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ORIGINAL ARTICLE

pH-dependent complex formation of amino acids with β-cyclodextrin and quaternary ammonium β-cyclodextrin

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Abstract Stability constants for the complexes of anionic, neutral (zwitterionic) and protonated forms of L- and D-enantiomers of eight amino acids with β-cyclodextrin and the positively charged quaternary ammonium β-cyclodextrin (QA-β-CD, DS = 3.6 ± 0.3) have been determined by spectrophotometric and pH-potentiometric methods. The highest stability constants have been obtained for the aromatic amino acids phenylalanine, tyrosine and tryptophan. Except the dianion of tyrosine and QA-β-CD, values for the anions in the range of 80–120 have been found, the stability constants for the zwitterionic forms are much smaller and complex formation is negligible with the protonated species. In the case of the other amino acids the differences are less pronounced. The results are interpreted in terms of hydrogen bonding, steric effects and electrostatic interactions between the amino acid moiety and the rims of the cyclodextrins, in addition to the inclusion of the side chain, and are supported by ^{1}H and ^{13}C NMR investigations on the systems containing L-phenylalanine and L-tyrosine. The differences between the complex formation constants of the L- and D-enantiomers do not exceed the limits of experimental error in most cases.

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Introduction

β-cyclodextrin (β-CD) is a well-known complexing agent for guests bearing sufficiently hydrophobic parts of appropriate size [1]. The main driving forces affecting the complex formation are van der Waals and hydrophobic interactions. In most cases the nonpolar part of the guest is located in the cavity, the polar substituent is near the wider rim of the ring, but sometimes the opposite orientation also occurs [2, 3]. Hydrogen bonding with the OH-groups of the rims may provide a significant contribution to the stabilization of the complexes [3, 4].

In the past decade several ionizable and permanently ionic derivatives have been synthesized, providing modified complexing abilities [5]. If the host or the guest molecules have a charge, electrostatic interactions gain increased importance. On the other hand, the substituents may cause steric hindrance against the inclusion [6].

In the (2-hydroxy-3-*N,N,N*-trimethylammonium)propyl-β-cyclodextrin chloride (quaternary ammonium β-cyclodextrin, QA-β-CD) the substituents bear permanent positive charges independently of the pH of the medium, and can provide strong electrostatic interactions with anionic guests. The degree of substitution (DS) and the substitution pattern of the derivatives can be different, depending on the circumstances of the preparation [7], and this may influence the complex formation properties significantly. In most cases the product is a mixture of derivatives with various substitution degrees. The average degree of substitution can be determined by ^{1}H-NMR method from the

ratio of the integrals of the trimethylammonium and C1 hydrogen signals.

Quaternary ammonium β-cyclodextrin is used mainly in the capillary electrophoresis as a chiral selector. It can be applied both in aqueous and nonaqueous media to separate the enantiomers of anionic, neutral and cationic analytes as well, among others amino acid derivatives and oligopeptides [8–10]. In spite of the wide application, well defined stability constants for the complexes are rarely available. The values obtained from electrophoresis measurements are apparent ones valid for the given buffer medium. Nevertheless, it is important to know the general behaviour because cyclodextrins are used not only in the separation sciences, but also in pharmaceutical and food industry [11–13].

The complex formation of amino acids with different cyclodextrins is especially interesting from the point of view of the applications in biological systems. The stability of the associates can be the result of various effects: the hydrophobic part can enter the cavity, while the hydrated amino acid moiety remains outside of the ring and can take part in electrostatic and hydrogen bonding interactions, depending on the different ionization states according to the pH of the solution. On the other hand, the enantiomers of these compounds permit of the investigation of chiral recognition.

Some data for the complex formation of β-cyclodextrin with amino acids can be found in the literature. The stoichiometry of the complexes has been found to 1:1, no associates of other ratios were detected with ESI–MS technics in the presence of different concentration ratios of the components [14]. The stability constants available in the literature spread over a wide range [15–21]. The main reason is that when determined at a given pH, the results may be average values of the stability constants of the differently protonated forms, but even for nearly neutral solutions of L-phenylalanine values between 85 and 3 can be found [15, 17–20, 22]. Very few data are available for tryptophan and for the D-enantiomers. The only consistent series of investigations covering the differently protonated forms of both enantiomers of several amino acids have been reported by Kahle and Holzgrabe [22], but their data are all substantially higher than those from other works, probably due to an approximation applied in the evaluation of the experimental data. No data can be found for the complexes with quaternary ammonium β-cyclodextrin.

Recently Chisholm and Wenzel [23] have dealt with the chiral discrimination of a large series of substrates, among others aromatic amino acids, with substituted cyclodextrins bearing 2-hydroxy-3-*N,N,N*-trimethylammonium substituents. In their case the substitution pattern was different and the average degree of substitution was much higher than in our work, and they did not deal with stability constants.

In the present work the interaction of β-cyclodextrin (β-CD) and quaternary ammonium β-cyclodextrin (QA-β-CD) with the L- and D-enantiomers of eight amino acids have been investigated, in order to reveal the role of the substituents in the complex formation. Individual stability constants for the complexes of the differently protonated species have been determined by spectrophotometric and pH-potentiometric methods. The results are supported by ^{1}H and ^{13}C NMR measurements.

Experimental

Materials

Cyclodextrins were obtained from Cyclolab Ltd. (Hungary) as a gift within the frame of a research collaboration. β-CD was used after recrystallization from hot water. QA-β-CD was used as obtained, the average degree of substitution, determined by ^{1}H NMR method, was 3.6 ± 0.3 per CD ring. The 2-hydroxy-3-trimethylamino-propyl substituents are attached mainly to the O(2) of the glucose units.

The investigated amino acids (L- and D-enantiomers from Serva and Sigma-Aldrich, respectively) were of analytical grade and used without further purification. The concentrations of the solutions were checked by the potentiometric titration.

All the other materials were commercial products of analytical grade and used without purification, except phenolphthalein which was recrystallized twice from an ethanol–water mixture.

Carbonate free NaOH-solution was prepared by the Sørensen method. All the solutions were made in doubly distilled water.

Deuterium oxide (99.9% purity) was purchased from Sigma-Aldrich.

Spectrophotometry

Spectrophotometric measurements were carried out with a Camspec M330 instrument in 10 mm cells, at 25 ± 0.5 °C temperature.

In alkaline medium phenolphthalein was used as indicator in a concentration of 3×10^{-5} M. The pH was adjusted to ~10.5 with 0.02 M Na_2CO_3. The concentration of the cyclodextrins varied stepwise from 0 to 2×10^{-4} M and that of the salts of amino acids (prepared in solution from weighed amounts of the acids by the addition of calculated amounts of NaOH) from 1×10^{-2} M to 5×10^{-2} M. The ionic strength was set to 0.25 M by the addition of NaCl. The absorbances were measured at $\lambda = 550$ nm.

Potentiometric measurements

Aliquots of 1.8×10^{-2}–1.0×10^{-1} M solutions of the salts of amino acids (freshly prepared the same way as for the spectrophotometric method) were titrated with 0.25 M HCl solution under stirring with nitrogen, in the absence and in the presence of 0.01 M β-CD or 0.01–0.025 M QA-β-CD. The ionic strength was adjusted to 0.25 M by the addition of NaCl. The titration was performed with a Radiometer VIT90 type video titrator fitted with a Radiometer pHC2701-7 combined glass electrode and a Radiometer ABU93 type automatic burette. This system was calibrated using two different buffers (pH 2.05 and 7.07) and checked using a third buffer (pH 9.27). The solutions were thermostatted at 25 ± 0.1 °C during the titrations.

^{1}H and ^{13}C NMR measurements

Appropriate amounts of L-phenylalanine, L-tyrosine and the dried cyclodextrins were weighed and dissolved in D_2O. The final concentrations were as follows: β-CD: 0.01 M, QA-β-CD: 0.05 M, phenylalanine: 0.01–0.04 M and 0.025–0.1 M, respectively, so the molar ratios in the mixtures varied from 1:1 to 1:4 in the case of β-CD and from 1:0.5 to 1:2 with QA-β-CD.

The spectra have been recorded in neutral solutions and in the presence of 0.02 M Na_2CO_3 as well, on a Bruker DRX 500 NMR spectrometer. 20 ppm wide ^{1}H NMR spectra were recorded with standard single pulse program. 64 K data points were acquired after the RF pulse with 30° flip angle. 16 scans were accumulated using 1.0 s repetition delay. 240 ppm wide ^{13}C NMR spectra were measured using 30° transmitter flip angle and waltz16 gated proton decoupling sequence. 64 K data points were acquired with 2.0 s repetition delay. Typically 1 K scans were collected. The temperature was kept constant at 300 ± 0.1 K.

Results and discussion

Evaluation of the experimental data and stability constants from spectrophotometry and potentiometry

Amino acids can be present in differently dissociated or protonated forms depending on the pH of the solution and each of them may form complexes with the cyclodextrins. The equilibria are closely coupled, the complex formation may cause an apparent shift in the protonation constants, which appears as a shift of the potentiometric titration curves [22, 24, 25].

The equilibria and the corresponding equilibrium constants can be characterised in a general form (charges omitted) as:

$$\begin{aligned} &pH^+ + qA \; + \; rD \leftrightarrow H_pA_qD_r \\ &\beta_{pqr} = [H_pA_qD_r]/([H^+]^p[A]^q[D]^r) \end{aligned} \quad (1)$$

where A stands for the completely dissociated amino acid and β-CD is symbolized by D. Square brackets denote equilibrium concentrations in mol dm^{-3} (M) units.

The most common stoichiometry of the CD-complexes is 1:1 [1, 14], and this was confirmed in our experiments, too, so in this case $q = r = 1$.

If the completely dissociated form of a monovalent acid (e.g. phenylalanine) is taken as the basic species, the stepwise formation constants of the differently protonated complexes can be obtained as follows:

$$A^- + D \leftrightarrow A^- \cdot D \quad K_{011} = [A^- \cdot D]/([A^-][D]) = \beta_{011} \quad (2)$$

$$\begin{aligned} &HA \; + \; D \leftrightarrow HA \cdot D \\ &K_{111} = [HA \cdot D]/([HA][D]) = \beta_{111}/\beta_{110} \end{aligned} \quad (3)$$

$$\begin{aligned} &H_2A^+ + \; D \leftrightarrow H_2A^+ \cdot D \\ &K_{211} = [H_2A^+ \cdot D]/([H_2A^+][D]) = \beta_{211}/\beta_{210} \end{aligned} \quad (4)$$

The total concentrations can be expressed by means of the overall stability constants:

$$\begin{aligned} c_H &= [H^+] + [HA] + 2[H_2A^+] + [HA \cdot D] + 2[H_2A^+ \cdot D] \\ &= [H^+] + \beta_{110}[H^+][A^-] + 2\beta_{210}[H^+]^2[A^-] \\ &\quad + \beta_{111}[H^+][A^-][D] + 2\beta_{211}[H^+]^2[A^-][D] \\ &= \sum p\beta_{pqr}[H^+]^p[A]^q[D]^r \end{aligned} \quad (5)$$

$$\begin{aligned} c_D &= [D] + [A^- \cdot D] + [HA \cdot D] + [H_2A^+ \cdot D] \\ &= \sum r\beta_{pqr}[H^+]^p[A]^q[D]^r \end{aligned} \quad (6)$$

$$\begin{aligned} c_A &= [A^-] + [HA] + [H_2A^+] + [A^- \cdot D] + [HA \cdot D] \\ &\quad + [H_2A^+ \cdot D] = \sum q\beta_{pqr}[H^+]^p[A^-]^q[D]^r \end{aligned} \quad (7)$$

Knowing the total concentrations and the measured equilibrium concentration of hydrogen ions, the stability constants can be calculated using Eqs. 2–7 by an iterative computer program searching for the best fit between experimental data and calculated values [25].

In the case of tyrosine, glutamic and aspartic acid the starting form has two negative charges because the phenolic –OH or the carboxylic group of the side chain is also dissociated. In the presence of tyrosine the acidic range could not be investigated because of the poor aqueous solubility of the apparently neutral compound, so the term for the third protonation step was omitted, the stability constant of the completely protonated complex cannot be determined this way. On the other hand, in the case of histidine, containing a nitrogen in the side chain, a third protonation step is possible resulting in a cation with double positive charges.

The shifts caused by the addition of CD in the apparent dissociation constants of the acid and so in the measured changes in the H^+-concentration depend largely on the ratio of the complex formation constants of two consecutively protonated species [24, 25]. As a consequence, pH-potentiometry itself cannot provide reliable values for the individual constants, it is advisable to determine one of them by an independent method.

Competitive spectrophotometric method using phenolphthalein as indicator was applied in alkaline solutions as a supplementary tool for the determination of the complex formation constants of the anions. Details of the method and the evaluation are described in Refs. [26, 27]. QA-β-CD forms a complex with phenolphthalein similarly to the unsubstituted β-cyclodextrin, its stability constant has been found to $K = (1.42 \pm 0.06) \times 10^5\ M^{-1}$.

The stability constants obtained from the spectrophotometric measurements are collected in Table 1.

These data were used as starting values in the evaluation of potentiometric titrations. The protonation of most amino acid anions is negligible at pH 10.5 (it mounts to about 10% for leucine, glutamic and aspartic acid), so the complex formation constants obtained from spectrophotometry can be attributed mainly to the anion. In the case of tyrosine it must be considered that dianions and monoanions are present together in relative concentrations of approximately 68 and 31%, respectively [28], so the data of Table 1 are apparent, averaged values. Protonation constants of the free guests were taken from the literature [28] and refined in the evaluation process for the titration of the pure acids, to fit the applied conditions. The final results were in fairly good agreement with the literature values.

Table 1 Stability constants (K/M^{-1}) of the different cyclodextrin complexes obtained from the spectrophotometric measurements

	β-CD	QA-β-CD
L-Phe	107 ± 6	93 ± 8
D-Phe	100 ± 9	94 ± 5
L-Tyr[a]	105 ± 4	180 ± 10
D-Tyr[a]	111 ± 6	180 ± 10
L-Trp	85 ± 3	112 ± 8
D-Trp	88 ± 6	118 ± 12
L-Leu	21.5 ± 3.5	17.5 ± 3.0
D-Leu	24 ± 4	23.5 ± 3.0
L-Asp	4.0 ± 0.7	37 ± 10
D-Asp	4.0 ± 0.4	5.3 ± 1.3
L-Glu	6 ± 2	30 ± 10
D-Glu	2.5 ± 1.3	3.0 ± 1.5
L-His	2.8 ± 0.5	3.7 ± 1.0
D-His	4.5 ± 1.0	4.8 ± 0.9
L-Thr	2.1 ± 0.5	2.1 ± 0.7
D-Thr	~0	2.1 ± 0.3

[a] cca 68% A^{2-}, 31% HA^- and 1% $H_2A^{\pm}$

The individual stability constants obtained for the complexes of the differently protonated forms of amino acids are summarized in Table 2. Most results for the β-CD-complexes are in fairly good agreement with literature values [15, 17, 18, 29, 30].

Based on the spectrophotometric results (Table 1), the investigated amino acids can be classified into three types. The highest stability constants have been obtained for phenylalanine, tyrosine and tryptophan, in accordance with the expectations: the presence of the aromatic side group is especially favourable for the complex formation. The lowest stabilities are found for the smallest or most hydrophilic compounds as threonine, and aspartic acid, glutamic acid and histidine. Similar classification was suggested by Miertus et al., based on theoretical modelling of the interactions [31]. It must be noted that in the case of glutamic and aspartic acids the evaluation of the spectrophotometric measurements was complicated by the formation of phenolphthalein–amino acid anion–β-CD ternary complex.

Complex formation of the differently protonated species with β-CD

The most remarkable fact looking at the results obtained for the aromatic group (Table 2) is that the complex stability constants of the apparently uncharged forms and β-CD are surprisingly low compared to those of other aromatic compounds e.g. benzoic acid or phenol [4, 32]. On the other hand, the formation constants for the anionic complexes are much higher than those of most simple aromatic anions (benzoate, phenolate, except *p*-nitro-phenolate) [32]. This is opposite to the general trends of β-CD-complexes [33], though similar observations were made by other authors, too [6, 17, 22].

To find an explanation, hydration and hydrogen bonding possibilities with the CD must be considered. It is important to emphasize that the apparently neutral amino acid molecules are not really chargeless but exist predominantly in zwitterionic forms [34]. It seems that this, containing a proton donating $-NH_3^+$ and a proton acceptor $-COO^-$ group can be better accommodated in the bulk aqueous structure, which is reflected in the good water solubility of phenylalanine, and the strong hydration acts against inclusion. The dissociated form, with proton accepting functional groups only, may be less favourable for the hydration but favourable for the two-point formation of H-bonds with the alcoholic OH-groups of the CD. Compared to benzoate or phenolate or other anions, an essential difference is the longer distance between the charged group and the aromatic ring, so the former one need not be

Table 2 Stability constants (K/M^{-1}) of the different cyclodextrin complexes obtained from the potentiometric measurements

	$A^{2-} \cdot D$	$A^{-} \cdot D$ ($HA^{-} \cdot D$)[a]	$HA^{\pm} \cdot D$ ($H_2A^{\pm} \cdot D$)[a]	$H_2A^{+} \cdot D$ ($H_3A^{+} \cdot D$)[a]	$H_3A^{2+} \cdot D$
L-Phe + β-CD		116 ± 12	10 ± 3	6 ± 4	
D-Phe + β-CD		103 ± 14	10 ± 3	11 ± 5	
L-Tyr + β-CD	102 ± 10	84 ± 19	4.5 ± 2.5		
D-Tyr + β-CD	108 ± 12	120 ± 18	30 ± 9		
L-Trp + β-CD		86 ± 11	7 ± 3	2.5 ± 2.0	
D-Trp + β-CD		94 ± 13	19 ± 9	8 ± 7	
L-Leu + β-CD		21 ± 2	5 ± 4	3 ± 1.5	
D-Leu + β-CD		28 ± 3.5	~0	0.9 ± 0.4	
L-Asp + β-CD	3.4 ± 0.2	14 ± 1.5	11 ± 6	~0	
D-Asp + β-CD	11.3 ± 2.0	4.6 ± 0.3	1.5 ± 0.6	~0	
L-Glu + β-CD	5.7 ± 0.3	19 ± 2	13 ± 7	~0	
D-Glu + β-CD	2.7 ± 0.1	2 ± 1	1.1 ± 0.2	~0	
L-His + β-CD		3.3 ± 0.2	1.2 ± 0.5	0.6 ± 0.3	~0
D-His + β-CD		5 ± 1	5 ± 1	0.4 ± 0.1	~0
L-Thr +β-CD		2 ± 1	2 ± 1.5	2.5 ± 1.1	
D-Thr + β-CD		2.8 ± 0.6	0.9 ± 0.5	2.0 ± 1.2	
L-Phe + QA-β-CD		100 ± 7	0.9 ± 0.5	3 ± 2	
D-Phe + QA-β-CD		107 ± 9	1.5 ± 1.5	1 ± 1	
L-Tyr + QA-β-CD	188 ± 22	126 ± 9	8 ± 3		
D-Tyr + QA-β-CD	190 ± 16	140 ± 6	9 ± 3		
L-Trp + QA-β-CD		107 ± 16	6.5 ± 1.5	2.5 ± 2	
D-Trp + QA-β-CD		105 ± 14	11 ± 7	2 ± 2	
L-Leu + QA-β-CD		21 ± 4	~0	~0	
D-Leu + QA-β-CD		27.5 ± 3.5	~0	0.6 ± 0.4	
L-Asp + QA-β-CD	37.5 ± 3.0	31 ± 9	3 ± 2	~0	
D-Asp + QA-βCD	10.2 ± 0.8	8.8 ± 0.7	11.4 ± 1.0	1.2 ± 0.5	
L-Glu + QA-β-CD	30 ± 5	23 ± 5	18 ± 5	~0	
D-Glu + QA-β-CD	3.8 ± 0.1	0.7 ± 0.2	~0	~0	
L-His + QA-β-CD		3.1 ± 0.3	4 ± 2	3.5 ± 2	~0
D-His + QA-β-CD		5.0 ± 0.3	0.9 ± 0.1	0.7 ± 0.5	0.7 ± 0.2
L-Thr +QA-β-CD		8.0 ± 1.5	4.8 ± 0.7	0.6 ± 0.3	
D-Thr + QA-β-CD		5.8 ± 1.4	4.1 ± 0.7	0.7 ± 0.1	

[a] For tyrosine, aspartic acid and glutamic acid

completely dehydrated to allow inclusion of the hydrophobic moiety.

The similarity of the values obtained for the different aromatic amino acids suggests that similar interactions are responsible for the complex formation: the dominating effect is the inclusion of the aromatic ring. In the case of tyrosine and unsubstituted β-CD, the stabilities of HA^{-}- and A^{2-}-complexes are very similar. It means that in this case the phenolic OH-group does not provide any significant contribution to the stability of the complexes, independently of its ionization state.

The complexes of the completely protonated forms are characterized by very low stability constants, which is in line with the common trends [33]. It must be noted that the contribution of these species is relatively low in the accessible pH-range (pH $\geq$ 2, while the corresponding protonation constants are ~200), so the pH-potentiometric method is less sensitive to these forms and the uncertainty of the values is higher.

Somewhat similar is the situation with leucine: the isobutyl side chain ensures appropriate space filling in the cavity.

It is worth mentioning that the difference between the complex formation of the anionic and neutral forms of the amino acids without aromatic groups is less pronounced than for the aromatic ones, moreover, in some cases (L-aspartic and glutamic acid) higher values have been found for the latter with β-CD. It shows that in the case of the smaller molecules, when the space filling is not so favourable and ensures weak inclusion only, the

contribution of other interactions with the more or less polar groups become predominant.

Comparison of β-CD and QA-β-CD

In most cases there is no remarkable difference between the complex formation with unsubstituted β-CD and QA-β-CD, although a strong electrostatic interaction, increasing the stability might be expected with anionic guests. It can be considered, however, that the positive charges are located on rather bulky substituents which can cause a considerable steric hindrance, depending on the conformation. These two opposite effects can compensate for each other and result in the nearly unchanged complex stability. A further explanation can be that the QA-β-CD molecule must be more flexible than β-CD because of the loss of intramolecular H-bonds between the secondary OH-groups of neighbouring glucose units. This may provide a possibility to diminish steric hindrance of the substituents, but, at the same time, decreases van der Waals contact inside the cavity.

A significant increase of the stability constants as compared to the native β-CD has been found only in the case of the anionic forms of tyrosine, L-aspartic acid and L-glutamic acid with QA-β-CD. This can be understood considering that in these cases hydrogen bonding is possible with both rims of the CD, in addition to the increased electrostatic attraction. This effect can overcompensate the steric hindrance of the substituents, resulting in deeper penetration of the guest molecule.

It is not surprising that QA-β-CD forms less stable complexes with the zwitterionic or cationic forms: the presence of positive charges on the guest is electrostatically unfavourable.

Differences between the enantiomers (?)

Concerning the enantioselectivity, as the data show, no major difference can be found between the complex formation of L- and D-enantiomers, not even in the case of tryptophan, in contrast to the results reported in Ref. [22]. The authors also admit that the enantiomer selection of tryptophan was not confirmed with chromatographic results. Some minor deviations are seen for the zwitterionic forms of tyrosine and tryptophan and for the monoanion of tyrosine, but—except tyrosine and β-CD—even these approach the limit of errors. Similarly small chiral discrimination was found for acetylated aromatic amino acids with native and aminated (ammonium) β-CD—the most significant for tryptophan [3, 6], and similarly for methyl esters of amino acids with 2-hydroxypropyl-β-CD [35]. Chisholm and Wenzel found no difference in the NMR signals of the enantiomers in the presence of β-CD, they found a very small discrimination with the substituted β-CD for histidine and the most expressed for tyrosine, especially in the methylene signals [23].

The most significant differences can be observed at aspartic and glutamic acids—containing a carboxylic group in the side chain—where higher values have been obtained for the L-enantiomers. The explanation can be similar to that mentioned previously: the relatively more significant contribution of the interactions between the chiral groups and the rims of the CD. From the pont of view of the application of cyclodextrins in the separation sciences it must be noted, however, that following of the nature of the chromatographic (and electrophoretic) processes the effect of small differences in the complex formation constants can be multiplicated and result in fairly good separation.

Results of ^{1}H and ^{13}C NMR experiments

The most surprising results of the potentiometric measurements were that the stability constants of the complexes with the neutral forms of the aromatic amino acids were unexpectedly low while those for the anions were much higher, and, on the other hand, that the equilibrium constants obtained with QA-β-CD were not much different in most cases.

^{1}H and ^{13}C NMR measurements were performed to get additional information concerning the surprising results obtained from the spectrophotometry and potentiometry and to gain deeper insight into the nature of the complex formation.

The equilibria are fast relative to the NMR time scale, so only averaged signals for the free and complexed components can be observed.

The most characteristic parts of the ^{1}H spectra are shown in Figs. 1, 2, and 3, and some data of the changes in ^{13}C chemical shifts are collected in Table 3. The shifts of some more interesting signals versus molar ratios of the components are demonstrated in Figs. 4, 5, and 6.

It is a generally accepted fact [36, 37] that when an aromatic guest is included in the CD-cavity, the signals of the host H3 and H5 protons, pointing inside, suffer a significant upfield shift ($\Delta\delta = \delta_{\text{average}} - \delta_{\text{free}}$ is negative). The ^{1}H shifts of the guest may be different, either positive or negative, depending on the position of the protons within the cavity [38] and the changes are often too small to allow definite conclusions [37, 39].

The interpretation of the ^{13}C signals is even more difficult. Generally the signals of carbon atoms which are deeper in the cavity undergo upfield shifts while those closer to the wider opening are shifted downfield, but these changes are usually small and more sensitive to conformational distortions [37]. Concerning the signals of the CD, large negative shifts are attributed to the

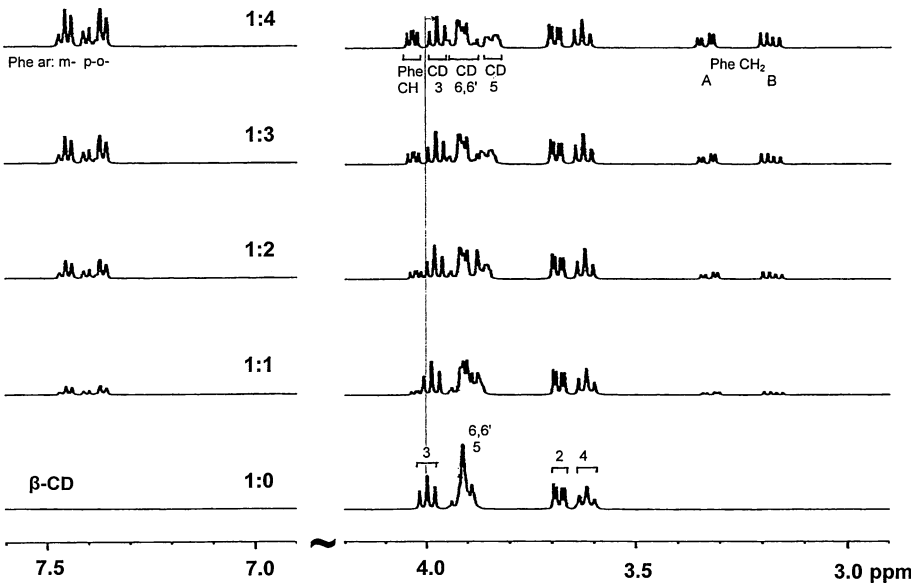

Fig. 1 ^{1}H NMR spectra recorded in neutral solutions of L-phenylalanine (Phe) and β-cyclodextrin of different CD:Phe ratios as indicated on the *lines*

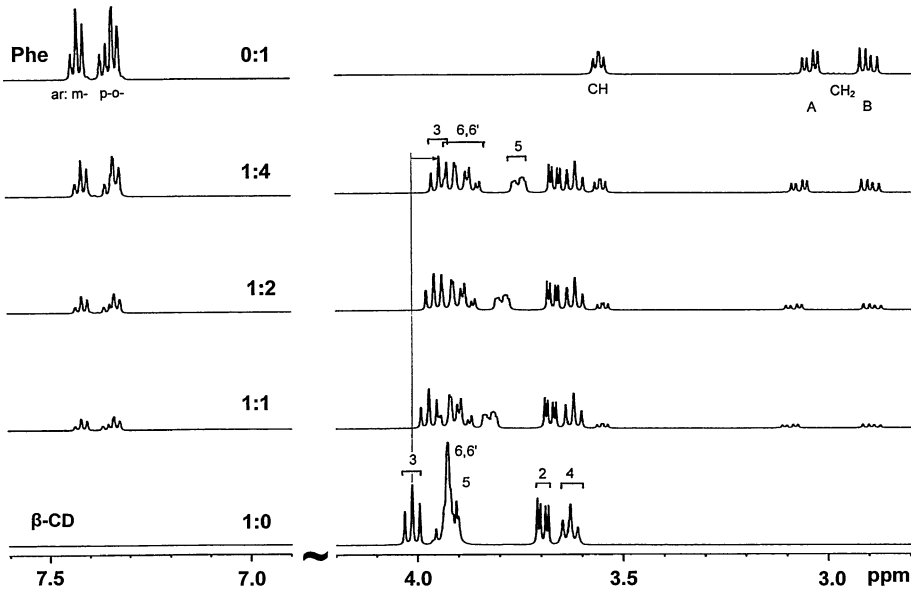

Fig. 2 ^{1}H NMR spectra of the L-phenylalanine–β-CD systems in alkaline solutions (CD:Phe ratios are indicated on the *lines*)

neighbourhood of aromatic rings or other groups with high electron density [40]. It must be noted that the assignment of the carbon signals of the CDs is not unanimous in the literature. Several authors determine the order of decreasing chemical shifts as corresponding to C3 $\gg$ C5 > C2 [e.g. 7], but according to Schneider et al. [37] the sequence is: C3 $\gg$ C2 > C5 and according to Yoshida et al. [40]: C2 $\gg$ C3 > C5.

The L-phenylalanine—β-CD systems

The most remarkable changes in the ^{1}H spectra recorded in neutral solutions (Fig. 1) are the upfield shifts of H3 (4.02–3.94 ppm) and H5 (3.94–3.80 ppm) signals of the β-CD on increasing concentration of phenylalanine, thus verifying the inclusion of the aromatic ring. The comparison with the spectra obtained in alkaline solutions (Fig. 2) justifies the results of pH-potentiometry: similar but much bigger shifts of the same signals can be observed, according to the higher stability of the complex.

The other important conclusion of the NMR results is that the amino acid moiety plays a determining role in the interactions. Relatively big shifts can be observed in the ^{1}H and ^{13}C signals of the CH_2-group (Figs. 4, 5), in the ^{13}C signals of the CH and first of all in that of the $-COO^{-}$ (Fig. 5; Table 3). The shifts of the aromatic moiety are mostly small. The CH_2-protons of phenylalanine are non-equivalent because of the proximity of amino and carboxylate groups. It is very interesting that the difference decreases with increasing CD/Phe ratio in neutral medium,

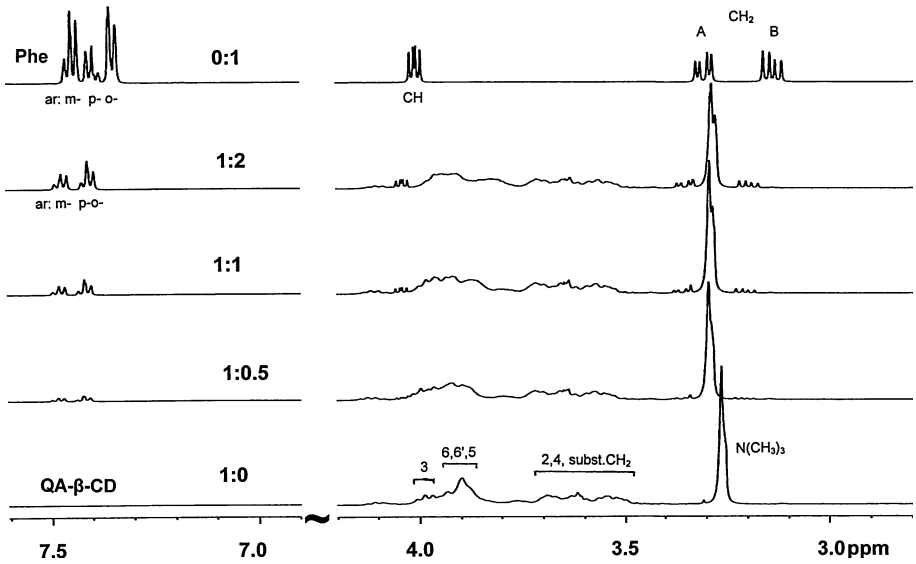

Fig. 3 ^{1}H NMR spectra of the L-phenylalanine–QA-β-CD systems in neutral solutions (CD:Phe ratios are indicated on the *lines*)

Table 3 ^{13}C NMR chemical shifts of L-phenylalanine (ppm)

Signal	Medium	δ_{free}	$\delta_{average} - \delta_{free}$					
			L-Phe: β-CD			L-Phe: QA-β-CD		
			4:1	2:1	1:1	2:1	1:1	0.5:1
COO^-	Neutral	174.33	−0.06	−0.07	[a]	−0.145	−0.150	−0.100
	Alkaline	182.65	−0.26	−0.33	−0.41	−0.92	−1.06	−1.18
CH_2	Neutral	36.74	+0.05	+0.09	[a]	+0.23	+0.29	+0.35
	Alkaline	41.03	+0.22	+0.37	+0.42	+0.52	+0.85	+0.94
CH	Neutral	56.42	+0.005	+0.010	+0.015	+0.11	+0.13	+0.14
	Alkaline	57.82	+0.05	+0.10	+0.12	+0.11	+0.24	+0.29
C1	Neutral	135.50	+0.025	+0.035	[a]	+0.22	+0.23	+0.29
	Alkaline	138.65	+0.015	+0.045	+0.060	+0.16	+0.31	+0.35
C2,6	Neutral	129.75	+0.010	+0.005	+0.005	+0.10	+0.09	+0.08
	Alkaline	129.84	−0.06	−0.12	−0.14	−0.16	−0.26	−0.29
C3,5	Neutral	129.50	−0.040	−0.050	−0.055	−0.01	−0.03	−0.05
	Alkaline	129.01	−0.03	−0.07	−0.08	−0.04	−0.06	−0.07
C4	Neutral	128.08	−0.020	−0.030	−0.035	−0.02	−0.02	−0.03
	Alkaline	127.06	~0	~0	+0.01	+0.07	+0.07	+0.09

[a] The signal is too small to be clearly identified

while an opposite change can be observed in alkaline solutions. This can be an indication of strong H-bonding interactions of amino and carboxylate groups with OH-groups of the CD, causing a hindrance against rotation. In neutral medium, however, an intramolecular H-bond is possible between the $-NH_3^+$ and $-COO^-$-groups, either directly [16] or more probably by the insertion of a water molecule, and this can be strengthened when the molecule is included in the CD-cavity. This concept is supported also by the changes in the ^{13}C signals of the $-COO^-$-group: large upfield shift in alkaline medium (Table 3) and a much smaller one in neutral. Most probably the hydrophilic part of the amino acid interacts with the secondary OH-groups at the wider rim of the ring. Similar interaction was suggested by Linde et al. [13], though they neglected the zwitterionic structure or dissociation of the carboxylic group. The role of H-bonding with the secondary OH-groups is supported also by Inoue et al. [41] and by Chisholm and Wenzel [23]. However, the opposite orientation cannot be excluded either. Sompornpisut et al. suggest the possibility of both arrangements on the basis of molecular docking studies [20]. Hydrogen bonding is also possible with the primary OH-groups of the CDs.

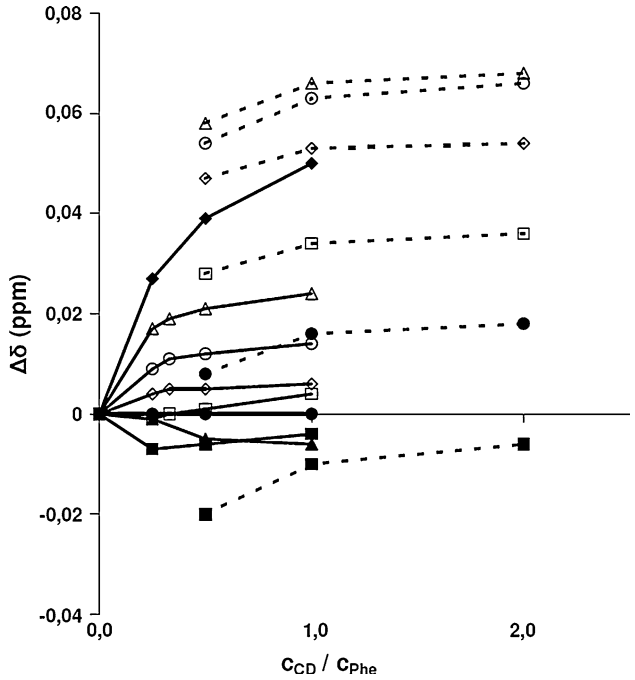

Fig. 4 Chemical shift changes ($\Delta\delta = \delta_{average} - \delta_{free}$) of some more interesting ^{1}H signals of L-phenylalanine with increasing CD-concentration. *Open symbols* refer to neutral solutions, *full symbols*: alkaline solution; *symbols* connected with continuous line mean β-CD, with *dashed line*: QA-β-CD; *diamond*: CH_2A, *triangle*: CH_2B, *circle*: Ar-ortho, *square*: Ar-meta. (*symbols* for CH_2 protons in alkaline medium of QA-β-CD fall outside the range of the graph)

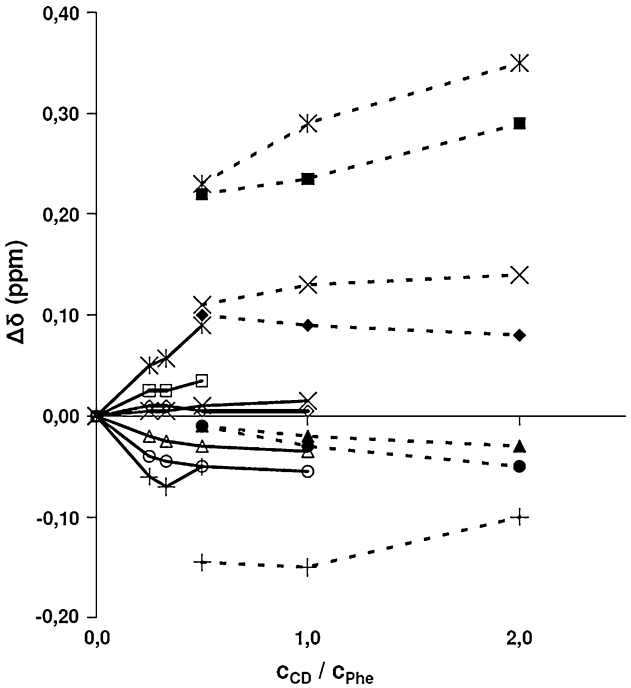

Fig. 5 Chemical shift changes of some more interesting ^{13}C signals of L-phenylalanine with increasing CD-concentration in neutral solutions. *Open symbols* connected with continuous lines refer to β-CD, *full symbols* with *dashed lines* mean QA-β-CD. *Plus*: COO^-, *times*: CH, *zhe* CH_2, *square*: Ar–C1, *diamond*: C2,6, *circle*: C3,5, *triangle*: C4

L-phenylalanine and QA-β-CD

The comparison of ^{1}H NMR signals of β-CD and QA-β-CD is not so simple because the substituted cyclodextrin is a mixture of molecules with different degrees of substitution and with different relative positions of the substituents, therefore the H3–H4 region of the ^{1}H spectrum is very complex and is in overlapping with the CH_2-signals of the substituents (Fig. 3). The ^{13}C spectra also contain broad, often multiplicated signals. Even though it seems that the behaviour of the H3 protons is similar. The ^{1}H and ^{13}C chemical signals of the L-phenylalanine change in a similar manner but to different extent in cases of the two cyclodextrins (Figs. 4, 5). It is an apparent contradiction that the changes of the signals belonging to the amino acid moiety and also of the aromatic C1 carbon are much larger than in β-CD, while the stability constants found by the potentiometric method are nearly identical. However, in the case of most aromatic signals the differences are small and the negative shifts of the meta and para carbons are smaller in neutral solutions (Fig. 5). These results suggest the formation of the inclusion complex, but with less deep penetration in QA-β-CD. The large shifts of the CH_2–, CH– and COO^--signals of phenylalanine can be attributed to steric effects caused by the substituents and to the proximity of positive charges, and just therefore the penetration cannot be so deep in QA-β-CD, thus explaining the similar stability constants.

It is surprising that chemical shifts of both ^{13}C and ^{1}H signals of the $-N(CH_3)_3$-group are negligible in basic medium. It can be explained considering that in alkaline solutions, in the absence of other guest, the positively charged groups can be in close contact with counter ions of the solution (chloride, hydroxide, carbonate), and in the complex these are partially exchanged for the carboxylate.

In neutral solutions of QA-β-CD and phenylalanine the $\Delta\delta$ values of most ^{1}H signals of the host (Fig. 6) (and also those of the phenylalanine C**H** and **C**OO^-) do not change monotonously as a function of the concentration ratio but pass over a maximum. This can indicate the existence of a 2:1 complex in the presence of excess cyclodextrin. However, it did not influence the potentiometric and spectrophotometric results because in those experiments the amino acid was always in excess.

The systems containing L-tyrosine

The conclusions drawn in the precedings for the complex formation of tyrosine also are supported by the NMR experiments made in alkaline solutions.

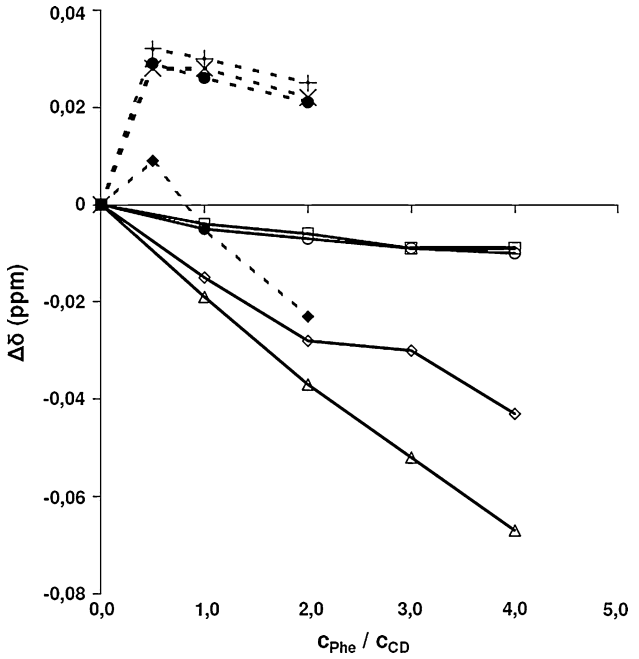

Fig. 6 Chemical shift changes of some more interesting ^{1}H signals of the cyclodextrins with increasing concentration of L-phenylalanine in neutral solutions. *Open symbols* connected with continuous lines refer to β-CD, *full symbols* with *dashed lines* belong to QA-β-CD. *Circle*: H1, *square*: H2, *diamond*: H3, *triangle*: H5, *times*: CH of the substituent of QA-β-CD, *plus*: NMe

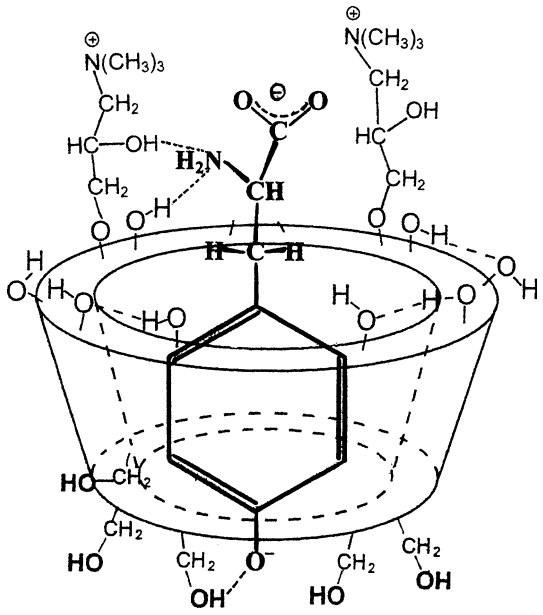

Fig. 7 Possible structure of the anionic complex of L-tyrosine and QA-β-CD (for the sake of clarity, only two of the substituents are represented and also some of the OH-groups are omitted)

The behaviour of the ^{1}H and ^{13}C signals of the CDs are similar in character as in the presence of phenylalanine, just the shifts are somewhat larger for the H1, H2 and H4. The most remarkable difference is the more expressed splitting and shift of the signals belonging to the –C(6)H_2 group.

The changes of ^{1}H and ^{13}C signals belonging to the amino acid moiety are similar but larger than those of phenylalanine, especially in the presence of QA-β-CD. At the molar ratio of Tyr:CD = 1:1, the ^{1}H $\Delta\delta$ values of the CH_2-group are +0.085 ppm and −0.081 ppm for the A and B signal groups, respectively, with β-CD and +0.218 ppm and −0.197 ppm with QA-β-CD, and significant upfield shift can be observed also in the C**H**-signal. In the presence of QA-β-CD $\Delta\delta = 1.4$ ppm for the **C**H_2 and −1.35 ppm for the carboxylate. In contrast to phenylalanine, significant negative shifts have been found for all ^{1}H and ^{13}C signals of the aromatic ring except the C1 carbon. Especially remarkable is the change of the signal belonging to C4 to which the phenolate –O^- is attached: $\Delta\delta = -0.4$ ppm in the presence of 1:1 molar ratio of QA-β-CD.

These results confirm the deeper insertion of the phenolate ring of tyrosine in the CD cavity and the stronger interaction between the functional groups of the amino acid anion and the primary alcoholic group(s) of the CD.

A possible structure of the complex of L-tyrosine with QA-β-CD is drawn in Fig. 7.

Conclusions

The complex formation behaviour of aromatic amino acids is different from other organic acids with aromatic groups: the most stable complexes are formed with the anions while the stability of the apparently neutral forms is very low both with β-CD and with QA-β-CD. The explanation can be found in the strong hydration of the zwitterionic species and the possibility of intramolecular hydrogen bonding within the guest. The amino acid moiety plays an important part in the interactions, most probably via hydrogen bonding with the OH-groups of the CD, and inclusion is helped by the longer distance between the polar group and the aromatic ring.

From the similarity of the stability constants (except tyrosine) obtained with the two cyclodextrins it can be concluded that in the case of QA-β-CD the electrostatic attraction of opposite charges is compensated for by steric hindrance and decreased van der Waals interaction owing to less deep penetration in the cavity and higher flexibility of the host. The higher stability found for the dianion of tyrosine can be explained by the fact that hydrogen bonds are possible with both rims of the CD at the same time, in addition to the effect of increased negative charge.

The amino acids without aromatic groups form complexes of low stability, and the contribution of interactions

between the more or less polar groups and the rims of the CD become more important.

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