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Noncovalent immobilized artificial membrane chromatography, an improved method for describing peptide–lipid bilayer interactions

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Abstract

A promising approach in assessing hydrophobic peptide-membrane interactions is the use of reversed-phase high-performance liquid chromatography. The present study describes the preparation and properties of a noncovalent immobilized artificial membrane (noncovalent IAM) stationary phase. The noncovalent IAM phase was prepared by coating the C_{18} chains of a reversed-phase HPLC column with the phospholipid ditetradecanoyl-*sn*-glycero-3-phosphocholine. Lipid coating was achieved by pumping a lipid solution in water–2-propanol through the column. The formation of a bilayer-like structure on the chromatographic surface was confirmed by calculating the phospholipid surface density of the stationary phase. The surface density was determined to be approximately 1.95 μmol m^{-2}, which is close to that of lipid vesicles. The coating was found to be stable in chromatographic elution systems containing less than 35% of acetonitrile. Employing this new technique, we determined interaction parameters of a set of helical antibacterial magainin-2-amide peptides with pairwise substitutions of adjacent amino acids by their D-enatiomers. The results demonstrate that the chromatographic retention behavior of peptides on noncovalent IAM stationary phase shows an excellent correlation with lipid affinities to phospholipid vesicles.

Keywords: Membranes; Immobilized artificial membrane chromatography; Stationary phases LC; Peptides; Phospholips; Magainin-2-amide

1. Introduction

Profound knowledge of processes determining the interaction of molecules with biological membranes may facilitate structure–activity studies of drugs and surface-active peptides, as well as prediction of drug transport across biological membranes [1–3]. The physicochemical characterization of lipid interactions of amphipathic peptide hormones and toxins is a prerequisite for understanding their action on biological membranes [4]. The measurement of partitioning coefficients on phospholipid vesicles, commonly used as model membrane systems, is attended by several problems. These include the necessity to label the peptides for determination of binding parameters by means of fluorescence or electron paramagnetic resonance spectroscopy, a limited range of measurable partition coefficients, and high experimental effort [3,5]. A promising approach for elucidating hydrophobic peptide-membrane interac-

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tions is the use of reversed-phase high-performance liquid chromatography (RP-HPLC) [6,7]. Although HPLC measurements are easily performed and good correlations were found between retention times and the membrane activities of peptides [7–9], RP-HPLC has a serious limitation. Due to the hydrophobic character of hydrocarbon chains, the RP-HPLC retention behavior mainly represents the hydrophobic component of the partitioning process. In contrast, membrane lipids are amphiphilic molecules, and hence interact with amphiphilic ligands by both hydrophobic and polar interactions.

To overcome these problems, several methods for immobilizing model membrane systems have been developed. The use of phospholipid-coated polystyrene-divinylbenzene beads has allowed for the detection of amphipathicity in peptides and proteins, as well as for the assessment of the binding parameters of several peptides and proteins [5]. Liposomes have been sterically immobilized in gel beads [10], and have been used for analysis of interactions between lipid bilayers and peptides corresponding to polypeptide segments of a membrane protein [11]. In order to study molecular interactions at membrane interfaces by HPLC immobilized artificial membrane (IAM) stationary phases have been prepared [12]. IAM stationary phases consist of phospholipid molecules (phosphatidylcholine, phosphatidylglycerol), which are covalently bound to propylamine-modified silica by the terminal carbons of their fatty acyl chains [13,14]. IAMs have been used to purify membrane proteins [15], and to obtain lipid binding constants of antibacterial peptides [16]. It has been shown that IAM chromatography provides better correlations than reversed-phase HPLC with respect to the partitioning of solutes into fluid membranes and the prediction of drug membrane permeability [17].

The present study describes the preparation and application of noncovalent immobilized artificial membrane stationary phases in which the C_{18} chains of a reversed-phase HPLC column and the alkyl chains of phospholipids form a bilayer-like structure. Employing this new technique, we determined chromatographic retention data of a set of antibacterial magainin peptides with pairwise substitution of adjacent amino acids by their D-enantiomers. Double D-substitution was previously reported to induce a localized destabilization of amphipathic α-helical structures, without changing other properties of the peptide [18]. In order to evaluate the method retention of the peptides on noncovalent IAM stationary phase was correlated with results of binding studies on phospholipid vesicles.

2. Experimental section

2.1. Materials

HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, USA). Water was purified with a Milli-Q system (Millipore, Eschborn, Germany). The eluents were degassed by continuous sparging with helium. All reagents were at least analytical-reagent grade. Trifluoroacetic acid (TFA) was supplied by Merck (Darmstadt, Germany). The Fmoc amino acids were obtained from Novabiochem (Bad Soden, Germany). 1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-phophadidyl-DL-glycerol (POPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA).

2.2. Peptide synthesis

Peptides were synthesized automatically by solid-phase methods using standard Fmoc chemistry on Tenta Gel S RAM resin (Rapp Polymere, Tübingen, Germany) in the continuous flow mode on a MilliGen 9050 peptide synthesizer [19]. Purification was carried out by preparative HPLC on PolyEncap A300, 10 μm, 250×20 mm I.D. (Bischoff Analysentechnik GmbH, Leonberg, Germany) to give final products >95% pure by HPLC analysis. All peptides were characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI II; Kratos, Manchester, UK) with the peptide content of lyophilized samples being determined by quantitative amino acid analysis (LC3000, Biotronik–Eppendorf, Germany).

2.3. Noncovalent IAM stationary phase

The IAM column was prepared by noncovalent immobilization of DMPC on a Vydac C_{18} column (Vydac 218TP5415; 150×4.6 mm I.D.; 1.12 g stationary phase; 100.9 m^2 surface area). The lipid loading was achieved by pumping a solution prepared from 20 mg ml^{-1} DMPC in 2-propanol–water (15:85, v/v), through the column, up to saturation. Prior to applying the lipid solution, the reversed-phase stationary phase was washed by pumping 10 ml of 2-propanol–water (15:85, v/v) through the column. The loading was performed at 25°C, and at a flow-rate of 1 ml min^{-1}, using a HPLC pump (PU-980, Jasco GmbH, Germany). The bound DMPC was determined from breakthrough curves of the lipid solution by UV detection at 220 nm (UV-975 detector, Jasco GmbH, Germany). Furthermore, the stationary phase bound DMPC was determined as follows: the loaded polymer was washed twice with defined volumes of chloroform. After centrifugation and separation of chromatographic material and supernatant, chloroform was evaporated, and the amount of lipid determined by phosphorus analysis [20]. To check the complete removal of bound lipid, the polymer was dried and used for phosphorus analysis. The determined amount of lipid, after washing steps, was less than 2% of the total bound lipid.

2.4. Noncovalent IAM chromatography

Chromatographic measurements were performed on a Jasco gradient HPLC system (Jasco GmbH, Germany), consisting of two PU-980 pumps, an AS-950 autoinjector, and a UV-975 detector operating at 220 nm. The sample concentration was 1 mg ml^{-1} of peptide in eluent A, and the injection volume was 20 μl. Runs were performed at 25°C (thermostated system), and at an eluent flow-rate of 1 ml min^{-1}. Mobile phases A and B consisted of 0.07 *M* KH_2PO_4 in water at pH 7.0, and 0.07 *M* KH_2PO_4 in 35% acetonitrile/65% water (v/v) at pH 7.0, respectively. Retention times of the peptides were determined using a linear gradient of 1 to 90% B in 40 min.

The isocratic retention times (t_R) are used to calculate the capacity factors k'_{IAM} using the following equation: $k'_{IAM}=(t_R-t_0)/t_0$, where t_R is the retention time of the peptide, and t_0 is the column void volume. Data for t_0 were obtained by injecting a liquid mixture with a volume composition different from that of the eluent. A linear relationship exists between ln k'_{IAM} and the organic mole fraction Φ, according to ln k'_{IAM}=ln $k'_0-S\Phi$, where S is a constant, and k'_0 is the capacity factor in the absence of the organic solvent [21]. Hence, ln k_0' can be determined by linear regression analysis. The capacity factor k'_0 is linear related to the equilibrium partition coefficient between lipid and aqueous phase according to $k'_0=\Phi K$, where Φ is the phase ratio of the column, which is a constant for a given column.

2.5. Small unilamellar vesicle preparation and determination of peptide-vesicle binding

A POPC lipid film was dried at 10^{-6} bar and 25°C for about 16 h using a turbomolecular pump (PT 50, Leybold, Germany). The lipid film was suspended by vortex mixing in buffer (10 m*M* Tris, 154 m*M* NaCl, 0.1 m*M* EDTA, pH=7.4), in order to give a final lipid concentration between 20 and 40 m*M*. The suspension was sonicated (under nitrogen, in ice water) for 25 min using a titanium tip ultrasonicator. Titanium debris was removed by centrifugation (5 min at 2000 *g*).

Relative peptide-vesicle binding was determined by CD spectroscopy on a Jasco 720 spectrometer at 23°C employing the following procedure: 1. Determination of the helicity of a peptide-vesicle suspension containing 11.5 m*M* POPC and 25 μ*M* peptide in buffer (10 m*M* Tris, 154 m*M* NaCl, 0.1 m*M* EDTA, pH=7.4) from the mean residue ellipticity [Θ] at 222 nm [22]. 2. Determination of the α-helical content of the lipid-bound peptide under conditions ensuring an almost complete peptide-vesicle binding. Complete binding was reached using vesicles composed of POPC/POPG, 3:1 (mol/mol) as well as POPG [23] 3. As the peptides were shown to be non-helical in buffer, and the helicity in the membrane bound state is known, the fraction of vesicle-associated peptide for a given peptide/lipid ratio can be calculated using the equation $F_{bound}=\alpha_{POPC}/\alpha_0$ (F_{bound}-fraction of bound peptide; α_{POPC}-

measured helicity; α_0-helicity of the completely lipid-bound peptide) [23].

3. Results and Discussion

3.1. Immobilization of DMPC and stability of phospholipid bound to the C_{18} phase

The coating of the C_{18} column with phospholipid was achieved by pumping a solution prepared from 20 mg ml^{-1} DMPC in 2-propanol–water (15:85, v/v) through the column. Fig. 1 shows the results of the frontal analysis. The curve obtained at a flow-rate of 1 ml min^{-1} is very sharp indicating a fast lipid transfer from the mobile to the stationary phase. Considering the dead volume of the column of 1.4 ml, the breakthrough volume was determined to be 6.4 ml. Thus, the final surface concentration of DMPC was 1.3 mg m^{-2} (1.95 μmol m^{-2}), or a nominal surface area of approximately 85 Å^2/molecule. A lipid density of 1.75 μmol of bound peptide per m^2 stationary phase surface was determined by phosphorus analysis. The calculated surface area occupied by the bound lipid is in the order of that of lipid in lipid bilayers (60–70 Å^2) [24,25]. These results confirm the formation of a stable stationary phase in which the C_{18} chains of the reversed-phase HPLC column and the alkyl chains of phospholipids form a bilayer-like structure on the chromatographic surface.

To assess the stability of phospholipid bound to the C_{18} phase under chromatographic conditions, the stationary phase was incubated in chromatographic solvent systems with increasing concentrations of acetonitrile (linear gradient 0–70% in 40 min, 1 ml min^{-1}) at pH 2.0 (0.1% TFA) and pH 7.0 (0.07 *M* KH_2PO_4). The dependence of the amount of released DMPC on the concentration of acetonitrile was measured by UV detection at 220 nm (Fig. 2) and by phosphorus analysis of the eluate. The result shows that no DMPC was found in the eluate at concentrations less than 35% of acetonitrile. Concentrations higher than 40% of acetonitrile lead to a significant release of DMPC as indicated by an increase UV absorption. Using linear gradient elutions up to 70% acetonitrile, the amount of released DMPC determined by phosphorus analysis was 50.7 mg (39% of the total amount of adsorbed phospholipid) and 32.5 mg (25% of the total amount of adsorbed phospholipid) or the first and second run, respectively. However, the stability of the DMPC loading in aqueous solutions was found to be appro-

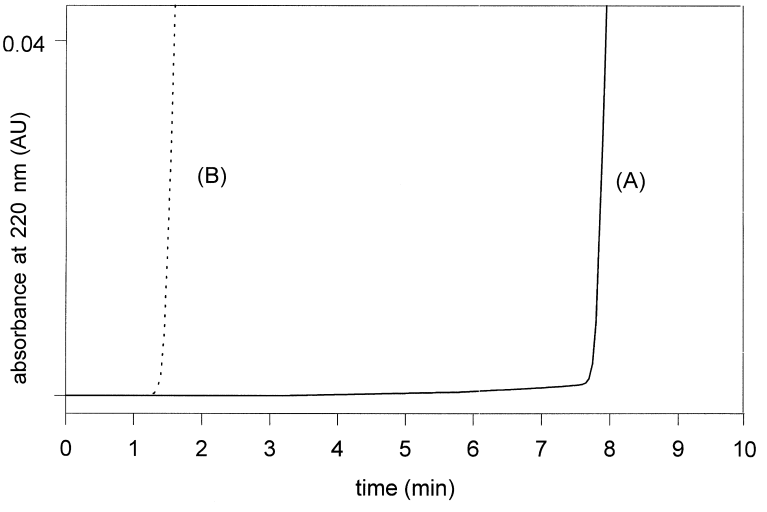

Fig. 1. DMPC breakthrough curve. A: Lipid loading on Vydac C_{18} up to saturation; B: lipid loading on the DMPC-coated column. Conditions: column, 150×4.6 mm I.D.; mobile phase, 2-propanol–water (15:85, v/v); DMPC concentration, 20 mg ml;$^{-1}$ UV detection, 220 nm; flow-rate, 1 ml min^{-1}.

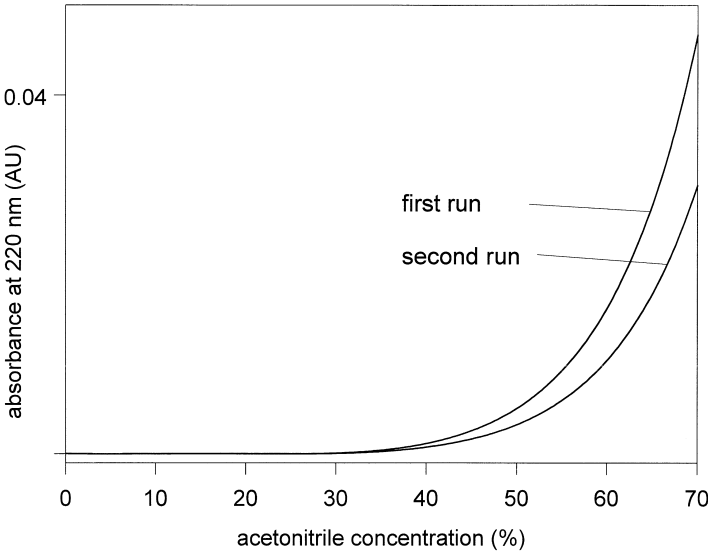

Fig. 2. The dependence of the amount of released DMPC on the concentration of acetonitrile. Conditions: column, 150×4.6 mm I.D.; mobile phase, linear gradient from 0 to 70% acetonitrile in 0.07 *M* KH_2PO_2, pH 7.0; UV detection, 220 nm; flow-rate, 1 ml min^{-1}.

priate for acetonitrile contents below 35%. In addition, the stability of the noncovalent DMPC column was evaluated in the presence of peptides. Multiple chromatographic runs of substance P in 0.07 *M* KH_2PO_4, pH 7.0, using a linear gradient of 1 to 30% acetonitrile in 40 min show a slight shift of retention times. Comparing the retention time of the first injection (21.2 min) with the retention time of the fifth injection (21.8 min), the result demonstrates that the stability of the lipid coating is sufficient for multiple column runs. The initial retention time can be restored by an additional loading of lipid performed by injection of 2 mg DMPC dissolved in 100 μl of 2-propanol–water (15:85, v/v). Therefore, the method allows determination of chromatographic retention times and partition coefficients of peptides.

3.2. Retention behavior of peptides

To estimate the hydrophobic character and the selectivity of the DMPC column, the peptide substance P and the amphipathic, antibacterial peptide magainin 2 amide (Table 1) were chromatographed on DMPC column and unmodified Vydac C_{18}. Because the peptides used in this study require acetonitrile in the mobile phase for elution, the measurements were performed in 0.07 *M* KH_2PO_4 buffer (pH 7.0) containing 0–30% (v/v) acetonitrile. The capacity factors (log k') were plotted as a function of the acetonitrile concentration in the eluent, as shown in Fig. 3. The data shows that the DMPC coated column is considerably less hydrophobic than the reversed-phase column: The amount of acetonitrile required to elute the peptides from the DMPC column (10–25%) was less than that required to elute the same peptide from the uncoated column (20–30%). The DMPC coated column shows an increased retention for the amphipathic magainin peptide, compared to substance P. This confirms the different selectivity of the phospholipid-coated stationary phase, in comparison with the reversed-phase column.

The increase in log k'_{IAM}, with decreasing acetonitrile concentration, was linear for all peptides. Consequently, the capacity factors of peptides on the phospholipid coated C_{18} phase could be linearly extrapolated to 100% aqueous phase content, yielding k'_0 (SP=56.8; magainin 2 amide=2540.2) values which are linearly related to the equilibrium partition coefficient between the immobilized artificial membrane phase and the aqueous phase.

Table 1
Synthetic peptides used in this study

Peptide	Sequence[a]
Substance P	RPKPQQFFGLM-amide
Magainin 2 amide	GIGKFLHSAKKFGKAFVGEIMNS-amide
D-Ile[2], D-Lys[4]- magainin 2 amide	G**I**G**K**FLHSAKKFGKAFVGEIMNS-amide
D-Lys[4], D-Phe[5]- magainin 2 amide	GIG**KF**LHSAKKFGKAFVGEIMNS-amide
D-Phe[5], D-Leu[6]- magainin 2 amide	GIGK**FL**HSAKKFGKAFVGEIMNS-amide
D-His[7], D-Ser[8]- magainin 2 amide	GIGKFL**HS**AKKFGKAFVGEIMNS-amide
D-Ala[9], D-Lys[10]- magainin 2 amide	GIGKFLHS**AK**KFGKAFVGEIMNS-amide
D-Lys[11], D-Phe[12]- magainin 2 amide	GIGKFLHSAK**KF**GKAFVGEIMNS-amide
D-Phe[12], D-Lys[14]- magainin 2 amide	GIGKFLHSAKK**F**G**K**AFVGEIMNS-amide
D-Ala[15], D-Phe[16]- magainin 2 amide	GIGKFLHSAKKFGK**AF**VGEIMNS-amide
D-Phe[16], D-Val[17]- magainin 2 amide	GIGKFLHSAKKFGKA**FV**GEIMNS-amide
D-Val[17], D-Glu[19]- magainin 2 amide	GIGKFLHSAKKFGKAF**V**G**E**IMNS-amide
D-Ile[20], D-Met[21]- magainin 2 amide	GIGKFLHSAKKFGKAFVGE**IM**NS-amide
D-Asn[22], D-Ser[23]- magainin 2 amide	GIGKFLHSAKKFGKAFVGEIM**NS**-amide

[a] The one-letter code of the D-amino acids is underlined.

3.3. Noncovalent IAM chromatography and binding studies to phospholipid vesicles

In order to test the method, the retention behavior of magainin 2 amide analogs on noncovalent DMPC column was compared with the lipid affinity to phosphatidylcholine (POPC) vesicles. Because double D-amino acid substitution was found to destabilize helical regions without changing other properties of the peptide, such as hydrophobicity, side chain functionality, or charge distribution [26], we synthesized and measured a double D-amino acid replacement set of magainin 2 amide. The analogs differed in the successive replacement of two adjacent amino acids by their corresponding D-amino acid (Table 1). Double D-replacement sets have been used to localize helical domains in neuropeptides [18,27] and to study the role of different peptide regions in membrane binding and permeabilization [23,28]. The retention times of the double D-analogs were determined at pH 7.0 (Figs. 4 and 5). The plot of the retention times of the double D-analogs

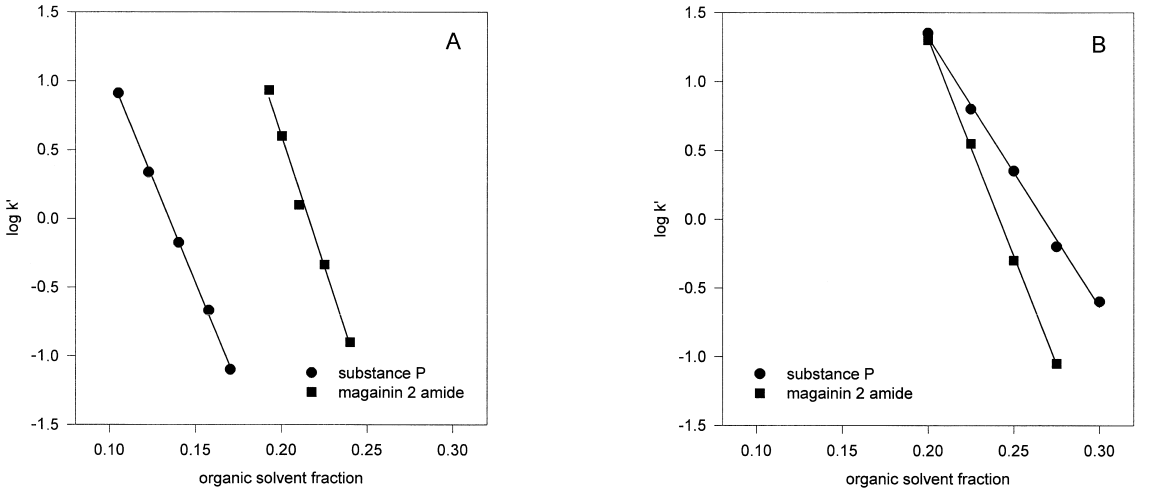

Fig. 3. Change in the capacity factors for substance P and D-Ala[9], D-Lys[10]-magainin 2 amide with increasing content of acetonitrile in water (0.07 *M* phosphate, pH 7.0) on (A) DMPC coated C_{18} column and (B) Vydac C_{18} column.

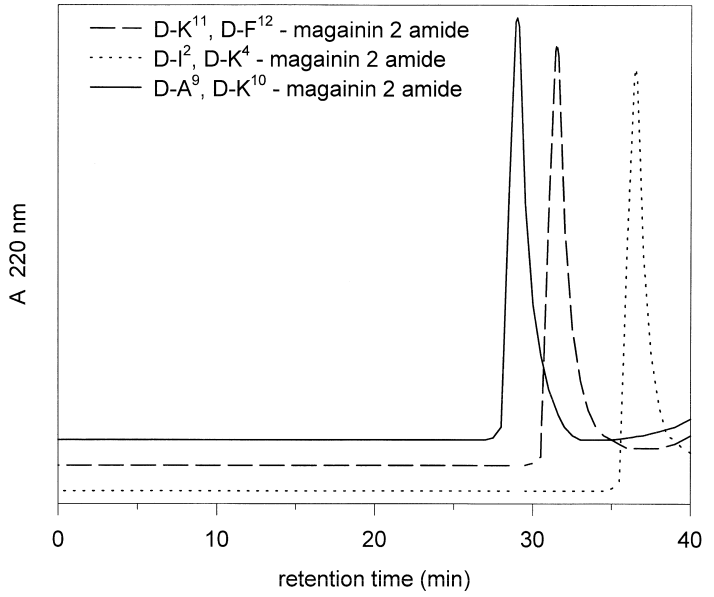

Fig. 4. HPLC chromatograms of magainin 2 amide analogs on DMPC coated C_{18} column. Conditions: linear AB gradient 1–90% B in 40 min (eluent A, 0.07 *M* KH_2PO_4 in water, pH 7.0; eluent B, 0.07 *M* KH_2PO_4 in acetonitrile–water (35:65, v/v), pH 7.0), 1 ml min^{-1}.

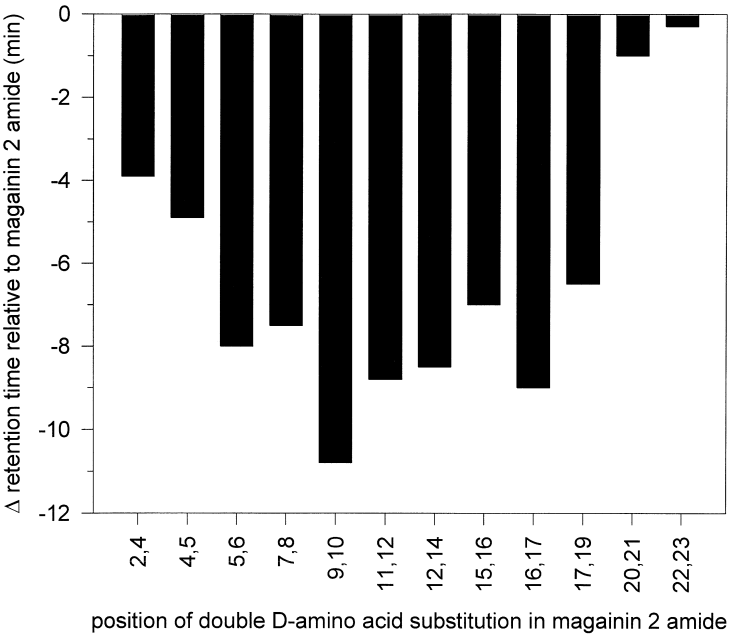

Fig. 5. Change in the retention time for the double D-isomers compared to magainin 2 amide (t_R=39.8 min). Conditions: DMPC coated C_{18} column, linear AB gradient 1–90% B in 40 min [eluent A, 0.07 *M* KH_2PO_4 in water, pH 7.0; eluent B, 0.07 *M* KH_2PO_4 in acetonitrile–water (35:65, v/v), pH 7.0], 1 ml min^{-1}.

relative to that of the all-L peptide vs. the position of D-amino acid replacement (retention time profile) shows that D-amino acids substitutions strongly influence the retention behavior. Substitution in any position reduces the retention time up to 11 min, compared to magainin 2 amide (38.8 min). This change in retention behavior is related to the ability of the peptide to form a helix in the bound state, is also in agreement with results showing that double D-amino acid substitution in any position destabilizes the helical structure of magainin [23].

In order to clarify if noncovalent IAM chromatography is an appropriate tool to assess the affinity of peptides to neutral lipid membranes, the retention behavior of the magainin double D-amino acid replacement set was compared with binding studies on phospholipid vesicles. Using CD-spectroscopy, the amount of POPC vesicle-bound peptide relative to magainin 2 amide was determined [see Experimental section for details]. The resulting binding profile reflects the individual apparent binding constants of the magainin analogs (Fig. 6). The data reveals that the inversion of the amino acid configuration leads to a significant drop of binding to phospholipid vesicles. The D-Ala^{9}, D-Lys^{10}-magainin analog, having the lowest retention time on the DMPC column, shows the lowest binding affinity of the double D-isomers. The correlation plot of both data sets (Fig. 7) reveals that the retention behavior on a DMPC column correlates well ($r=0.958$) with peptide binding data on POPC vesicles. The results demonstrate that noncovalent IAM chromatography on modified reversed-phase HPCL column can be used to predict binding of membrane-active peptides to phospholipid vesicles.

4. Conclusion

Immobilized artificial membrane chromatography stationary phases consisting of phospholipid molecules (DMPC) which are noncovalenty bound to the C_{18} chains of reversed-phase silica can be prepared by pumping lipid solution through the column. The formation of a bilayer-like structure on the chromatographic surface, and the stability of the DMPC loading, allows determination of chromatographic retention times and partition coefficients of peptides.

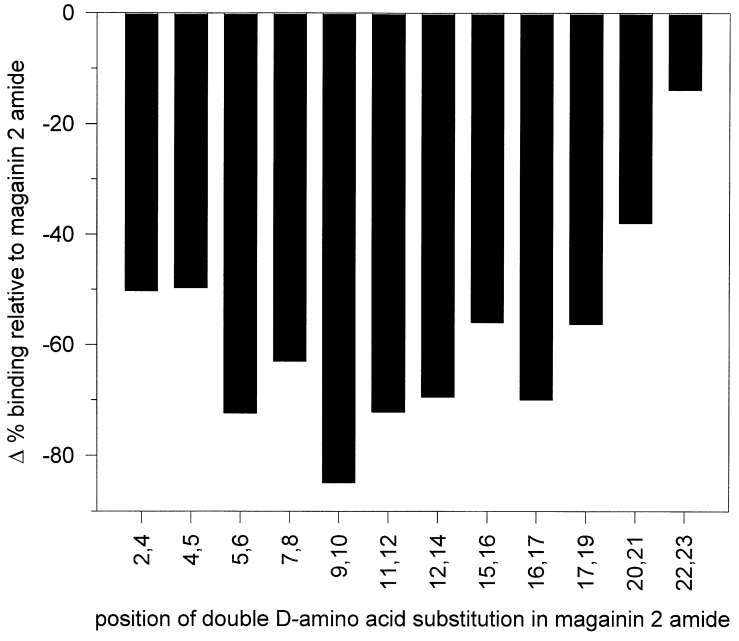

Fig. 6. Change in the POPC small unilamellar vesicle-bound peptide fraction for the D-isomers compared to magainin 2 amide. Conditions: 11.5 m*M* POPC, 25 μ*M* peptide in buffer (10 m*M* Tris, 154 m*M* NaF, 0.1 m*M* EDTA, pH.4).

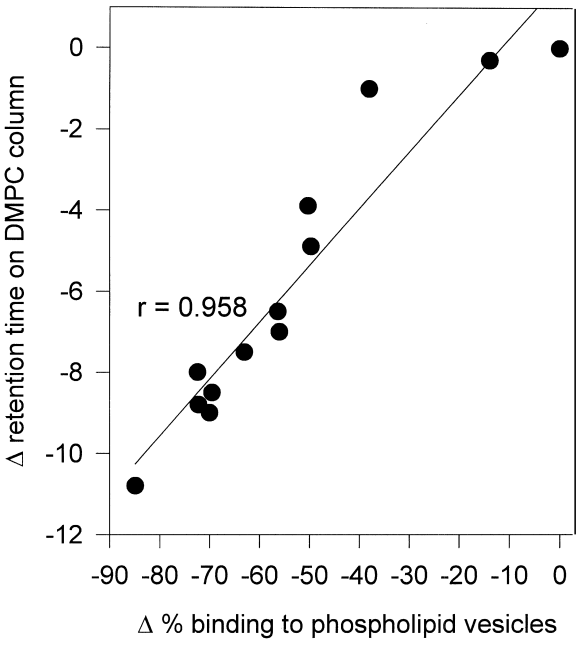

Fig. 7. Correlation plot Δ retention time on DMPC column *vs.* Δ% binding to phospholipid vesicles. See Fig. 5 and Fig. 6 for experimental details.

The retention behavior on noncovalent DMPC stationary phases shows an excellent correlation with binding parameters on phospholipid vesicles, as demonstrated for magainin 2 amide analogs. In conclusion, noncovalent IAM chromatography may be an useful tool to assess the lipid affinity of membrane-active peptides.

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