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Interaction of immunologically-active lipopeptides with membranes

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Synthetic tripalmitoyl-*S*-glycerylcysteinyl (Pam_3Cys) peptides are derived from the N-terminal part of bacterial lipoprotein and constitute polyclonal B-lymphocyte and macrophage activators. In order to elucidate the primary events of leukocyte activation, we investigated the biophysical interaction of lipopeptides containing spin labels or fluorescent markers with phosphatidylcholine vesicles or immune cells. Utilizing fluorescence microscopy and FACS analysis we found, that the surface of cells, after incubation with a fluorescein-labelled lipopeptide, was highly fluorescent. In addition, capping and patching was observed. Furthermore, fluorescence quenching experiments and electron paramagnetic resonance studies using vesicles incubated with lipopeptides suggested, that the peptide moiety and other more polar molecules linked to the lipo-amino acid are exposed to the hydrophilic compartment. These results show that in lipopeptide conjugates the Pam_3Cys moiety acts as an efficient membrane anchor for molecules covalently coupled to it. The sequestering of the fatty-acid chains of the lipopeptide within the membrane is an early step of interaction, which might induce the uptake of the lipopeptide into the cell and the stimulation of immunocompetent cells.

Introduction

Many structural and functional bacterial lipoproteins have been described so far, which contain *S*-(2,3-dihydroxypropyl)cysteine (*S*-glycerylcysteine) as the N-terminal amino acid [1]. In these lipoproteins the two hydroxy-groups and, with one exception [2], also the amino group of this unusual amino acid are acylated with various fatty acids. The triacyl-*S*-glycerylcysteinyl moiety anchors the lipoprotein in the bacterial membranes of Gram-positive and Gram-negative bacteria.

Synthetic lipopeptides carrying N-terminally the lipo-amino acid *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteine (Pam_3Cys) [3] are derived from the N-terminus of bacterial lipoprotein [4]. They activate B-lymphocytes [5], macrophages [6] and neutrophils [7,8] and constitute highly potent, non-toxic immunoadjuvants [9]. On the basis of lipopeptide–antigen conjugates, a novel carrier/adjuvant system for the induction of antigen- and hapten-specific antibodies [10–14,41] and a new concept for the design of totally synthetic low-molecular mass vaccines was developed [15–18].

The interaction of cells with lipopeptides is of interest for the understanding of the molecular mechanisms involved in the activation of B-lymphocytes and macrophages, which are still largely unknown. In this

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Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMPC; dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EELS, electron energy loss spectroscopy; EPR, electron paramagnetic resonance; FACS, fluorescence activated cell sorter; FDMS, field desorption mass spectrometry; FITC, fluorescein isothiocyanate; HMD, 1,6-diaminohexane; ES-MS, electrospray mass spectrometry; MEM, minimal essential medium; PBS, phosphate-buffered saline; Pam, palmitoyl; Pam_3Cys, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteine; SUV, small unilamellar vesicles; TDM, 4,4′-bis(dimethylamino)diphenylmethane; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TFA, trifluoroacetic acid; T_c, crystalline-liquid crystalline phase-transition temperature.

study, we tried to elucidate the first step of the activation cascade directly after incubation with the lipopeptides. We studied the interaction of small synthetic lipopeptides, containing the unique membrane anchor Pam_3Cys and marker molecules, with membranes using different spectroscopic techniques.

Materials and Methods

Chemicals

All solvents and dicyclohexylcarbodiimide were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid, *N*-ethylmorpholine, 4,4′-bis(dimethylamino)-diphenylmethane, *tert*-butanol, thioanisol, potassium iodide, potassium chloride and sodium thiosulfate were purchased from Fluka (Ulm, Germany), N_α-*tert*-butyloxycarbonyl-4-tryptophan-*N*-4-hydroxysuccinimide ester from Bachem (Bubendorf, Switzerland), 16-(9-anthroyloxy)palmitic acid from P.L. Biochemicals (Milwaukee, WI, USA), DMPC, DPPC, DOPC and fluorescein isothiocyanate isomer I from Sigma (Deisenhofen, Germany), 4-isothiocyanato-TEMPO and 4-amino-TEMPO were obtained from Aldrich (Steinheim, Germany). Sephadex LH-20 was from Pharmacia (Freiburg, Germany). The lipo-amino acid Pam_3Cys-OH [3] was obtained from Rapp Polymere (Tübingen, Germany). The lipopeptides Pam_3Cys-HMD × TFA, Pam_3Cys-Ser-Lys × TFA and Pam_3Cys-Ser-$(Lys)_4$ × 3 TFA were synthesized [19].

Thin-layer chromatography

Silica gel plates 60 F_{254} (Merck, Darmstadt, Germany) and the following solvent systems were used in solvent saturated glass chambers for determining R_f values at room temperature: I, chloroform/methanol/water (65:25:4, v/v/v); II, chloroform/acetic acid/water (90:10:1, v/v/v); III, ethyl acetate saturated with water; IV, butanol/acetic acid/water (2:1:1, v/v/v). For detection ninhydrin, chlorine/4,4′-bis(dimethylamino)diphenylmethane (TDM) and water were used.

Spectroscopic methods

NMR. ^{13}C-NMR spectra were recorded in $CDCl_3$ on a WM 400 (Bruker-Physik, Karlsruhe, Germany).

Mass spectrometry. Field desorption mass spectra (FD-MS) were recorded on a Varian MAT 711A (Varian, Bremen, Germany) at an ion source temperature of 50°C. Electrospray mass spectra were recorded on a Sciex API III triple quadrupole mass spectrometer with IonSpray™ interface (Sciex, Thornhill, Ontario, Canada).

Fluorescence activated cell sorter. The FACS analyses were done on a FACS SCAN (Becton Dickinson, Heidelberg, Germany).

Electron paramagnetic resonance spectroscopy. EPR spectra were taken on Varian E-line instruments at a microwave frequency of 9.5 GHz, which were linked to an ER 140 data system (Bruker-Physik, Karlsruhe, Germany). Modulation of magnetic field was kept below 1/3 of the line-width. Samples were not degassed and 60 × 14 × 0.3 mm flat cells were used in a Varian E238 cavity.

Fluorescence spectroscopy. Measurements were made on a Perkin Elmer LS-5 spectrofluorometer.

Synthesis of FITC-labelled Pam_3Cys-Ser-$(Lys)_4$-OH

Pam_3Cys-Cys-Ser-$(Lys)_4$ × 3 TFA [19] (18.5 mg, 10 μmol) and FITC (15.6 mg, 40 μmol) were dissolved in *tert*-butanol/pyridine (1:1 (v/v), 1 ml). The solution was stirred for 72 h. The solvent was removed in vacuo, and the residue chromatographed on Sephadex LH-20 (chloroform/methanol 1:1, v/v, column 45 × 1 cm). The first yellow fraction being eluted as a broad band (ninhydrin and chlorine/TDM positive) was collected and lyophilized from *tert*-butanol. 12.6 mg of a yellow powder were obtained. Amino-acid analysis: Ser 1(1); Lys 4.1 (4).

Synthesis of Pam_3Cys-TEMPO

Pam_3Cys-OH (91 mg; 0.1 mmol) was activated with dicyclohexylcarbodiimide (21 mg; 0.1 mmol) and *N*-hydroxybenzotriazol (15 mg) in chloroform (1.5 ml). 4-amino-TEMPO (17 mg; 0.1 mmol) and *N*-ethylmorpholine (13 μl, 0.1 mmol) were added. The mixture was stirred for 6 h. The solvent was evaporated, and the residue chromatographed on Sephadex LH-20 (chloroform/methanol 1:1 (v/v), column 40 × 1.5 cm). The fractions containing the spin-labelled lipopeptide (chlorine/TDM positive) were combined and evaporated to dryness. The residue was lyophilized from *tert*-butanol. Yield: 76 mg (72%). R_f(II) = 0.60; R_f(III) = 0.65. The compound, dissolved in dichloromethane, shows a triplet in the EPR experiment characteristic for TEMPO. $C_{63}H_{120}N_3O_7S$ (1063.7).

Synthesis of Pam_3Cys-HMD-TEMPO

To a solution of Pam_3Cys-HMD × TFA [19] (50.4 mg; 49 μmol) in dichloromethane (0.5 ml) 4-isothiocyanato-TEMPO (12.5 mg, 59 μmol) and triethylamine (5 μl, 50 μmol) were added. The mixture was stirred for 16 h. The solvent was evaporated, and the residue chromatographed on Sephadex LH-20 (chloroform/methanol 1:1 (v/v), column 40 × 1.5 cm). The fractions containing the spin-labelled lipopeptide (chlorine/TDM positive) were combined and evaporated to dryness. The residue was lyophilized from *tert*-butanol. Yield: 21 mg (35%, referring to Pam_3Cys-HMD × TFA). R_f(II) = 0.67; R_f(III) = 0.71. The compound, dissolved in dichloromethane, shows a triplet in the EPR experiment characteristic for TEMPO.

$C_{70}H_{134}N_5O_7S_2$ (1222.0). ES-MS: $(M + H)^+$, 1223.
Calc.: C, 68.80; H, 11.05; N, 5.73; S, 5.25.
Found: C, 68.75; H, 10.87; N, 5.58; S, 5.40.

Synthesis of Pam_3Cys-Ser-Lys(TEMPO)-OH

To a solution of Pam_3Cys-Ser-Lys × TFA [19] (62 mg, 50 μmol) in dichloromethane/*tert*-butanol (0.5 ml, 1:1, v/v) 4-isothiocyanato-TEMPO (12.8 mg; 60 μmol) and triethylamine (7 μl, 50 μmol) were added. The mixture was stirred for 14 h. The solvent was evaporated, and the residue chromatographed on Sephadex LH-20 (chloroform/methanol 1:1 (v/v), column 40 × 1.5 cm). The fractions containing the spin-labelled lipopeptide (chlorine/TDM positive) were combined and evaporated to dryness. The residue was lyophilized from *tert*-butanol. Yield: 49 mg (73%, referring to Pam_3Cys-HMD × TFA). $R_f(I) = 0.54$; $R_f(II) = 0.39$; $R_f(IV) = 0.79$. The compound, dissolved in dichloromethane, shows a triplet in the EPR experiment, which is characteristic for TEMPO.
$C_{73}H_{137}N_6O_{11}S_2$ (1339.1). ES-MS: $(M + H)^+$, 1340; $(M + Na)^+$, 1362; $(M + K)^+$, 1377.
Calc.: C, 65.48; H, 10.31; N, 6.28; S, 4.79.
Found: C, 65.63; H, 10.19; N, 6.12; S, 4.93.

Synthesis of Pam_3 Cys-Ser-HMD ← (Boc-Trp)

Pam_3Cys-Ser-HMD × TFA [19] (100 mg, 82.7 μmol), Boc-Trp-OSu (66.4 mg, 165.4 μmol) and *N*-ethylmorpholine (10 μl, 83 μmol) were stirred for 17 h in DMF. The solvent was evaporated to dryness and the residue recrystallized from methanol at 4°C. The protected lipopeptide was purified on Sephadex LH-20 (chloroform/methanol 1:1; column 55 × 2 cm). Yield: 78 mg (68%). $R_f(II) = 0.68$; $R_f(III) = 0.53$.

^{13}C-NMR (100.16 MHz; 240 mg/ml $CDCl_3$; 5000 scans); δ (ppm): 170.1/170.8/173.4/173.5/174.3 (carbonyl signals; not assigned); 136.3 (Trp-C-3a); 127.3 (Trp-C-2); 123.5 (Trp-C-7a); 121.8 (Trp-C-4); 119.3 (Trp-C-6); 118.6 (Trp-C-5); 111.4 (Trp-C-7); 110.1 (Trp-C-3); 155.5 Boc-CO; 79.8 Boc-C_q; 69.0, 70.0 (*S*-glyceryl CH-O); 63.5, 63.7 (*S*-glyceryl CH_2-O); 62.3 (Ser-C_β); 55.4 (Trp-C_α); 55.1 (Ser-C_α); 52.7, 53.2 (Cys-C_α); 38.8/38.9 (HMD-C-1,6)); 36.2 (*N*-Pam-C-2); 34.4 (Cys-C_β); 34.0/34.3 (*O*-Pam-C-2; 2 ×); 32.2/32.9 (*S*-glyceryl CH_2-S); 31.9 (Pam-C-14; 3 ×); 29.1/29.3/29.5/29.6 (Pam-C-4-13; 30 ×); 28.6, 28.7, 28.8 (HMD-C-2-4); 28.3 Boc-CH_3 (3 ×); 25.5 (Trp-C_β); 25.4 (*N*-Pam-C-3); 24.8/24.9 (*O*-Pam-C-3; 2 ×); 22.6 (Pam-C-15; 3 ×; 14.0 (Pam-C-16; 3 ×).

$C_{79}H_{140}N_6O_{11}S_2$ (1382.1). ES-MS: $(M + H)^+$, 1383.
Calc.: C, 68.66; H, 10.21; N, 6.08; S, 2.32.
Found: C, 68.39; H, 10.56; N, 6.32; S, 1.99.

Synthesis of Pam_3Cys-Ser-HMD ← (H-Trp) × TFA

Pam_3Cys-Ser-HMD ← (Boc-Trp) (20 mg, 14.5 μmol) was dissolved in a mixture of thioanisol, trifluoroacetic acid and dichloromethane (1:4:5, v/v:v). After 20 min the solvent was removed in vacuo and the residue chromatographed on Sephadex LH-20 (column 40 × 1 cm, chloroform/methanol 1:1, v/v). The unprotected lipopeptide was obtained as a colourless powder by lyophilization from *tert*-butanol. Yield: 15 mg (76%) $R_f(I) = 0.70$; $R_f(II) = 0.14$.
$C_{74}H_{132}N_6O_9S_2$ (1282.0). ES-MS: $(M + H)^+$, 1283.

Phospholipid vesicles

Phospholipids (DPPC, DOPC, DMPC) were dissolved in chloroform/methanol (1:1, v/v), and nitrogen was used to dry them as a film on the wall of an 80-ml beaker. The sample was placed under vacuum for about 10 h. After the addition of the appropriate volume of buffer (0.1 M Tris-HCl, pH 7.4), the lipid samples were sonicated with a probe-type sonicator (MSE Soniprep 150) for 6–10 × 30 s bursts (temperature above the phase transition temperature of the lipid), with cooling periods of 30 s between each burst. Under these conditions all samples achieved optical clarity. The final suspension contained 0.5 mM phospholipid in 0.1 M Tris-HCl buffer (pH 7.4). In the iodide quenching experiments 0.5 M KCl was added to keep the ionic strength constant [20].

The preparation of small unilamellar vesicles (SUV) from soy-bean lecithin used for the EPR studies is described elsewhere [21].

Incubation of cells with labelled lipopeptides

For fluorescence microscopy investigations and the FACS analyses the B-lymphocyte cell line BCL_1 and the macrophage cell line $P388D_1$ were used. Cells were separated from the cell culture medium by centrifugation and then washed three times with MEM to remove cell-bound proteins (centrifugation 5 min, 250 × *g*). The pellet ($1.5 \cdot 10^6$ cells) was suspended in PBS (1 ml) containing the FITC-labelled lipopeptide ($2.5 \cdot 10^{-5}$ M; opalescent solution obtained by ultrasonication of the aqueous lipopeptide suspension). The cell suspension was incubated for 30 min at 0°C in the presence of NaN_3, or at 37°C in the absence of NaN_3 (theoretical average labelling ratio about $1 \cdot 10^{10}$ molecules/cell). The cells were washed twice with PBS to remove unbound lipopeptide, resuspended in PBS (300 μl) with or without 0.02% NaN_3 and then investigated without further fixation by fluorescence microscopy. For FACS analysis, the cells were incubated in the presence of 0.02% NaN_3 at 0°C with lipopeptide concentrations between $2 \cdot 10^{-5}$ and $1 \cdot 10^{-8}$ M.

Quenching experiments

Stock solutions (1 mM) of Pam_3Cys-Ser-HMD ← (Boc-Trp) and Pam_3Cys-Ser-HMD ← (H-Trp) × TFA were prepared in 2-propanol. 20 μl of these stock solutions were added to 3 ml of vesicle suspension (0.5

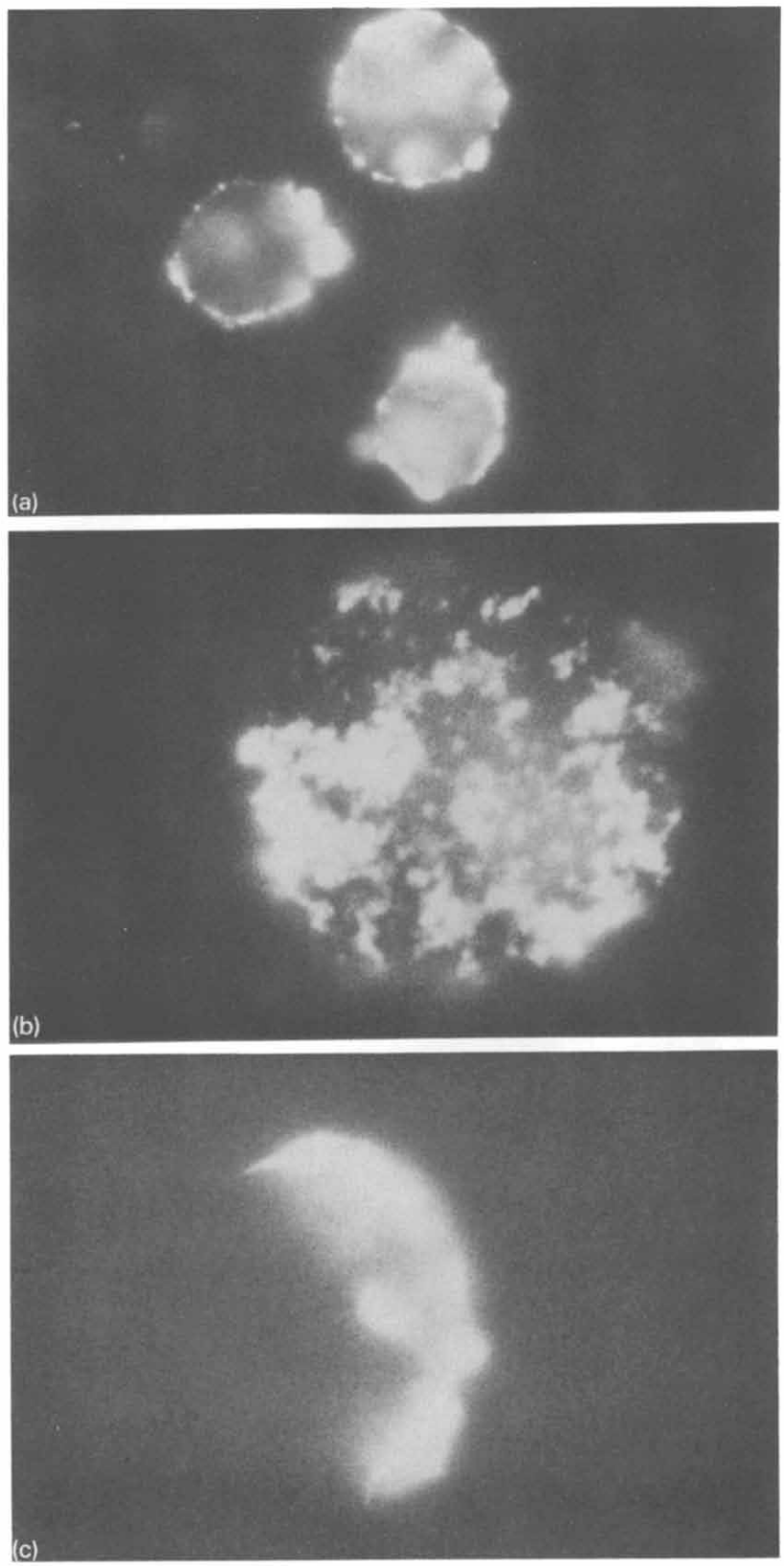

Fig. 1. Fluorescence microscopic investigation of cells of the B-lymphocyte cell line BCL_1 incubated with 52 μg/ml Pam_3Cys-Ser-[$Lys_4(FITC)_{1-4}$]-OH for 30 min (a) at 4°C in the presence of azide and (b and c) at 37°C in the absence of azide. The cells incubated at 37°C in the absence of sodium azide showed patching (b) and capping (c).

mM phospholipid/6.7 μM Pam_3Cys-compound; molar ratio 75:1). The sample was stirred and annealed [22] afterwards at 50°C or alternately at 15 and 50°C (in time intervals of 10 min) for about 30–60 min. Some samples were sonicated after the addition of the Pam_3Cys-compound in order to distribute the Pam_3-Cys-compound between the inner and outer leaflet of the phospholipid bilayer (cf. Fig. 6). Stock solutions of KI (0.5 M) were prepared in 0.1 M Tris-HCl (pH 7.4). A small amount of $S_2O_3^{2-}$ (about 10^{-4} M) was added to the iodide solution to prevent I_3^- formation, which absorbs in the wavelength region of Trp fluorescence (filter effect) and may cause chemical reactions [23]. Aliquots (6×25 μl) of the quencher stock solution were added to 3 ml of the vesicle suspension in a cuvette. Excitation/emission wavelengths were 290/325 nm for the Trp lipopeptides. Quenching titrations were corrected for dilution effects and scattering contributions.

Bimolecular quenching is described by the Stern-Volmer equation:

$$I_0/I = 1 + K_{sv}[Q] = \tau_0/\tau = 1 + k_q\tau_0[Q] \quad (1)$$

where I_0 and I are the fluorescence intensities, τ_0 and τ the fluorescence lifetimes in absence and presence of the quencher Q, respectively, K_{sv} is the Stern-Volmer constant, and k_q is the bimolecular quenching constant. The accessibility of a fluorophore to a quencher can be determined using a modified Stern-Volmer equation as proposed by Lehrer [24]:

$$I_0/\Delta I = 1/([Q]f_aK_Q) + 1/f_a \quad (2)$$

where $\Delta I = I_0 - I$ and f_a is the fraction of fluorescence which is accessible to the quencher. Eqn. 2 is valid for the restricted case of a protein containing many tryptophanyl residues some being accessible with a quenching constant K_Q and the remainder being totally inaccessible ($K_Q = 0$). According to Eqn. 2 the reciprocal intercept of a straight line obtained by plotting $I_0/\Delta I$ vs. $1/[Q]$ represents the fraction of fluorescence accessible to a collisional quencher [24].

Iodide was chosen as a suitable quencher for these determinations because it is a dynamical quencher of high efficiency. Ionic strength effects due to the addition of iodide ion were minimized by conducting experiments at high ionic strength [25]. A central premise for all quenching experiments with vesicles is that the bilayer is impermeable to the iodide ion. There are several reports dealing with that problem [23,25,26]. In the present case an impermeability for the I^- ion can be assumed, because the intensity did not change with course of time in the presence of quencher. Penetration of the bilayer into the intravesicular space would have led to time-dependence of the quenched intensity.

Results and Discussion

Fluorescent microscopy and FACS analysis

Pam_3Cys-peptides are specific B-cell and macrophage activators, but do not activate T-lymphocytes. We investigated the binding of lipopeptides to two tumor cell lines, a B-cell line (BCL_1) and a macrophage cell line ($P388D_1$). The cells were incubated with a $2.5 \cdot 10^{-5}$ M solution (about 50 μg/ml) of the fluorescent conjugate Pam_3Cys-Ser-[$Lys_4(FITC)_{1-4}$]-OH in PBS in the presence and absence of NaN_3. At this concentration the lipopeptide is a potent mitogen and activates murine spleen cells to proliferate as determined by the incorporation of [^{3}H]thymidine into DNA. The labelling, however, led to a slightly-reduced capability in comparison to the non-labelled Pam_3Cys-Ser-$(Lys)_4$ (data not shown). After removal of non-cell-bound lipopeptide the cells were analyzed by fluorescence microscopy.

The surface of the cells of both cell lines was strongly fluorescent, indicating the binding of the lipopeptide to their cell membranes (Fig. 1a). When cells were incubated at 37°C in the absence of sodium azide, areas of high fluorescence either distributed on the cell surface ('patching'; Fig. 1b) or concentrated at one pole of the cell ('capping'; Fig. 1c) was observed. Capping and patching are well-known processes usually induced by cross-linking of surface proteins of cells via antibodies [27,28]. To our knowledge, however, there are no reports on the induction of capping and patching by low-molecular-mass compounds in the absence of cross-linking molecules. The binding or association of lipopeptides to defined membrane proteins of murine spleen cells including proteins of the major histocompatibility complex, as already described [29], could be involved in this process. As capping was suppressed by sodium azide, this seems to be a physiological energy-dependent process (as in the case of antibody-induced capping) rather than an unspecific phase-separation process.

In addition, the two labelled cell species BCL_1 and $P388D_1$ were investigated in a fluorescence-activated cell sorter (FACS). They were found to be highly fluorescent (data not shown). The differences in the binding affinity of the lipopeptide to the B-cell and the macrophage cell lines were only marginal. Since the membranes of *Paramecium* could also be effectively stained (Klumpp, S. and Metzger, J.W., unpublished data), we conclude that tripalmitoyl-*S*-glycerylcysteinyl compounds have a high affinity for any membrane. Hence, the first steps of interaction of lipopeptides with cells include the non-specific attachment of lipopeptides to the cell surface. Evidence, that a specific recognition step is involved in leukocyte activation, too, is obtained by the observation that two lipopeptides containing diastereomers of Pam_3Cys activate B-

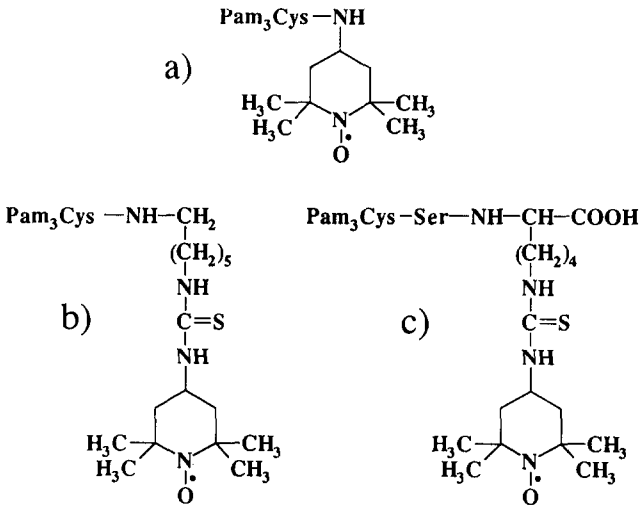

Fig. 2. Structural formulae of the spin-labelled lipopeptides Pam_3-Cys-TEMPO (a), Pam_3Cys-HMD-TEMPO (b) and Pam_3Cys-Ser-Lys(TEMPO)-OH (c).

lymphocytes to a different degree [16,30]. Whether the stereospecific signalling step already occurs on the cell surface or only inside the target cell still remains to be clarified.

Electron paramagnetic resonance spectroscopy

Lipopeptides containing the spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were used for EPR studies. The EPR spectrum of the stable nitroxide radical TEMPO, consists of three equidistant lines of about the same height caused by the interaction of the unpaired electron with the nitrogen nucleus ($I = 1$). As the EPR spectrum is sensitive to changes in the polarity of the environment of the label and to motional parameters of the side-chain, it constitutes a molecular probe for the location of a labelled molecule in a membrane.

Three lipopeptides containing TEMPO were synthesized (for structural formulae, see Fig. 2). Pam_3Cys-TEMPO was obtained by coupling of Pam_3Cys-OH with dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole to 4-amino-TEMPO. Pam_3Cys-HMD-TEMPO and Pam_3Cys-Ser-Lys(TEMPO)-OH were obtained by reaction of 4-isothiocyanato-TEMPO with the free amino group of Pam_3Cys-HMD × TFA and Pam_3Cys-Ser-Lys-OH × TFA, respectively. The resultant conjugates differ in their lipophilicity and in the length of the spacer between the label and the Pam_3Cys moiety.

All three compounds were only scarcely soluble in water. In ethanolic solution they showed the expected three-line EPR spectra. Sonication of these solutions did not change the spectra. Spectra of Pam_3Cys-TEMPO in aqueous 'solution' (obtained after injection of an ethanolic stock solution of the lipopeptide into water and prolonged sonication of the resultant suspension) always showed a very broad signal, which we

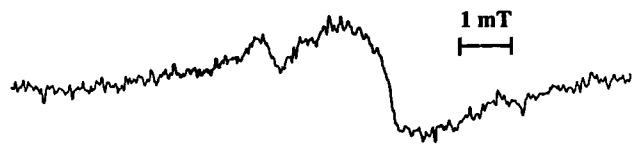

Fig. 3. EPR-spectra (9.5 GHz, 20 mW, modulation 0.15 mT) of Pam_3Cys-TEMPO in aqueous lecithin vesicle suspension. (lipopeptide concentration: 10 μM; 0.5 mM lecithin, 0.1 M Tris-HCl (pH 6.7), $T = 24$°C).

attribute to spins in micelles undergoing exchange broadening, since dilution with 200-fold excess of unlabelled lipopeptide restored a narrow three-line spectrum (not shown). A second (small) population showed narrow lines in aqueous 'solution'. Similar spectra were obtained with lipopeptides containing spacers. The degree of hindrance of rotation depended on the length of the spacer-group between the lipo-amino acid and the label (see below).

Lecithin vesicles were incubated with the spin-labelled lipopeptides, and spectra were measured at room temperature, i.e., above the transition temperature of the membranes used. The EPR spectra obtained were similar to those obtained from aqueous 'solutions' (Figs. 3 and 4), but they were essentially unchanged by the addition of unlabelled lipopeptide. These spectra can be understood as the sum of mainly two signals. Firstly, a rather broad signal showing strong hindrance of rotation. This population is inaccessable to paramagnetic broadening with chromium oxalate [21] or reduction with sodium ascorbate and hence not accessible from the aqueous compartment of the sample. This component dominates with Pam_3Cys-TEMPO and is small with the derivatives bearing the spacers HMD and Ser-Lys. Secondly, a rather freely rotating population with rotational correlation times depending on the length of the spacer-group, which is accessible from the aqueous compartiment, i.e., reducible by ascorbate and broadened by chromium oxalate. Rota-

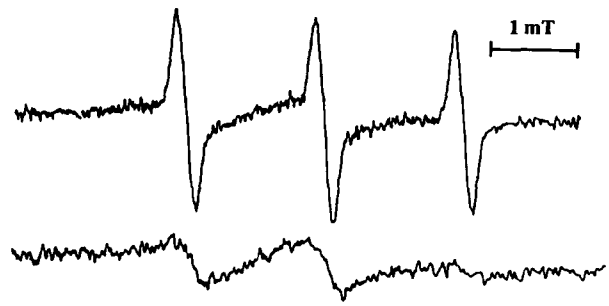

Fig. 4. EPR-spectra (9.5 GHz, 20 mW, modulation 0.15 mT) of Pam_3Cys-HMD-TEMPO in aqueous lecithin vesicle suspension before (a) and after (b) addition of chromium oxalate (lipopeptide concentration: 10 μM; 0.5 mM lecithin, 0.1 M Tris-HCl (pH 6.7), chromium oxalate concentration 20 mM, $T = 24$°C). A similar EPR-spectrum was obtained for the spin-labelled lipopeptide Pam_3Cys-Ser-Lys(TEMPO)-OH.

tional correlation times were estimated according to [31] from such spectra after subtracting the spectrum of a similar sample in the presence of chromium oxalate. This procedure produced a spectrum of three narrow lines. The theory of these measurements is valid for $T_c < 10^{-9}$ s and assumes isotropic rotation. Fig. 4 shows the effect of chromium oxalate [21] on the two populations in small unilamellar vesicles incubated with Pam_3Cys-HMD-TEMPO. Only the narrow lines vanish, which indicates that two distinct populations exist and only one is accessible by the quencher and therefore resides in the aqueous compartiment.

The spectrum depicted in Fig. 3 may contain some minor contribution from exchange-broadened signals. Very similar composite spectra have been obtained with spin-labels attached to amphiphilic peptides [32] and alamethicin [33], where broad lines indicating slow rotation were usually attributed to molecules bound to a membrane while narrow lines seem to originate from monomeric species in aqueous solution. Note that chromium oxalate does not affect spectra of spins residing on the surface of phospholipid vesicles [33]. Both populations were also present on cells of the macrophage cell line $P388D_1$ incubated with the spin-labelled lipopeptides and could be shown to persist at 0°C for 6 h with cells (data not shown).

Rotational correlation times in aqueous solution (after addition of unlabelled lipopeptide) were $> 10^{-9}$ s with Pam_3Cys-TEMPO (population visible after dilution with Pam_3Cys-OH to suppress exchange broadening), about $4 \cdot 10^{-11}$ s with Pam_3Cys-HMD-TEMPO, and $2.5 \cdot 10^{-11}$ s with Pam_3Cys-Ser-Lys(TEMPO). These values are in agreement with the motional parameters expected for segmental motion of the nitroxide-moieties in the derivatives bearing spacer groups. In micelles and when bound to membranes (chromium oxalate inaccessible part of signal), there is a hindrance in rotation by a factor > 20 (rough estimate), even in the presence of a hydrophilic spacer, which points to a very ordered structure at the membrane surface.

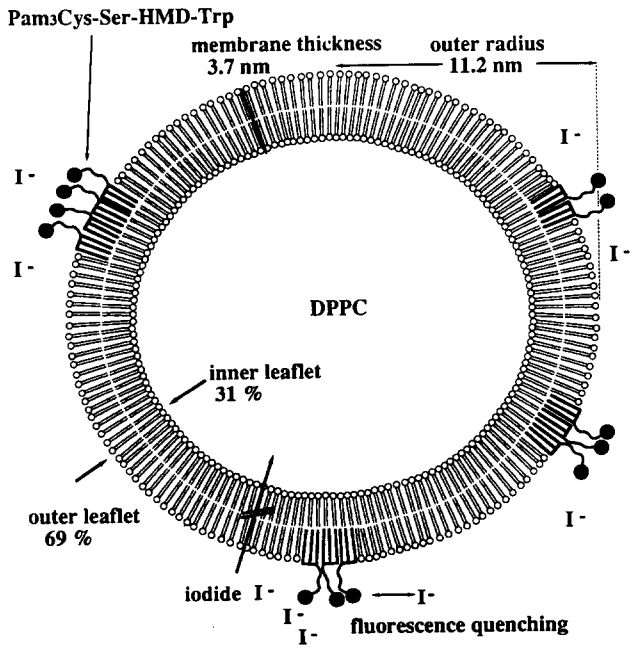

Fig. 5. Fluorescence quenching of a tryptophan-containing lipopeptide with iodide. The values for the distribution of phosphatidylcholine on the outside and inside layers, the membrane thickness and the calculated outer diameter are taken from Ref. 36.

The hyperfine splitting due to the nitrogen nuclear spin can be obtained from the spectra. Contributions of motional and exchange-broadening will lead to smaller splittings and so only a lower limit can be estimated. This parameter is a measure of the polarity of the environment of the spin-population in question. The compounds with Ser-Lys- and HMD-spacer produced a splitting of 1.625 mT in ethanol and of 1.675–1.685 mT in the populations detectable in the presence of vesicles. This last figure is indistinguishable from the splitting of the small amount of narrow signal seen in water. These findings show that both populations face a very polar environment.

Fluorescence quenching experiments

Phosphatidylcholine vesicles incubated with the lipopeptides Pam_3Cys-Ser-HMD ← (Boc-Trp) and Pam_3Cys-Ser-HMD ← (Trp) × TFA, which contain tryptophan in the peptide part, were investigated in order to obtain additional information about the the accessibility of the peptide moiety to the aqueous phase (Fig. 5). The accessibility of the fluorophore was determined by iodide quenching using modified Stern-Volmer plots (see Materials and Methods) [24].

The effects of the following factors on the accessibility of Trp in the lipopeptides Pam_3Cys-Ser-HMD ← (Boc-Trp) and Pam_3Cys-Ser-HMD ← (Trp) × TFA were investigated: the effect of annealing, temperature ($T < T_c$, $T > T_c$), the choice of phosphatidylcholines (DMPC, DPPC and DOPC), and the effect of sonication of vesicles during and after incubation with the lipopeptides.

Pam_3Cys-Ser-HMD ← (Boc-Trp) was added to DPPC vesicles and the sample was shaken briefly. The fluorescence intensity attained a maximum value within seconds and in the course of 90 min decreased slightly by 5 to 10% until it reached a constant value (data not shown). We interpret this result as an indication for the rapid uptake of the lipopeptide into the vesicle membrane. However, since the supernatant after the incubation of cells with FITC-labelled Pam_3Cys-Ser-$(Lys)_4$-OH (see Fluorescence microscopy studies) was still fluorescent, it can be assumed that at least for more amphiphilic lipopeptides a fraction resides in the aqueous compartment, in micelles or multilayer structures. The temporally decreasing fluorescence intensity may be caused by the fluorophore moving from a less

polar to a more polar environment. A lateral movement of the lipopeptide molecules in the bilayer of the vesicle, caused by a tendency of Pam_3Cys to aggregate in the vesicle membrane (Fig. 6), could be one of the reasons for this effect. In the lipopeptide aggregates the fluorophore molecules would be closer together causing concentration quenching and thus a decreased fluorescence intensity. Constant fluorescence intensity was reached faster when the vesicle preparation was annealed at 50°C or alternately in intervals of 10 min at 15 and 50°C for about 60 min after addition of the Pam_3Cys-compound. Annealing is known to remedy structural defects in vesicles [22]. Higher temperatures ($> T_c$) probably accelerate the distribution of the lipopeptide on the vesicle surface.

Accessibilities can be obtained as the reciprocal intercepts in plots of $I_0/\Delta I$ vs. $1/[Q]$ (see Materials and Methods). Experiments performed with identical batches of vesicles incubated with the Pam_3Cys-compound gave reproducible values for the accessibilities only if the vesicles were annealed for about 60 min above T_c after incorporation of the lipopeptide. However, when using a different batch of vesicles or when the same vesicle batch was incubated at a later point of time, different accessibilities were obtained. The deviation was dependent on the range in which the measured $1/f_a$ lay, being greater as f_a decreased. The deviation for intercepts that ranged between 1 and 2 (corresponding to 100–50% accessibility) was up to 20% whereas the deviation calculated from intercepts between 2 and 3 (corresponding to 50–33.3% accessibility) was only about 5%. Without annealing, correlation coefficients for the plot $I_0/\Delta I$ vs. $1/[Q]$ were worse and the calculated accessibilities were much lower than with annealing (Table I). Further problems dealing with the determination of accessibilities by fluorescence quenching are discussed in Ref. 34.

The results of the iodide quenching experiments are given in Table I. The following conclusions can be drawn from Table I:

(1) Annealing, in general, improves the reproducibility of the experiment and leads to a higher accessibility of the fluorophore, probably because it leads to a more ordered structure on the vesicle surface (cf. Fig. 6).

(2) All experiments were carried out at 30°C and 47°C.

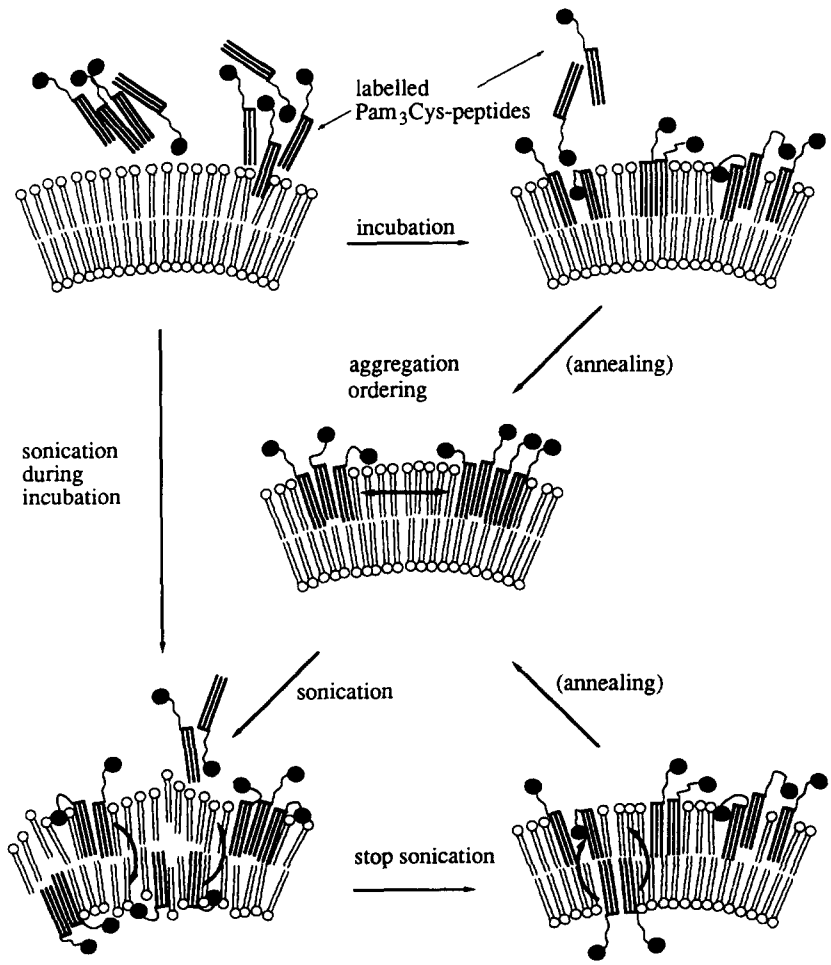

Fig. 6. Pam_3Cys-peptides in vesicle membranes: possible effects of annealing and sonication.

TABLE I

Accessibilities of the Trp fluorophore in lipopeptides Pam_3Cys-Ser-HMD ← (Boc-Trp) (I) and Pam_3Cys-Ser-HMD ← Trp (II) (in %).

Accessibilities were obtained by fluorescence quenching with iodide (5 M KI, 0.5 mM phosphatidylcholine in 0.1 M Tris-HCl/0.5 M KCl containing 6.7 μM lipopeptide).

Lipid (T_c)	Not sonicated		Sonicated		Temperature
	I	II	I	II	
DOPC (−20°C)	56.4	75.2	55.9	64.1	
DMPC (22°C)	50.0	65.0	66.0	67.5	30°C
DPPC (42°C)	56.4	75.9	54.2	68.2	
	23.5 *		32.7 *		
DOPC (−20°C)	84.9	85.0	83.2	100.0	
DMPC (22°C)	76.2	76.6	78.3	67.0	
			54.5 *		47°C
DPPC (42°C)	77.0	75.4	82.0	82.3	
			65.2 *		

* Vesicles were not annealed.

In most cases the accessibility was higher at the higher temperature. Increasing temperature increases the diffusion rate of the quencher and therefore the quenching efficiency of collisional quenching.

(3) The fluorescence intensity of DPPC-vesicles incubated with the Boc-Trp-lipopeptide was examined in the presence and absence of I^- (KI concentration 0.19 M). In both cases increasing temperature is accompanied by decreasing intensity. Near T_c (for DPPC, $T_c = 42°C$) only in the presence of quencher a significant change of intensity is observed (Fig. 7; ordinate is the relative intensity referring to the intensity at 22.5°C). This suggests that the state of the phospholipid influences the penetration of the iodide into the surface of the membrane and/or the arrangement of the Pam_3Cys-molecules.

(4) The choice of the phospholipid used for the preparation of the vesicles did not reveal a regular influence on the Trp accessibility, though the diameter, the curvature, the membrane thickness and T_c differs in DMPC (22°C), DPPC (42°C) and DOPC (−20°C) [35]. The curvature of all vesicles, however, is high in comparison to that of cells. It might be possible that Pam_3Cys-compounds avoid the inner leaflet with higher curvature and remain in the outer leaflet. With this assumption, there should not be striking differences in the accessibilities between the different phospholipids. However, it should be noted, that monolayer experiments carried out previously showed, that the surface areas of the Pam_3Cys moiety is not larger than the area which is required for three individual fatty acids [36].

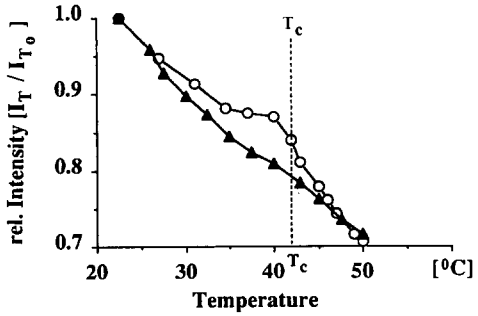

Fig. 7. Temperature dependence of the relative fluorescence intensity of 0.5 mM DPPC-vesicles (0.1 M Tris-HCl (pH 7.4)/0.5 M KCl) containing 6.7 μM Pam_3Cys-Ser-HMD ← (Boc-Trp) (excitation 290 nm, emission 325 nm) in absence (▲) and presence (○) of 0.19 M KI).

(5) Sonication of the vesicles after incubation with the lipopeptide compounds should lead to a lower accessibility of the Trp than in the unsonicated version, because a fraction of the lipopeptide should remain in the inner leaflet of the bilayer, where it is inaccessible to the quencher (cf. Fig. 6). Such a tendency, however, was not observed (Table I). The accessibility in most cases remained the same with or without sonication, but sometimes also increased or decreased in an obviously non-regular way. As mentioned above, it can be assumed that the lipopeptide tends to remain in the outer leaflet because of the higher curvature in the inner one. Immediately after sonication the lipopeptide molecules may start flipping from the inner to the outer leaflet (Fig. 6). This assumption can be supported by the observation that the fluorescence intensity of a freshly-sonicated sample decreased immediately after interrupting the sonication of the sample. Only after 1–2 h it did reach a constant value. This process could be accelerated by annealing of the vesicle suspension above T_c of the corresponding phospholipid for about 30 min.

(6) The accessibilities of Pam_3Cys-Ser-HMD ← (Boc-Trp) and the positively-charged Pam_3Cys-Ser-HMD ← (H-Trp) do not differ much at 47°C. At 30°C, however, the accessibility of the charged lipopeptide is higher in all three phospholipid vesicles used in this study (Fig. 8). At 30°C the fluorophore of the charged lipopeptide is already highly exposed whereas the same accessibility for the Boc-protected lipopeptide is only attainable by raising the temperature. There are only small differences for both at 47°C, though the polarity of the charged lipopeptide is much higher. Probably the length of the spacer Ser-HMD between Pam_3Cys and Trp is responsible for this effect. It is possible that a back bending of the Trp-HMD moiety to the membrane surface where it might interact with the negatively charged membrane and thereby reduce its accessibility for iodide (an evalu-

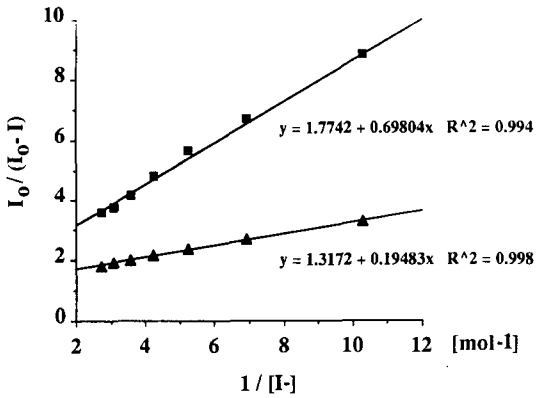

Fig. 8. Modified Stern-Vollmer plot [24] for the iodide quenching of the fluorescence of DPPC vesicles incubated with the lipopeptides Pam_3Cys-Ser-HMD ← Trp(Boc) (■) and Pam_3Cys-Ser-HMD ← Trp (▲), $T = 30°C$. The vesicles were annealed, but not sonicated after incubation with the lipopeptides. The fraction of fluorescence accessible to iodide (obtained as the reciprocal intercept of a straight line obtained by plotting $I_0/\Delta I$ vs. $1/[Q]$) was 56% for the Boc derivative and 76% for the deprotected lipopeptide.

ation of charge effects on iodide quenching is given in Ref. 37) occurs in the case Pam_3Cys-Ser-HMD ← (Boc-Trp) at 30°C. At higher temperatures both compounds might stick in the phospholipid bilayer as shown in Fig. 5 resulting in a high accessibility of the fluorophore.

Conclusions

Immunologically-active lipopeptides containing N-terminally the lipoamino acid Pam_3Cys are taken up rapidly into the membranes of vesicles and cells. By sequestering their fatty acid chains within the lipid bilayer, Pam_3Cys acts as a membrane anchor for the peptide and polar molecules attached to it, which remain accessible from the aqueous compartment. Recent studies using electron energy loss spectroscopy and perfluoropropionylated lipopeptides showed that the lipopeptides do not remain on the surface of cells, but enter the cell plasma, the nuclear membrane and even the nucleus within a few minutes after incubation of the cells with the lipopeptide [38–40]. Such an uptake is a prerequisite for the intracellular processing of synthetic lipopeptide vaccines [12–17,41] based on Pam_3Cys-OH, which is followed by presentation of the peptide antigens on MHC class-I and II molecules to elicit T-cell receptor response.

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