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Influence of the structure of cyclodextrins and amino acid sequence of dipeptides and tripeptides on the pH-dependent reversal of the migration order in capillary electrophoresis

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Abstract

The pH-dependent reversal of the migration order in cyclodextrin (CD)-mediated capillary electrophoresis (CE) enantioseparations of dipeptides and tripeptides has been studied between pH 2.5 and 3.5 using β-CD and several of its neutral derivatives. The occurrence of the phenomenon depended on both the structure of the CD and the amino acid composition and sequence of the peptides. While an inversion was observed for several peptides when native β-CD, dimethyl-β-cyclodextrin or trimethyl-β-cyclodextrin were added to the run buffer, no alteration of the order occurred in the presence of permethyl-β-cyclodextrin or hydroxypropyl-β-cyclodextrin. Most peptides that displayed a change of the migration behavior upon increasing the buffer pH contained Phe at the C-terminus. An ionizable carboxyl group in the peptide structure was a prerequisite. As seen with other uncommon migration effects in CE, the pH-dependent reversal of the migration order occurred in the pH region of the pK_a values of the peptide carboxyl functions.

Keywords: Enantiomer separation; pH effects; Chiral selectors; Buffer composition; Cyclodextrins; Peptides

1. Introduction

In chiral capillary electrophoresis (CE) a reversal of the migration order is an important issue. In contrast to chromatographic techniques, where peak tailing is usually observed, peak fronting as well as peak tailing can be observed in CE. Depending on the migration of the minor component compared to the migration of the major component it is often desirable to change the migration order in order to lower the detection limit and the limit of quantitation [1,2] and to increase the reproducibility [3]. This is especially true when only small mobility differences between the impurity and the major component are observed.

Several techniques which can be applied for a reversal of the enantiomer migration order in CE have been summarized in a review [4] and a book chapter [5]. Recently, the pH-dependent reversal of the migration order of the enantiomers of small peptides has been observed as a new phenomenon [6–8]. Using β-CD as chiral selector in the presence of urea, the LL enantiomer of the dipeptides Ala–Phe and Leu–Phe migrated faster than the respective DD enantiomers at pH 2.7 whereas the DD forms migrated faster at pH 3.5. This behavior was explained by differences in the binding constants between the peptides and β-CD at the respective pH values [7]. Interestingly, the DL and LD isomers of the dipeptides did not change the migration order. A similar

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behavior was studied in detail for the tripeptide enantiomers Gly–β-L-Asp–D-PheNH$_2$ and Gly–β-D-Asp–L-PheNH$_2$ and carboxymethyl-β-cyclodextrin (CM-β-CD) as chiral selector using a polyacrylamide-coated capillary [6,8]. At pH 3.60 the DL enantiomer was complexed stronger by CM-β-CD than the LD enantiomer resulting in the migration order DL before LD. In contrast, at pH 5.25 the LD isomer was complexed preferentially leading to the migration order LD before DL. The chiral recognition ability of CM-β-CD towards Gly–β-L-Asp–L-PheNH$_2$ and Gly–β-D-Asp–D-PheNH$_2$ or towards the corresponding α-Asp tripeptides was not altered by an increase of the buffer pH [8]. While studying the reversal of the migration order upon variation of the concentration of hydroxypropyl-β-cyclodextrin (HP-β-CD), pH-dependent reversal of the migration order was also observed for the enantioseparation of dansylated Phe and Trp at low concentrations of the chiral selector although this topic was not specifically addressed [9].

As only few examples have been published on this phenomenon to date, the present study was conducted in order to gain further insight on factors such as amino acid composition and sequence of the peptides and the nature of the CDs on the pH-dependence of the migration order of small peptides.

2. Experimental

2.1. Chemicals

β-Cyclodextrin (β-CD), permethyl-β-cyclodextrin (PM-β-CD, average substitution degree 1.8), hydroxypropyl-β-cyclodextrin (HP-β-CD, average substitution degree 4.2) were from Wacker (Munich, Germany). Heptakis-2,6-dimethyl-β-cyclodextrin (DM-β-CD) was obtained from Hewlett-Packard (Waldbronn, Germany), heptakis-2,3,6-trimethyl-β-cyclodextrin (TM-β-CD) was purchased from Aldrich (Deisenhofen, Germany). Gly–L-Phe, Gly–D-Phe, L-Ala–L-Phe, D-Ala–D-Phe, Gly–L-Ala–L-Phe, L-Ala–L-Tyr, L-Phe–L-Phe, D-Phe–D-Phe, L-Phe–L-Ala, L-Ala–L-Tyr, L-Ala–L-Trp and L-Ala–L-Leu were from Bachem (Heidelberg, Germany). All other peptides were prepared by reaction of the respective *N*-benzyloxycarbonyl-protected amino acid *N*-hydroxysuccinimide with the second amino acid in dimethylformamide [10] followed by hydrogenolytic deprotection. The enantiomers of Asp–PheOMe and Asp–PheNH$_2$ were prepared as described [6]. 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.

2.2. Instrumentation

All experiments were performed on a Beckman P/ACE 5510 instrument (Beckman–Coulter, Unterschleissheim, Germany) equipped with a diode array detector at 20°C using 50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The effective length of the capillaries was 40 cm, the total length was 47 cm. UV detection at 215 nm was performed at the cathodic end. Sample solutions (100 μg/ml peptide dissolved in water) were introduced at the anodic end at a pressure of 0.5 p.s.i. for 3 s (1 p.s.i.=6894.76 Pa). Separations were performed in 50 m*M* sodium phosphate buffer. The pH was adjusted using 100 m*M* phosphoric acid after the addition of the CDs. Buffers containing β-CD were prepared with 2 *M* urea. Between the analyses the capillaries were washed for 1 min with 100 m*M* phosphoric acid and for 3 min with the run buffer. The migration order of the peptide enantiomers was confirmed by spiking with the enantiomerically pure individual peptides. The electric currents in the case of β-CD were 59 μA and 47 μA at pH 2.5 and pH 3.5, respectively, in the case of PM-β-CD 43 μA and 36 μA, in the case of DM-β-CD 42 μA and 32 μA, in the case of TM-β-CD 43 μA and 33 μA, and in the case of HP-β-CD 47 μA and 40 μA.

3. Results and discussion

3.1. General aspects of the chiral separations

The CE separation of the peptide enantiomers by native β-CD and its neutral derivatives PM-β-CD, DM-β-CD, TM-β-CD and HP-β-CD was investigated under standard conditions using phosphate buffer, pH 2.5 and 3.5, respectively, which contained

20 mg/ml of each CD. No attempt was made to optimize the system with regard to the CD concentration, buffer pH, buffer molarity or buffer composition. The study was restricted to the LL and DD enantiomers of the peptides. The respective DL and LD epimers were not investigated. The results are summarized in Table 1. The peptide enantiomers could be separated with at least one of the CD derivatives at one of the two buffer pH values studied. β-CD, TM-β-CD and especially DM-β-CD were the most versatile chiral selectors for the present set of peptides while PM-β-CD and HP-β-CD displayed lower chiral recognition abilities towards the compounds under the experimental conditions applied. The enantiomers of Ala–Phe were readily separated by β-CD, DM-β-CD and TM-β-CD at both pH values, while the stereoisomers of the ''inverse'' Phe–Ala were only resolved by DM-β-CD at pH 2.5 under the standard conditions applied illustrating the influence of the amino acid sequence of peptides on CD-mediated chiral separations. Interestingly, the migration order of Phe–Ala was reversed compared to Ala–Phe in this example. When the concentration of DM-β-CD was raised to 50 mg/ml resolution of the enantiomers of Phe–Ala was also observed at pH 3.5. Small differences such as formation of a peptide amide or ester may also affected the chiral recognition by the CDs. For example, in the presence of TM-β-CD the LL enantiomer of Ala–PheOMe migrates before the DD stereoisomer at both pH values, while the DD isomer migrated faster than the LL enantiomer in the case of Ala–$PheNH_2$. TM-β-CD displayed opposite chiral recognition towards several dipeptides compared to the other CDs, especially β-CD and DM-β-CD. A similar observation has been made for many non-peptide compounds and may be attributed to the distorted shape of the cyclodextrin cavity of TM-β-CD compared to the other native and derivatized CDs [11,12]. The migration order of Phe–Phe was identical, i.e., DD before LL, for HP-β-CD and TM-β-CD at pH 2.5 while it is opposite (LL before DD) when β-CD is used as chiral selector.

3.2. pH-Dependent reversal of the migration order

Reversal of the migration order upon changing the pH of the run buffer from 2.5 to 3.5 was observed in

Table 1
Migration order of the peptide enantiomers at pH 2.5 and 3.5 using 20 mg/ml of the respective cyclodextrin[a]

Peptide	β-CD		PM-β-CD		DM-β-CD		TM-β-CD		HP-β-CD	
	pH 2.5	pH 3.5	pH 2.5	pH 3.5	pH 2.5	pH 3.5	pH 2.5	pH 3.5	pH 2.5	pH 3.5
Gly–Phe	Ø	D>L	D>L	Ø	D>L	D>L	***D>L***	***L>D***	D>L	D>L
Ala–Phe	***LL>DD***	***DD>LL***	Ø	Ø	***LL>DD***	***DD>LL***	***DD>LL***	***LL>DD***	Ø	Ø
Phe–Ala	Ø	Ø	Ø	Ø	DD>LL	DD>LL[b]	Ø	Ø	Ø	Ø
Ala–Tyr	***LL>DD***	***DD>LL***	Ø	Ø	***LL>DD***	***DD>LL***	***DD>LL***	***LL>DD***	Ø	DD>LL
Phe–Phe	***LL>DD***	***DD>LL***	Ø	Ø	Ø	DD>LL	***DD>LL***	***LL>DD***	DD>LL	DD>LL
Gly–Ala–Phe	Ø	DD>LL	DD>LL	Ø	DD>LL	DD>LL	***DD>LL***	***LL>DD***	DD>LL	Ø
Ala–Gly–Phe	Ø	Ø	–	–	DD>LL	Ø	***DD>LL***	***LL>DD***	DD>LL	Ø
Ala–Trp	Ø	Ø	LL>DD	Ø	LL>DD	LL>DD	DD>LL	Ø	Ø	Ø
Ala–Leu	LL>DD	LL>DD	LL>DD	nd	LL>DD	LL>DD	Ø	Ø	LL>DD	LL>DD
Ala–cHAla[c]	Ø	DD>LL	Ø	Ø	Ø	DD>LL	***DD>LL***	***LL>DD***	Ø	Ø
Ala–PheOMe	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD
Ala–$PheNH_2$	Ø	Ø	LL>DD	LL>DD	LL>DD	LL>DD	DD>LL	DD>LL	LL>DD	LL>DD
Glu–$PheNH_2$	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	Ø	Ø	LL>DD	LL>DD
Asp–PheOMe	***LL>DD***	***DD>LL***	LL>DD	nd	LL>DD	LL>DD	LL>DD	Ø	LL>DD	LL>DD
Asp–$PheNH_2$	Ø	DD>LL	LL>DD	nd	LL>DD	LL>DD	Ø	Ø	Ø	Ø
Gly–Asp–$PheNH_2$	DD>LL	DD>LL	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

[a] The faster migrating enantiomer is listed first. pH-Dependent reversal of the migration order is indicated in bold italic.
[b] Concentration of DM-β-CD: 50 mg/ml.
[c] Alanyl-cyclohexylalanine.
Ø, No separation.
nd, Not detectable within 50 min.

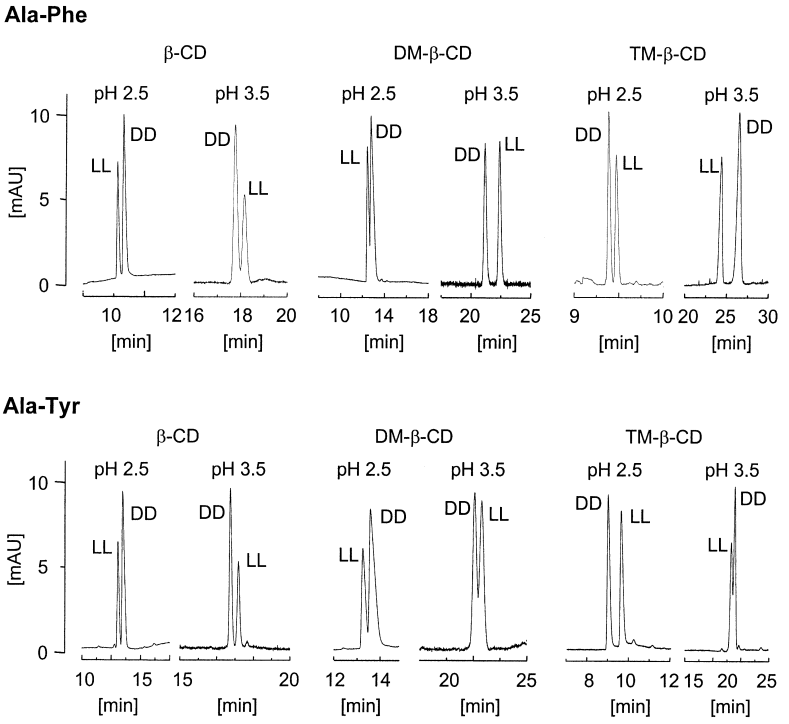

Fig. 1. Electropherograms of the chiral separation of Ala–Phe (top) and Ala–Tyr (bottom) at pH 2.5 and 3.5 in the presence of 20 mg/ml β-CD, DM-β-CD and TM-β-CD. Conditions: 47 cm (40 cm effective length) fused-silica capillary, 50 m*M* sodium phosphate buffer, 25 kV.

several cases and has been highlighted in Table 1. Fig. 1 shows the electropherograms of the CD-mediated separation of the enantiomers of Ala–Phe and Ala–Tyr. Fig. 2 illustrates the migration behavior of the Ala–Tyr enantiomers in the presence of 20 mg/ml TM-β-CD over the pH range 2.4 to 3.8. At pH 3.2 comigration of the enantiomers occurs.

Both, the structure of the CD and the structure of the peptide influence the occurrence of a reversal of the migration order upon increasing the pH from 2.5 to 3.5. With respect to the structure of the CDs, reversal of the migration order was found for many peptides when TM-β-CD was used. In almost all cases where the enantiomers could be separated at

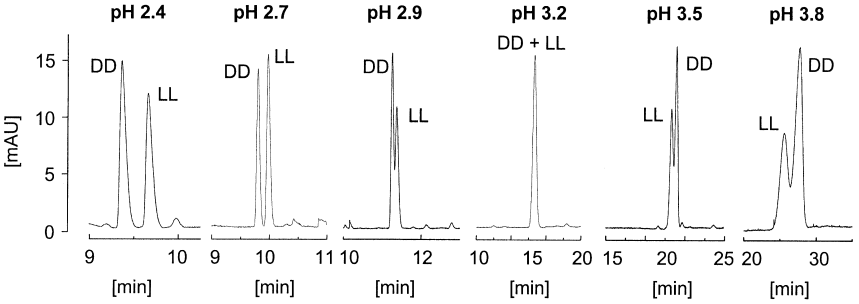

Fig. 2. pH-Dependent reversal of the migration order of the enantiomers of Ala–Tyr between pH 2.4 and 3.8. Conditions: 47 cm (40 cm effective length) fused-silica capillary, 50 m*M* sodium phosphate buffer, 25 kV.

pH 2.5 and 3.5 a concomitant change of the migration order occurred. β-CD and DM-β-CD displayed opposite chiral recognition towards several peptide enantiomers at both pH values while the phenomenon was not observed for the randomly substituted PM-β-CD and HP-β-CD. This may be due to the fact that β-CD, DM-β-CD and TM-β-CD are defined derivatives whereas PM-β-CD and HP-β-CD are a mixture of compounds differing in the degree of substitution.

The amino acid composition and sequence of the peptides greatly affected the pH-dependent inversion of the migration order. Although the number of compounds is limited, some ''structure–separation relationships'' may be discussed based on the present set of data. Phe at the C terminus appears to favor an opposite migration sequence at pH 2.5 and pH 3.5. Thus, inversion of the order of the enantiomers of Ala–Phe and Phe–Phe was observed for almost all CDs that were able to discriminate between the stereoisomers. In contrast, Phe–Ala was hardly separated at all. No inverse order was also observed for Ala–Leu although the enantiomers were resolved by almost all CDs. The pH-dependent reversal of the migration order has also been described previously for Ala–Phe and Leu–Phe between pH 2.0 and 3.5 in the presence of 36 m*M* β-CD [7]. Introduction of an OH substituent in the phenyl ring does not affect the separations significantly as Ala–Tyr displayed the same behavior as Ala–Phe. Upon changing Ala to the achiral amino acid Gly or addition of a Gly residue to Ala–Phe yielding Gly–Ala–Phe reversal was only observed in the presence of TM-β-CD. Increasing the distance between the two chiral centers from two atoms to four atoms by inserting Gly between Ala and Phe to yield the tripeptide Ala–Gly–Phe reversal was again only observed in the presence of TM-β-CD. The same is true when the aromatic ring is ''hydrogenated'', i.e., changing Phe to cyclohexylalanine (cHAla). ''Enlarging'' the spatial size of the aromatic moiety by changing Phe to Trp led to a general decrease of the chiral recognition abilities of the CDs towards the peptide. No inversion of the migration order could be observed. Upon derivatization of the terminal carboxyl group by formation of an ester or amide no pH-dependent reversal of the migration order was found despite the fact that the enantiomers of Ala–PheOMe and Ala–$PheNH_2$ were readily separated by several CDs. If a carboxyl group was introduced in the side chain by changing Ala to Asp a reversed migration order at pH 2.5 and 3.5 was observed in the case of the separation of Asp–PheOMe by β-CD. Thus, an ionizable carboxyl group appears to be a prerequisite for the occurrence of the pH-dependent reversal of the migration order. This inversion is observed in the buffer pH range of the pK_a values of the terminal carboxyl groups of small peptides which range between 2.5 and 3.8 [13,14]. A pK_a of 3.2 has been reported for the side chain carboxyl function of L-Asp–L-PheOMe [15]. It has been generally observed that the most effective changes of the migration behavior of closely related compounds in CE apparently including the pH-dependent reversal of the migration order occur within the pK_a region of ionizable groups. This is in accordance with the literature where inversion of the migration sequence of the LL and DD enantiomers of Ala–Phe and Leu–Phe in the presence of β-CD was observed between pH 2.7 and 3.5 [7] and between pH 3.6 and 5.25 in the case of the CM-β-CD-mediated resolution of the tripeptide enantiomers Gly–β-L-Asp–D-$PheNH_2$ and Gly–β-D-Asp–L-$PheNH_2$ in a polyacrylamide-coated capillary [6,8]. pH-Dependent reversal of the migration order has been attributed to a change of the relative strength of the complexation of peptide enantiomers by a CD due to a variation of the charge pattern of the peptides [6,8]. The enantiomer that formed weaker complexes at lower pH was bound more strongly at higher pH reversing the observed migration order. In a recent study Rizzi and Kremser also described a pH-dependent inversion of the relative ratio of the binding constants between HP-β-CD and dansylated Phe (Dns-Phe) and Trp (Dns-Trp) [9]. At pH 2.5 the complexation constant for the D-enantiomers of both compounds was larger than the constant for the respective L-enantiomer. In contrast, the L-enantiomers were complexed stronger at pH 3.8 (Dns-Phe) and 4.1 (Dns-Trp), respectively, resulting in reversal of the migration order at low CD concentrations. However, at high concentrations of the chiral selector no reversal of the migration order was observed. This was attributed to the fact that the enantioselective pK_a shifts induced by the formation of the diastereomeric complexes between the enantiomers and the chiral selector resulted in a higher

electrophoretic mobility of the complex containing the stronger bound enantiomer counteracting the normally retarding effect of a stronger complexation constant on the analyte mobility. The effect was observed in the buffer pH range across ± 2 units around the pK_a of the analytes stressing the importance of this pH region for uncommon migration behavior.

Vigh and co-workers developed theoretical models for enantiomer resolutions of acidic and basic electrolytes as well as neutral solutes using neutral and charged selectors [16–18]. The theories also predict that the migration order of enantiomers can be reversed by varying the pH of the run buffer. However, these models are primarily based on mobilities and do not predict a change of the stereoselectivity of the complexation between selectors and peptides that were used to interpret the reversal observed previously [7,8]. However, in the light of the findings of Rizzi and Kremser [9] detailed studies including complexation constants and complex mobilities will be required for a detailed interpretation of the mechanisms of the pH-dependent reversal of the migration order.

4. Conclusions

pH-Dependent reversal of the migration order can be observed in CD-mediated chiral separations of small peptides and amino acid derivatives. The occurrence is dependent on both, the structure of the CD and the amino acid composition and amino acid sequence of the peptides. Phe at the C-terminus seems to favor an inversion of the migration order. A ionizable carboxyl group is a prerequisite as the reversal occurred in the pH region of the pK_a values of the peptide carboxyl functions as often seen for other uncommon migration phenomena.

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