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The 21-residue surfactant peptide $(LysLeu_4)_4Lys(KL_4)$ is a transmembrane α-helix with a mixed nonpolar/polar surface

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Abstract The 21-residue peptide KLLLLKLLLLKLL-LLKLLLLK (KL_4) has been synthesized and analyzed regarding its secondary structure and orientation in lipid environments. Fourier transform infrared and circular dichroism spectroscopy shows that the peptide exhibits approximately 80% α-helical content both in dodecylphosphocholine micelles and in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/phosphatidylglycerol (PG) 7:3 (w/w) bilayers. The positively charged lysine residues are evenly distributed over the entire, otherwise nonpolar, circumference of the helix. This is in sharp contrast to the uneven distribution of polar and nonpolar residues in amphipathic helices. Fourier transform infrared spectroscopy of the peptide inserted in DPPC/PG bilayers shows that the helical axis is oriented parallel to the lipid acyl chains. These data do not support a previous hypothesis that the KL_4 peptide interacts with peripheral parts of a phospholipid monolayer and mimics the pulmonary surfactant protein SP-B, which is composed of several amphipathic α-helices. KL_4 accelerates the spreading of phospholipid mixtures at an air/water interface but does so less efficiently than other transmembranous helical polypeptides studied.

Key words: Pulmonary surfactant; SP-B; SP-C; Synthetic peptide; Lipid-associated peptides; Secondary structure; Transmembrane α-helix; Membrane protein

1. Introduction

Low levels of pulmonary surfactant in premature infants cause respiratory distress syndrome (RDS) [1], which is a disease with high morbidity and mortality (see [2]). The pulmonary surfactant system consists of several lipid and protein constituents combined in different morphological entities. In the alveolar type II cell, surfactant lipids and proteins are packed together in dense lamellar bodies which, in the alveoli, are converted to a lattice of tubular myelin bilayers. Eventually tubular myelin spreads into a film of mainly 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at the alveolar air/liquid interface. This film is responsible for lowering the alveolar surface tension, thus preventing lung collapse at end-expiration. Four surfactant proteins (SP)-A, -B, -C and -D have been described to date (see [3,4]). Efficient treatment of RDS is achieved by airway instillation of animal lung surfactant preparations containing lipids, predominantly DPPC, and small amounts (1–2 weight %) of the strictly hydrophobic proteins SP-B and SP-C [5].

SP-B, a homodimer of two 79-residue polypeptide chains [6,7], has an α-helical content of about 45% as shown by Fourier transform infrared (FTIR) [8] and circular dichroism (CD) spectroscopy [9]. SP-B has been proposed to contain four amphipathic helices interacting preferentially with superficial parts of a phospholipid bilayer [9]. SP-C is a 35-residue polypeptide [10] with palmitoyl groups thioester-linked to the cysteine residues in the N-terminal part of the molecule [6]. FTIR spectroscopy revealed that SP-C adopts mainly an α-helical conformation with its α-helix oriented parallel to the lipid acyl chains [11,12]. These restults are supported by those obtained by NMR spectroscopy showing that SP-C in mixed organic solvents forms a very regular α-helix between positions 9 and 34 [13] which is perfectly suited to span a DPPC bilayer [14]. The small sizes of SP-B and SP-C have raised the possibility of producing bioactive analogues, for formulating synthetic peptide-containing surfactant preparations. Cochrane and Revak designed a 21-residue polypeptide, KL_4, with a repetitive sequence of leucine and lysine residues [15–18]. This peptide mimics the pattern of hydrophobic and hydrophilic residues in SP-B and was supposed to stabilize the phospholipid bilayer by interactions with the lipid headgroups and superficial parts of the acyl chains [15].

We now analyze the secondary structure and orientation of KL_4 in phospholipids. We conclude that it is a transmembrane peptide with a non-amphipathic but mixed nonpolar/polar surface. These features are compared to those of the native hydrophobic surfactant polypeptides and possible implications for structure–activity relationships are discussed.

2. Experimental procedures

2.1. Peptide synthesis and purification

The KL_4 peptide (KLLLLKLLLLKLLLLKLLLLK, [15]) was synthesized on a 0.5 mmol scale using *tert*-butyloxycarbonyl chemistry in an Applied Biosystems 430A instrument. Treatment with anisol/*para*-thiocresol/hydrogen fluoride 1:1:10 (by vol) for 1 h at −4°C cleaved the peptide from the resin and deprotected the lysine residues. After removal of protecting groups and scavengers with diethylether, the peptide was extracted from the resin with dichloromethane/trifluoroacetic acid (TFA) 3:1 (v/v) [19]. After evaporation of solvents and resolubilization in ethanol, the peptide was purified on a C_{18} column (2.2×25 cm, Vydac) using a linear gradient of 95% ethanol/0.1% TFA in 80% methanol/0.1% TFA and a flow of 10 ml/min. This yielded one major peak of KL_4 identified by amino acid analysis, N-

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Abbreviations: ATR, attenuated total reflection; CD, circular dichroism; DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; FTIR, Fourier transform infrared; PG, phosphatidylglycerol; TFA, trifluoroacetic acid.

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terminal amino acid sequence analysis and laser-desorption mass spectrometry.

The SP-C/bacteriorhodopsin hybrid peptide (SP-C/BR) (LRIPCCPVNLKRFYAITTLVAAIAFTLYLSLLLGY), where the first 12 residues are identical to porcine SP-C and the remaining 23 residues correspond to the transmembrane part of the second helix of bacteriorhodopsin, was synthesized and purified as described [20]. Native SP-C (LRIPCCPVNLKRLLVVVVVVVLVVVVIVGALLMGL with both cysteines palmitoylated [6]) was purified from porcine lungs [21].

2.2. Circular dichroism spectroscopy

For analysis of secondary structure in phospholipid micelles, the KL_4 peptide was solubilized in 20 mM dodecylphosphocholine (DPC [20]; critical micelle concentration ~1 mM [22])/50 mM sodium phosphate buffer, pH 6.0, and diluted to 10 mM DPC in the same buffer, giving a final peptide concentration of about 20 μM. CD spectra between 260 and 184 nm were recorded at room temperature with a Jasco-720 instrument using a scan speed of 20 nm/min, a response time of 2 s, a band width of 1.0 nm, a sensitivity of 20 mdeg and a resolution of 2 data points/nm. The residual molar ellipticity was calculated after determination of the KL_4 concentration by amino acid analysis and expressed in kdeg × cm^2/dmol.

2.3. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy

KL_4 (8 nmoles) in ethanol was added to 270 nmoles of DPPC/PG 7:3 (w/w) in chloroform. The solvents were evaporated under nitrogen and solvent traces were removed by keeping the residue under reduced pressure overnight. The peptide/lipid film was hydrated in 100 μl of 2 mM HEPES, pH 7.4 by vortexing at 45°C. Peptide-containing vesicles were isolated by centrifugation in a continuous sucrose gradient (2–30%) at 100 000 × *g* for 16 h at 4°C. The location of phospholipids in the gradient was identified by a colorimetric test for choline (Boehringer) and peptide-containing fractions were pooled and dialysed extensively against 2 mM HEPES, pH 7.4. ATR-FTIR spectra were recorded with a Perkin-Elmer 1720X FTIR spectrophotometer at a resolution of 4 cm^{-1}. The internal reflection element was a germanium plate with an aperture angle of 45°, yielding 25 internal reflections. 128 scan cycles were averaged for each spectrum. Films of KL_4 and KL_4 associated with DPPC/PG 7:3 (w/w) were obtained by slowly evaporating the sample on the germanium plate under nitrogen at room temperature. Hydrogen to deuterium (H/D) exchange of readily accessible amide protons were obtained by flushing the sample on the ATR plate sealed in a universal sample holder with D_2O-saturated N_2.

Fourier self-deconvolution was applied in the amide I′ (the prime indicating that an H/D exchange has been performed) spectral region (1700–1600 cm^{-1}) to reveal the different secondary structure elements. To quantify the percentage of each resulting band, a least square iterative curve-fitting was performed to fit Lorentzian line shapes to the unconvoluted spectrum between 1700 and 1600 cm^{-1}. The percentage of each secondary structure component was determined from the Lorentzian band areas in the respective frequency region (see [12,23] for details).

Additional spectra were recorded with parallel (0°) and perpendicular (90°) polarized incident light with respect to the normal to the ATR plate. The mean angle between the helix axis and a normal to the ATR plate was calculated from the dichroic ratio R_{ATR} = A90°/A0°. The $\gamma_w(CH_2)$ transition at 1200 cm^{-1}, the dipole of which lies parallel to the all-*trans* hydrocarbon chains, was used to characterize the lipid acyl chain orientation (see [23] for details).

2.4. Measurement of surface activity

Surface properties of the lipid/peptide preparations were measured at 30°C with a Wilhelmy balance (Biegler, Vienna) equipped with a teflon trough (18 cm^2), containing a 20 ml hypophase of 150 mM NaCl. DPPC, PG and palmitic acid (PA), 68:22:9 (by weight, [24]), were dissolved in chloroform/methanol 98:2 (v/v), and added to the dried polypeptide (lipid:polypeptide ratio 50:1, w/w). After sonication, the solvents were evaporated under nitrogen and the residue was suspended by gentle ultrasonication (50 W, 48 kHz) in 150 mM NaCl at a lipid concentration of 10 mg/ml. 0.1 ml of the surfactant preparation was added as a droplet onto the hypophase and surface pressure was recorded continuously for 1 min. The presented values are the mean of three different recordings.

3. Results

3.1. Secondary structure of KL_4 in phospholipid micelles and bilayers

KL_4 exhibits a high degree of α-helical structure in DPC micelles with the CD spectrum exhibiting minima at 222 and 208 nm, indicative of α-helical secondary structure (Fig. 1). From the molar ellipticities at 222 and 208 nm [25] an α-helical content of 80% is estimated. The FTIR spectrum of KL_4 in DPPC/PG 7:3 (w/w) bilayers shows a peak maximum at 1656 cm^{-1}, in the spectral region associated with helical structure. Deconvolution and curve fitting between 1700 cm^{-1} and 1600 cm^{-1} [8,12,23] yields 84% of α-helices for lipid-associated KL_4. KL_4 is helical also in non-lipid environments; in trifluoroethanol it gives a CD spectrum which is similar to that obtained in DPC micelles, and FTIR spectroscopy of KL_4 alone, i.e. after it has been spread on the germanium plate from an organic solvent solution, also results in 84% helical content. Inspection of the spacing of the charged Lys side-chains and the nonpolar aliphatic Leu side-chains in helical KL_4 (Fig. 2) reveals a mixed nonpolar/polar surface. This is in sharp contrast to the spatial separation of polar and nonpolar side-chains along an amphipathic helix.

3.2. Orientation of KL_4 in a phospholipid bilayer

The distribution of charged and aliphatic residues in α-helical KL_4 raised questions regarding its preferred orientation in a phospholipid bilayer. A transmembranous orientation was expected to produce unfavourable interactions between the charged lysine amino groups and the lipid acyl chains, while an orientation perpendicular to the lipid acyl chains, the preferred orientation of amphipathic helices, was expected to give adverse interactions between the leucines and the polar phospholipid head-groups and the surrounding aqueous phase. KL_4 associated with DPPC/PG 7:3 (w/w) vesicles [15] was analyzed by FTIR spectroscopy with 0° and 90° polarized incident light. The dichroic spectrum (90°–0°) clearly shows that the amide I′ band and the $\gamma_w(CH_2)$ band are strongly 90° polarized from the positive deviations centered at 1657 cm^{-1}, in the helix region, and at 1200 cm^{-1}, respectively (Fig. 3). These positive deviations reflect a preferential perpendicular orientation of both these dipole transitions relative to the germanium surface. From the dichroic ratios of 1.7 for the α-helix and 4.1 for the $\gamma_w(CH_2)$ transition

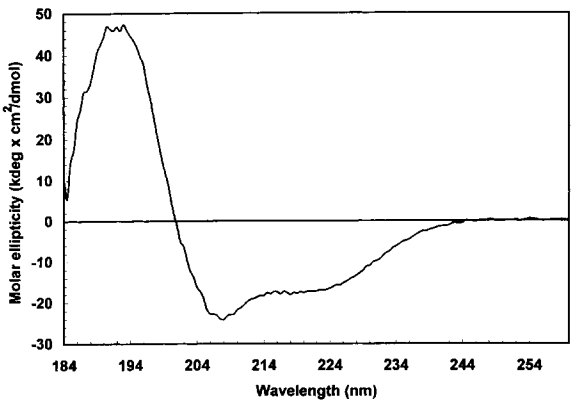

Fig. 1. CD spectrum of KL_4 in DPC micelles. The spectrum was recorded with a 21 μM solution of KL_4 in 10 mM DPC/50 mM sodium phosphate buffer, pH 6.0 at 22°C.

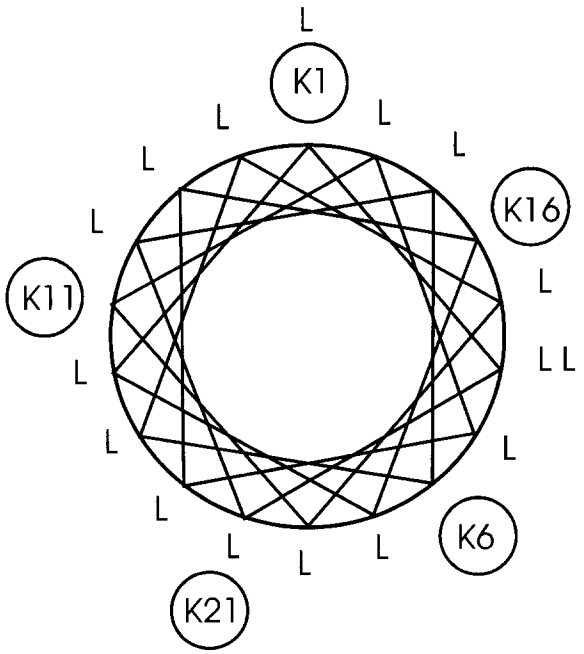

Fig. 2. Helical wheel presentation of the KL_4 amino acid sequence. The view is along the helical axis. The lysine residues are circled and identified by their sequence location to emphasize their even distribution over the entire helical circumference.

it is calculated that the helix axis and the all-*trans* lipid acyl chains make a maximum tilt from a normal to the ATR plate of 27° and 20°, respectively. This means that the KL_4 peptide is oriented practically parallel to the lipid acyl chains in a DPPC/PG bilayer. In other words, the approximately 30 Å long KL_4 peptide (assuming all-helical structure) is oriented in a transmembranous fashion in the approximately 40 Å DPPC/PG fluid bilayer. In this orientation only the centrally situated lysine (Lys-11) is forced to accomodate its charged amino group in an entirely nonpolar, hydrocarbon, environment since the amino groups of the remaining lysines with their side-chains directed towards the bilayer surface, will be able to interact with the phospholipid head-groups. Evidently, the single unfavourable interaction is compensated for by the many favourable interactions between aliphatic leucine side-chains and the lipid acyl chains.

3.3. *Effects of KL_4 on the spreading of lipids at an air/water interface*

Spreading kinetics of 2% (w/w) of KL_4 in a DPPC/PG/PA 68:22:9 (by weight) mixture at an air/water interface was compared to that of native SP-C and SP-C/BR [20], both with transmembranous α-helices in similar phospholipid mixtures [11,12,14,26]. KL_4 is less efficient than native SP-C and SP-C/BR in accelerating the spreading of the lipid mixture and also reaches a lower surface pressure after 1 min (Fig. 4). Thus, a transmembranous α-helix as such is apparently not sufficient to give optimal spreading properties of lipid/peptide mixtures.

4. Discussion

KL_4, with helix-promoting [27,28] leucine and lysine residues only, exhibits a helical secondary structure in phospholipid micelles and bilayers. The helix presents a regular surface best characterized as mixed nonpolar/polar. This helical peptide, in spite of a lack of an all-hydrophobic part, adopts a transmembranous orientation. This is in sharp contrast to the mechanism of action first proposed for SP-B and KL_4 [15] which involves simultaneous interactions between the positively charged lysine side-chains and phospholipid head-groups on the one hand and interactions between leucine side-chains and superficial parts of lipid acyl chains on the other. Such interactions are difficult to envision with the KL_4 helical surface and orientation in a phospholipid bilayer now found, suggesting another mechanism of action for KL_4 in pulmonary phospholipids.

The secondary structure and transmembranous orientation of KL_4 prompts investigation of its spreading properties in a lipid mixture, since another transmembrane helix (SP-C/BR) with a primary structure which is unrelated to any of the pulmonary surfactant proteins has significant effects on lipid spreading kinetics [20,26]. KL_4 does accelerate the spreading of lipids at an air/water interface but it is less effective than both native SP-C and the SP-C/BR hybrid in this respect. Perhaps the most prominent differences between KL_4 on the one hand and native SP-C and SP-C/BR on the other are that (i) the KL_4 helix is shorter (about 30 Å compared to 37 Å in native SP-C [13,14] and presumably also in SP-C/BR with the

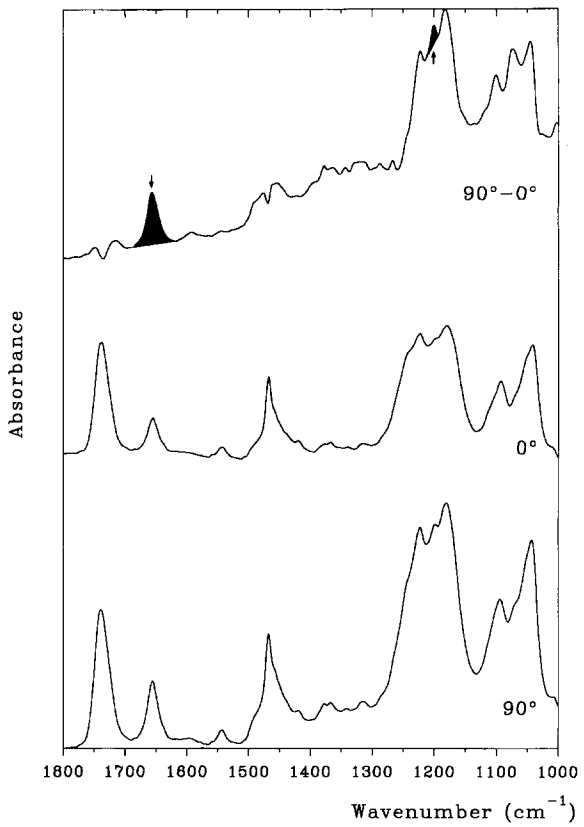

Fig. 3. Dichroic spectrum of deuterated KL_4 in oriented DPPC/PG 7:3 (w/w) bilayers. The dichroic spectrum (top tracing) represents the difference between the spectra recorded with 90° and 0° polarized light. The recorded spectra are drawn with identical ordinate scale; the dichroic spectrum is expanded threefold in the ordinate direction. The arrows indicate the protein amide I′ band at 1657 cm^{-1} and the phospholipid $\gamma_w(CH_2)$ band at 1200 cm^{-1}. The sample was deuterated for 30 min.

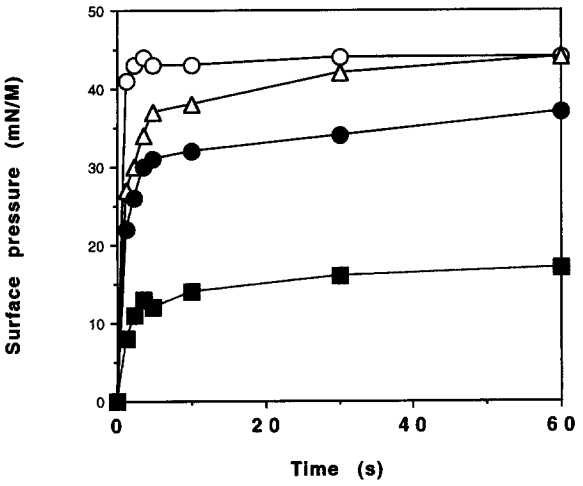

Fig. 4. Spreading kinetics of KL_4. Plots of surface pressure versus time with 2% (w/w) of KL_4 (●), SP-C/BR (△), or native SP-C (○) in DPPC/PG/PA 68:22:9 (by weight) compared to the lipids alone (■).

same number of residues and similar α-helical content as native SP-C [20]) and (ii) KL_4 has a symmetric distribution of positively charged residues along the helix while native SP-C and SP-C/BR have basic residues exclusively at the N-terminal end of the helix. The latter may give KL_4 a more static and uniform interaction with a phospholipid bilayer compared to native SP-C and SP-C/BR which both are expected to exhibit a 'mobility gradient' [14] due to greater lateral mobility in the C-terminal end than in the N-terminal end. We speculate that such effects may contribute to the observed differences in spreading kinetics between KL_4 and native SP-C and SP-C/BR.

In summary, we have shown that the 21-residue peptide KL_4 is a transmembranous helix with a mixed polar/nonpolar surface. The helix is oriented parallel to the lipid acyl chains and the structure and orientation of KL_4 indicate that its mechanism of action is similar to that of SP-C, in contrast to previous suggestions [15–18]. This knowledge is useful for designing peptide analogues for further studies of the mechanism of action of pulmonary surfactant polypeptides.

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