# NMR Spectroscopic and Molecular Modelling Studies on Cyclodextrin–Dipeptide Inclusion Complexes

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Cyclodextrins (CDs) are often used to separate the enantiomers of chiral drugs by HPLC, GC, and capillary electrophoresis (CE). For dipeptides containing one aromatic amino acid, a reversal of the migration order is observed in CE upon increasing the pH value of the background electrolyte from 2.5 to 3.5. In order to understand the mechanism of chiral recognition of this phenomenon, NMR experiments, namely complexation-induced chemical shifts (CICS) and ROESY, were performed. Comparing the CICSs obtained for the pairs Diac-β-CD/Ala-Phe and HDAS-β-CD/Ala-Phe and Diac-β-CD/Ala-Tyr and HDAS-β-CD/Ala-Tyr revealed very small changes, thereby indicating a rather weak interaction of the respective guest molecules with their host. The CICS for β-CD and HS-β-CD confirmed an inclusion of the aromatic moiety into the CD cavity. It is shown that, at pH 2.5, the D,D-enantiomer of Ala-Tyr immerses deeper into the cavity of β-cyclodextrin than the L,L-enantiomer, and this was subsequently confirmed by molecular dynamics simulations. Furthermore, at pH 3.5 the immersion is shallower, as shown by the $^1$H and ROESY NMR findings and confirmed by the molecular dynamics simulations. The immersion depths of the dipeptides were calculated by defining a plane in the cavity to gain information about the different behaviour of the studied chemical species. The employed procedure is easily generalised to other host-guest complexes and is expected to improve data evaluation in other cases, too.


## Introduction

Native cyclodextrins are produced by the fermentation of starch with *Bacillus macerans* amylase.[1] Cyclodextrin derivatives may be chemically modified for various purposes. Derivatisation of the hydroxyl groups leads to a more hydrophobic or hydrophilic behaviour. In addition, the cyclodextrins can be converted to branched, polymeric, noncyclic or chromophore-modified products.[2]

Cyclodextrins are able to form inclusion complexes, with organic or inorganic molecules acting as guests inside the cavity.[2] Due to their chiral cavity, they are commonly used as chiral selectors in HPLC, GC and capillary electrophoresis (CE). A careful choice of the appropriate cyclodextrin or cyclodextrin derivative makes countless enantioseparations possible.[3–5] In capillary electrophoresis, negatively charged chiral selectors play an important role because in the normal separation mode at acidic pH they migrate in the opposite direction to the analyte and, as a consequence, provide more interaction possibilities than the native and uncharged cyclodextrins.

Systematic capillary electrophoretic studies on separating the enantiomers of various dipeptides with a great range of native and derivatised cyclodextrins at different pH values have already been reported.[6–11] When separating the enantiomers of, for example, Ala-Phe or Ala-Tyr, a reversal of the migration order of the enantiomers with a β-cyclodextrin (β-CD)-containing buffer occurred when changing the buffer pH from 2.5 to 3.5.[6,12] With the single-isomer derivatives heptakis-6-sulfo-β-cyclodextrin (HS-β-CD), heptakis-2,3-diacetyl-β-cyclodextrin (Diac-β-CD) and heptakis-2,3-diacetyl-6-sulfo-β-cyclodextrin (HDAS-β-CD), no reversal of the migration order was observed (Table 1).[7,8,11]

Table 1. Migration order of the Ala-Phe and Ala-Tyr enantiomers at pH 2.5 and 3.5 in the presence of 20 mg mL$^{-1}$ of the respective cyclodextrin.

| | β-CD | | HS-β-CD | |
|---|---|---|---|---|
| | pH 2.5 | pH 3.5 | pH 2.5 | pH 3.5 |
| Ala-Phe | ***L,L* > *D,D*** [a] | ***D,D* > *L,L*** | L,L > D,D | L,L > D,D |
| Ala-Tyr | ***L,L* > *D,D*** | ***D,D* > *L,L*** | L,L > D,D | L,L > D,D |
| | Diac-β-CD | | HDAS-β-CD | |
| | pH 2.5 | pH 3.5 | pH 2.5 | pH 3.5 |
| Ala-Phe | D,D > L,L | ns[b] | D,D > L,L | D,D > L,L |
| Ala-Tyr | ns | ns | D,D > L,L | D,D > L,L |

[a] The faster-migrating enantiomer is listed first. pH-dependent reversal of the migration order is indicated in bold italic. [b] ns: no separation.

The purpose of this study was to gain insight into the corresponding chiral recognition mechanism. The inclusion

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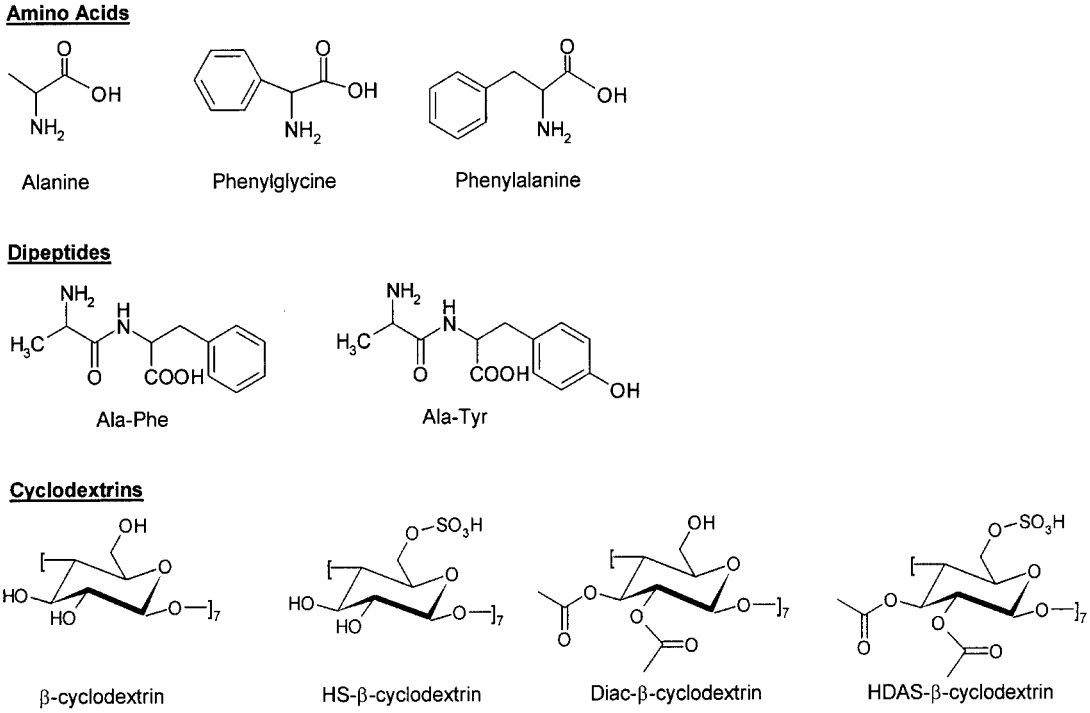


Figure 1. Compounds investigated.

complexes of β-CD and its single-isomer derivatives HS-β-CD, Diac-β-CD and HDAS-β-CD with alanyl-based aromatic dipeptides (Figure 1) were investigated by $^1$H NMR spectroscopy.

Special attention was given to the pH-dependent determination of complexation-induced chemical shifts (CICS) in aqueous buffers. In order to avoid large water signals in the $^1$H NMR spectra, either a presaturation water suppression method or lyophilised analyte solutions redissolved in deuterium oxide were used. Both methods were compared regarding commonalities and differences in the complexation-induced chemical shifts, and reliability. Furthermore, ROESY experiments and molecular dynamics simulations were carried out to gain more information about the respective inclusion mode.

## Results and Discussion

### $^1$H NMR Experiments

The CICS for the aqueous samples and the lyophilised samples redissolved in $D_2O$ are quite similar. However, for the dipeptide hydrogen H5, the signal in $D_2O$ is a doublet of doublets, as expected, whereas the same signal in $H_2O$, measured with a presaturation technique, is split more strongly and shows different coupling constants. Because this particular signal is located close to the water signal, it might be influenced by the saturation process of the water signal, which takes place with a power of 60 dB. Another possible explanation could be that the signals for the protonated nitrogen atoms, which are overlapped by the water signal, could be influenced by the saturation process and, thus, lead to distortion of the hydrogen H5 signal, which is very close to the nitrogen atoms in the molecular context. All other coupling constants remain the same in both solvents. However, the coupling constants of both dipeptide and cyclodextrin do not change upon complexation, thereby indicating that the conformation of either molecule remains the same upon complexation. This might be due to the fact that the cavity provides enough space for taking in the aromatic ring of the dipeptide.

A comparison of the CICS obtained for the pairs Diac-β-CD/Ala-Phe and HDAS-β-CD/Ala-Phe (data not shown) and Diac-β-CD/Ala-Tyr and HDAS-β-CD/Ala-Tyr (Figure 2) revealed very small changes, thus indicating a rather weak interaction of the respective guest molecules with their host. Thus, it is impossible to gain insight into the complex structure. The weak interaction is mirrored in the capillary electrophoresis results: with the exception of Ala-Phe at pH 2.5, no separation is observed in the case of Diac-β-CD complexes.[11]

#### *Influence on the Cyclodextrin Hydrogen Atoms*

For β-CD and HS-β-CD with Ala-Phe, the CICS for the cyclodextrin hydrogens H3′ and H5′ are quite high (Figure 3), thus indicating an inclusion of Ala-Phe into the cyclodextrin cavity. Since the CICS of H5′ are always higher than the CICS of H3′, the phenyl ring presumably dives deeply into the cavity.

HS-β-CD induces rather large changes in chemical shifts upon complexation with both dipeptides (even higher than for β-CD) and, along with this, an additional interaction for the hydrogen H1′, which is located at the wider rim of the cyclodextrin. At each pH value, the CICS for HS-β-CD

  

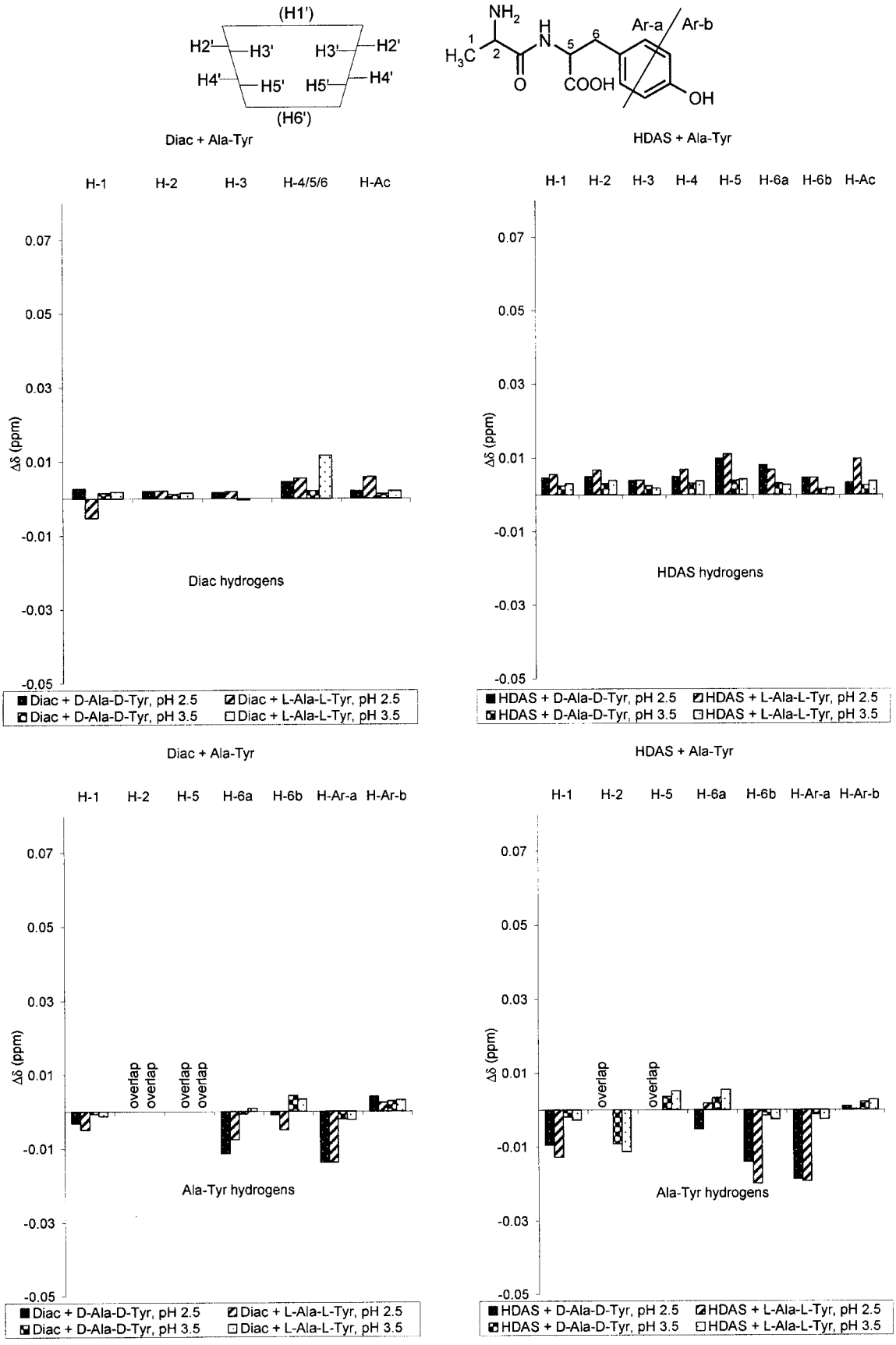


Figure 2. CICS for Ala-Tyr with Diac-β-CD and HDAS-β-CD and a scheme showing the CD and Ala-Tyr hydrogens.

  

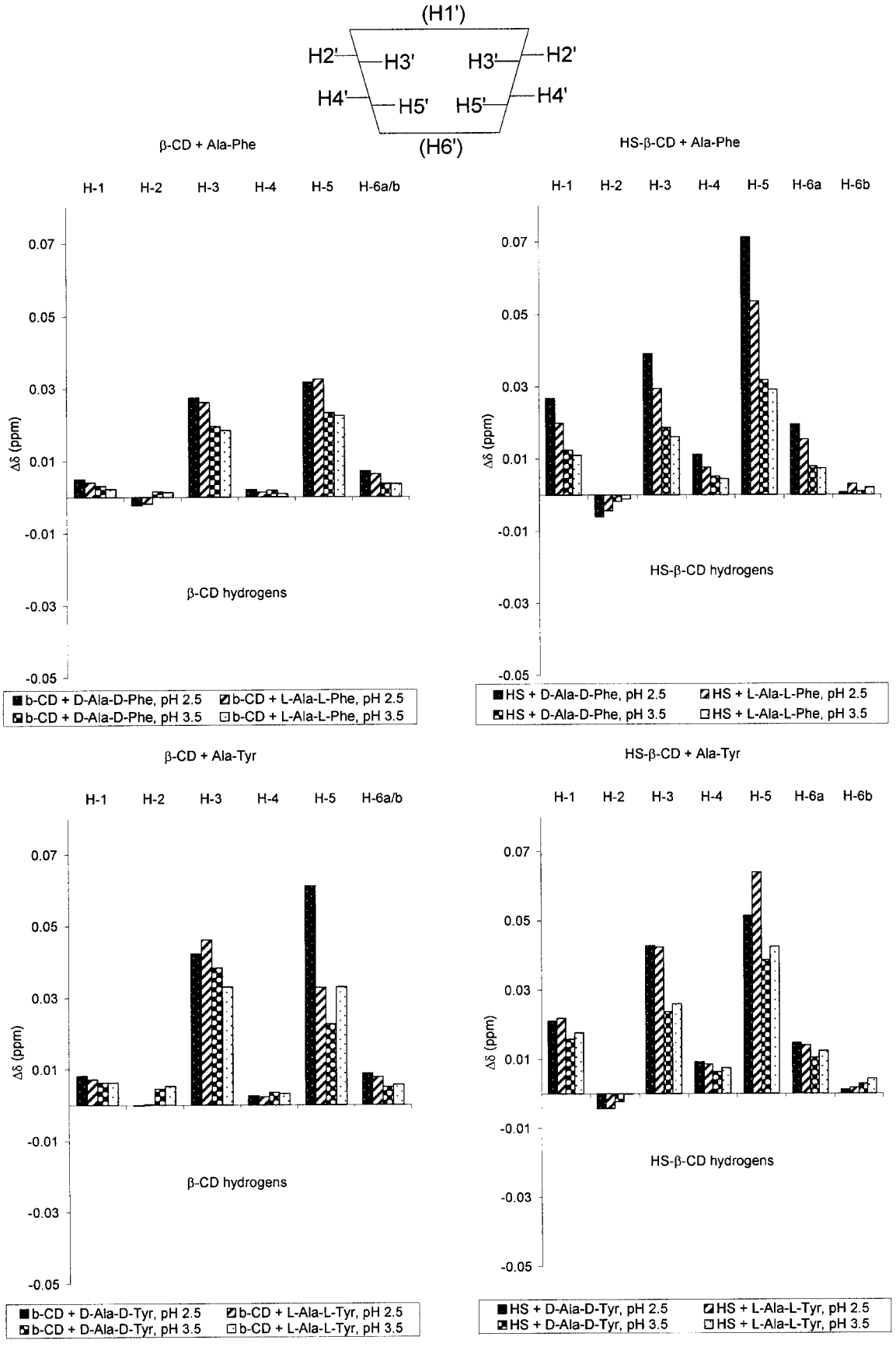


Figure 3. CICS for Ala-Phe and Ala-Tyr with β-CD and HS-β-CD and a scheme showing the CD hydrogens.

  

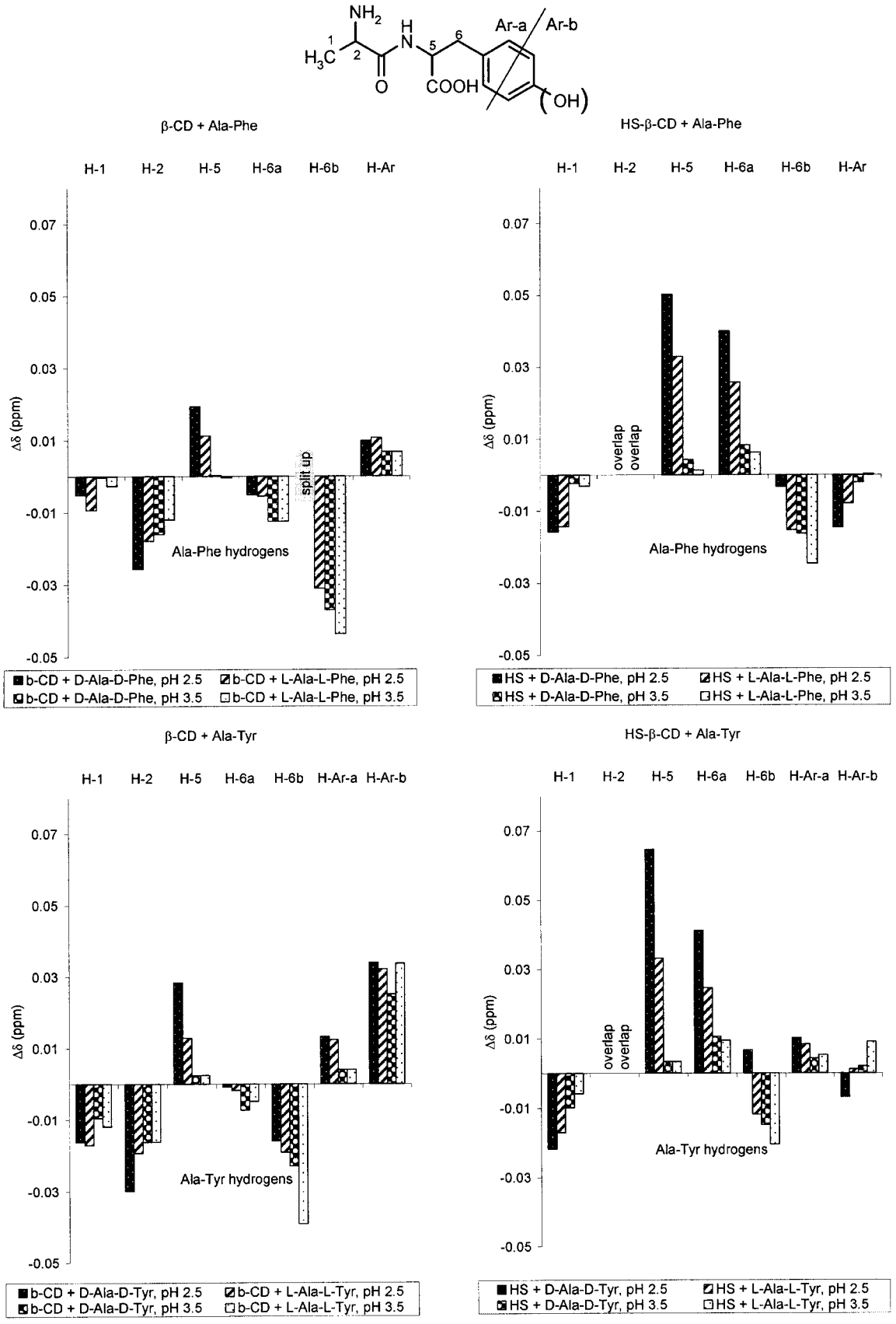


Figure 4. CICS for Ala-Phe and Ala-Tyr with β-CD and HS-β-CD and a scheme showing the dipeptide hydrogens.

  

are larger when interacting with the D,D-enantiomer of Ala-Phe, which indicates a stronger binding than for the L,L-enantiomer. With β-CD, the CICS are very similar for both enantiomers, so no statements about differences between the enantiomers are possible.

Upon complexation with β-CD or HS-β-CD, Ala-Tyr causes even higher CICS for the hydrogens inside the cyclodextrin cavity than Ala-Phe (Figure 3). As observed with Ala-Phe, HS-β-CD shows additional interactions with the hydrogen H1′. The CICS for HS-β-CD are very similar for both enantiomers, and only H5′ shows a stronger interaction with the L,L-enantiomer. A different picture emerges for β-CD: here, the CICS for hydrogen H3′ are higher for the L,L-enantiomer at pH 2.5 and higher for the D,D-enantiomer at pH 3.5. For hydrogen H5′, the opposite occurs: the CICS are higher for the D,D-enantiomer at pH 2.5 and higher for the L,L-enantiomer at pH 3.5. Considering that hydrogen H5′ is located deeper inside the cavity than H3′, one can conclude that at pH 2.5, the D,D-enantiomer of Ala-Tyr immerses deeper into the cavity, and at pH 3.5 the L,L-enantiomer immerses deeper into the cavity.

### *Influence on the Dipeptide Hydrogen Atoms*

Considering the Ala-Phe hydrogens, there are indeed differences between the enantiomers at both pH values and with both β-CD and HS-β-CD (Figure 4). Upon complexation with β-CD, the CICS for the D,D-enantiomer of Ala-Phe are larger for hydrogens H2 and H5. For the benzylic hydrogen H6-b, the CICS for the L,L-enantiomer are higher. With increasing pH, the CICS for the benzylic hydrogens increase and the CICS for the hydrogens of the aliphatic side-chain decrease, indicating stronger interactions with the phenyl part of the peptide. With HS-β-CD, the CICS of the benzylic hydrogens and hydrogen H5 of Ala-Phe are even more distinct than with β-CD, with a clear preference for the D,D-enantiomer, which seems to be bound more strongly.

Ala-Tyr shows large CICS for the aromatic and benzylic hydrogens upon complexation with β-CD (Figure 4) and high CICS for the benzylic hydrogens and hydrogen H5 upon complexation with HS-β-CD. At pH 2.5, the latter are higher for the D,D-enantiomer; thus, the D,D-enantiomer is bound more strongly to HS-β-CD than the L,L-enantiomer.

The CICS patterns of the dipeptide hydrogens of both Ala-Phe and Ala-Tyr look very much alike upon complexation with either β-CD or HS-β-CD; thus, a similar mode of inclusion occurs for both dipeptides with the respective cyclodextrin.

Since the CICS are generally larger at pH 2.5 than at pH 3.5, it can be concluded that the interaction is stronger at the lower pH value, leading to a shallower immersion into the cyclodextrin cavity at pH 3.5. The p$K_a$ value of the carboxyl group of both Ala-Phe and Ala-Tyr was measured to be 3.1, so at pH 2.5 there are 80% of the cationic species and 20% of the zwitterionic species existent. On the other hand, at pH 3.5 there are 70% of the zwitterionic species and 30% of the cationic species present. This leads to the conclusion that the cationic species provides stronger interaction possibilities along with more possible interaction sites than the zwitterionic species of the dipeptide.

For alanine, no significant CICS with either cyclodextrin could be observed (data not shown). For phenylalanine, the CICS were qualitatively the same, but less pronounced than those of Ala-Phe (data not shown). Comparing the CICS of the amino acid with the corresponding dipeptide reveals the dipeptide to be a stronger binder to β-CD and HS-β-CD and indicates that the extension of the aliphatic side-chain by a peptidic moiety seems to increase the number of possible interaction sites and results in larger CICS. Tyrosine, also a single amino acid associated with the dipeptides in question, was not investigated due to its poor solubility.

Phenylglycine, which shows no interaction with cyclodextrins in potentiometric studies,[13] was also investigated as a negative control and showed no significant CICS with either cyclodextrin (data not shown).

## ROESY Experiments

To gain further insight into the present chiral recognition mechanism, 2D ROESY experiments were carried out to study the interactions of the D,D- and L,L-enantiomers of Ala-Tyr with β-CD and HS-β-CD, respectively, at pH 2.5 and 3.5. Ala-Tyr was chosen because it produces two doublets for the aromatic hydrogens, caused by *para* substitution, which can easily be assigned: the hydrogens Ar-a and Ar-b (Figure 5) each appear as a doublet, which offers additional interpretation possibilities on the orientation of the aromatic ring. In contrast, Ala-Phe shows an unresolved multiplet for the aromatic hydrogens, which is difficult to analyse.

Additional interactions between the benzylic hydrogens of D-Ala-D-Tyr and the cyclodextrin hydrogens H2′ and H4′, which are located outside the cavity, are rather common and are generally explained as being due to the occurrence of a dipolar transfer via their J coupling to H3′ and H5′ of the cyclodextrin rather than to the formation of an external host-guest complex.[14]

Strong cross signals between the aromatic hydrogens of Ala-Tyr and the hydrogens H3′ and H5′ (Figure 5) of both cyclodextrins, which are located inside the cavity, confirm an inclusion of the aromatic moiety into the cyclodextrin cavity.[15] Ala-Tyr penetrates the cavity at the wider rim with the phenyl ring first. This is shown by the fact that the cross-signals between Ar-b (Ala-Tyr) and H6′ (β-CD) are larger than the cross-signals between Ar-a and H6′ (β-CD).

For β-CD, the cross-signals between Ar-a (Ala-Tyr) and H3′ (β-CD) are larger than the cross signals between Ar-b (Ala-Tyr) and H3′ (β-CD) and the cross-signals between Ar-b (Ala-Tyr) and H5′ (β-CD) are larger than the cross-signals with H3′ (β-CD). These facts additionally confirm the penetration of the cyclodextrin cavity at the wider rim with the phenyl ring of the dipeptide first.

Increasing the pH from 2.5 to 3.5 leads generally to weaker cross-signals of the aromatic dipeptide hydrogens

  

with H5′ (β-CD). It is possible that the aromatic moiety does not penetrate the cyclodextrin cavity as deeply at pH 3.5 as at pH 2.5, which is in accordance with the CICS findings.

At pH 2.5, the D,D-enantiomer immerses deeper into the cavity than the L,L-enantiomer, as shown by the weak interactions with H3′ (β-CD), which is located above H5′ (β-CD), and the strong interactions of the aromatic hydrogens with H5′, which is located deeper inside the cavity. Furthermore, there are stronger cross-signals for the benzylic hydrogens with the hydrogens located inside the cavity, which confirms the deeper penetration of the β-CD cavity by the D,D-enantiomer. At pH 3.5, there are virtually no differences between the enantiomers. This can be confirmed by capillary electrophoresis experiments, which lead to no separation of the enantiomers of Ala-Tyr at pH 3.5 in a β-CD-containing buffer.[16]

The cross-signals occurring upon complexation with HS-β-CD are somewhat difficult to interpret. They are not consistent with a deeper inclusion of either enantiomer, although small differences between the enantiomers occur at both pH values. Thus, the complexation behaviour of Ala-Tyr with HS-β-CD cannot be derived.

### Molecular Dynamics Simulations

To gain further insight into the binding modes of the enantiomers of Ala-Phe and Ala-Tyr to β-CD, molecular dynamics (MD) simulations were carried out. The simulations were performed with each enantiomer of Ala-Phe and Ala-Tyr at every possible state of protonation, i.e. cation, zwitterion and anion (see Figure 6). Since the p$K_a$ value of the carboxyl group of both Ala-Phe and Ala-Tyr was mea-

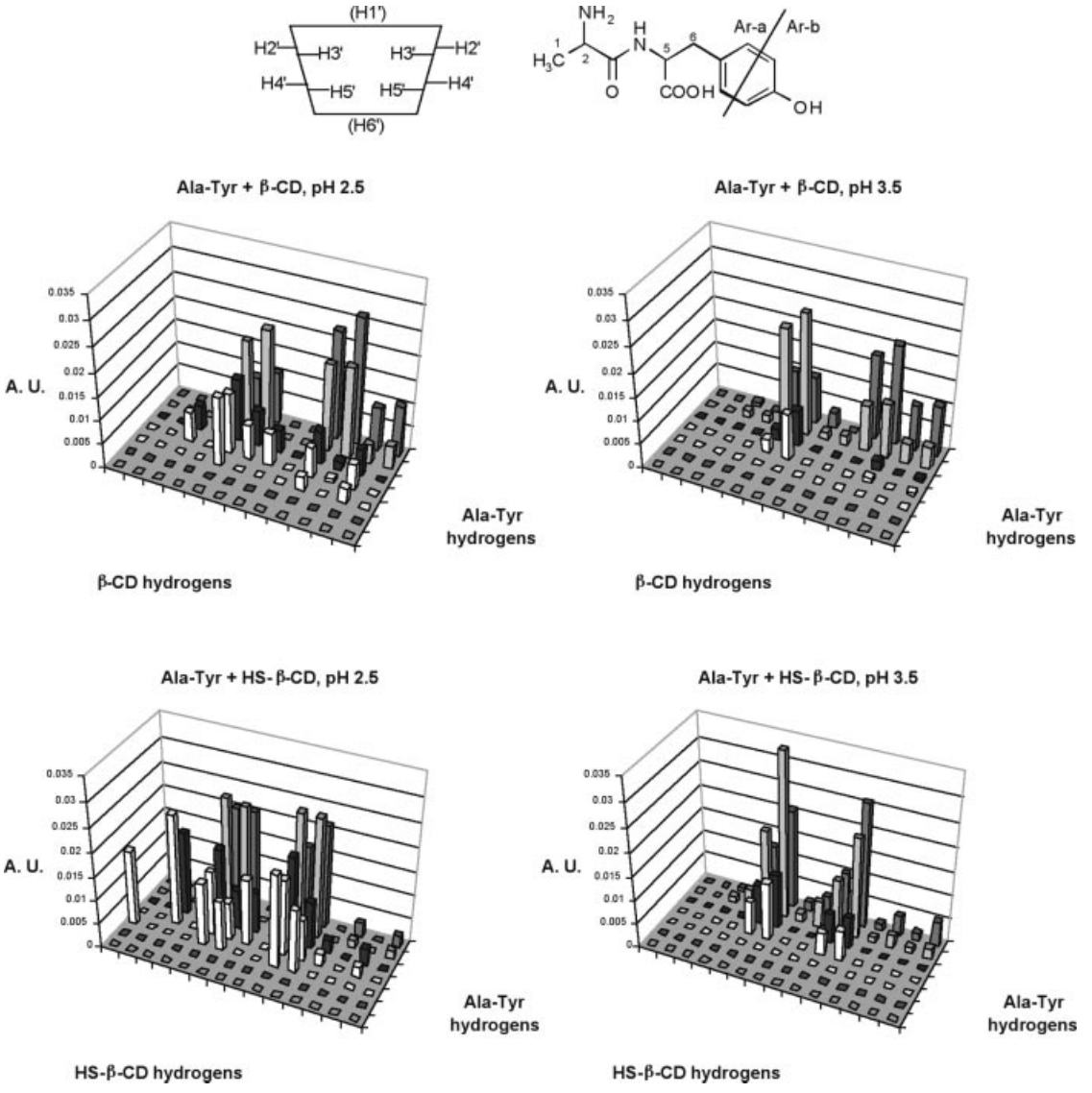


Figure 5. Relative cross-signal intensities of ROESY experiments for Ala-Tyr with β-CD and HS-β-CD (signal intensities are in arbitrary units).

  

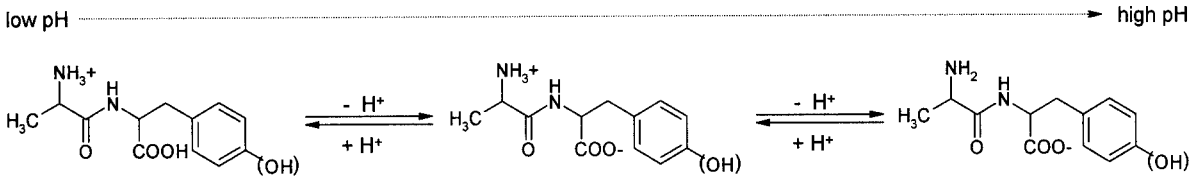


Figure 6. The different states of protonation for the dipeptides.

sured to be 3.1 (see $^1$H NMR results), the simulations for the cation and the zwitterion mirror the conditions at those pH values relevant for the previous NMR experiments quite well.

Since the dipeptide does not change its conformation upon complexation (see $^1$H NMR results), it is legitimate to energy-minimise the dipeptide molecule first and then place it into the cyclodextrin cavity.

For the first time MD simulations over such a long timeframe were carried out systematically for a larger series of different complexes of enantiomers at varying protonation states. Many MD simulations studying cyclodextrin inclusion complexes have been reported in the literature (for an overview see ref.[17]). However, in all cases they were either shorter or they were applied to a single inclusion complex only. Moreover, many earlier simulations were run in vacuo which renders comparisons between different complexes extremely difficult, if not impossible.

The data flood generated by the simulations carried out requires a careful selection of the presented parameters. The primary goal was to characterise the immersion depth of the studied ligand. Initial experiments aimed at measuring the distances between a single atom in the host and in the ligand revealed that the immersion depth is characterised insufficiently by this procedure because it provides a blurred picture of the whole complex. To better characterise immersion depth a plane was fitted in two different ways into the cyclodextrin cavity. The variability of the plane with respect to its location in space was extremely low whether the first ($p_1$,$p_3$,$p_5$) or the second ($p_2$,$p_4$,$p_6$) set of reference atoms was chosen (see Experimental Section). The stable location of the plane in the cyclodextrin cavity made it an ideal reference point for characterising immersion depth. Moreover, compared to the distance to a single atom in the cavity, the distance of the ligand to the plane better resembles the mechanism by which the NMR spectroscopic data are generated more (recall that in NMR the average interaction of the ligand with all chemically equivalent C5′ atoms is observed). To the best of our knowledge this approach has not yet been applied to cyclodextrins.

Despite being simple this approach simplified and improved the interpretation of the respective experimental data. The approach is easy to generalize and can be used for characterising the immersion depth for many other inclusion complexes.

The assignments of the dipeptide and β-CD carbons, which are necessary to calculate the distance, $d_i$ (immersion depth), are shown in Figure 7.

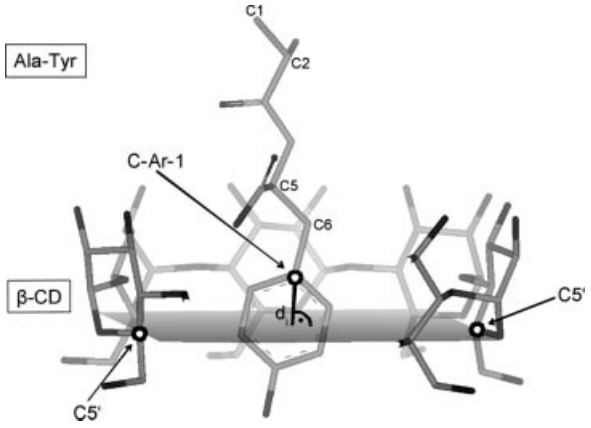


Figure 7. Plane fitted to C5′ (β-CD), immersion depth, $d_i$, and assignments of dipeptide and CD carbons. The CD cavity is clipped at the front to clearly show the position of the dipeptide.

The simulations for the cationic species of Ala-Phe, which mimic the conditions at pH 2.5, where the cationic species predominates, confirm a deeper inclusion of the D,D-enantiomer into the β-CD cavity. In contrast to this, the simulations for the zwitterion of Ala-Phe suggest a deeper penetration of the L,L-enantiomer (see Figure 8).

Since the dipeptide molecule was placed manually into the β-CD cavity and then energy-minimised, the first 250 ps of the simulation were regarded as an equilibration time and are therefore clipped in Figure 9, which shows the immersion depths, $d_i$, of CAr-a; C5 exhibits a similar immersion depth. It can be seen in part A of this figure that the immersion depths of the D,D- and L,L-enantiomers of the Ala-Tyr cation are quite similar. On average, the D,D-enantiomer immerses a bit more deeply into the β-CD cavity, which is consistent with the $^1$H and ROESY NMR findings. Part B shows clear differences in immersion behaviour between the enantiomers of the Ala-Tyr zwitterion. As opposed to the D,D-enantiomer, which shows a more or less constant immersion depth, the L,L-enantiomer dives deeply into the cavity at first. In a stepwise fashion the immersion depth is reduced in the course of time. These differences between the enantiomers cannot be seen in the NMR experiments, since at pH 3.5 a ratio of 50% cation and 50% zwitterion occurs. To see these effects would have required experiments at a pH of at least 4.5. It can be seen in parts C and D that the cationic Ala-Tyr species shows the largest immersion depth on average. The cation is followed by the zwitterion and the anionic species. This rank order is con-

  

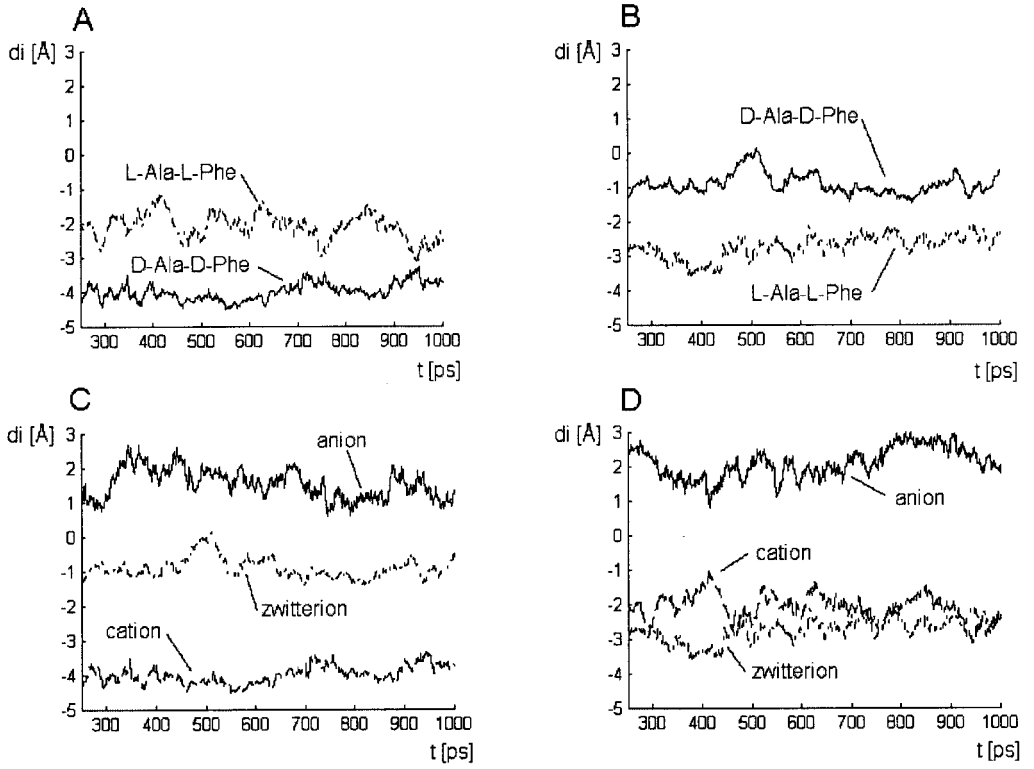


Figure 8. Immersion depths ($d_i$) for CAr-a of the D-Ala-D-Phe cation (solid) and L-Ala-L-Phe cation (dotted) (A), D-Ala-D-Phe zwitterion (solid) and L-Ala-L-Phe zwitterion (dotted) (B), D-Ala-D-Phe cation (dotted), D-Ala-D-Phe zwitterion (dash-dotted) and D-Ala-D-Phe anion (solid) (C), and L-Ala-L-Phe cation (dotted), L-Ala-L-Phe zwitterion (dash-dotted) and L-Ala-L-Phe anion (solid) (D).

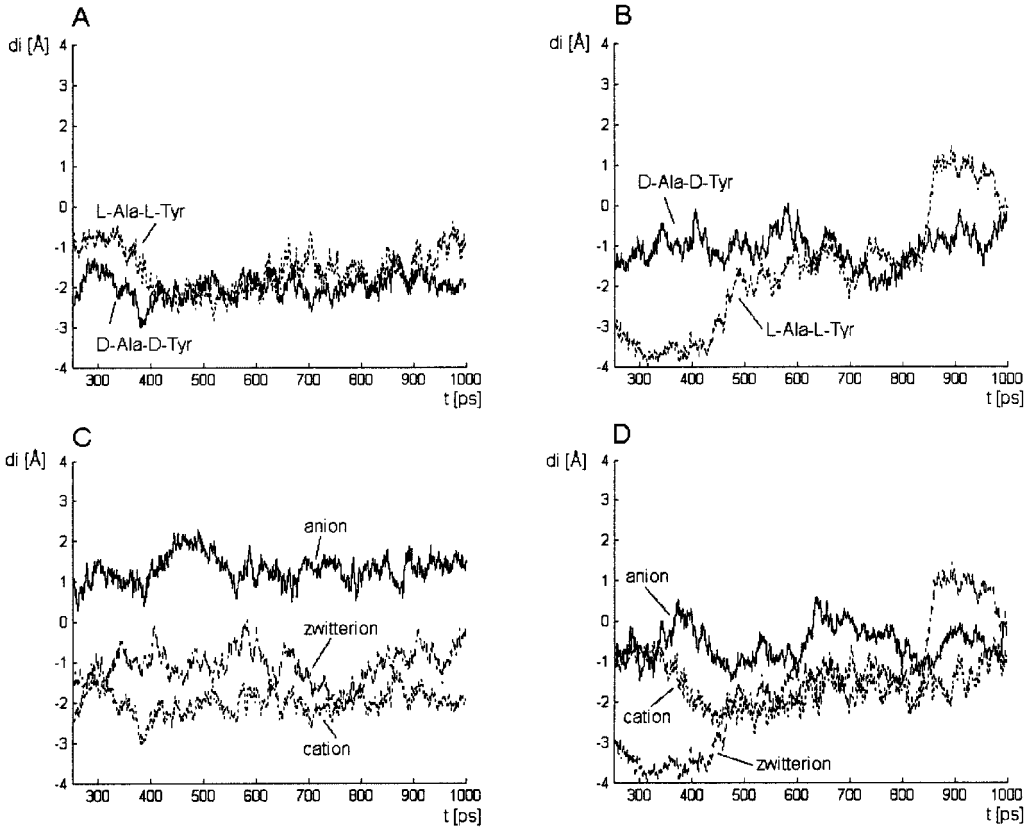


Figure 9. Immersion depths ($d_i$) for CAr-a of: A) D-Ala-D-Tyr cation (solid) and L-Ala-L-Tyr cation (dotted); B) D-Ala-D-Tyr zwitterion (solid) and L-Ala-L-Tyr zwitterion (dotted); C) D-Ala-D-Tyr cation (dotted), D-Ala-D-Tyr zwitterion (dash-dotted) and D-Ala-D-Tyr anion (solid); D) L-Ala-L-Tyr cation (dotted), L-Ala-L-Tyr zwitterion (dash-dotted) and L-Ala-L-Tyr anion (solid).

  

Table 2. Comparison between $^1$H NMR spectra (CICS), ROESY and CE experiments of Ala-Tyr.

| | $^1$H NMR (CICS) | ROESY | CE |
|---|---|---|---|
| β-CD pH 2.5 | D,D immerses deeper into the cavity | D,D immerses deeper into the cavity | D,D migrates slower |
| β-CD pH 3.5 | L,L immerses deeper into the cavity | No significant difference between D,D and L,L | L,L migrates slower |
| HS-β-CD pH 2.5 | Stronger interactions with D,D (dipeptide hydrogens) | No significant difference between D,D and L,L | D,D migrates slower |
| HS-β-CD pH 3.5 | No significant difference between D,D and L,L | No significant difference between D,D and L,L | D,D migrates slower |

sistent with the $^1$H and ROESY NMR findings. As mentioned before, the L-Ala-L-Tyr zwitterion behaves slightly different.

Alanine and phenylglycine, which show no interaction with cyclodextrins in potentiometric studies,[13] were also investigated as a negative control. After being placed in the β-CD cavity, neither enantiomer (D and L) of phenylglycine interacts with the cavity and, as a consequence, they leave the cavity immediately. Alanine appears to be much too small for proper interactions and "dances" in and about the cyclodextrin cavity (data not shown).

Table 2 shows that all the experimental data compiled in this study are in good agreement with respect to the previous CE experiments.[7,8] Deep immersion of the dipeptide enantiomers always comes along with a longer migration time, thus indicating a stronger complexation.

## Conclusions

The complexation-induced chemical shifts observed in $^1$H NMR experiments of samples containing the enantiomers of Ala-Phe or Ala-Tyr and β-CD or heptakis-6-sulfo-β-CD are consistent with an inclusion of the aromatic moiety into the cyclodextrin cavity, which was confirmed by the 2D ROESY NMR experiments. It has been shown that at pH 2.5, the D,D-enantiomer of Ala-Tyr immerses deeper into the cavity of β-CD, and this was confirmed by the molecular dynamics simulations. Furthermore, at pH 3.5, the immersion is shallower, as shown by the $^1$H and ROESY NMR findings and confirmed by the molecular dynamics simulations. MD simulations have proved helpful in supplementing the experimental results. They ease interpretation and facilitate understanding of the chiral interactions studied here by visualising immersion depth in a reliable manner.

## Experimental Section

**NMR Methods:** The $^1$H NMR experiments were performed on a Bruker Avance 400 FT NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 400.13 MHz using the XWIN NMR program package version 3.5 running on Microsoft® Windows™ NT 4.0. For the spectra 128 scans with a frequency range of 4006.410 Hz were collected into 48 K data points, giving a digital resolution of 0.16 Hz per point. An appropriate window function was applied before Fourier transformation in order to enhance the spectral resolution. The ROESY experiments were performed on a Bruker DMX 600 FT NMR spectrometer operating at 600.13 MHz using the XWIN NMR program package version 2.0 running on Microsoft® Windows™ NT 4.0. The spinlock pulse was 200 or 300 ms. All samples were measured in $H_2O$ or $D_2O$ at 300 K.

Stock solutions consisted of 12 mM cyclodextrin, amino acid or dipeptide in phosphate buffer with the respective pH and were mixed 1:1 before measuring. When using the water suppression method, the pH 2.5 and 3.5 buffers consisted of 50 mM sodium phosphate in $H_2O$, pH adjusted with 10% phosphoric acid. Water suppression was carried out using the presaturation method with the transmitter offset adjusted to the water resonance. Spectra were referenced to the maleic acid peak at $\delta$ = 6.3 ppm, which was introduced in a Wilmad (Buena, NJ, USA) stem coaxial insert as external reference (50 mM in $D_2O$). When performing the lyophilisation prior to the NMR measurement, the pH 2.5 and 3.5 buffers consisted of 50 mM sodium phosphate in $H_2O$, pH adjusted with 10% phosphoric acid. This solution was lyophilised. The analyte was redissolved in $D_2O$ and again lyophilised. This procedure was repeated twice. Spectra were referenced to the maleic acid peak at $\delta$ = 6.3 ppm, which was introduced in a Wilmad (Buena, NJ, USA) stem coaxial insert as external reference (in $D_2O$).

The $^1$H spectra were analysed concerning CICS (Complexation-induced Chemical Shifts) and $\Delta\delta$ values were determined according to [Equation (1)].

$$\Delta\delta \text{ value} = \delta \text{ (uncomplexed substance)} - \delta \text{ (1:1 mixture)} \quad (1)$$

The ROESY experiments (Bruker roesytp) were accomplished by lyophilisation prior to the NMR measurement and a Schott (Mainz, Germany) NMR tube without a coaxial insert. The 2D plots were analysed by normalising the cross-signal intensities, which are represented by the 2D integrals. Each integral value was divided by the integral value of the C1′/C4′ cross signal of the cyclodextrin, which does not change upon complexation.

**Chemicals:** β-CD was a gift from Wacker Chemie (Burghausen, Germany). HS- and HDAS-β-CD were purchased from Beckman (Fullerton, CA, USA), Diac-β-CD was prepared according to a published procedure.[18] D- and L-Amino acids were purchased from Fluka (Buchs SG, Switzerland), and D-Ala-D-Phe, L-Ala-L-Phe and L-Ala-L-Tyr from Bachem (Weil am Rhein, Germany). D-Ala-D-Tyr

  

was prepared by reaction of the *N*-benzyloxycarbonyl-protected amino acid *N*-hydroxysuccinimide with the second amino acid, in dimethylformamide,[6,19] followed by hydrogenolytic deprotection, by the group of Prof. Dr. G. K. E. Scriba at the University of Jena, Germany. The latter and L-Ala-L-Tyr were a gift from Prof. Dr. G. K. E. Scriba, University of Jena, Germany, which is gratefully acknowledged.

**Molecular Modelling Methods:** First of all an initial 3D structure of each β-CD complex containing the specific dipeptide was built. A similar structure [β-CD complexed with *N*-acetyl-L-phenylalanine clathrate[20] was found in the Cambridge Structural Database (CCD code: AGAZIR[21])]. The respective file was retrieved from CCD and modified using the Accelrys DS Viewer Pro[22] as follows. A single complex was extracted and saved as template. Next, each dipeptide structure, which was converted from 2D to 3D in DS Viewer Pro, was placed manually in the centre of the cyclodextrin cavity in the same manner as the structure of the original ligand (*N*-acetyl-L-phenylalanine clathrate) by aligning their molecular backbones. The phenylalanine substructure of the original ligand and that of the dipeptide were superimposed for this task with the phenylalanine substructure pointing to the narrower end of the cyclodextrin cavity, as in Figure 7. The original ligand was removed afterwards. That way a single file for each dipeptide studied complexed with β-CD was obtained.

These structures were transferred to Tripos SYBYL[23] using OpenBabel[24] (file format conversion). The molecules were solvated in a water box of two solvent layers surrounding the complex by applying SYBYL's standard procedure. The average number of water molecules was about 4100. The SYBYL-generated output was converted to NAMD[25,26] input files using VEGA[27–29] and in-house written software. NAMD, which was used for the molecular dynamics simulations, was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign. It was run on a Dual 3-GHz Xeon machine with the integrated CHARMM[30] force field.

Geometric restraints were not applied to the entire complex during the simulation. At the beginning of the simulation each of the aforementioned inclusion complexes was energy-minimised for 1000 fs in time steps of 0.5 fs (i.e. 2000 steps). Afterwards the molecular dynamics simulation of the complex in the water box was carried out for 1 ns (1000 ps) in time steps of 0.5 fs at a temperature of 300 K. Temperature and pressure were kept constant (NPT simulation) using Langevin dynamics as implemented in NAMD. A uniform dielectric constant of 1, and a cut-off for non-bonded forces with a switching function starting at a distance of 10 Å and reaching zero at 13.5 Å was used. During the simulation a trajectory state was written to a file every 500 fs for further analysis. For every complex seven atoms − six from the cyclodextrin (C5′), termed $p_1$ to $p_6$ and one from the included dipeptide (CAr-1; see Figure 7) − were taken and read into MATLAB[31] using MATDCD.[32] Next, a plane was fitted to C5′ (cyclodextrin carbons) to calculate the distance $d_i$ (Figure 7) representing the immersion depth of the dipeptides.

The $d_i$ values are given in the text. The values for $d_i$ were calculated as follows. Starting from the Cartesian coordinates of the cyclodextrin C5′ atoms for the points $p_1$ to $p_6$ two fits were computed. The first used point $p_1$(first), $p_3$(second) and $p_5$(third) and the second employed $p_2$(first), $p_4$(second) and $p_6$(third). Both fits used the same coordinates of atom CAr-1 of each dipeptide. The equations for computing the immersion depth ($d_i$) are defined as follows:

$$r_1 = p_{second} - p_{first} \quad (2)$$

$$r_2 = p_{third} - p_{first} \quad (3)$$

$$f = r_1 \times r_2 \quad (4)$$

$$v = [p_E - p_{first}]T \cdot f \quad (5)$$

$$d_i = \frac{v}{\|f\|} \quad (6)$$

where $p_E$ represents the cartesian coordinates of the dipeptide atom. The operator "×" refers to the usual cross product and the operator ||...|| returns the Euclidian length of the respective vector.

The distance $d_i$ was computed for each previously stored trajectory state. Since $d_i$ represents the position of the dipeptide CAr-1 atom relative to the plane, positive as well as negative values may result. For positive $d_i$ values C-Ar-1 is located above the plane and vice versa. The final immersion depth was taken as the average of the two $d_i$ values resulting from the two different fits (see above). Eventually, it turned out that using only one fit does not change the results substantially.

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