0.17 (0.24)	1149 (36.6)
Lys 0.32	0.27 (0.05)	0.23 (0.09)	0.18 (0.14)	0.12 (9.20)	1209 (17.7)

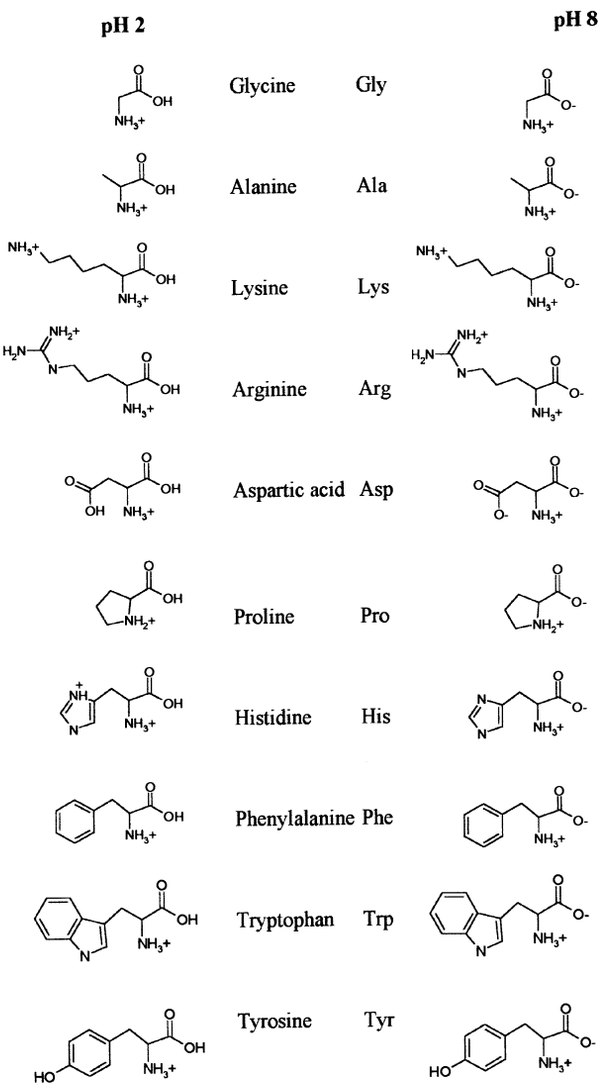

Fig. 1 Formulae of amino acids and ionisation states at pH 2 and 8.

with phosphate buffer of pH 8 (prepared by mixing 96.6 mL of a 0.01 mol kg^{-1} NaH_2PO_4 solution and 3.4 mL of a 0.01 mol kg^{-1} NaH_2PO_4 solution) and at pH 2 adjusted with hydrochloric acid. The pH was verified on a pH-meter calibrated with two different buffer solutions. The solutions' molalities were, prior to titration, in the range 0.0005–0.01 mol kg^{-1} for the calixarene and 0.005 mol kg^{-1} for the amino acids. The titration was carried out by introducing 300 μL of amino acid buffered solutions into NMR tubes and by adding increasing amounts (0 to 450 μL) of calixarene buffered solutions, completing to 1 mL with buffer. An NMR spectrum of each tube was recorded.

RP-HPLC analysis

The mobile phases (A: an acetonitrile–water–trifluoroacetic acid mixture containing **SC[4]A** at concentrations of 0.16, 0.32, 0.65. and 1.3 mM and B: methanol–water–trifluoroacetic acid mixture containing **SC[4]A** at concentrations of 0.25, 0.5, 0.65 and 1.07 mM) were prepared by dissolving **SC[4]A** in the mobile phase acetonitrile–water–trifluoroacetic acid (70 : 30 : 0.5, v/v) solution at ambient temperature (20 °C). Each of the concentrations was analysed five times. The concentration of amino acids in the injected solutions (identical to mobile phase) was 10 mM. The amount of the sample injected was 20 μL. Each of the samples was analysed five times. All chromatograms were obtained at 38 °C. The flow rate was 0.4 mL min^{-1}, and the UV detector was operated at 254 nm. The dead time (t_0) was measured with $NaNO_2$. Mobile phases with **SC[4]A** as additive were equilibrated for 3 h before analysis.

Results and discussion

The influence of the SC[4]A mobile phase on retention of amino acids

The effect of the **SC[4]A** additive to the mobile phase on the retention time, t_R, and the capacity factor, k', of amino acids (Fig. 1) was studied. The capacity factors, k', and retention times, t_R, of amino acids were determined and are presented in Tables 1 and 2. The presence of **SC[4]A** additive in the mobile phase led to decreasing capacity factors for the amino acids.

Determination of stability constants

Stability constants of calixarene complexes with organic molecules in solutions are usually determined by NMR spectrometry.[14,15] Recently the RP-HPLC method was used to study the binding properties of calixresorcinarenes[2] and calixarenes.[3]

As with calix[4]resorcinarenes,[2] calix[4]arene,[16,17] and calix[8]-arene,[3] the introduction of **SC[4]A** into the mobile phase results in a decrease of the retention times and the capacity factors of the amino acids (Table 1 and 2), indicating host–guest inclusion complex formation in both the acetonitrile–water (mobile phase A) and the methanol–water solution (mobile phase B). To determine the composition of the complexes formed, the dependence of the $1/k'$ values of the amino acids on the calixarene concentration in the mobile phase was studied. As shown in Fig. 2 and Fig. 3, for mobile phase A and B, respectively, all the compounds investigated show a linear dependence of $1/k'$ as a function of calixarene concentration in the range 0.16–1.3 mM, indicating the formation of the host–guest complexes with a 1 : 1 stoichiometry.[16,17] From the dependence obtained for $1/k'$ with the concentration of the calixarene additive, the stability constants of the complexes were calculated from eqn. (1)[16]

$$1/k' = 1/k'_o + [\text{Host}]/K_D \times k'_0 \quad (1)$$

Table 2 Capacity factors, k', and stability constants, K_A, measured for amino acids with a calix[4]arene additive in mobile phase B (CH_3OH–H_2O–CF_3COOH with **SC[4]A** additive)

	k'_0 (No additive)	$10^3k'$ ($\Delta k'$)/M^{-1} 0.25	0.50	0.64	1.07	K_A/M^{-1} (RSD, %)
Asp	1.45	1.40 (0.05)	1.37 (0.08)	1.35 (0.10)	1.30 (0.15)	113 (5.26)
Gly	1.54	1.50 (0.04)	1.45 (0.09)	1.42 (0.12)	1.34 (0.20)	128 (24.5)
Tyr	2.29	2.18 (0.11)	2.06 (0.23)	2.0 (0.29)	1.85 (0.44)	210 (17.5)
Phe	4.50	4.0 (0.50)	3.60 (0.9)	3.30 (1.20)	2.75 (1.75)	588 (18.2)
Ala	3.17	2.68 (0.49)	2.32 (0.85)	2.19 (0.98)	1.82 (1.35)	675 (20.6)
His	2.33	2.0 (0.33)	1.66 (0.67)	1.60 (0.73)	1.33 (1.0)	717 (6.46)
Lys	5.17	4.37 (0.80)	3.45 (1.72)	3.0 (2.17)	2.48 (2.69)	1221 (23.2)
Trp	6.10	4.51 (1.59)	3.50 (2.60)	3.0 (3.10)	2.41 (3.59)	1518 (13.7)
Pro	7.75	5.75 (2.0)	5.0 (2.75)	4.33 (3.42)	3.72 (4.03)	1138 (22.4)
Arg	15.01	8.90 (6.71)	6.25 (7.59)	5.5 (8.41)	3.9 (11.23)	2587 (30.9)

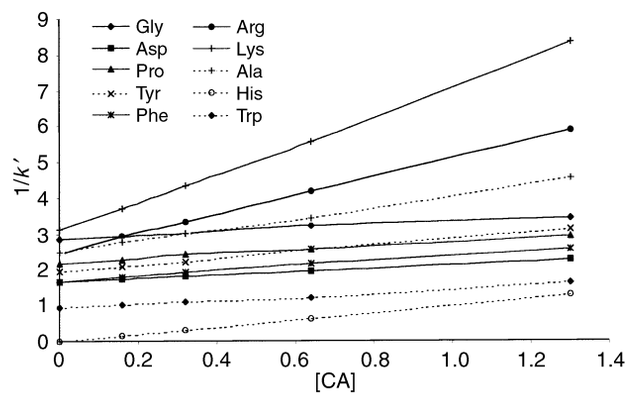

Fig. 2 Plots of $1/k'$ for Lys (1), Gly (2), Arg (3), Pro (4), Tyr (5), Trp (6), Phe (7) for mobile phase A.

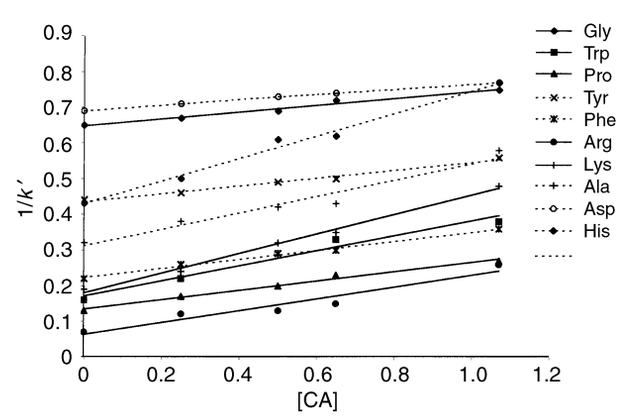

Fig. 3 Plots of $1/k'$ for Gly (1), Tyr (2), Lys (3), Trp (4), Phe (5), Pro (6), Arg (7) for mobile phase B.

where k'_0 is the capacity factor in the absence of a host, [Host] is the concentration of **SC[4]A** in the mobile phase and K_D is the dissociation constant of the complex.[16]

In the chromatographic column containing the amino acid (AA) and calixarene **SC[4]A** equilibria exist between the mobile phase and stationary phase.

$$(\text{guest})_s \overset{K_S}{\rightleftharpoons} (\text{guest})_m + (\text{host})_m \underset{K_D}{\rightleftharpoons} (\text{host–guest})_m \overset{K_C}{\rightleftharpoons} (\text{host–guest})_s$$

The equilibria constants K_S, K_D and K_C are given as (i) distribution constant K_S of amino acid AA between stationary phase S and mobile phase m [eqn. (2)];

$$K_S = \frac{[(AA)_S]}{[(AA)_m]} \quad (2)$$

(ii) dissociation constant of the complex calixarene–amino acid SCA–AA [eqn. (3)];

$$K_D = \frac{[(\mathbf{SC[4]A})_m]\,[AA]_m}{[(\mathbf{SC[4]A})\text{–}AA]_m} \quad (3)$$

(iii) distribution constant of K_C **SC[4]A**–AA [eqn. (4)].

$$K_C = \frac{[(\mathbf{SC[4]A}\text{–}AA)_S]}{[(\mathbf{SC[4]A}\text{–}AA)_m]} \quad (4)$$

In the proposed scheme, the distribution of **SC[4]A** between the phases may be negligible. Under the analysis conditions the column is saturated with calixarene. Due to saturation of the column with calixarene the distribution equilibrium of the amino acid calixarene complex on to the stationary phase may be neglected because the sorption of this complex must be similar to the sorption of the calixarene itself. The capacity factor of an amino acid may be presented as shown in eqn. (5) where φ denotes the phase ratio of the column. The total concentration of $[(\mathbf{SC[4]A})_T]$ in the mobile phase is given by eqn. (6). Thus, eqn. (5) may be presented as shown in eqn. (7).

$$K' = \varphi \frac{[(AA)_S]}{[(AA)_m] + [(\mathbf{SC[4]A}\text{–}AA)_m]} \quad (5)$$

$$[\mathbf{SC[4]A}]_T = [(\mathbf{SC[4]A})_m] + [(\mathbf{SC[4]A}\text{–}AA)_m] \quad (6)$$

$$K' = \varphi \frac{K_{Aa} \times K_D}{K_D + [\mathbf{SC[4]A}]_T - [(\mathbf{SC[4]A}\text{–}AA)_m]} \quad (7)$$

When the AA concentration is very small compared with the calixarene's concentration eqn. (8) can be used.

$$[\mathbf{SC[4]A}]_T - [(\mathbf{SC[4]A}\text{–}AA)_m] = [\mathbf{SC[4]A}] \quad (8)$$

Furthermore, $K_S\varphi$ is equal to the capacity factor (k'_0) determined in the absence of **SC[4]A**. Then eqn. (6) may be presented as shown in eqn. (9).

$$1/k' = 1/k'_0 + \frac{[\mathbf{SC[4]A}]_T}{K_D \times k'_0} \quad (9)$$

In the selected region of **SC[4]A** concentrations the relationship of $1/k'$ to the **SC[4]A** concentrations $[(\mathbf{SC[4]A})_T]$ (Fig. 2 and 3) confirms formation of the 1:1 complexes for amino acids investigated.

The choice of amino acid was dictated by a number of factors: favourable electrostatic interactions for Lys and Arg, non-favourable electrostatic interactions for Asp, a hydrophobic folded shape in the case of Pro (which may mimic the solid-state interactions observed for Lys with sulfonatocalix[4]arene),[12] aromatic functions (which are known to be favour-

able for calixarene complexation) for Phe and Tyr, and possibly for Trp (which is too large to fit the cavity of **SC[4]A**), Gly was used as a blank. Ala was also chosen in view of the future possibility of the construction of a soluble model of the α-helical peptides known to bind heparin, thus permitting "model" studies of the heparinoid behaviour of **SC[4]A**. The structures and ionisations states at pH 2 and 8 of the amino-acids used in this study are given in Fig. 1.

As shown in Table 1, Tyr and Phe, the two amino acids with simple aromatic side chains exhibit in mobile phase A essentially identical K_A values of 433 and 448 M^{-1} respectively. Complexation of mono- and *para*-di-substituted benzene rings within the cavity of calix[4]arene is well known [15] and the complex of **SC[4]A** with the trimethylanilinium ion is known, with an apparent K_A value of 5600 M^{-1}.[6] Direct comparison with this value is difficult due to the difference in the pH values between the two measurements. For Trp, in which the aromatic side chain is sterically much larger, the K_A value observed is, as expected, smaller than those of Phe and Tyr in mobile phase A: 282 M^{-1}. The two positively charged amino acids Lys and Arg show the highest K_A values of 1209 and 1149 M^{-1} respectively. It is apparent that in mobile phase A, the favourable electrostatic interactions between the positively charged amino acids and the negatively charged sulfonatocalix[4]arene lead to tight ion-pair binding in the complex. As expected in the case of Asp where unfavorable electrostatic interactions will be present, the lowest K_A was observed, 171 M^{-1}. A K_A of 541 M^{-1} is observed for His, intermediate between Pro and the positively charged amino acids. The amino acid Gly, chosen as a negative blank as it contains no side chain, shows a low K_A of 180 M^{-1} by RP-HPLC. Surprisingly in mobile phase A, Ala shows quite strong binding to **SC[4]A**, with a K_A of 648 M^{-1}.

The K_A and capacity factors values for mobile phase B are summarised in Table 2. Comparing with the values obtained in mobile phase A, the complexation behaviour of the amino acids can be divided into two groups; firstly, those for which the K_A value remains the same or decreases slightly Gly (128 *vs.* 180 M^{-1}), Phe (588 *vs.* 448 M^{-1}), Tyr (210 *vs.* 433 M^{-1}) and Lys (1221 *vs.* 1209 M^{-1}) and those for which a very strong increase in K_A is observed, Trp (1518 *vs.* 282 M^{-1}), Pro (1138 *vs.* 285 M^{-1}) and Arg (2587 *vs.* 1149 M^{-1}). It is evident that the interaction mechanism for these last three amino acids must be different from the other amino acids and also change between the two mobile phases. Looking at the structural similarities between Trp, Pro and Arg (Fig. 1) it is tempting to ascribe these differences to the presence in these three amino acids of a secondary amino function. However, it is probably better to treat Pro differently from Arg and Trp. For Arg and Trp a double interaction involving both the secondary amine and π–π interactions between the aromatic groups of the **SC[4]A** macrocycle with, respectively, the guanidinium function of Arg and the aromatic ring of Trp, are probably responsible for the changes in K_A, with the change in solvent polarity as the driving force.

For Pro in this more polar medium, the hydrophobic interactions between the folded conformation of the cyclic amino acid and the **SC[4]A** cavity give rise to a K_A similar to that of Lys (1138 *vs.* 1221 M^{-1}), which suggests that in this medium, the role of the electrostatic interactions is less important than that of the hydrophobic interactions. Interestingly we have recently observed [18] that for the two mixed dipeptides Arg-Lys and Lys-Arg, the Lys is in both cases prefentially included in the **SC[4]A** cavity, in spite of introduction of an unfavourable electrostatic interactions for Arg-Lys, in which the Lys fragment carries a terminal carboxylate group; again this results from the hydrophobic interactions in the side chain. Once more in mobile phase B, the weakest complexation is with the acidic amino acid Asp with a K_A of 113 M^{-1}. ^{1}H NMR experiments at pH 2 show no shift in the protons' signals, giving an effective K_A of 0 M^{-1}.

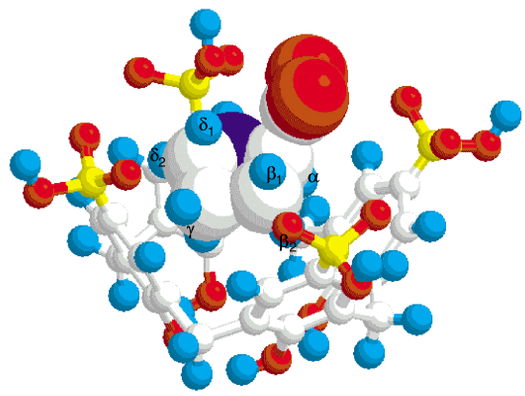

Fig. 4 Model of proline–**SC[4]A** complexation.

In the case of His, a much smaller variation in K_A is observed between the two mobile phases (541 M^{-1} in mobile phase A and 717 M^{-1} in mobile phase B). This may be explained by the more polar nature of the imidazole ring compared to the Pro five-membered ring.

Two of the amino acids bear simple aromatic side chains: Phe and Tyr. In the case of Phe essentially no change in the K_A value occurs between the two mobile phases; here the driving force for inclusion will be simple aromatic–aromatic interactions. However, for Tyr the K_A value decreases in the more polar, H-bonding solvent of mobile phase B. This must arise from solvation of the phenolic OH group of Tyr, reducing the capacity for inclusion.

For Lys it may be that a balance in the energy between ion-pair interactions arising in mobile phase A and hydrophobic interactions coupled with weakened electrostatic interactions in mobile phase B leads to an apparent lack of change in the observed K_A values (1209 M^{-1} and 1221 M^{-1}). As expected for Gly, which does not possess a side chain capable of interaction with the cavity of **SC[4]A** a small decrease in K_A is consistent with a diminution in the importance of ion-pair effects in mobile phase B.

Again relatively strong binding is observed for Ala which has a K_A of 675 M^{-1}. The change between phase A and phase B is similar to that observed for Phe, suggesting a simple hydrophobic association of the short alkyl side chain is the driving force for binding. The crystal structure of the Lys–**SC[4]A** complex [12] shows that the Lys side chain is folded on binding into the cavity. Therefore, it might be expected that Pro, in which the five-membered ring forms the side chain, could mimic the folded Lys conformation, and thus bind tightly to **SC[4]A**. ^{1}H NMR studies of the complexation of Pro with **SC[4]A** show that this amino acid indeed interacts with the host at pH 2 and pH 8. The association constants determined at pH 2 and 8 are respectively 100 and 70 M^{-1}. The K_A is somewhat higher than those observed by Arena for other amino acids, but is an order of magnitude lower than those values previously observed by us for Lys. For the three amino acids studied by NMR at pH 2 and pH 8, the K_A values at pH 2 are always higher. This observation arises from the removal of repulsive interactions between the anionic carboxylate group and the anionic sulfonate functions of the calixarene.

Splitting of the non-equivalent δ protons of Pro was observed in the NMR experiment at pH 2. This implies that Pro adopts a specific conformation in the cavity of **SC[4]A** (Fig. 4). The splitting increases with increasing concentration of **SC[4]A** implying that on inclusion into the calixarene cavity, the δ protons are situated in quite different environments. In contrast, the β protons show a decrease in the chemical inequivalence (Fig. 5). If in the case of lysine it was the δ and ε protons that had the highest chemical shifts, then in the case of proline, these are now the γ and δ protons. Comparison with the X-ray structure of the lysine complex shows that one of the δ protons

Table 3 Stability constants K_A of the amino acids with **SC[4]A** calculated by different methods

Amino acid	RP-HPLC Mobile phase A, pH 2	RP-HPLC Mobile phase B, pH 2	^{1}H NMR pH 8	^{1}H NMR pH 2	Microcalorimetry pH 8	Microcalorimetry pH 1
Val	—	—	16[14]		—	—
Gly	180	128	—		—	—
Ala	648	675	0[14]		—	—
Phe	448	588	63[14]		—	—
His	541	717	20[14]		—	—
Tyr	433	210	0[14]		—	—
Lys	1209	1221	760[18]	1700[18]	735[11]	1400[11]
Asp	171	113	—	0	—	—
Pro	285	1138	70	100	—	—
Trp	282	1518	25[14]		—	—
Arg	1149	2587	1490[18]	3900[18]	1520[11]	2830[11]

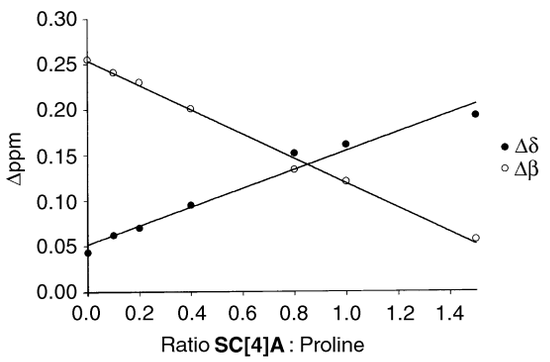

Fig. 5 Splitting of non-equivalent δ and β protons for Pro.

will be directed towards a phenyl ring with the other directed down the cavity. The decrease for the β protons shows that their chemical environment becomes less different, implying that one of them is close to a sulfuric group with the other one close to the carboxylic acid of the proline, leading to a similar polar environment. In free proline, only one β proton is in close proximity to the carboxylic group.

In order to compare the association constants determined by RP-HPLC with those derived from NMR,[10,13] and microcalorimetry,[11] all of the known K_A values for amino acid complexation by **SC[4]A** are presented in Table 3. Given the differences in conditions (pH 2 in this work, pH 8 in refs. 10 and 13; solvent polarities, $CH_3CN–H_2O$ or $MeOH–H_2O$ here and 0.1 M PBS[10] in refs. 10 and 13 and experimental nature RP-HPLC *vs.* NMR or microcalorimetry[11]) considerable prudence is necessary in the comparison. Both in the current work for Asp and Pro and in the previous work of Arena, the values of K_A obtained from ^{1}H NMR are an order of magnitude lower than those obtained by RP-HPLC. However, for Lys and Arg much larger K_A values were obtained from ^{1}H NMR. This again would appear to emphasise the importance of strong electrostatic ion-pair binding. Taking this experimental discrepancy into account, some interesting points are found.

Studies of Lys and Arg with **SC[4]A** by ^{1}H NMR[10,11] and microcalorimetry[11] and also in the current work all show strong interactions. As expected in the more polar solvent system in mobile phase B the interactions for Arg are stronger than those for Lys and this is in accordance with NMR and microcalorimetry. The K_A values in all three experimental methods are approximately twice as large for Arg and Lys. The numerical divergence arises from the difference in the experimental methodology. For Phe and Tyr, the values found here for high polarity show Phe > Tyr, which is in accordance with the ^{1}H NMR data[14] where Phe is bound but Tyr is apparently not. As we noted above in polar systems, the purely aromatic side chain of Phe should be a better group for inclusion than the phenolic side chain of Tyr. For Trp the weak association is found, at pH 8, by ^{1}H NMR ($K_A = 25\ M^{-1}$). This is very much lower than the value found here at pH 2 but the difference should arise from a lack of protonation of the secondary amine present on the side chain of Trp at pH 8. Ala shows relatively strong binding under RP-HPLC conditions, in contrast to the ^{1}H NMR experiments in which zero binding is observed.

The association constants observed in this work, show that the values obtained by HPLC are, with the exception of those for Lys and Arg, approximately an order of magnitude greater than those obtained by NMR titration. There is some general correlation between the trends in values of K_A observed by the two methods.

The experimental conditions between the two methods used in this study are quite different, both in terms of the solvent systems used and, especially, in the nature of the interactions involved. In the case of the NMR titrations, the molecules interact in solution and the only competition for inclusion that may occur is between the phosphate buffer and the guest molecule. However, in the case of the HPLC experiments, the measurements depend on how the amino acids are removed from the hydrophobic environment of the C_{18} chains coupled on to the surface of the silica column.

It is therefore to be expected that there will be strong divergence in the results obtained by the two methods. Interestingly, from a biological point of view, both approaches will give useful results for studying the interactions between the calixarenes and various amino acids. In the case of the K_A values derived from ^{1}H NMR titration these results could correspond to those in which the amino acids were found in a hydrophilic environment, *e.g.* in circulating proteins of peptide fragments. The K_A values derived from HPLC measurements may be more relevant to interactions between the calixarenesulfonates and amino acids in more hydrophobic conditions or on a surface, *e.g.* for membrane bound systems.

Conclusion

In conclusion we have shown that RP-HPLC is a valid tool for the study of the complexation process of amino acids with **SC[4]A**. The various interactions parameters, including ion-pair formation, electrostatic interactions, hydrophobic interactions and aromatic–aromatic interactions, can be used to explain the relative binding strengths of the amino acids.

Comparison with results obtained by other methods, such as ^{1}H NMR, is possible if the different experimental conditions are taken into account.

Work is currently being undertaken to extend the work to various biologically relevant peptide systems and, in particular, to peptides known to act as receptors for heparin.

Acknowledgements

O. I. K. acknowledges the support of the IBCP (CNRS) in carrying out this work.

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