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Comparison of Cyclodextrin-Dipeptide Inclusion Complexes in the Absence and Presence of Urea by Means of Capillary Electrophoresis, Nuclear Magnetic Resonance and Molecular Modeling

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The use of capillary electrophoresis (CE) modified with cyclodextrin (CD) for the separation of stereoisomers of peptides is well established. To increase the solubility of β-CD, urea is often added to the buffer which may influence the complexation of a CD with a guest molecule. The aim of the present study was to investigate the influence of urea on the complexation between dipeptides and β-CD using Ala-Phe and Ala-Tyr as model compounds. For this purpose three different analytical methods were employed: capillary electrophoresis (CE), ^{1}H-NMR spectroscopy and molecular dynamics simulations (MD). Electropherograms of the peptide enantiomers were different in the presence and absence of urea. For example, at pH 2.5 in the absence of urea the enantiomers of Ala-Tyr are not separated in contrast to the use of buffers containing urea. Applying "complexation-induced chemical shift (CICS)" in NMR spectroscopy and rotating frame Overhauser enhancement spectroscopy (ROESY) revealed differences in the complexation of the peptide enantiomers by β-CD in the absence and presence of urea suggesting the stabilization of the complex through the phenolic hydroxyl group of tyrosine. MD simulations for different complexes were carried out with consideration of both water and urea molecules in solution. Simulations were performed for 1 ns. In conclusion, NMR spectroscopy and MD methods help to understand the structure of peptide-CD complexes and the separation and migration behavior in CE.

Introduction

The pH-dependent reversal of the migration order of the enantiomers of dipeptides and tripeptides in capillary electrophoresis (CE) has been reported recently. Li and Waldron found opposite migration order of the LL/DD enantiomeric pairs of Ala-Phe and Leu-Phe at pH 2.7 and pH 3.5 when using native β-cyclodextrin (β-CD) as chiral selector.[1] Reversed migration order was also found for the tripeptide enantiomers Gly-β-L-Asp-D-PheNH$_2$ and Gly-β-D-Asp-L-PheNH$_2$ at pH 3.6 and 5.3 in the presence of carboxymethyl-β-cyclodextrin (CM-β-CD).[2] Subsequent systematic studies on the enantioseparation of various dipeptides and tripeptides by neutral and charged CD derivatives under standardized conditions revealed that the pH-dependent reversal of the migration order appeared to be a rather common phenomenon for CD-mediated peptide enantioseparations.[3] It is dependent on the structure of the peptides (amino acid sequence) as well as the structure of the CD.

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Besides for β-CD, the pH-dependent reversal of the enantiomer migration order was observed for uncharged β-CD derivatives,[3,4] β-CD derivatives with carboxyl substituents[5] or sulfate groups[6] as well as for the quaternary ammonium derivative (2-hydroxypropyl)trimethylammonium-β-CD.[7] Interestingly, the pH-dependent change of the enantiomer migration order has only been demonstrated for β-CD and its derivatives while the effect has not been observed yet for α-CD or γ-CD and their hydroxypropyl derivatives although enantioseparations of peptides were achieved with these cyclodextrins.[8]

Mechanistic studies revealed two general mechanisms for the pH-dependent reversal of the enantiomer migration order. The first is based on a change of the chiral recognition of the CD towards the peptide enantiomers expressed as complexation constants depending on the dissociation state of the peptide. For example, the LL enantiomers of Ala-Phe and Leu-Phe were complexed weaker by β-CD at pH 2.7 compared to the respective DD enantiomers and, subsequently, migrated faster than the DD isomers whereas at pH 3.5 the DD enantiomers were complexed stronger resulting in a change of the enantiomer migration order.[1] A similar behavior was observed between pH 2.5 and 3.5 for Ala-Tyr and Phe-Phe in the presence of 2,3,6-trimethyl-β-cyclodextrin (TM-β-CD),[9] for Ala-Phe and Ala-Tyr using *heptakis*-6-sulfo-β-cyclodextrin (HS-β-CD) between pH 2.5

and 5.3[6] as well as for the tripeptide enantiomers Gly-β-L-Asp-D-PheNH$_2$ and Gly-β-D-Asp-L-PheNH$_2$ between pH 3.6 and 5.3 using CM-β-CD as chiral selector.[2]

The second mechanism is based on differences in the mobilities of the diastereomeric peptide-CD complexes while the chiral recognition of the CD towards the peptide enantiomers remains unchanged by a variation of the buffer pH. Thus, compared to the LL stereoisomer the DD enantiomer of Ala-Tyr is complexed stronger at pH 2.5 and 3.5 by β-CD (i.e. the chiral recognition of β-CD towards the enantiomers remains unchanged) despite the fact that L-Ala-L-Phe migrated faster than D-Ala-D-Phe at pH 2.5 while it migrated slower than the DD enantiomer at pH 3.5.[9] However, the mobilities of the diastereomeric complexes between β-CD and the LL and DD enantiomers were essentially identical at pH 2.5 but the mobilities differed at pH 3.5. The stronger complexed DD isomer exhibited the higher mobility compared to the LL epimer which counteracted the stronger complexation of the DD enantiomer by β-CD resulting in a faster effective migration of the DD enantiomer at pH 3.5. The observation may be explained by a shift of the protonation equilibria of the enantiomers upon complexation by the CD as demonstrated for dansylated Phe and Trp.[10] This causes different charge densities of the diastereomeric complexes resulting in different mobilities of the complexes. Complex mobility as the basis for a pH-dependent reversal of the enantiomer migration order has also been attributed for some peptides in the presence of *heptakis*-(2,6-dimethyl)-β-cyclodextrin,[9] CM-β-CD[5] and sulfated β-CD derivatives.[6] As shown by simulation performed by Rizzi, differences in the mobilities of the analyte enantiomer-chiral selector complexes as small as 1% can significantly affect chiral separations.[11]

The chiral recognition mechanisms and the structures of the peptide-CD complexes have not yet been explored extensively. Initial ^{1}H-NMR spectroscopy studies on the complexation-induced chemical shifts (CICS) of combinations of the dipeptides Ala-Phe and Ala-Tyr with β-CD, *heptakis*-(2,3-di-*O*-acetyl)-β-cyclodextrin (DIAC-β-CD), HS-β-CD and *heptakis*-(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS-β-CD) indicated the formation of inclusion complexes in the case of β-CD and HS-β-CD with the aromatic moiety protruding into the CD cavity.[12] Thus, high downfield shifts in the presence of the dipeptide enantiomers were observed in the case of β-CD and HS-β-CD for the protons H3 and H5 located inside the CD cavity as well as significant upfield shifts for the benzyl H atoms combined with considerable shifts of the aromatic hydrogen atoms of the peptides. The structures of β-CD and the dipeptides studied, and the assignment of the H atoms are displayed in Figure 1.

In contrast, the CICS patterns obtained for DIAC-β-CD and HDAS-β-CD did not provide substantial evidence on the peptide-CD complex as they were rather randomly shifted.[12]

The structures of guest host complexes involving CDs can be analyzed by various physico-chemical methods including NMR techniques.[13] For example, rotating frame

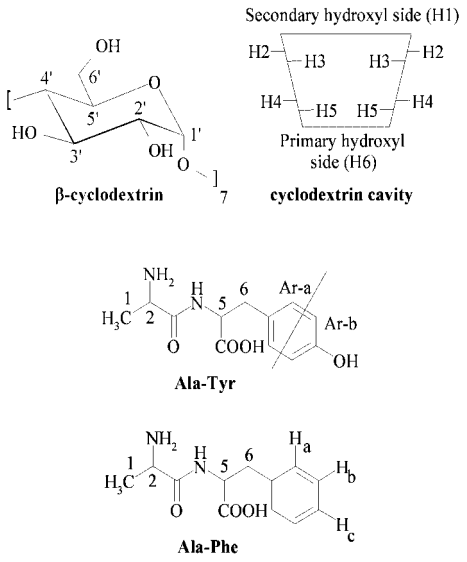

Figure 1. Assignments of dipeptide and β-CD H atoms. Schematic display of the β-CD torus.

nuclear Overhauser effect (ROESY) experiments have been successfully applied to elucidate the structures of guest-CD complexes including complexes involving amino acids,[14,15] and peptide derivatives[16] and have previously confirmed the formation of an inclusion complex between Ala-Phe and Ala-Tyr with β-CD.[17]

To the best of our knowledge no investigation of the structure of the analyte-CD complexes has been performed to date which considers the influence of urea on the complex structure. The improvement of peak shape and separation efficiency upon addition of urea has been described for micellar electrokinetic chromatography[18] and microemulsion electrokinetic chromatography.[19] High concentrations of urea and methylated urea derivatives (4–7 M) significantly improved β-CD mediated enantioseparations of dansylated amino acids in CZE.[11] Except for dansylated Phe, this phenomenon was only observed for β-CD while deterioration of the enantioseparations was observed in the case of γ-CD or methylated β-CD derivatives.[11] Taken together, none of these publications investigated the structures of the complexes. Thus, the aim of the present study was to compare the CE separation of Ala-Phe and Ala-Tyr in the presence and absence of urea with the structure of the peptide-CD complexes using ROESY experiments as well molecular dynamics simulations (MD). Urea was suggested as a donor and acceptor of hydrogen bonds as already described for water molecules by Stezowski et al.,[20,21] by the use of crystallographic studies. Since we were unable to crystallize complexes between β-CD and the dipeptides, we used the methods described above.

Results and Discussion

Capillary Electrophoresis

Due to the limited aqueous solubility of β-CD, enantioseparations utilizing β-CD as chiral selector are often per-

formed in buffers containing 2 M urea. As buffer additives can affect the separation selectivity in CE the β-CD-mediated separation of the enantiomers of the dipeptides Ala-Phe and Ala-Tyr (see Figure 1) was investigated in the presence and absence of urea. Representative electropherograms of the chiral separations of the dipeptides at pH 2.5 and 3.5 at a concentration of β-CD of 15 mg/mL in the presence and the absence of urea are summarized in Figure 2. The selected concentration of the chiral selector β-CD is slightly below the aqueous solubility limit of 18 mg/mL so that experiments in the absence of urea became possible.

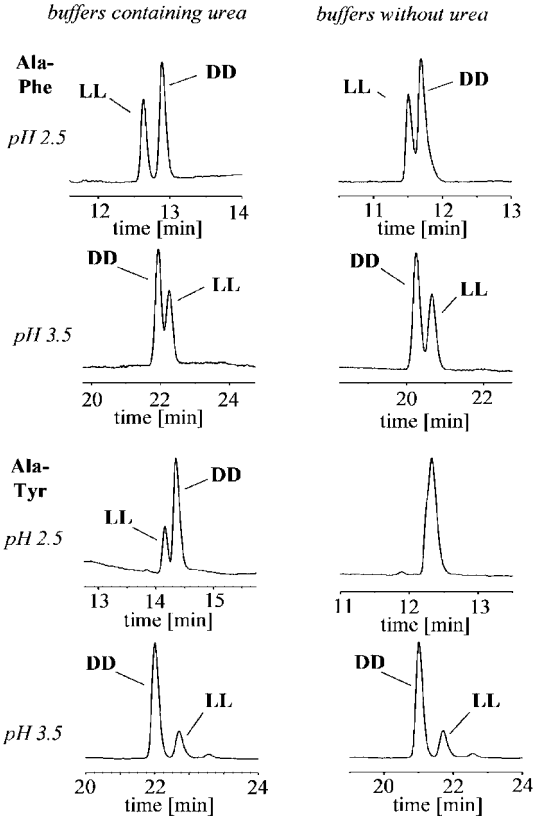

Figure 2. Chiral separations of the enantiomers of Ala-Phe and Ala-Tyr at pH 2.5 and 3.5 using β-CD as selector in the presence and absence of urea.

With the exception of Ala-Tyr at pH 2.5 in the absence of urea, β-CD provided enantioseparations of the peptides under all experimental conditions applied. At pH 2.5 the resolution was generally higher when urea was present. The improvement of β-CD-mediated enantioseparations of dansylated amino acids upon addition of 7 M urea has also been reported using a borate buffer, pH 9.[22] In contrast, at pH 3.5 a small but insignificant improvement of the enantioseparation could be observed for urea-free buffers. The pH-dependent reversal of the enantiomer migration order is not affected by urea when increasing the buffer pH from pH 2.5 to 3.5. In the electropherogram of Ala-Tyr at pH 2.5 in the presence of urea, a small shoulder can be seen indicating that the LL stereoisomer migrates before the DD enantiomer but due to the relatively low concentration of the chiral selector a separation was not observed under the applied conditions.

CICS Measurements

In order to examine the structural differences between Ala-Phe- and Ala-Tyr-β-CD complexes as well as differences of the peptide enantiomer-β-CD complexes in the presence or absence of urea at a molecular level, ^{1}H-NMR spectra of the peptides in buffered, aqueous solutions were recorded and the CICS ($\Delta\delta$ values) were calculated. The assignments of both the β-CD and dipeptide H atoms are displayed in Figure 1. As described by Kahle et al.,[17] the $\Delta\delta$ values of the β-CD H atoms H3′ and H5′ which are located inside the CD cavity displayed a large upfield shift upon addition of the dipeptides. Likewise, the CICS patterns of the Ala-Tyr H atoms showed large $\Delta\delta$ values of the lower aromatic region (Ar-B) adjacent to the phenolic hydroxyl group and the benzylic H atoms (H6a, H6b) (data not shown). This indicates that the phenyl ring of the dipeptides penetrated into the CD cavity from the wider, secondary side. A section of the ^{1}H NMR spectra of the dipeptide D-Ala-D-Tyr is displayed in Figure 3 demonstrating the effect of urea and β-CD upon the chemical shifts of the aromatic signals of the dipeptide.

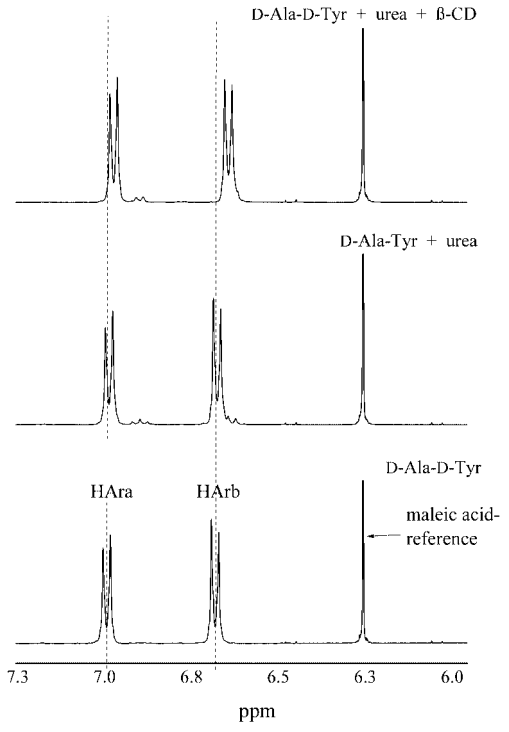

Figure 3. ^{1}H-NMR spectra of the aromatic region of D-Ala-D-Tyr (HAra and HArb) at pH 2.5. Pure D-Ala-D-Tyr (lower), D-Ala-D-Tyr with urea (middle) and with urea and β-CD (upper).

Similar CICS were observed for both enantiomers, at both pH values and in the presence and absence of urea. Moreover, the coupling constants of the respective peptide H atoms did not change in any case indicating that the con-

formation of the dipeptides was not altered upon complexation with the CD and by the addition of urea.

Comparing the CICS of the β-CD protons upon complexation with Ala-Phe and Ala-Tyr revealed that the urea apparently has no significant influence in the case of Ala-Phe (Figure 4) while differences were noted for Ala-Tyr (Figure 5). Since the only difference between Ala-Phe and Ala-Tyr is the phenolic OH-group this moiety seems to be the key structure.

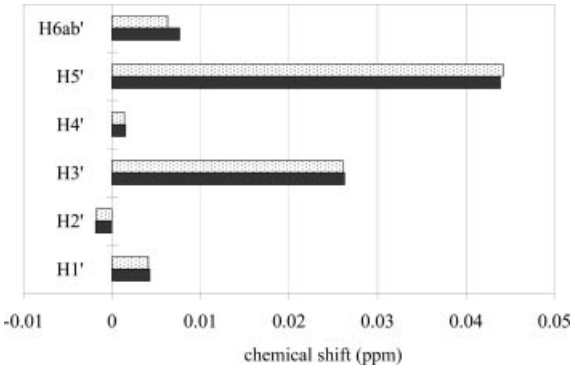

Figure 4. CICS of β-CD H atoms in presence of L-Ala-L-Phe with (solid) and without (dotted) urea at pH 2.5. ^{1}H-NMR recorded at 400 MHz in H_2O applying water suppression.

In the presence of urea, D-Ala-D-Tyr displayed stronger interactions with the H3′ and H5′ CD protons inside the cavity compared to L-Ala-L-Tyr at both pH values. This indicates a deeper insertion of D-Ala-D-Tyr into the cavity which is also reflected by the higher complexation constants at both pH values for the DD stereoisomer than the LL enantiomer as determined by CE.[9] However, the CICS of especially H5′ and H6ab′ of Ala-Tyr decreased significantly by changing to pH 3.5, whereas the CICS of L-Ala-L-Tyr increased.

Comparing the CICS obtained in the absence of urea, differences can be observed with regard to the CICS obtained in buffers containing urea and with respect to the two pH values investigated as well. Comparing the data in the presence and absence of urea at pH 2.5, the interaction between the protons inside the cavity of β-CD and Ala-Tyr enantiomers was similar for H5′ (although a somewhat stronger interaction could be noted for L-Ala-L-Tyr in comparison to D-Ala-D-Tyr) but different for H3′. Both experimental conditions resulted in higher absolute CICS values but the DD enantiomer appeared to interact stronger in the presence of urea while the LL enantiomer exhibited larger CICS values in the absence of urea. At pH 3.5, the observed CICS were similar with respect to the Ala-Tyr enantiomers for H3′ (although higher values were also found in the absence of urea) while the pattern differed for H5′. Minor differences could also be observed for the other CD protons. Overall, this can be interpreted as an indication that the complexes differ when urea is added to the buffer solutions compared to the absence of urea. The effect appears to be more pronounced at pH 2.5 than at pH 3.5. Thus, it may be hypothesized that urea is not just a solubility enhancer but participates in the complex formation, especially at pH 2.5.

Rotating Frame Overhauser Enhancement Spectroscopy

In order to gain further insight into the structure between β-CD and the Ala-Tyr enantiomers ROESY experi-

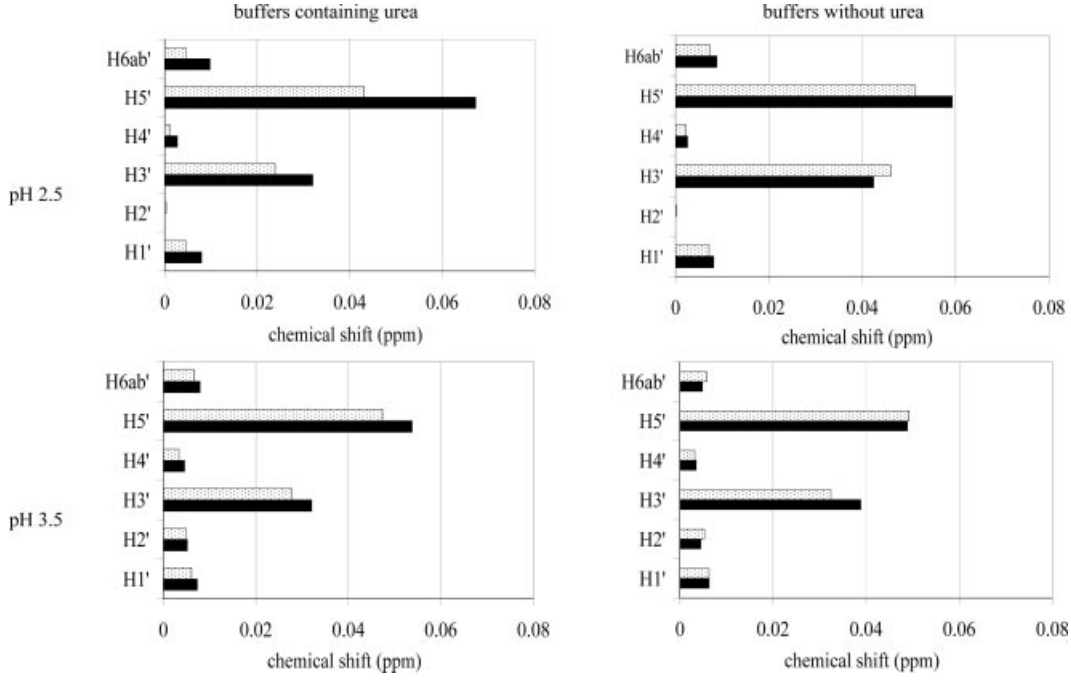

Figure 5. CICS of β-CD H atoms in presence of L-Ala-L-Tyr (dotted) and D-Ala-D-Tyr (solid). ^{1}H NMR measured at 400 MHz in H_2O applying water suppression by presaturation.

ments were performed as this is a well established method to investigate intermolecular interactions between adjacent H atoms of β-CD and a guest molecule.[23] Changes of these interactions initiated by changing the buffer conditions may provide more detailed information about a modified complexation mode than the CICS measurements because the integral values of the cross-signals are directly proportional to the distance of H atoms and, therefore, small differences in distances can be detected. The spectra were qualitatively and quantitatively analyzed by determining the integral values of the intermolecular cross-peaks. All investigated cross-peaks were referenced to the H1′-H4′ intramolecular cross-peak of β-CD assuming that the distance of these H atoms did not change under all experimental conditions.

A comparison of ROESY data obtained for the D-Ala-D-Tyr and D-Ala-D-Phe is summarized in Figure 6 showing the crosspeaks of H3′, H5′, and H6ab′ of β-CD with the aromatic dipeptide H atoms in *meta*-position (HArb of Ala-Tyr and H_b of Ala-Phe, respectively) upon addition of urea. The stronger effect of the Ala-Tyr protons HArb on H5′ and H6ab′ indicated that the aromatic moiety of Ala-Tyr is located deeper in the cavity than the phenyl ring of Ala-Phe. This was further supported by the relatively smaller intensity of the HArb-H3′ crosspeak, indicating a larger distance between the wider secondary opening of the cyclodextrin torus and the lower aromatic part of Ala-Tyr. As the structural difference between Ala-Phe and Ala-Tyr is the phenolic hydroxyl group, the phenol appears to be the primary reason for the deeper penetration of D-Ala-D-Tyr in the cavity of β-CD compared to D-Ala-D-Phe which is in accordance with the CICS findings (see above).

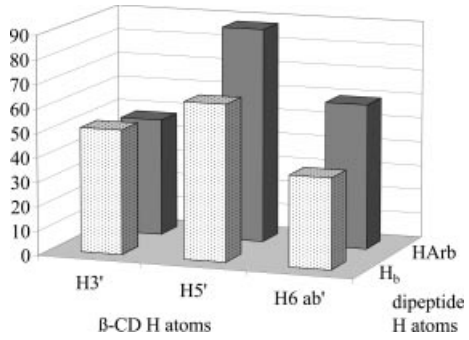

Figure 6. Values of the integrals of the ROESY crosspeaks between β-CD, D-Ala-D-Phe (dotted), and D-Ala-D-Tyr (solid) in addition of urea at pH 3.5. ROESY recorded at 600 MHz in D_2O applying 64 scans.

ROESY has also been applied to study the complexes of the enantiomers of Ala-Tyr and β-CD. The cross-signal integrals are displayed in Figure 7. The crosspeak pattern for the enantiomers at pH 2.5 in the absence of urea did not significantly differ from each other, indicating almost identical positions of the stereoisomers in the CD cavity. This also reflects the low chiral differentiation of β-CD towards the peptide enantiomers observed in CE under

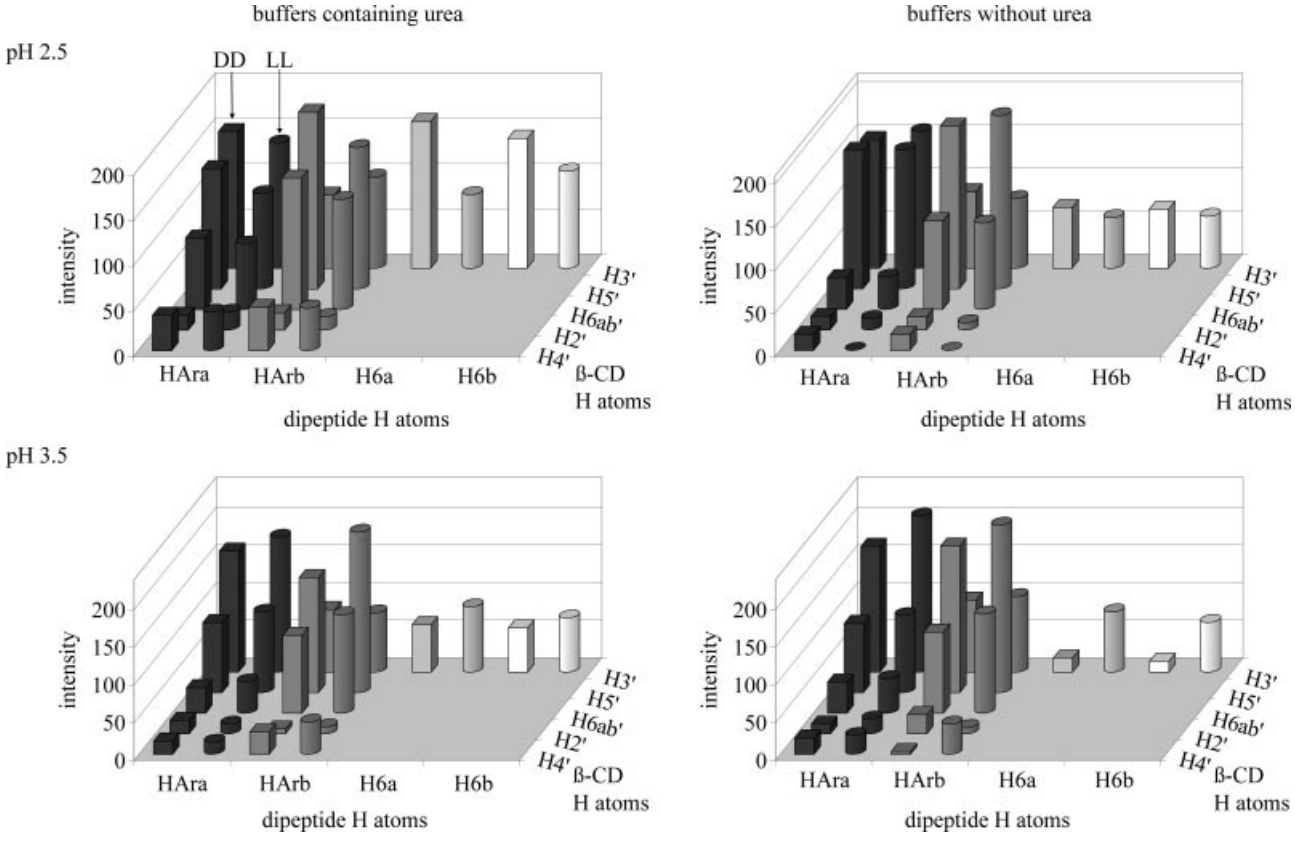

Figure 7. Values of the integrals of the ROESY crosspeaks between β-CD, D-Ala-D-Tyr (bars), and L-Ala-L-Tyr (cylinders), respectively. ROESY measured at 600.13 MHz in D_2O applying 64 scans.

these experimental conditions (Figure 2). In contrast, in the presence of urea, the HAra, HArb, H6a and H6b atoms of the D-Ala-D-Tyr exhibited stronger crosspeaks than the corresponding H atoms of the LL enantiomer. This indicates a deeper insertion of D-Ala-D-Tyr in the CD cavity compared to L-Ala-L-Tyr. A deeper penetration is further supported by the weaker crosspeaks of H3′of β-CD and the phenolic H atoms of D-Ala-D-Tyr.

The weaker crosspeaks between the H3′ protons in the "upper" wider part of the β-CD torus and protons HArb of D-Ala-D-Tyr compared to the LL enantiomer further support a deeper penetration of the DD stereoisomer than the LL isomer. This is consistent with the corresponding CICS data described above where also larger shifts were observed for the DD enantiomer.

At pH 3.5, the complexation of the Ala-Tyr enantiomers appeared to be less affected by the addition of urea than at pH 2.5 (Figure 7). The integral values with and without urea were quite similar. However, due to the relative reduction of the crosspeaks between H3′ with H6a, H3′ with H6b and the increase of the H5′-HArb crosspeaks, the complexation mode of D-Ala-D-Tyr appeared to be slightly different in absence of urea compared to that in presence of urea.

Comparing the integral values of the ROESY crosspeaks at pH 2.5 and pH 3.5 similar peptide enantiomer-CD complex structures can be assumed in the absence of urea. The relative intensities of the crosspeaks of HAra and HArb peptide protons with H3′, H5′ and H6ab′ β-CD protons were comparable while differences existed for the peptide protons H6a and H6b with H3′. Thus, the complex structure may not change when the pH of the buffer is raised. In contrast, significant differences for the crosspeaks were observed in buffers containing urea when increasing the pH from 2.5 to 3.5. Larger crosspeaks between peptide and CD protons were found for D-Ala-D-Tyr at pH 2.5 while more intense crosspeaks were noted for L-Ala-L-Tyr at pH 3.5. A larger crosspeak correlates with a tighter fit of the peptide H atoms to β-CD H atoms indicating a deeper penetration of the DD enantiomer at pH 2.5 and of LL at pH 3.5. However, there is no evidence for a stronger binding of the dipeptide to β-CD but for a different mode of complexation so that the ROESY measurements are not in contradiction to the CE determination of the respective binding constants. The data indicated a stronger complexation for D-Ala-D-Tyr at both pH values, the observed reversal of the enantiomer migration order was interpreted as the result of the higher electrophoretic mobility of the D-Ala-D-Tyr-β-CD complex compared to the complex of L-Ala-L-Tyr.[9] The ROESY data indicate an altered complexation mode being responsible for this change of the electrophoretic mobility. In the absence of urea the difference of the mobility between the respective enantiomers does not seem to be as distinctive as in the presence of urea.

One has to keep in mind that the ROESY experiments were performed in D_2O while CE and CICS measurements were performed water. D_2O and water differ in their physico-chemical properties including the dielectric constants. However, the dissociation equilibria of both the dipeptides and buffer substances should be quite similar in D_2O and H_2O.[24]

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 L-Ala-L-Tyr, D-Ala-D-Tyr and D-Ala-D-Phe as described in the methods section. The behaviour of L-Ala-L-Phe and D-Ala-D-Phe in MD simulations is described detailed in Kahle et al.[17] Considering the fact that these dipeptides are almost not influenced by urea (see Figure 9) only the simulation for D-Ala-D-Phe has been analysed in detail. This is consistent with the NMR results.

Furthermore it was necessary to consider the dissociation equilibrium of the peptides. At pH 2.5 the compounds exist primarily as protonated, positively charged species whereas at pH 3.5 a considerable fraction exists as zwitterionic uncharged species. To reflect this situation the peptides were modelled as protonated species reflecting the situation at pH 2.5 and as zwitterionic species in order to resemble the situation at pH 3.5.

The data generated by the simulations required careful selection of the parameters. The primary goal was to characterize the immersion depth of the dipeptides. Initial experiments aimed at measuring the distances between a single atom in the CD host and the peptide guest revealed that the immersion depth is not sufficiently characterized by this procedure because it provided a blurred picture of the whole complex. To better characterize the immersion depth a plane was fitted in two different ways into the CD cavity. The variability of the position of the plane in the CD 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 Exp. Sect.). This "stable" location of the plane in the CD cavity made it an ideal reference point for characterizing the immersion depth of the peptide enantiomers (Figure 8).

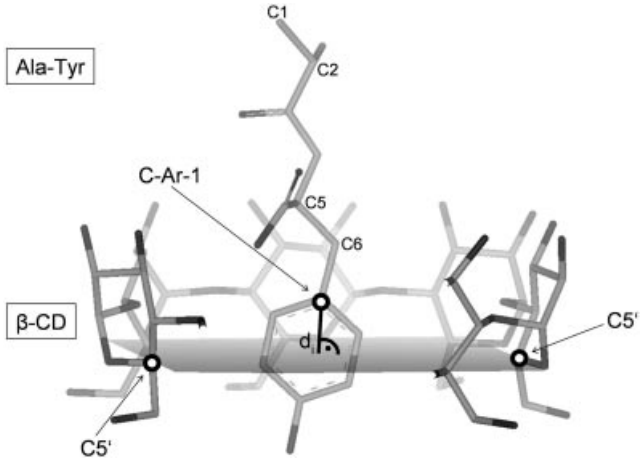

Figure 8. Plane fitted to C5′ (β-CD), immersion depth d_i and assignments of dipeptide and CD carbons. The CD cavity is clipped at the front to clearly show the position of the dipeptide (cf. ref.[17]).

Moreover, in comparison to the distance to a single atom of a guest in the cavity, the distance of the ligand relative to the plane better resembled the mechanism by which the NMR spectroscopic data were generated. However, it should be kept in mind that a NMR experiment only generates data of the average interaction of a ligand with the CD cavity C5′ atoms, i.e. the fitted plane.

Since the dipeptide did not change its conformation upon complexation as confirmed by ^{1}H NMR, the dipeptide enantiomers were energy-minimized first and subsequently placed into the CD cavity. Because the dipeptide molecules were placed manually into the β-CD cavity and subsequently energy-minimized, the first 250 ps of the simulation were considered as the equilibration time and are therefore not shown in the respective figures. A detailed description of the workflow for dipeptide minimization, insertion, solvation and consecutive complex optimization can be found in the recent publication by Kahle et al.[17] Figure 9 summarizes the immersion depth, d_i, of the Ala-Tyr enantiomers and D-Ala-D-Phe into the β-CD cavity in the presence and absence of urea at pH 2.5 as a fully protonated species. Generally, a deeper immersion of the analytes in the presence of urea is noted. The deeper immersion may also be interpreted as a stronger complexation which is in accordance with the NMR measurements described above. Moreover, the stronger complexation may also contribute to the longer migration times observed in the CE experiments in buffers containing urea besides the increased buffer viscosity upon addition of urea.

CICS and ROESY experiments indicated differences in the interaction between β-CD and Ala-Phe and Ala-Tyr, respectively, in urea containing buffers. This is also reflected in the MD simulations as shown in Figure 10. Although identical starting points were applied to D-Ala-D-Phe and D-Ala-D-Tyr the latter shows deeper penetration into the CD cavity compared to the Ala-Phe stereoisomer when urea is included in the MD simulations. In the absence of urea no difference were found with regard to d_i of the analytes (Figure 10).

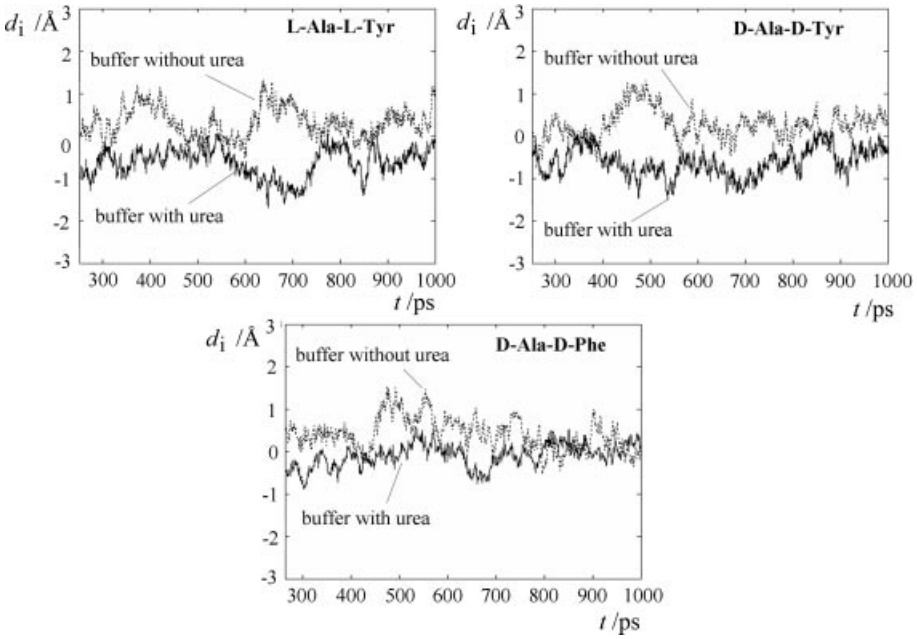

Figure 9. Development of the immersion depth d_i, of L-Ala-L-Tyr, D-Ala-D-Tyr, and D-Ala-D-Phe obtained by MD simulations in the presence of urea (solid line) and in the absence of urea (dotted line) at a pH of 2.5.

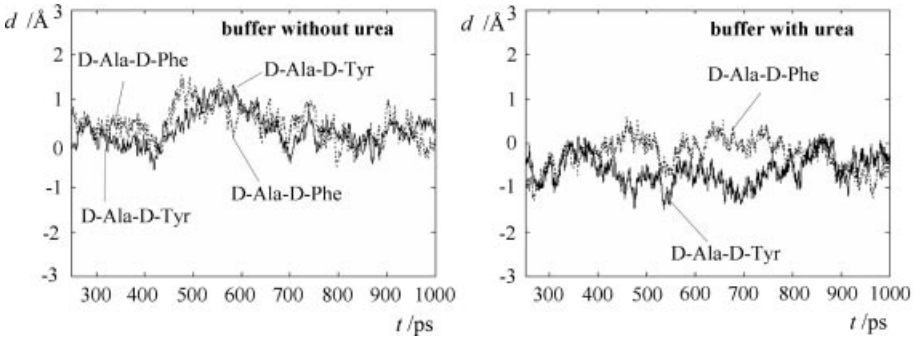

Figure 10. Comparison of the MD simulation immersion depth d_i of D-Ala-D-Tyr (solid line) and D-Ala-D-Phe (dotted line) in the presence of urea (right) and the absence of urea (left) at pH 2.5.

The structural difference between Ala-Phe and Ala-Tyr is the phenolic hydroxyl group. Thus, it may be hypothesized that an interaction between urea and the phenolic moiety is responsible for this observation. The hydroxyl group is capable of acting as a hydrogen-bond donor as well as a hydrogen-bond acceptor and probably interacts by a hydrogen bond with one or more urea molecules which can also act as a hydrogen-bond donors and/or acceptors. A possible structure is shown in Figure 11 to illustrate the concept. Other complexes may certainly exist. As a consequence the complex of the dipeptide in the CD cavity is additionally stabilized in comparison to Ala-Phe. This may be an explanation for the results obtained by CE and NMR spectroscopy and strongly supports the theory that urea does not only act as a solubility enhancer but also participates in the process of complex formation. This is consistent with higher binding constants determined by CE for Ala-Tyr[9] compared to Ala-Phe.[1]

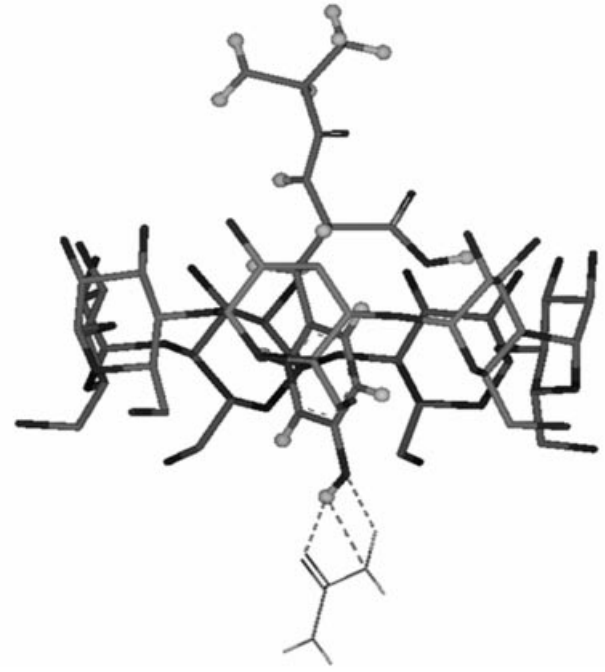

Figure 11. Hypothetical structure of the D-Ala-D-Tyr-CD complex in presence of urea: the interaction between a urea molecule and the hydroxyl moiety of tyrosine stabilizes the complex.

The MD simulations did not reveal significant differences between the immersion depths, d_i, of the Ala-Tyr enantiomers under either simulated conditions (data not shown) although the chiral recognition is different as enantiomer separations are observed in CE especially in the presence of urea (Figure 2). In the absence of urea the electropherogram shows a slight shoulder indicating that chiral recognition occurs but the concentration of the CD is not high enough to translate into a chiral separation. Differences between the enantiomers have also been observed in the NMR experiments. The reason for this discrepancy is currently not known. Maybe applying simulations over a longer period of time will indicate differences in complex formation. On the other hand, the peptide enantiomer-CD complexes may differ in their size (solvated volume) and/or dissociation equilibrium while the immersion depth is not significantly different. This would also result in differences in charge density which translates into chiral separations in CE.

Conclusions

NMR experiments, i.e. CICS and ROESY measurements, as well as MD simulations have been performed in order to understand chiral separations of peptide enantiomers in CE using β-CD as chiral selector in the presence and absence of urea in the background electrolytes. Under the conditions investigated urea appears to act not only as a solubility enhancer for the poorly water-soluble β-CD but participates in the formation of the complex especially in the case of Ala-Tyr. Although the exact structure of such complexes is unknown hydrogen bond formation between the phenolic hydroxyl group of the Try moiety and urea appears to be the most likely explanation for the observations in CE and NMR experiments.

Experimental Section

General: β-CD was from Wacker Chemie GmbH (Munich, Germany), L-Ala-L-Phe, D-Ala-D-Phe, and L-Ala-L-Tyr were from Bachem AG (Heidelberg, Germany). All other chemicals were of analytical grade. Buffers and sample solutions were prepared in double-distilled, deionized water, filtered (0.47 μm), and degassed by sonication.

Synthesis of D-Ala-D-Tyr: D-Ala-D-Tyr was prepared according to standard peptide chemistry procedures.[25] Briefly, to 200 mg D-Tyr(Bzl)OH (0.74 mmol) and 150 mg triethylamine in 10 mL dimethylformamide were added 240 mg Z-D-Ala-OSu (0.75 mmol) in 5 mL dimethylformamide at room temperature and the solution was stirred overnight. Upon evaporation of dimethylformamide under reduced pressure the residue was dissolved in 10 mL ethyl acetate and extracted 3 times with 5 mL ice-cold 0.5 M hydrochloric acid. The organic phase was dried with sodium sulfate and evaporated yielding an amorphous white powder which was dissolved in 20 mL methanol. 50 mg palladium on charcoal was added and the solution was hydrogenated overnight. Following filtration the solvent was removed under reduced pressure yielding 110 mg (68.3%) of a white residue. ^{1}H NMR (250 MHz, D_2O): δ = 7.07 (d, J = 10 Hz, arom. CH), 6.76 (d, J = 10 Hz, arom. CH), 4.32 (m, CH Phe), 3.90 (q, J = 7.5/14 Hz, CH Ala), 3.03 and 2.82 (each dd, J = 5.3/14 Hz and J = 8.7/14 Hz, CH_2), 1.41 (d, J = 7.5 Hz, CH_3) ppm. ^{13}C NMR (62.9 MHz, D_2O): δ = 177.6 (CO), 169.7 (CO), 154.1 (CH), 130.5 (CH), 129.5 (CH), 115.3 (CH), 56.8 (CH), 48.9 (CH), 36.4 (CH_2), 16.3 (CH_3) ppm. ESI-MS: m/z = 275.2 ([M + Na]$^+$), 253.2 ([M + H]$^+$). The stereochemical purity of the peptide was ensured by CE.

Capillary Electrophoresis: All experiments were performed on a Beckman MDQ instrument (Beckman Coulter GmbH, Unterschleißheim, Germany) equipped with a diode array detector at 25 °C using 50 μm ID, 360 μm OD fused-silica capillaries (Polymicro Technologies, Phoenix, Arizona, USA). The effective length of the capillaries was 40.0 cm; the total length was 50.2 cm. UV detection at 200 nm was performed at the cathodic end. Sample solu-

tions (100 μg/mL peptides dissolved in water) were injected at a pressure of 3.45 kPa (0.5 psi) for 3 s. All separations were performed in 50 mM sodium phosphate buffer. The corresponding pH was adjusted using 100 mM phosphoric acid after the addition of the cyclodextrins. Between the analyses the capillaries were washed 5 min with phosphate buffer containing 2 M urea, 1 min with 100 mM phosphoric acid, and 3 min with the respective run buffer.

NMR Experiments: 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/point. An appropriate window function of 0.3 Hz 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 at pH 2.5 or 300 ms at pH 3.5, respectively. Each spectrum consisted of a matrix of 2 K (F1 and F2) data points covering a sweep width of 4882 Hz. All samples were measured in H_2O or D_2O, respectively, at 300 K.

Stock solutions containing 12 mM cyclodextrin or dipeptide in phosphate buffer at the respective pH were mixed at a ratio of 1:1 prior to the experiments. In the respective studies 681 mM urea was added. For ^{1}H NMR measurements water suppression was carried out using the presaturation method (zgpr) with the transmitter offset adjusted to the water resonance. When using the water suppression method, the pH 2.5 and 3.5 buffers consisted of 50 mM sodium phosphate in H_2O, the respective pH was adjusted with 10% phosphoric acid. Spectra were referenced to the maleic acid peak at 6.3 ppm, which was introduced in a Wilmad stem coaxial insert (Buena, NJ, USA) as an external reference (50 mM in D_2O). The ^{1}H spectra were analyzed with regard to CICS (*c*omplexation-*i*nduced *c*hemical *s*hifts) and $\Delta\delta$ values were determined according to

$\Delta\delta$ value = δ (uncomplexed ligand) – δ (1:1 mixture)

The ROESY experiments were accomplished after lyophilization of the sample solutions prepared as described above prior to the NMR measurements. The residues were re-dissolved in D_2O and again lyophilized. This procedure was repeated twice before transferring the final solution to a Schott (Mainz, Germany) NMR tube without a coaxial insert. The 2D plots were analyzed with respect to cross signal integrals, which were scaled to the glycosidic H1-H4 crosspeak.

Molecular Modeling: Molecular dynamics simulations were preformed according to Kahle et al.[17] with the differences that Packmol[26] was used instead of SYBYL for generating the solvent box and the larger solvent system around the cyclodextrin. First, 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[27] reported in the Cambridge Structural Database (CCD code: AGAZIR[28]), was retrieved and modified using the Accelrys DS Viewer Pro[29] 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 center 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 substructures of the original ligand and of the dipeptide were superimposed for this task with the phenylalanine substructure pointing to the narrow rim of the cyclodextrin cavity (see Figure 8). The original ligand was subsequently removed. Using this protocol a single file for each dipeptide complexed with β-CD was obtained.

These structures were transferred to Packmol[26] using OpenBabel[30] (file format conversion). The molecules (one cyclodextrin and one dipeptide) were solvated by employing the standard procedure of Packmol in a box with 9167 water and 113 urea molecules to represent the experimental conditions, i.e. an urea concentration of 681 mM. The respective molecule quantities were also calculated using Packmol. The Packmol-generated output was converted to NAMD,[31,32] input files using VEGA,[33–35] 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. The computations were run on a cluster of 20 3-GHz Pentium-4 PCs with the integrated CHARMM[36] 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-minimized 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/urea 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 nonbonded forces with a switching function starting at a distance of 10 Å and reaching zero at 13.5 Å were employed. During the simulation, every 500 fs a trajectory state was written to a file for further analysis. For every complex seven atoms – six from the cyclodextrin (C5′), termed p_1 to p_6, one from the included dipeptide (C-Ar-1) (see Figure 8) – were taken and read into MATLAB[37] using MATDCD.[38] Next, a plane was fit to the C5′ (cyclodextrin carbons) to calculate the distance d_i (see Figure 8) representing the immersion depth of the dipeptides.

The values for the distance 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 computation used point p_1 (first), p_3 (second), p_5 (third), the second employed p_2 (first), p_4 (second), p_6 (third). Both fits used the same coordinates of atom C-Ar-1 of each dipeptide. The equations for computing the immersion depth (d_i) are defined as follows:

$$r_1 = p_{\text{second}} - p_{\text{first}} \quad (1)$$

$$r_2 = p_{\text{third}} - p_{\text{first}} \quad (2)$$

$$f = r_1 \times r_2 \quad (3)$$

$$v = [p_E - p_{\text{first}}]^T \cdot f \quad (4)$$

$$d_i = \frac{v}{\|f\|} \quad (5)$$

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 C-Ar-1 atom relative to the plane, positive as well as negative values may result. For positive d_i C-Ar-1 is located above the plane and vice versa. The final immersion depth d_i was taken as the average of the two d_i values resulting from the two different fits (see above). Eventu-

ally, it turned out that using only one fit did not change the results substantially.

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