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Role of helix formation for the retention of peptides in reversed-phase high-performance liquid chromatography

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Abstract

In order to get insight into the role of helix formation for retention in reversed-phase HPLC, we have studied the isocratic retention behavior of amphipathic and non-amphipathic potentially helical model peptides. Plots of the logarithmic capacity factor in absence of organic solvent ($\ln k_0$) versus $1/T$ were used to derive the enthalpy, ΔH^0, the free energy, ΔG^0, the entropy of interaction, ΔS^0, and the heat capacity change, ΔC_p. Retention of all peptides was accompanied by negative ΔC_p revealing that hydrophobic interactions play a large role independent of peptide sequence and secondary structure. ΔH^0 was negative for the amphipathic analogs and was attributed mainly to helix formation of these peptides upon interaction with the stationary phase. In contrast, ΔH^0 was considerably less exothermic or even endothermic for the non-amphipathic analogs. The differences in helix formation between the individual analogs were quantified on the basis of thermodynamic data of helix formation previously derived for peptides in a hydrophobic environment. Correlation of the helicity with the free energy of stationary phase interaction revealed that helix formation accounts for ~40–70% of ΔG^0, and is hence in addition to the hydrophobic effect a major driving force of retention.

Keywords: Helix formation; Thermodynamic parameters; Heat capacity; Peptides

1. Introduction

Over the last decade, reversed-phase HPLC has become increasingly popular not only for the purification and characterization of peptides and proteins, but also for the study of conformational changes accompanying peptide–protein interactions with a hydrophobic/hydrophilic interface. An interface-induced secondary structure formation plays a major role for peptides possessing the potential to assume an amphipathic α-helix or β-sheet structure [1–6]. Amphipathic peptides interact with the hydrophobic phase in such a way that the hydrophobic part of the structure strongly interacts with the reversed stationary phase while the polar groups remain in contact with the hydrophilic mobile phase. HPLC studies revealed that the formation of a hydrophobic binding domain in amphipathic helices considerably increases the retention time in comparison to non-amphipathic analogs [1,3]. The study of the formation and stabilization of amphipathic secondary structures is of particular interest, because amphipathic α-helices and β-sheets are a common secondary structure element in globular and mem-

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brane proteins. Furthermore, amphipathic structures are a typical membrane-binding motif present in most membrane-interacting proteins and peptides [7,8]. Insight into the driving forces of peptide–hydrophobic phase interaction and secondary structure formation may therefore help to understand the determinants of protein folding and membrane binding.

The interaction of hydrophobic amino acid side chains with a non-polar stationary phase is by far the main driving force of the retention process in reversed-phase HPLC [9,10]. However, additional contributions may in principle come from electrostatic interactions, arising form the transfer of dipoles and charges from an environment of a high to a low dielectric constant, from changes in Van der Waals interactions between the stationary phase chains and from conformational transitions accompanying the interaction of peptides with the stationary phase. Van der Waals interactions and the random coil–helix transition were recently shown to contribute considerably to the free energy of binding of amphipathic peptides to lipid membranes [11,12]. Despite the existence of numerous HPLC studies, addressing the interactions of amphipathic and non-amphipathic peptides with reversed stationary phases, only few studies focused on a determination of the thermodynamic parameters of retention and on an interpretation of the results with regard to the driving forces [13,14].

It is hence of major concern of the present study (i) to elucidate the thermodynamic parameters of the interaction of amphipathic and non-amphipathic potentially α-helical model peptides with a reversed stationary phase and (ii) to interpret the results with respect to the energetic contributions involved in the retention process. This paper especially focuses on the contribution of helix formation to the retention process of peptides. By studying the isocratic retention behavior of model peptides having the potential to assume an amphipathic α-helical conformation, we have determined the relevant thermodynamic parameters of interaction of these peptides with a reversed-phase column, i.e. the standard free energy of interaction, ΔG^0, the enthalpy change, ΔH^0, the entropy change, ΔS^0, and the heat capacity change, ΔC_p. In order to get insight into the contribution of amphipathicity and helix formation to these interaction parameters, we have additionally studied peptides capable of forming a non-amphipathic helix and peptides possessing a largely reduced helix-forming potential. The latter peptides were so-called double D-isomers, i.e. peptides with two L-amino acids in the middle of the peptide sequence substituted by their corresponding D-enantiomers. Double D-substitution was previously shown to result in a distinct local disturbance of the helical conformation and in a reduced capability to interact with HPLC stationary phases and with phospholipid membranes [2,3,15–17].

Our results reveal that, in addition to the hydrophobic effect, the formation of an α-helical conformation upon interaction with the stationary phase is a major driving force of the retention process. The strong interaction of amphipathic helical peptides with the stationary phase is therefore caused by the fact that these peptides can, at the same time, form an α-helical conformation and establish an optimal contact between hydrophobic peptide groups and the hydrophobic stationary phase.

2. Experimental

2.1. Peptide synthesis

Peptides were synthesized on a 433A peptide synthesizer (PE Biosystems, Weiterstadt, Germany) by solid-phase methods using standard Fmoc (N^{α}-9-fluorenylmethoxycarbonyl) chemistry on TentaGel S RAM resin (Rapp Polymere, Tübingen, Germany) as described previously [2]. The N-terminus was acetylated using a mixture of acetic anhydride–DIEA–dimethyl formamide (1:2:7) for 30 min. The peptides were cleaved from the resin support with a solution of 2% triisopropylsilane, 5% phenol, and 5% water in trifluoroacetic acid for 3 h. The crude products were precipitated with diethyl ether and purified by preparative reversed-phase chromatography on PolyEncap A300 (10 μm, 250×20 mm I.D., Bischoff Analysentechnik, Leonberg, Germany), using an acetonitrile–0.1% trifluoroacetic acid solvent system. The purified peptides (>95% according to HPLC analysis) were characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Voyager-DE STR, Perseptive

Biosystems, Framingham, MA, USA) using an α-cyano-4-hydroxycinnamic acid matrix, which gave the expected $[M+H]^+$ mass peaks for each peptide. The peptide content of lyophilized samples was determined by quantitative amino acid analysis (LC 3000, Biotronik-Eppendorf, Maintal, Germany).

2.2. Circular dichroism measurements

Circular dichroism (CD) spectra were measured at 25°C on a J-720 spectropolarimeter (Jasco, Tokyo, Japan) in a quartz cell of 0.1-cm path length between 190 and 260 nm. The instrument was calibrated with an aqueous solution of (+)-10-camphorsulfonic acid. Peptide concentrations were $2\cdot10^{-5}$ *M* in 0.01 *M* KH_2PO_4, pH 7.0. Spectra were the average of a series of five scans made at 0.1-nm intervals. The fraction of helix (f) which is assumed to be linearly related to the measured ellipticity at 222 nm ($[\Theta]_{222}$) was calculated using $f=[\Theta]_{222}-[\Theta]_{coil}/[\Theta]_{helix}-[\Theta]_{coil}$. $[\Theta]_{helix}=(-44000+250T)\ (1-3/N)$ and $[\Theta]_{coil}=2220-53T$ represent the mean residue ellipticity of a complete helix and complete random coil, respectively, where T is the temperature in °C and N is the number of amino acid residues [18].

2.3. Reversed-phase HPLC

Chromatographic measurements were performed on a Jasco HPLC system (Jasco, Germany), consisting of two PU-980 pumps, an AS-950 autoinjector, and a UV-975 detector operating at 220 nm. Runs were carried out on silica encapsulated with butylacrylamide (250×4.6 mm I.D., 5 μm, Bischoff Analysentechnik, Germany). The phase was prepared by copolymerization of vinyl-derivatized silica (Nucleosil 5 μm, 300-Å pore diameter) with butylacrylamide as previously described [19]. The sample concentration was 1 mg/ml of peptide in the eluent. The injection volume was 20 μl. Runs were performed at temperatures between 8 and 85°C (thermostated system), and at an eluent flow-rate of 1 ml/min. Mobile phase A was 0.1% trifluoroacetic acid in water and B was 0.1% trifluoroacetic acid in acetonitrile–water (1:1, v/v). Isocratic retention times were determined using eluents consisting of 0.1% trifluoroacetic acid in water containing different concentrations of mobile phase B. The isocratic retention times (t_R) are used to calculate the capacity factors k' using the following equation: $k'=(t_R-t_0)/t_0$, where t_R is the retention time of the peptide, and t_0 is the column void time. Data for t_0 were obtained by injecting a liquid mixture with a volume composition different from that of the eluent.

2.4. Theory

RP-HPLC studies of peptides usually employ mobile phases of a mixture of water and organic solvent and are generally analyzed using the linear solvent strengths model [20,21]. According to this theory, a linear relationship exists between the mole fraction of organic solvent ψ and the capacity factors k':

$$\log k' = \log k - S\psi \quad (1)$$

where k is the capacity factor in absence of the organic solvent and S is the slope of a plot of $\log k'$ versus the organic solvent content of the mobile phase. The S value can be related to the hydrophobic contact area between the peptide and the stationary phase [22]. The k value is a measure of the affinity of a peptide for the stationary phase in the absence of an organic solvent and is related to the partition coefficient K according to

$$k = K\Phi \quad (2)$$

where Φ is the phase ratio, i.e. the volume ratio of the stationary phase and the mobile phase of the column, which is a constant for a given column and was estimated to be 0.1, as previously described [13]. The standard free energy of interaction with the stationary phase ΔG^0 can be obtained from

$$\Delta G^0 = -RT \ln K = -RT(\ln k - \ln \Phi) \quad (3)$$

where T is the temperature and R is the universal gas constant. Combination of Eq. (3) with $\Delta G^0=\Delta H^0-T\,\Delta S^0$ yields

$$\ln k = -\Delta H^0/RT + \Delta S^0/R + \ln \Phi \quad (4)$$

When ΔH^0 and ΔS^0 are independent of temperature, plots of $\ln k$ versus $1/T$ (Van 't Hoff plots) are linear and a direct determination of both thermodynamic parameters is possible. However, a major

driving force of retention in RP-HPLC is the hydrophobic effect, which is characterized by a removal of hydrophobically bound water from hydrophobic solute surfaces upon binding to the stationary phase. This desolvation is accompanied by a decrease in the heat capacity of the system resulting consequently in a large temperature dependence of the interaction enthalpy [23]. Therefore, Van 't Hoff plots of the interaction of peptides with a hydrophobic surface are expected to be non-linear. To account for the non-linearity, the integrated form of the Van 't Hoff equation can be used:

$$\ln k = \frac{\Delta H^0}{R} \cdot \left(\frac{1}{T_0} - \frac{1}{T}\right) + \frac{\Delta C_p}{R} \cdot \ln \frac{T}{T_0} + \frac{\Delta C_p T_0}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_0}\right) + \ln k_0 \quad (5)$$

where T_0 is the reference temperature (35°C), ΔH^0 is the enthalpy change at T_0, ΔC_p is the heat capacity change and $\ln k_0$ is $\ln k$ at T_0. A fit of Eq. (5) to the Van 't Hoff plot finally yields ΔH^0 and ΔC_p. Knowledge of the phase ratio allows then a calculation of ΔS^0. It should be mentioned that the phase ratio given in the literature for a reversed-phase stationary phase ($\Phi \sim 0.1$) is only a rough estimate. Since the values of ΔG^0 and ΔS^0 depend critically on Φ, the parameters determined should only be used to discuss differences in the retention of peptides studied with the same chromatographic column.

3. Results

3.1. Peptide design

We have designed and synthesized 18-amino acid model peptides possessing the potential to form either ideally amphipathic or non-amphipathic α-helices (Table 1). Peptide aA (amphipathic peptide A) consists of six hydrophobic leucine residues, which form the hydrophobic core of the amphipathic helix, six polar threonine residues, three positively charged lysine and three glutamic acid residues (Fig. 1). The peptide has a charge of +3 under the condition of HPLC, since the Glu residues are protonated. An additional stabilization of a helical conformation is possible by hydrogen bond formation between Lys and Glu side chains [24]. The mean residue hydrophobicity of the peptide is −0.170 as calculated from the Eisenberg hydrophobicity scale (Table 1) [25]. The hydrophobic moment of the peptide, assuming an α-helical conformation, was estimated to be 0.387. Peptide nA (non-amphipathic A) has the same amino acid composition as aA. However, the amino acids are distributed in such a way that the hydrophobic moment is zero in the α-helical conformation (non-amphipathic helix).

Similar design principles have been employed for the peptide pair aB (amphipathic peptide B) and nB (non-amphipathic peptide B). Again, both peptides

Table 1
Sequence, charge, hydrophobicity, hydrophobic moment and α-helix propensity of peptides

Peptide	Sequence[a]	Charge[b]	Hydrophobicity,[c] h	Hydrophobic moment,[c] μ	Helix propensity,[d] ΣP_α	Fraction of helix[e]
aA	Ac-LKTLT ETLKE LTKTL TEL-NH_2	+3	−0.170	0.387	20.91	0.99
nA	Ac-LLKTT ELLKT TELLK TTE-NH_2	+3	−0.170	0.000	20.91	1.00
aB	Ac-LKTLA TALTK LAKTL TTL-NH_2	+3	−0.025	0.342	22.17	0.83
nB	Ac-LLKTT ALLKT TALLK TTA-NH_2	+3	−0.025	0.000	22.17	0.96

[a] The one-letter code for amino acid residues is used.
[b] Peptide charge at pH 2, i.e. under condition of reversed-phase HPLC (0.1% trifluoroacetic acid).
[c] The mean residue hydrophobicity as well as the hydrophobic moment were calculated using the Eisenberg consensus scale of hydrophobicity [25].
[d] Helix propensity was calculated using the Chou-Fasman helix propensity parameters [26].
[e] Measured by CD spectroscopy; conditions: 10 m*M* phosphate buffer, 50% TFE, pH 7.0, peptide concentration $2 \cdot 10^{-5}$ *M*.

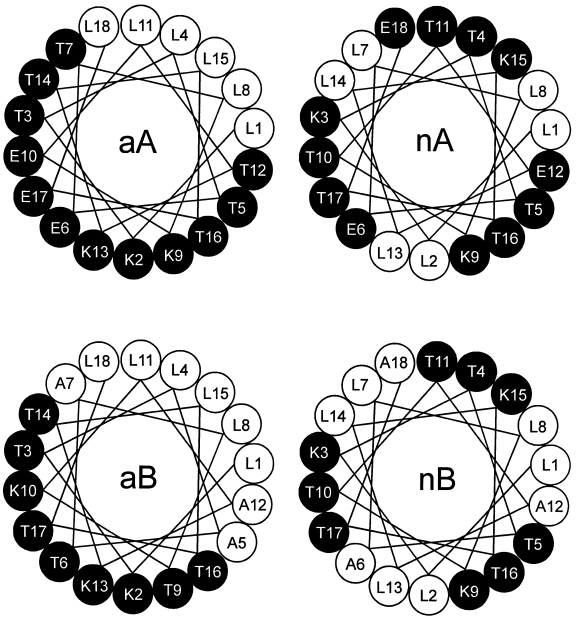

Fig. 1. Helical wheel projection of peptides aA, nA (A set) and aB, nB (B set). The black circles refer to polar amino acids, whereas the white circles refer to hydrophobic residues.

bear a formal charge of +3 due to the presence of three Lys residues. The mean residue hydrophobicity of the peptides is −0.025. Hence the peptides are somewhat more hydrophobic than the aA/nA pair. The amphipathic analog aB has a hydrophobic moment of 0.342, which is somewhat smaller than that of peptide aA. All four peptides synthesized are expected to have a high helix-forming tendency according to the Chou-Fasman parameters (Table 1) [26]. Furthermore, the N-terminus of the peptides is acetylated and the C-terminus is amidated. This suppresses unfavorable dipole-charge interaction in the helical conformation thereby increasing helix stability [27]. Circular dichroism measurements revealed that all peptides are nearly unstructured in aqueous solution (data not shown) but assume α-helical conformations in presence of 50% of the helix-stabilizing solvent trifluoroethanol (Table 1). Therefore, the analogs are able to undergo a random coil–α-helix transition [28].

In addition to these two peptide pairs, we have synthesized double D-isomers of all peptides (d-aA, d-nA, d-aB, and d-nB). In these analogs, two adjacent amino acids in middle of the sequence (position 9 and 10) are substituted by their corresponding D-enantiomers. Double D-substitution was previously shown to lead to a local disturbance of helical conformations without changing other important peptide properties such as hydrophobicity and side chain functionality [2]. For amphipathic peptides it was shown that double D-substitution results in a pronounced decrease of the retention times in reversed-phase HPLC [2,3,28]. Since double D-substitution solely reduces the helix propensity of a peptide, double D-analogs can be used in reversed-phase HPLC as a probe indicating the formation of a helical conformation in the corresponding all L-amino acid peptide.

In the following we will refer to peptides of the same amino acid composition as one peptide set. Peptide set A consists of peptides aA, nA, d-aA, and d-nA and peptide set B consists of peptides aB, nB, d-aB, and d-nB.

3.2. HPLC-retention behavior

Isocratic retention times of all peptides were determined on a polymer-encapsulated silica based stationary phase coated with butylacrylamide at temperatures between 8 and 85°C. Because of the relatively small size of the hydrophobic ligand (C_4) this column possesses only a moderate hydrophobicity allowing the use of comparably small concentrations of organic modifier for elution. Polymer-encapsulated silica phases are suitable for the chromatography of amphipathic peptides with basic amino acids and hydrophobic domains [3,34]. Furthermore, a short chain reversed-phase surface offers the advantage that temperature induced structural transitions are unlikely to occur.

The capacity factors k' have been calculated from the retention times obtained with acetonitrile–water mixtures with organic solvent contents of between 10 and 40% (v/v) (Fig. 2). At a specific temperature, k' values were obtained for three to four different acetonitrile concentrations. As an example, Fig. 3 shows the dependence of log k' on the acetonitrile concentration for the amphipathic peptide aA (Fig.

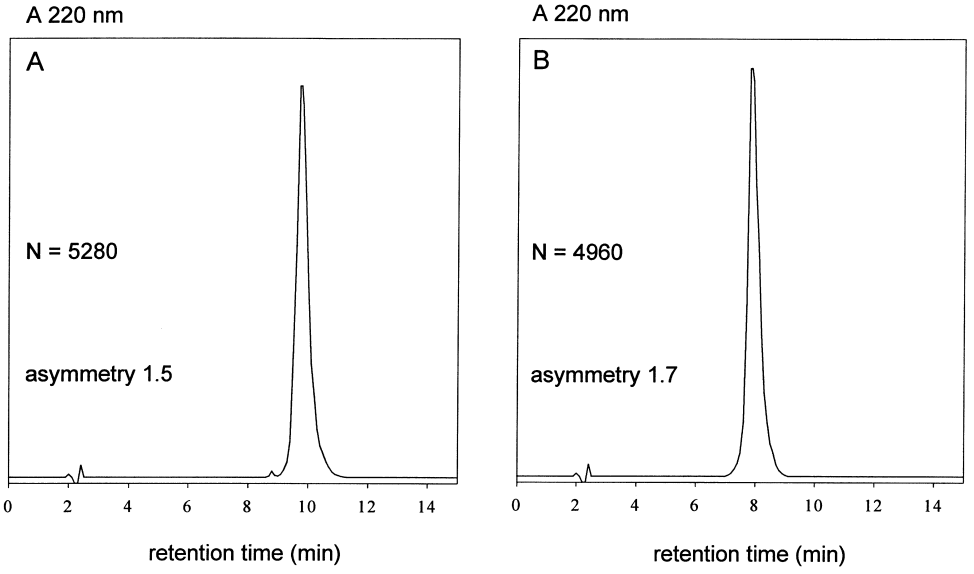

Fig. 2. Reversed-phase HPLC chromatograms of (A) the amphipathic peptide aA and (B) the non-amphipathic peptide nA on polymer-encapsulated silica column. Experimental conditions: eluent A–eluent B (45:55) for peptide aA, eluent A–eluent B (65:35) for peptide nA; eluent A, 0.1% aqueous trifluoroacetic acid; eluent B, 0.1% trifluoroacetic acid in acetonitrile–water (1:1, v/v); 1 ml/min, 25°C.

3A), the non-amphipathic peptide nA (Fig. 3B) and the double D-isomer d-aA (Fig. 3C). From this figure, three important results can be obtained. (i) Despite the same hydrophobicity, the amphipathic peptide aA interacts much more strongly with the stationary phase than the non-amphipathic analog nA and peptide d-aA, as revealed by the higher acetonitrile content needed to elute peptide A. (ii) At temperatures above 35°C, the capacity factors were found to decrease with increasing temperature reflecting a reduced capability of the peptides to interact with the stationary phase. A similar behavior was generally observed in reversed-phase chromatography of small molecules [29–31]. (iii) A linear relation exists between log k' values and the acetonitrile content (correlation coefficients $r^2 > 0.991$) allowing calculation of the capacity factor k in absence of organic solvent according to Eq. (1). The same characteristics were also observed for peptides aB and nB as well as for the double D-analogs d-nA, d-aB, and d-nB (not shown). The extrapolated capacity factors in absence of organic solvent ($\ln k$) at 35°C are summarized in Table 2. For peptide set A, $\ln k$ decreases in the order aA > d-aA > nA > d-nA. The same order of capacity factors was observed for peptide set B (aB > d-aB > nB > d-nB). The existence of a linear relation between log k' and the organic solvent content Ψ additionally allows calculation of the S values (see Table 2 for 35°C). According to the solvophobic theory, the S value is proportional to the hydrophobic contact area between peptide and stationary phase [22]. The S values are highest for both amphipathic peptides and are reduced by ~10–25% for the non-amphipathic analogs as well as for the double D-analogs. Using a phase ratio of $\Phi = 0.1$, standard free energies of interaction can be calculated from the capacity factors according to Eq. (3) (Table 2).

Fig. 4 (aA, nA, d-aA, d-nA) and Fig. 5 (aB, nB, d-aB, d-nB) show the Van 't Hoff plots, i.e. the dependence of $\ln k$ on $1/T$. For all peptides, nonlinear Van 't Hoff plots have been observed and most Van 't Hoff plots go through a maximum in the temperature range investigated. This behavior indicates that the interaction of the peptides with the stationary phase cannot be described by a constant ΔH^0 over the whole temperature range (7–85°C). Therefore, ΔH^0 is a function of the temperature and the retention process is accompanied by a change in the heat capacity ΔC_p. The individual thermody-

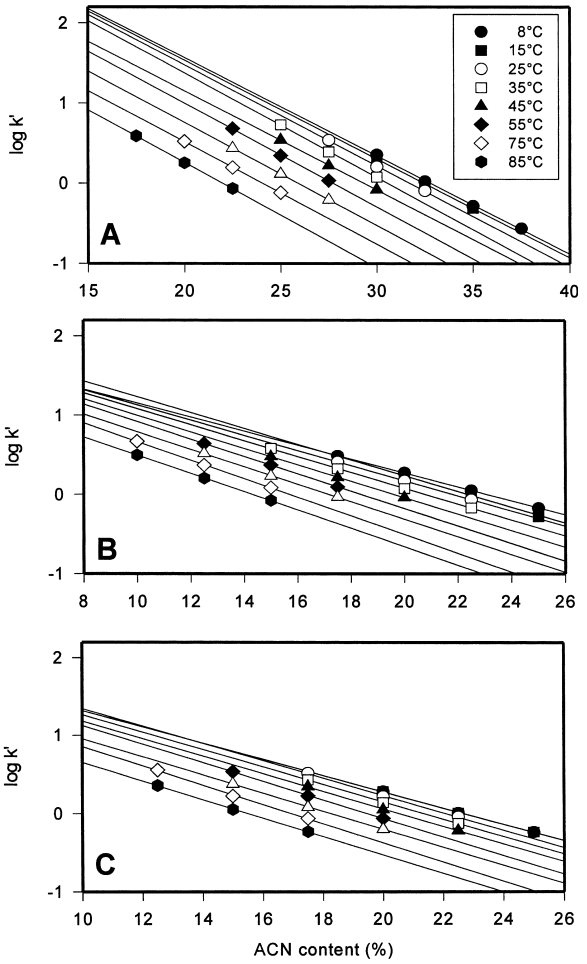

Fig. 3. Dependence of the logarithm of the capacity factor on the acetonitrile content at different temperatures. (A) Peptide aA, (B) peptide nA and (C) peptide d-aA.

namic quantities ΔH^0 (35°C) and ΔC_p can be calculated for each peptide from a fit of Eq. (5) to the experimental data. The best fits are included in Figs. 4 and 5 as solid lines. Knowledge of ΔG^0 and ΔH^0 allows finally a calculation of ΔS^0. All thermodynamic parameters at the reference temperature of 35°C are summarized in Table 2. The data reveal that the interaction of all peptides with the stationary phase was accompanied by a large negative heat capacity change, ΔC_p, of between -300 and -900 J/mol K. Negative heat capacity changes are the main hallmark of the hydrophobic effect [23], which is considered to be a main driving force of retention in reversed-phase HPLC. Negative ΔC_p values were recently also reported for the retention of dansylated amino acids in hydrophobic interaction chromatography [32]. However, it should be emphasized that the ΔC_p values observed in this work do not correlate with the $\ln k$ and ΔG^0 values of the peptides. The interaction of both ideally amphipathic peptides aA and aB with the stationary phase is characterized by a large exothermic enthalpy change at the reference temperature of 35°C. Peptides with a reduced amphipathicity or with a disturbance of a helix due to double D-substitution give rise to smaller exothermic enthalpies or even to endothermic enthalpy changes. Interestingly, the interaction enthalpy increases in a similar order as the free energy for both peptide sets (aA<d-aA≈nA<d-nA and aB<d-aB<nB<d-nB).

4. Discussion

In order to get insight into the role of helix formation for the retention process we have determined thermodynamic parameters for the interaction of peptides with a hydrophobic stationary phase. It was previously shown for a variety of different potentially amphipathic helical peptides that retention qualitatively correlates with peptide amphipathicity [1–3]. Recent studies provided direct evidence for the formation of amphipathic α-helical structures of the stationary phase-bound peptides during reversed-phase HPLC [33–35].

In the present study, the two peptides having the potential of forming an ideally amphipathic helix (peptides aA and aB) show indeed the highest $\ln k$ values and hence the lowest ΔG^0 values. This confirms qualitatively the preferred interaction of amphipathic peptides with a hydrophobic stationary phase. The retention is drastically reduced by a change in the primary structure which completely abolishes the amphipathicity of the helices (peptides nA and nB) or by a disturbance of the helix formation due to double D-substitution (peptides d-aA and d-aB). A qualitative explanation for this behavior is usually given by the existence of a preferred hydrophobic binding domain [1]. The formation of a large hydrophobic binding domain, as present in amphipathic α-helices, allows an effective

Table 2
Thermodynamic interaction parameters of peptides at 35°C

Peptide	ln k[a]	ΔG^{0} [b] (kJ/mol)	ΔH^{0} [b] (kJ/mol)	ΔS^{0} [b] (J/mol K)	ΔC_p [b] (J/mol K)	S[a]
aA	9.17	−29.4	−23.6	18.8	−816	30.0
d-aA	5.47	−19.9	−5.3	47.4	−731	25.6
nA	4.70	−17.9	−5.9	28.6	−471	23.2
d-nA	3.81	−15.6	11.5	88.0	−961	22.5
aB	7.33	−24.7	−29.3	−14.9	−302	24.1
d-aB	4.62	−17.7	4.6	72.4	−960	23.7
nB	3.65	−15.2	15.2	98.7	−796	19.8
d-nB	3.35	−14.5	16.4	100.3	−865	20.3

[a] The S value and ln k were determined from the linear regression of plots of log k' versus the organic solvent fraction using Eq. (1).
[b] ΔG^{0} and ΔS^{0} have been calculated using Eq. (3) and $\Delta G^{0} = \Delta H^{0} - T\Delta S^{0}$. ΔH^{0} and ΔC_p were obtained from fits of Eq. (5) to the Van 't Hoff plots (cf. Figs. 4 and 5).

hydrophobic contact between the peptides and the reversed-phase stationary phase. In contrast, compact hydrophobic domains cannot be formed in non-amphipathic peptides (nA and nB) and in peptides where helix formation is disturbed by D-amino acid substitutions. Despite the general suitability of this concept to compare qualitatively the retention times of peptides with different potential to form an amphipathic helix, two arguments suggest that this concept is not sufficient for a comprehensive understanding of the retention process:

(i) With regard to the hydrophobic effect the amphipathic α-helix has to be compared with an extended conformation. It can be assumed that also in an extended peptide conformation the side chains can be arranged in such a way that the hydrophobic residues strongly interact with the stationary phase

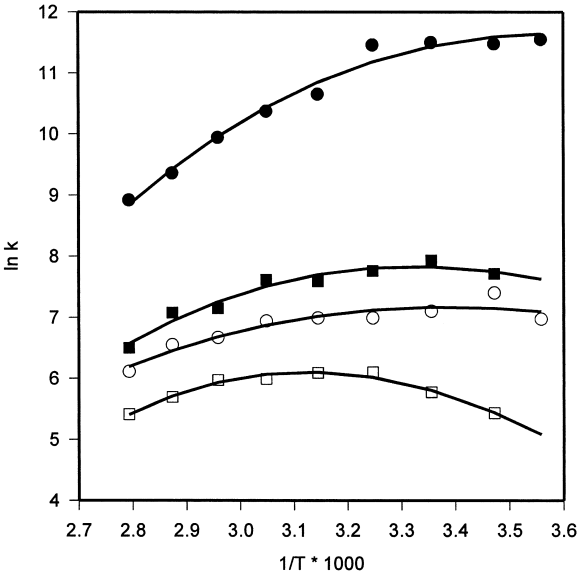

Fig. 4. Van 't Hoff plots of retention data obtained with peptides of the A set: (●) peptide aA, (■) peptide d-aA, (○) peptide nA and (□) peptide d-nA. The solid lines correspond to the best fit of Eq. (5) to the experimental data.

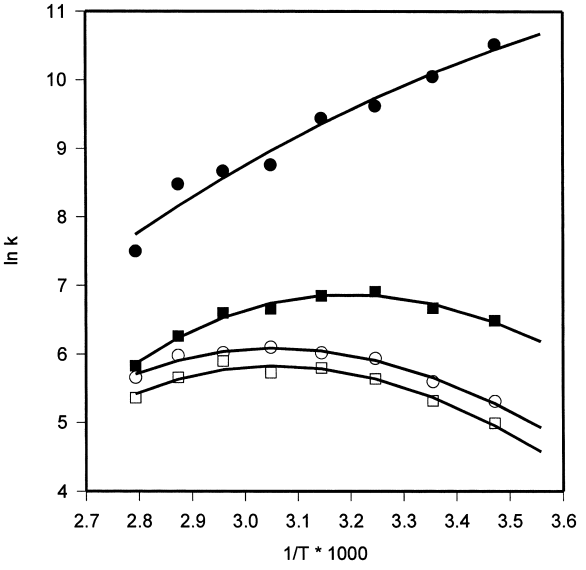

Fig. 5. Van 't Hoff plots of retention data obtained with peptides of the B set: (●) peptide aB, (■) peptide d-aB, (○) peptide nB and (□) peptide d-nB. The solid lines correspond to the best fit of Eq. (5) to the experimental data.

while the hydrophilic residues remain in contact with the solvent. Hence, it is not straightforward to assume hydrophobic interactions to be more efficient in an amphipathic helix.

(ii) HPLC studies of a non-amphipathic model peptide and its complete double-D-amino acid substitution set at a C_4 phase revealed a pronounced decrease in the retention times for the double D-isomers compared to the all L-peptide [3]. A decrease of capacity factor was also observed for the double D-analogs of the non-amphipathic peptides in the present work (d-nA and d-nB). This can only be explained by an at least partial helix formation of the non-amphipathic all L-peptides nA and nB upon interaction with the stationary phase. However, helix formation of non-amphipathic peptide does not result in the formation of a preferred binding domain, and therefore, must be attributed to a higher stability of a helix versus an extended conformation in the more hydrophobic environment of the stationary phase. Hence, helix formation per se, i.e. the formation of intra-molecular hydrogen bonds, favorably contributes to the overall thermodynamics of stationary phase interaction.

In order to understand the thermodynamic parameters of the interaction of the peptides with the stationary phase we have therefore to consider the contributions of both the hydrophobic effect and helix formation.

A striking feature of the retention process of all peptides investigated in this study is the large negative ΔC_p, which is a main hallmark of the hydrophobic effect (Table 2) [23]. The hydrophobic effect, i.e. the dehydration of hydrophobic solute surfaces upon transfer from an aqueous into a hydrophobic phase, is a major driving force of the retention process in reversed-phase HPLC [9,10]. The importance of the hydrophobic effect for the retention of all analogs in this study is reinforced by the *S* values, which are related to the hydrophobic contact areas between peptides and stationary phase. The peptides of the A set show *S* values between 22.5 and 30.0 and the analogs of the B set between 19.8 and 24.1 (Table 2). Hence, a pronounced hydrophobic contact exists between peptide hydrophobic groups and the stationary phase not only for the amphipathic peptides but also for the non-amphipathic analogs and the double D-isomers.

A remarkable feature of the interaction of the amphipathic peptides aA and aB with the stationary phase is the large negative ΔH^0 value. The enthalpy change attributed to the hydrophobic effect is close to zero around room temperature [36]. However, the coil–α-helix transition of peptides was reported to be characterized by a distinctly exothermic enthalpy change [12,37,38]. Therefore, we suggest that ΔH^0 of the amphipathic analogs is dominated by a stationary phase-induced coil–helix transition. The helix-forming tendency of small peptides is generally low in an aqueous environment, since intramolecular hydrogen bonds compete with hydrogen bonds to water. This is also valid for peptides aA and aB, which adopt mainly a random coil conformation in aqueous solution as revealed by CD spectroscopy. Interaction of amphipathic peptides with a hydrophobic/hydrophilic interface such as a biological membrane or an RP-HPLC stationary phase, induces helix formation [1,33,35]. In water, helix formation was found to be driven by a negative enthalpy of between -3.8 and -5.4 kJ/mol per residue. The negative enthalpy was mainly attributed to differences in the enthalpic states between intra- and intermolecular hydrogen bonds [37,38]. In the more hydrophobic environment of a trifluoroethanol–water mixture and in a lipid membrane, helix formation was accompanied by a somewhat less negative enthalpy change of $\Delta H_{helix} = -2.9$ to -3.3 kJ/mol per residue [12,39]. It is instructive to apply these enthalpy values to the helix formation of the amphipathic peptides at the stationary phase. Assuming $\Delta H_{helix} = -3.3$ kJ/mol per residue and a helicity of 99% (~18 residues) and 83% (~15 residues), as observed in 50% trifluoroethanol for peptides aA and aB, respectively, the contribution of helix formation to ΔH^0 can be estimated to be about -59 kJ/mol for aA and -50 kJ/mol for aB. For the sake of the argument, we assume now that the differences in ΔH^0 between the peptides are mainly caused by differences in the helicity. This is a reasonable assumption, because the enthalpy contribution of the hydrophobic effect is about zero, and contributions from the stationary phase, including Van der Waals interactions, should not vary much, since the hydrophobic contact area between peptide and stationary phase is only moderately changed within a peptide set (only 20–25% changes in *S* value, but also

changes in the sign of ΔH^0; cf. Table 2). Incorporation of two D-amino acids in position 9 and 10 of peptide aA increases ΔH by 18.3 kJ/mol suggesting a decrease in helicity of ~30%, which corresponds to about five residues. A similar drop in helicity is caused by changing the sequence of peptide aA to a non-amphipathic sequence (nA: ΔH increased by 17.7 kJ/mol). However, it should be noted that according to this calculation the non-amphipathic analog nA still forms a helix comprising ~70% of the residues ($18-5=13$ residues) when interacting with the stationary phase. This is in accordance with results of HPLC studies of a double D-amino acid replacement set of a non-amphipathic model peptide [3]. From the reduced retention time of the double D-isomers compared to the all L-analog, it was concluded that the all L-peptide is helical at the stationary phase. Furthermore, this is in line with the finding that a further increase in ΔH^0 is observed after incorporation of two D-amino acids in the sequence of the non-amphipathic analog nA. The change in ΔH^0 corresponds to a further reduction of the helicity by about six residues compared to nA ($13-6=7$ residues remaining in a helical conformation in d-nA). Qualitatively the same modification in helicity can be calculated for peptide set B. However, incorporation of D-amino acids in peptide aB leads already to a larger drop in the helicity (from 15 to five amino acids) than observed for peptide set A.

To assess the contribution of helix formation to the free energy of interaction, Fig. 6 shows a plot of ΔG^0 versus the number of residues estimated to be in a helical conformation as derived from the ΔH^0 analysis. For both peptide sets, ΔG^0 has the tendency to increase with decreasing helicity. A linear relation between ΔG^0 and helicity is obtained for peptide set B. From the slope of the regression line, the contribution of helix formation to the free energy of stationary phase interaction is calculated to be about -0.7 kJ/mol and residue. The intercept of -13.8 kJ/mol corresponds to the ΔG^0 of a peptide which remains in an extended conformation upon stationary phase interaction. A comparison with ΔG^0 of the helical peptide aB (-24.7 kJ/mol) reveals that helix formation accounts for ~45% of the free energy. The remaining contribution of -13.8 kJ/mol can mainly be attributed to the hydrophobic effect and, possibly, to electrostatic interactions and to changes in the packing of the hydrophobic chains of the stationary phase (Van der Waals interactions).

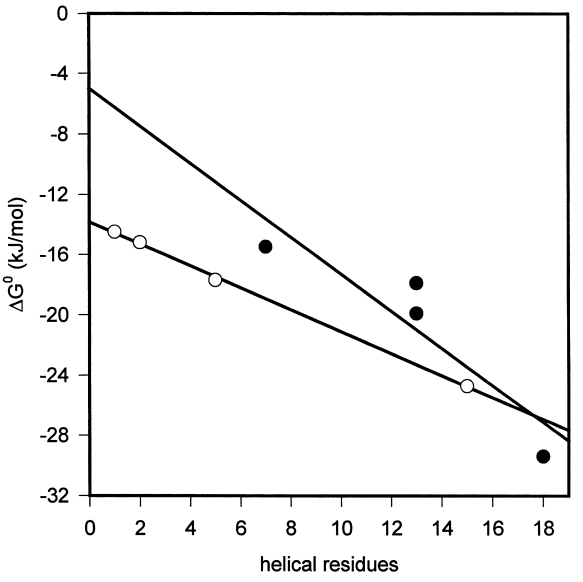

Fig. 6. Dependence of the free energy of interaction ΔG^0 on the estimated peptide helicity at the stationary phase. The number of helical residues was estimated on basis of the differences in ΔH^0 as described in the text. Symbols: (●) peptide of set A; (○) peptides of set B.

In contrast to the B set, the relation between ΔG^0 and helicity for the A peptide set can only poorly be described by a linear relation ($r^2=0.8$). This is probably caused by the different ability of the analogs to form hydrogen bonds between Lys and Glu (e.g. peptide aA: $2\times i$, $i+4$ and $2\times i$, $i+3$ interactions possible; peptide nA: $5\times i+3$). Hydrogen bonds between positively charged Lys (i) and uncharged Glu residues ($i+3$ or $i+4$) were found to contribute about -1.2 kJ/mol to the free energy of helix formation of model peptides in water [24]. Therefore, side chain–side chain interactions may give additional contributions to ΔH^0 and ΔG^0, which are not considered in the present analysis and may result in deviations from a linear behavior. Nevertheless, a linear regression analysis was performed in order to estimate at least the order of magnitude of the contribution of helix formation to ΔG^0. From the slope of the regression line the approximate free energy of helix formation is estimated to be -1.2 kJ/mol per residue. It should be emphasized that this large negative value includes

the contribution of the formation of hydrogen bonds between Lys and Glu residues. The non-helix contribution to ΔG^0, as estimated from the intercept, is about −5 kJ/mol. For peptide aA, helix formation (including Lys–Glu side chain interactions) accounts for ~80% of the free energy of interaction.

It should be noted that the non-helix contribution to the negative free energy, which includes the hydrophobic effect, is smaller for peptide set A (−5.0 kJ/mol) than for the B set (−13.8 kJ/mol). This is in accordance with the fact that the B set contains nine hydrophobic amino acids ($h = -0.025$) while the A set contains only six ($h = -0.170$; Table 1). The data calculated for the contribution of helix formation to the free energy of interaction with the hydrophobic stationary phase (A set: $\Delta G^0_{\text{helix}} = -1.2$ kJ/mol; B set: $\Delta G^0_{\text{helix}} = -0.7$ kJ/mol) are in general agreement with data recently derived for the interactions of amphipathic helical peptides with lipid membranes. The contribution of helix formation to the free energy of membrane-binding of the antibacterial peptide magainin 2 amide was found to be −0.6 kJ/mol and residue [12]. In a similar study, it was reported that helix formation contributes −1.7 kJ/mol per residue to the free energy of binding of the bee venom peptide melittin [40]. It should be emphasized that the analysis performed above is based on the assumption that the enthalpy of helix formation is −3.3 kJ/mol per residue and that any changes in ΔH between different analogs of one peptide set are solely caused by differences in the helicity. Any deviations from these assumptions would result in changes in the absolute values estimated for helix formation and for the other contribution to ΔG^0. The data should hence only be considered as a rough estimate of the contribution of helix formation to the overall thermodynamic of stationary phase interaction.

In summary, in this work we have shown that in addition to the hydrophobic effect the stationary phase induced coil–helix transition is a major driving forces of the retention process in reversed-phase HPLC. For amphipathic peptides, both helix formation and the hydrophobic effect can effectively contribute to the retention process at the same time. The coil–helix transition accounts for ~80 and 45% of the free energy of interaction of the helix-forming amphipathic peptides aA and aB, respectively. In peptide aA the large contribution of helix formation to ΔG is in part caused by Lys–Glu side chain interactions. In double D-isomers, however, helix formation is reduced because of sterical reasons, but the peptide can still adopt a conformation which allows an interaction of hydrophobic peptide chains with the stationary phase (extended conformation). The decreased retention of double D-isomers is then predominantly caused by a reduced helix formation. The situation is different in the non-amphipathic peptides nA and nB. These peptide have the potential to assume α-helical conformations. However, a non-amphipathic helix does not allow effective hydrophobic interactions between peptide side chains and the stationary phase. The retention of these peptides is hence reduced compared to the amphipathic analogs, since helix formation and an effective hydrophobic contact cannot be established at the same time. Whether retention of such peptides is dominated by helix formation or by an effective hydrophobic contact might depend on the specific peptide sequence. For the non-amphipathic peptides investigated in this study, helix formation was reduced compared to the amphipathic analogs in order to establish an effective hydrophobic contact.

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