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Membrane-active properties of α-MSH analogs: aggregation and fusion of liposomes triggered by surface-conjugated peptides

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Abstract

Reaction of the melanotropin hormone analogs [Nle^4,D-Phe^7]-α-MSH and [Nle^4,D-Phe^7]-α-MSH(4–10), which were extended at their N-terminus by a thiol-functionalized spacer arm, with preformed liposomes containing thiol-reactive (phospho)lipid derivatives resulted in the aggregation of the vesicles and in a partial leakage of their inner contents. This aggregation/leakage effect, which was only observed when the peptides were covalently conjugated to the surface of the liposomes, was correlated with the fusion of the vesicles as demonstrated by the observed decrease in resonance energy transfer between probes in a membrane lipid mixing assay. A limited fusion was confirmed by monitoring the mixing of the liposome inner contents (formation of 1-aminonaphthalene-3,6,8-trisulfonic acid/*p*-xylene bis(pyridinium bromide) complex). The membrane-active properties of the peptides could be correlated with changes in the fluorescence emission spectra of their tryptophan residue, which suggested that after their covalent binding to the outer surface of the liposomes they can partition within the core of the bilayers. A blue shift of 10 nm was observed for [Nle^4,D-Phe^7]-α-MSH which was correlated with an increase in fluorescence anisotropy and with changes in the accessibility of the coupled peptide as assessed by the quenching of fluorescence of its tryptophan residue by iodide (Stern–Volmer plots). These results should be related to the previously described capacity of α-MSH, and analogs, to interact with membranes and with the favored conformation of these peptides which, via a β-turn, segregate their central hydrophobic residues into a domain that could insert into membranes and, as shown here, trigger their destabilization.

Keywords: α-Melanocyte-stimulating hormone; Membrane-active peptide; Membrane fusion; Liposome

Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; BOP, 2,3-bis[(*Z*)-octadec-9-enyloxy]propanol; CF, 5(6)-carboxyfluorescein; Chol, cholesterol; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol; DPX, *p*-xylene bis(pyridinium bromide); MSH, melanocyte-stimulating hormone; [NDP]-α-MSH, [Nle^4,D-Phe^7]-α-MSH; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; PC, phosphatidylcholine; PCH, hydrogenated soybean PC; PG, phosphatidylglycerol; RET, resonance energy transfer

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1. Introduction

α-Melanocyte-stimulating hormone (α-MSH; Table 1B) binds with high affinity to melanocortin G protein-coupled receptors (MCR) and in humans at least five subtypes of MCR have been identified in various tissues and cells including melanocytes [1]. Although it is primarily responsible for the control of skin pigmentation, this tridecapeptide is endowed with many other biological and lesser defined functions such as in nervous system as a neurotransmitter and in the immune system where it seems able to modulate immune responses and inflammatory processes [2,3]. The occurrence of MCR as a membrane marker of human melanoma cells [4] is also of great interest as a target for the localization and therapy of these malignant cells. Accordingly several strategies of clinical relevance based on the use of α-MSH and analogs as ligands for targeting melanoma cells have been designed in e.g. scintigraphy and radioligand constructs or delivery of chemotherapeutic agents [1]. During recent decades much effort has been devoted to elucidating the structure–activity of the melanotropins. Thus, numerous analogs have been synthesized including cyclized peptides leading to the discovery of the paramount importance of the 'core' sequence His^6-Phe-Arg-Trp^9 for biological activity [5,6]. Similarly the two- and three-dimensional structures of α-MSH and analogs when bound to their receptor has also been investigated and conformations such as β-turns that involve this central 'message' region are presently invoked [7]. Early on, 'superpotent' agonists have been developed such as [Nle^4,D-Phe^7]-α-MSH ([NDP]-α-MSH, **1**), an analog of the natural hormone in which residues at positions 4 and 7 have been substituted by non-natural amino acids (Table 1B), and the truncated analog [NDP]-α-MSH(4–10) (**2**) [5,6]. These peptides are characterized by a higher affinity for the melanocortin receptor and a lesser susceptibility to proteases [8], and to explain their unusually long lasting biological effect [9] an interaction with the lipid phase of membranes was postulated ('stickiness' or 'sequestration'). Indeed, several publications, mostly on model systems such as liposomes, have clearly indicated that α-MSH was able to interact and penetrate membranes. A two-step process was described: an initial electrostatic interaction between the positively charged peptide with negatively charged (phospho)-lipids of the membrane making it possible to reach an appropriate local concentration is followed by the penetration of the peptide in the hydrophobic core of the bilayer [10–14]. No consequence for the membrane structure of this penetration has, however, been described.

During our recent work on the targeting of melanoma cells with liposomes carrying antitumor drugs we have covalently coupled the two superpotent α-MSH analogs **1** and **2** to the outer surface of preformed vesicles according to a classical strategy outlined in Table 1A. This resulted in a net aggregation of the liposomes and in the partial leakage of their inner content. We have therefore undertaken a study to better characterize and understand these activities. The present work deals with these new properties of α-MSH analogs which were found, in analogy to the mode of action of membrane-active peptides [15–18], to destabilize the bilayers of liposomes and trigger their fusion. This behavior, which is only observed when peptides are conjugated to the outer surface of the vesicles, is reminiscent of the activity of fusion peptide sequences of viral proteins which are also associated with a membrane-anchored subunit [19]. The results described in this study add a new dimension to the potential of these quite unique bioactive peptides which have been used e.g. to promote cationic lipid-based gene delivery [20].

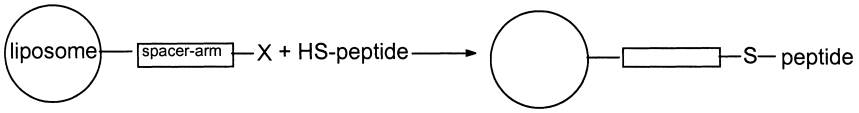
2. Materials and methods

2.1. Materials

The 3-thiopropionate *N*-derivatives of NDP-[NDP]-α-MSH (**1**) and [NDP]-α-MSH(4–10) (**2**) were obtained from Neosystem (Strasbourg, France). Egg yolk phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol), hydrogenated soybean PC (PCH), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG) and fluorescein-5′-isothiocyanate-dextran 77 kDa were obtained from Sigma (L'Isle d'Abeau Chesnes, France). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE)-PEG_3-maleimide and DPPE-bromoacetyl derivatives were prepared in our laboratory as described previously [21]. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-

Table 1
Coupling of peptides to liposomes

A - Coupling of HS-peptides to preformed liposomes[a]



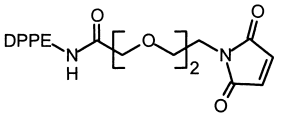
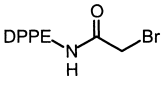
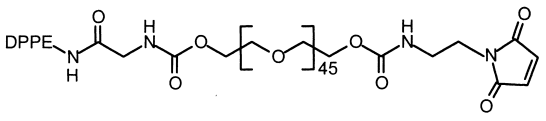
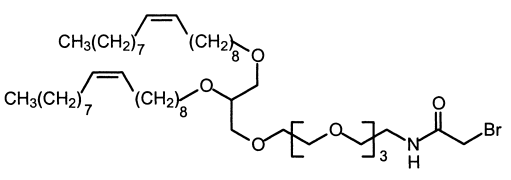
B - Peptide derivatives used in this study[b]

1	HS-CH_2CH_2CONH-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
2	HS-CH_2CH_2CONH-Gly-Nle-Glu-His-D-Phe-Arg-Trp-Gly-NH_2
α-MSH	AcNH-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

[a]α-MSH analogs **1** and **2**, derivatized at their N-terminus with thiol-functionalized linkers, were conjugated (see text) to preformed liposomes containing thiol-reactive groups X such as maleimide or bromoacetyl (Table 2).
[b]The linkers are underlined.

phosphatidylethanolamine (*N*-NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE), 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) (disodium salt) and *p*-xylene bis(pyridinium bromide) (DPX) were purchased from Molecular Probes. 5,6-Carboxyfluorescein (Eastman-Kodak) and calcein (Prolabo) were purified respectively on Sephadex LH-20 (Pharmacia)

Table 2
Structure of thiol-reactive (phospho)lipid derivatives used in this study

Compounds	Structure
DPPE-PEG_3-Mal	
DPPE-AcBr	
DPPE-PEG_{45}-Mal	
BOP-PEG_4-AcBr	

[22] or by recrystallization from ethanol/water. All other reagents were of analytical grade. Ellman's method was used to determine thiol-reactive functions [23].

2.2. Synthesis of lipid derivatives

BOP-PEG$_4$-AcBr was prepared by coupling 2,3-bis[(*Z*)-octadec-9-enyloxy]-(amino-11-trioxa-3,6,9-undecyl)-3-propane (BOP-PEG$_4$-NH$_2$) to *N*-succinimidyl bromoacetate [24] essentially as described by Frisch et al. [21]. R_f 0.51 (CH_2Cl_2:CH_3OH, 9:1). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, CH_3), 1.28 (d, 22 CH_2), 1.50–1.60 (m, 2 CH_2CH_2O), 1.92–2.06 (m, 4 $CH_2CH=CH$), 3.43–3.67 (m, CH_2NHCO, 11 CH_2O, $(CH_2)_2CHO$), 3.87 (s, CH_2Br), 5.28–5.40 (m, 2 $CH=CH$), 7.10 (ls, NHCO). ^{13}C-NMR ($CDCl_3$, 75 MHz) major peaks at δ 32.59 (CH_2Br), 39.91 (CH_2NHCO), 77.8 $(CH_2)_2CHO$), 129.80 and 129.91($CH=CH$), 165.73 (CONH). High resolution mass spectra (FAB^+): *m*/*z* calculated for $C_{49}H_{94}NO_7Br$ 889.20; found 890.6 $(M+H)^+$. The synthesis of DPPE-PEG$_{45}$-Mal will be reported elsewhere (Frisch et al.).

2.3. Preparation of liposomes

A solution of lipids in chloroform/methanol (9:1, v:v) was evaporated and the resulting film dried under high vacuum. The film was then rehydrated in indicated buffers at concentrations of 1–10 μmol lipid ml^{-1} by vortexing and the resulting suspension was sonicated (1 h at 25°C), under a continuous flow of nitrogen, using a titanium probe-type sonicator (Vibra Cell; Sonics and Materials, Danbury, CT, USA). Probe debris was removed by centrifugation. When needed, the obtained liposomes were extruded nine times through 0.1 μm pore size polycarbonate membranes (Nucleopore). In the case of liposomes composed of PCH/DPPG/Chol/DPPE-PEG$_3$-Mal (70/20/50/10; molar ratios), the lipid film was rehydrated by five freeze/thawing cycles at −80°C/+25°C. This preparation was then further sonicated (1 h) at 70°C and extruded. These different procedures yielded vesicles with a 110 ± 15 nm mean diameter as determined by quasi-elastic light scattering (Coulter sub-micron particle analyzer; Coulter Electronics, Hialeah, FL, USA). Liposomes loaded with 5(6)-carboxyfluorescein (CF), calcein, ANTS or DPX were prepared and separated from free dyes by gel filtration (Sephadex G75, Pharmacia). Liposomal concentrations were determined using the phosphate assay [25].

2.4. Fluorescence and light scattering measurements

Fluorescence and light scattering were recorded in a ratio mode, at 25°C, on a SLM-8000 spectrofluorimeter computerized with a Biokine ASCII program (Biologic, Claix, France). A stirring device enabled constant mixing of the medium and the temperature was controlled with a thermostated circulating water bath.

2.5. Determination of CF or calcein leakage

The fluorescence was recorded after addition, under mild agitation, of the peptides (2:1 molar excess peptide vs. surface-accessible thiol-reactive functions) to dye-loaded liposomal suspensions (50–500 μM phospholipid in given buffers, final volume: 1 ml, temperature: 25°C). Excitation and emission wavelengths were set at 490 and 520 nm, respectively. To measure the total fluorescence intensity corresponding to 100% dye release, 100 μl of Triton X-100 (5% w:v in HEPES or borate buffers) was added to the vesicles. The percentage of dye release caused by the peptides was calculated using the equation: $(F-F_0)\times 100/(F_t-F_0)$, where F is the fluorescence intensity measured in the presence of the peptide and F_0, F_t respectively the intensities obtained before the addition of the peptides and after Triton X-100 treatment [26]. F_t values were corrected for dilutions caused by the Triton X-100 addition.

2.6. Vesicle aggregation

The aggregation was followed by monitoring the right-angle light scattering of vesicle suspensions, with excitation and emission monochromators of the fluorimeter both set at 400 nm. The change of scattered light, after addition of peptides, was recorded during 60–90 min at 25°C.

2.7. Rate of reaction of the peptides **1** or **2** with the liposomal thiol-reactive functions

The composition of the liposomes used for this study was PC/PG/Chol/DPPE-PEG_3-Mal (65/20/50/15) (L1) in 10 mM HEPES (pH 6.5 or 7.4) buffer containing 140 mM NaCl (B1) for the maleimide function and PC/Chol/BOP-PEG_4-AcBr (85/50/15) (L2) in 40 mM borate buffer containing 130 mM NaCl (pH 9.0) (B2) for the bromoacetyl function. Peptides **1** or **2** (90 nmol) were added to the vesicle suspensions (100 μM phospholipids; final volume: 6 ml) at 25°C. The reaction progress was measured by determination at given times of residual thiols on 500 μl aliquots with 1.5 ml Ellman's reagent (2 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 1 mM EDTA in 0.2 M sodium phosphate buffer, pH 7.27) [23]. The mixture was immediately filtered through a 0.2 μm filter (Gelman Sciences) and the absorbance read at 412 nm against a blank obtained with liposomes in the absence of peptides. A standard curve was established with 2-mercaptoethanol for each buffer. The rates were corrected for the spontaneous peptide oxidation (dimerization) at each pH value [27].

To determine the yield of peptide coupling to the outer surface of the vesicles, the conjugation step was followed by the addition of a 10-fold excess of 2-mercaptoethanol to deactivate the remaining thiol-reactive functions. After 1 h of this treatment, the liposomes were dialyzed extensively against a 10 mM HEPES buffer (pH 7.4) containing 145 mM NaCl to eliminate unconjugated peptides and reagents. Estimation of liposome-associated peptides was obtained by hydrolyzing an aliquot of the preparation (100 μl liposome suspension, 100 μl 12 M HCl, 110°C, 12 h) followed by quantification of the number of amino groups with fluorescamine [28]. Control liposomes (i.e. unconjugated liposomes) were prepared similarly without the peptide coupling step.

2.8. Tryptophan fluorescence measurements

The SLM-8000 spectrofluorimeter in the T-format was used to measure the steady-state fluorescence anisotropy $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$. The excitation wavelength was 280 nm and 355 nm Schott interference filters were used for the emitted light. A home-built device ensured automatic rotation of the excitation polarizer, allowing a continuous average measurement of r. Tryptophan emission spectra was recorded, from 300 to 440 nm with an excitation wavelength of 280 nm for the peptides, either free in buffers B1 and B2 or in the presence of control liposomes PC/PG/Chol (80/20/50) and PC/Chol (100/50), and for the peptides covalently coupled to the liposomes L1 and L2. Trp fluorescence intensity measurements were also performed in the presence of potassium iodide. Increasing concentrations of KI (from a 400 mM aqueous stock solution) were added to 0.5 ml of B2 containing free peptide **1** and control liposomes PC/PG/Chol (80/20/50) and to peptide **1** coupled to PC/PG/Chol/BOP-PEG_4-AcBr liposomes (with 75/20/50/5 molar ratios chosen to limit the aggregation of the vesicles) in buffer B2. Potassium chloride was added to bring a total salt concentration to a constant value of 200 mM for all samples (final volume 1 ml) [29]. The final pH was maintained at 9.0. The collisional quenching was assessed with the Stern–Volmer relation, $F_0/F = K_{sv}[Q]+1$, where F_0 is the fluorescence intensity in the absence of I^- and F the observed intensity at a given concentration [Q] of I^-; K_{sv} is the Stern–Volmer quenching constant. K_{sv} is readily obtained from the slopes of plots F_0/F vs. [Q]; this value decreases when the probability of collision with the quencher decreases, i.e. when the fluorophore is shielded from the quencher. Fluorescence intensity was measured at 340 nm. Values were corrected for the vesicle blank (scatter) when peptides were in the presence of liposomes.

2.9. Liposome fusion assay by lipid mixing

Lipid mixing was monitored by the *N*-NBD-PE/*N*-Rh-PE energy transfer assay [30]. Labeled liposomes containing each probe at 0.9 mol% (vs. phospholipids), which results in the quenching of the *N*-NBD-PE fluorescence signal, were prepared and mixed in a 1:9 mol ratio with probe-free vesicles at a final phospholipid concentration of 100 μM in B1 or B2, respectively for the maleimide or bromoacetyl functionalized thiol-reactive lipid derivatives. Liposome–liposome lipid mixing/fusion was triggered by addition of peptide **1** or **2** and the probe diffusion/dilution was evidenced by an increase of the *N*-NBD-PE

fluorescence resulting from self-quenching relief [31]. The fluorescence before peptide addition was taken as 0% and the 100% fluorescence was determined using an equivalent concentration of vesicles containing 0.09 mol% of both probes ('mock' vesicles). The excitation wavelength was 460 nm and the increase in *N*-NBD-PE fluorescence, after addition of the peptide, was monitored as a function of time at 525 nm.

2.10. Liposome fusion assay by internal content mixing

The ANTS/DPX fusion assay was carried out as described by Ellens et al. [32]. Liposomes L1 (100 μM phospholipids) contained either 25 mM ANTS, 95 mM NaCl and 10 mM HEPES buffer (pH 6.5), or 90 mM DPX, 55 mM NaCl and 10 mM HEPES buffer (pH 6.5) were prepared. Control vesicles corresponding to a complete content mixing and which contained 12.5 mM ANTS, 45 mM DPX, 75 mM NaCl and 10 mM HEPES buffer (pH 6.5) were also made. ANTS fluorescence intensity was monitored at 505 nm (emission) using an excitation at 355 nm. For fusion assays a 1:1 molar ratio of DPX-containing liposomes and ANTS-containing liposomes in buffer B1 was used. The initial fluorescence of the combined suspension was taken as 100% fluorescence level (or 0% fusion). The decrease in fluorescence triggered by peptide addition, due to contents mixing and the formation of an ANTS/DPX complex with reduced quantum yield, reflected the rate of the vesicle fusion. The fluorescence level corresponding to 100% of content mixing, i.e. 100% leakage-free fusion, was determined from 100 μM liposomes containing the coencapsulated ANTS and DPX.

3. Results

3.1. Coupling of α-MSH peptide analogs to the surface of liposomes

The conjugation of [NDP]-α-MSH(1–13) (**1**) and [NDP]-α-MSH(4–10) (**2**) to the surface of vesicles (Table 1) was performed according to a strategy we have developed before for the preparation of liposome-based synthetic vaccines [33,34]. Accordingly: (i) the peptides were extended at their N-terminus by a thiol-functionalized spacer arm; this was shown before to bear no consequence on the affinity of these ligands for their receptor [35,36], (ii) thiol-reactive derivatives (e.g. maleimide or bromoacetyl functions) of phosphatidylethanolamine [21] or other lipophilic anchors (see Table 2) were incorporated in the (phospho)lipid composition of the liposomes, (iii) the conjugation of the thiolated peptides to the surface of preformed liposomes was performed under mild and well controlled pH and temperature conditions. When using low proportions of thiol-reactive derivatives in the preparation of the vesicles or short spacer arms such as DPPE-AcBr, i.e. conditions which limit vesicle aggregation (see below), the quantity of peptide coupled to the liposomes represented consistently 50% (± 5%) of the total amount of incorporated maleimide or bromoacetyl derivatives; thus under these specific conditions the coupling reactions were nearly quantitative with respect to any thiol-reactive function exposed on the surface of the vesicles.

3.2. Conjugation of α-MSH analogs triggers the aggregation and leakage of liposomes – phenomenological aspects

When **1** was added to negatively charged unilamellar liposomes (PC/PG/Chol/DPPE-PEG_3-Mal) that encapsulated CF or calcein, we were confronted with a fast (min range) and extensive aggregation of the vesicles (Fig. 1B), i.e. the suspension became milky and in the most extreme cases this was followed by the formation of precipitates. As assessed by the increase in fluorescence, this was accompanied by a partial release of the vesicle inner contents that plateaued at about 15% of total dye release (Fig. 1A). Similar results, i.e. aggregation and leakage, were observed with liposomes containing the same proportion of BOP-PEG_4-AcBr, another thiol-reactive PE derivative that requires basic conditions (pH 9.0) to covalently react with the thiol function of the peptide (Fig. 1A,B). In this case however, an increased lag phase due to a slower peptide coupling rate (see below) was observable. Because the α-MSH analog used here carries a net positive charge, we also verified the potential involvement of charge–charge interactions in these effects. Covalent cou-

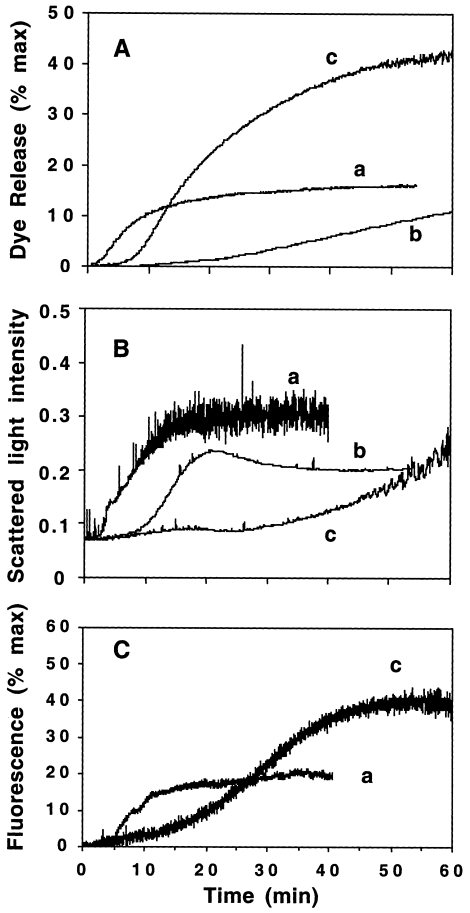


Fig. 1. Representative time courses of aggregation, leakage and lipid mixing triggered by covalent coupling of **1** to the surface of liposomes. Peptide **1** was added at a 2:1 molar ratio vs. surface-exposed thiol-reactive functions to suspensions (25°C) of: (a) PC/PG/Chol/DPPE-PEG_3-Mal (65/20/50/15) liposomes in 10 mM HEPES buffer, 140 mM NaCl, pH 6.5, (b) PC/PG/Chol/BOP-PEG_4-AcBr (65/20/50/15) and (c) PC/Chol/BOP-PEG_4-AcBr (85/50/15) liposomes in 40 mM sodium borate buffer, 130 mM NaCl, pH 9.0. Final (phospho)lipid concentration 100 μM in 1 ml. (A) Leakage of CF (all vesicles contained 40 mM dye) was monitored at 520 nm (excitation 490 nm) and is given as % of the maximal dye release obtained in the presence of detergent (see text). (B) Light scattering intensity increase due to aggregation (at 400 nm). (C) Lipid mixing fusion assay. The liposomes were mixed in a lipid molar ratio of 9:1 with vesicles of same composition (i.e. labeled vesicles) containing both *N*-NBD-PE and *N*-Rh-PE probes (each at 0.9 mol% vs. phospholipids). Peptide **1** was added at time zero. In this probe dilution assay the increase of *N*-NBD-PE fluorescence due to quenching relief was monitored at 525 nm (excitation 460 nm). The maximal fluorescence (100%) was determined with a mixture of liposomes containing each probe at appropriate concentrations (see text).

pling of peptide **1** to zwitterionic (neutral) liposomes (PC/Chol/BOP-PEG_4-AcBr) also provoked the aggregation of the vesicles and their extensive leakage (about 40%) (Fig. 1A,B). This rules out, a priori, that electrostatic interactions play an important role in the destabilization of these vesicles. We have also tested the influence of the bilayer lipid packing with liposomes such as PCH/DPPG/Chol/DPPE-PEG_3-Mal where the phospholipids carry saturated fatty acids ($T_c > 25$°C). On addition of peptide **1**, an immediate and extensive content release was also observed with these vesicles (not shown).

Finally, we verified the effect of pH on these phenomena. The conformation of many membrane-active peptides and their corresponding membrane-destabilizing effects are sensitive to pH [37]; thus many virus-derived fusogenic peptides are only active under acidic conditions [19,38]. In Fig. 2 are given representative leakage time courses, as a function of pH, of liposomes containing 15 mol% DPPE-PEG_3-Mal after addition of peptide **1**. No pH threshold value was observed between pH 5 and 7.4 and the higher rates observed at pH 7.4 could be explained by a faster coupling reaction between **1** and the maleimide function [21,27].

These properties of α-MSH analog **1**, which were totally unpredicted, could not be reproduced with control vesicles that lacked thiol-reactive lipids (PC/PG/Chol) or with liposomes where the maleimide function had been reacted first with e.g. 2-mercaptoethanol; in both cases the vesicles remained perfectly

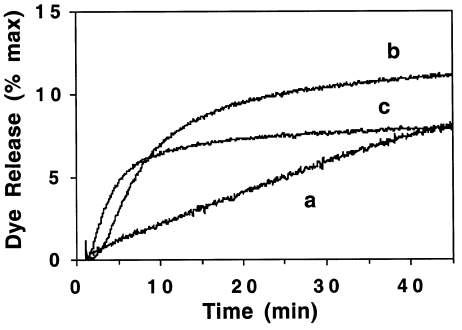


Fig. 2. Influence of pH on calcein leakage time course. Peptide **1** was added at a 2:1 molar ratio vs. surface-exposed thiol-reactive functions to PC/PG/Chol/DPPE-PEG_3-Mal (65/20/50/15) liposomes (100 μM phospholipid; 25°C), having encapsulated 50 mM calcein, in 10 mM sodium citrate buffer, 140 mM NaCl, pH 5.0 (a) or 10 mM HEPES buffer, 140 mM NaCl, pH 6.5 (b) and 7.4 (c) (see Fig. 1A).

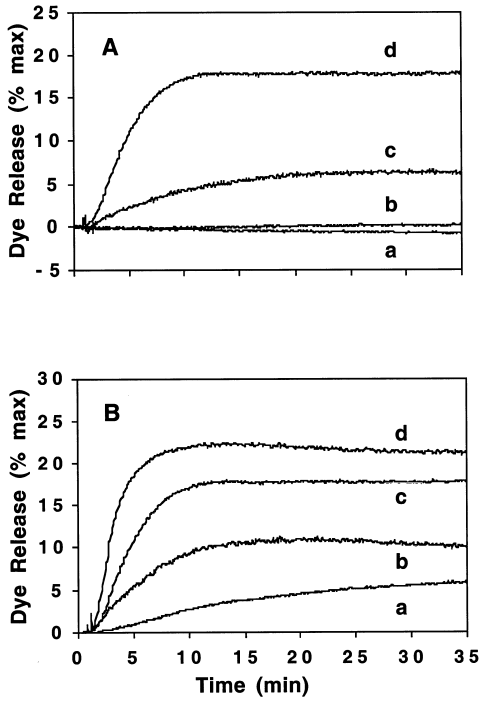


Fig. 3. Parameters influencing the release of CF from liposomes by covalent coupling of peptide **1**. Peptide **1** was added (25°C) to PC/PG/Chol/DPPE-PEG$_3$-Mal (78.5–65/20/50/1.5–15) liposomes, having encapsulated 40 mM CF, in 10 mM HEPES buffer, 100 mM NaCl, pH 7.4. (A) The proportions of DPPE-PEG$_3$-Mal in the vesicles (100 μM) were 1.5 (a), 5 (b), 10 (c) and 15 mol% (d) and **1** was added at 2 mol equivalents. (B) The proportion of DPPE-PEG$_3$-Mal in the vesicles was 15 mol% and the liposomes (100 μM phospholipid) were reacted with **1** at 0.67 (a), 1.2 (b), 2 (c) and 3 (d) mol equivalents. These representative time courses were recorded at 520 nm (see Fig. 1A).

stable in the presence of free peptide **1**. It seems therefore that the covalent coupling of **1** to the surface of the liposomes was responsible for these phenomena.

3.3. Parameters influencing liposome aggregation and leakage triggered by the coupling of α-MSH analogs

3.3.1. Spacer arm length

We first studied the influence of the spacer arm length between the peptide and the liposome surface. The first alternative construct was PC/PG/Chol/DPPE-AcBr (65/20/50/15) in which the thiol-reactive derivative carries a very short linker (see Table 2). Reaction with **1** also resulted in an aggregation that was paralleled by a leakage of 5% of the dye. The other spacer arm tested was carried by DPPE-PEG$_{45}$-Mal. When incorporated into PC/PG/Chol/DPPE-PEG$_{45}$-Mal (70/20/50/10) liposomes, this thiol-reactive derivative was expected to fulfill a double role: (i) because of the length of the PEG$_{45}$ chain, the peptide that is coupled to its distal end gains a great spatial mobility, and (ii) the hydrophilic PEG$_{45}$-chain provides a property of 'stealthiness' to the vesicles that carry it [39], i.e. these vesicles are much less prone to aggregation and to destabilization for example by opsonins. Coupling of **1** to these liposomes resulted in an aggregation comparable to that observed with vesicles containing DPPE-PEG$_3$-Mal but, in contrast to all other cases examined so far, this phenomenon was not accompanied by a leakage of the vesicles (not shown).

3.3.2. Effect of the surface ligand density

The sensitivity of the aggregation and leakage to the surface density of the thiol-reactive functions, and therefore on the amount of peptide that could be coupled, was then studied. To that end PC/PG/Chol/DPPE-PEG$_3$-Mal liposomes were prepared in which the relative proportions of DPPE-PEG$_3$-Mal were varied from 1.5 to 15 mol%. As indicated in Fig. 3A, a threshold was observed at 1.5 mol% of

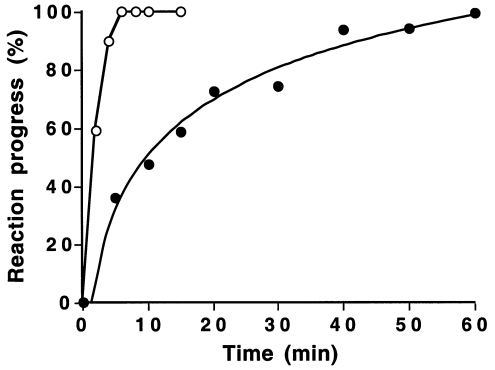


Fig. 4. Rates of reaction between peptide **1** with thiol-reactive derivatives anchored in liposomes. The reactions were performed at 25°C in the presence of a two-fold molar excess of **1** vs. surface-exposed thiol-reactive derivatives with PC/PG/Chol/DPPE-PEG$_3$-Mal (65/20/50/15) (○) and PC/Chol/BOP-PEG$_4$-AcBr (85/50/15) (●) liposomes (100 μM (phospho)lipid final concentration), in respectively 10 mM HEPES, 140 mM NaCl, pH 6.5 and 40 mM sodium borate, 130 mM NaCl, pH 9.0 buffers. Reaction progress was measured on aliquots by determination of residual peptide thiols with Ellman's reagent; 100% represents the disappearance of 50% of added thiols corrected for the spontaneous peptide oxidation (see text).

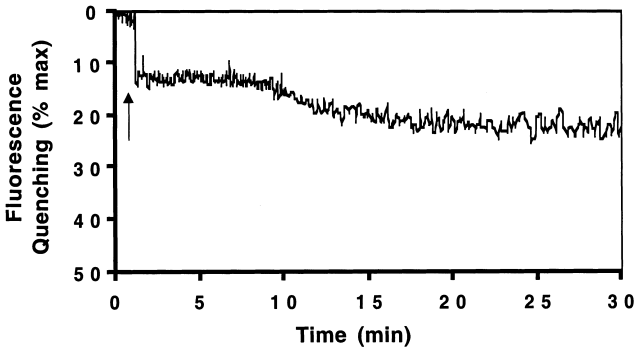


Fig. 5. Representative time course of internal content mixing upon coupling of α-MSH analog. Peptide **1** was added (arrow) at a 1.5:1 molar ratio vs. surface-exposed thiol-reactive function (25°C) to a 1:1 mixture in 10 mM HEPES buffer (pH 6.5) containing 145 mM NaCl of liposomes (PC/PG/Chol/DPPE-PEG_3-Mal; 65/20/50/15) containing either 25 mM ANTS or 90 mM DPX (final phospholipid concentration: 100 μM). The quenching of ANTS fluorescence due to the formation of the ANTS/DPX complex was monitored at 505 nm (excitation 355 nm). The initial fluorescence was taken as 100% and the minimal fluorescence (maximal quenching), corresponding to a 100% leakage-free fusion, was obtained with a preparation of liposomes containing coencapsulated ANTS and DPX (see text).

the thiol-reactive lipid derivative; thus essentially no leakage of the vesicles that contained CF was measurable on addition of peptide **1** whereas when conjugated at 5 mol% the leakage remained still minimal. Loss of internal content became really important at 10 mol% of DPPE-PEG_3-Mal and at 15 mol% it plateaued at about 15%. Interestingly, a correlation between the initial rates of vesicle leakage and its final extent seems to exist (Fig. 3A). Next, liposomes that contained 15 mol% DPPE-PEG_3-Mal were reacted with varying amounts of peptide **1**. We have shown before that this type of reaction could be analyzed, in first approximation, by second-order rate equations [27]. As shown in Fig. 3B, an apparent correlation of the leakage rates – and extent – with the concentration of the peptide, i.e. its rate of coupling, could indeed be observed. The smallest leakage rate was found with a sub-optimal concentration of the peptide, i.e. with 0.66 eq. of **1**, which, assuming that the coupling is quantitative, would be equivalent to the rate observed with vesicles containing about 10 mol% of DPPE-PEG_3-Mal. This seems to be the case: compare Fig. 3A and B.

Altogether these observations indicate that: (i) the rate and extent of membrane destabilization are dependent on the density of the conjugated peptides, (ii) a minimal peptide density (>5 mol%) must exist on the surface of the vesicles to observe the leakage, and importantly, (iii) α-MSH-targeted vesicles can be made that do not readily aggregate, albeit with a limited surface ligand density (~ 5 mol%).

3.4. Correlation between rates of aggregation/leakage of the liposomes and the rate of covalent coupling of the α-MSH analogs

To demonstrate the potential correlation between covalent coupling of peptides **1** to the surface of the vesicles with their aggregation/leakage we have taken advantage of the difference of reactivity, in aqueous media, of the maleimide and bromoacetyl groups with thiols carried by e.g. peptides. Thus in the 6.5–7.5 pH range the maleimide reacts very rapidly and quantitatively whereas, comparatively, the bromoacetyl reacts much more slowly [27]. To observe

Table 3
Tryptophan fluorescence blue shift and fluorescence anisotropy of peptides **1** and **2**, either free in the presence of liposomes or liposome-coupled[a]

Conditions	$\Delta\lambda_{max}$ (±2 nm)[b]		Anisotropy values ($r \pm 0.002$)	
	Peptide		Peptide	
	1	**2**	**1**	**2**
PC/Chol/BOP-PEG_4-AcBr (85/50/15)				
Free peptide	NS[c]	NS	0.032	0.030
Coupled peptide	3.0	4.5	0.054	0.050
PC/PG/Chol/DPPE-PEG_3-Mal (65/20/50/15)				
Free peptide	NS	NS	0.024	0.019
Coupled peptide	9.0	10.5	0.082	0.075
PC/PG/Chol/DPPE-AcBr (65/20/50/15)				
Free peptide	NS	NS	0.020	0.020
Coupled peptide	NS	NS	0.040	0.040
PC/PG/Chol/DPPE-PEG_{45}-Mal (70/20/50/10)				
Free peptide	–	–	0.031	0.023
Coupled peptide	–	–	0.037	0.043

[a]Liposomes (100 μM (phospho)lipids) and 1 mol eq. peptide vs. thiol-reactive anchor were in B1 buffer pH 6.5 or B2 buffer pH 9.0, at 25°C. Coupling time: 60 min (maleimide) and 90 min (bromoacetyl). Free peptides were incubated similarly with control liposomes, i.e. vesicles in which the thiol-reactive functions were first reacted with 2-mercaptoethanol or which lacked the thiol-reactive anchors.

[b]In the absence of lipids (in buffer solution), all peptides display a $\lambda_{max} = 352 \pm 2$ nm.

[c]Not significant.

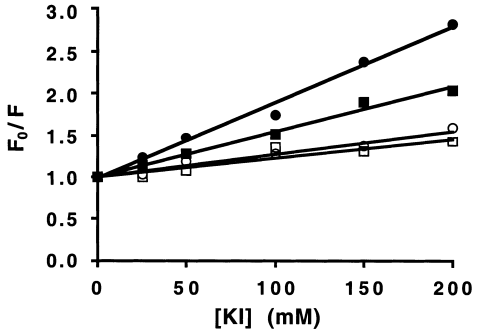


Fig. 6. Stern–Volmer plots of tryptophan fluorescence quenching by I^- of the α-MSH analogs. Peptides **1** (●, ○) and **2** (■, □) and liposomes (PC/PG/Chol/BOP-PEG$_4$-AcBr; 75/20/50/5) were incubated for 90 min in 40 mM borate buffer, 130 mM NaCl (pH 9.0), at 25°C, before measurement. Trp fluorescence intensity of free peptides (closed symbols) or coupled to liposomes (open symbols) was recorded at 340 nm ($\lambda_{exc} = 280$ nm) in the presence of increasing concentrations of KI and were corrected for the scattered blank of vesicles. The results are expressed as F_0/F, where F_0 denotes the fluorescence intensity in the absence and F the fluorescence intensity in the presence of the aqueous quencher. Phospholipid concentration 100 μM; peptide concentration 2.5 μM.

the coupling of the latter group, basic pH must be used (e.g. pH 9.0). In Fig. 4 are represented the coupling of **1** (2 molar eq.), at 25°C, to liposomes containing 15 mol% of DPPE-PEG$_3$-Mal or BOP-PEG$_4$-AcBr at pH 6.5 and 9.0 respectively. In agreement with our previous observations [27], in the first case the coupling took place within 6–8 min whereas in the second it plateaued after 40–60 min. The extent of peptide coupling is similar in both cases and essentially all the thiol-reactive groups present at the surface of the vesicles had reacted. Under neutral conditions, where the coupling of peptides **1** to BOP-PEG$_4$-AcBr takes place at very slow rates, essentially no aggregation or leakage of the vesicles could be observed (not shown); however, a good correlation could be found between the coupling of the peptides to the liposomes containing DPPE-PEG$_3$-Mal and their aggregation/leakage (compare Figs. 4 and 1A,B). For the zwitterionic vesicles, however, the leakage occurs faster that the complete coupling of e.g. peptide **1**. It seems therefore that if the coupling of the peptide to the surface of the liposomes is a prerequisite for their aggregation/leakage a limited amount (< 15 mol%) is enough to trigger these phenomena.

3.5. Leakage and aggregation are correlated with liposome fusion

The occurrence of a loss of liposomal content that is correlated with an aggregation of the vesicles can be indicative of fusion events. We therefore wanted to verify whether the α-MSH analog used in this study, through destabilization of the membrane bilayers, was endowed with fusogenic properties. In a first approach the fusion of liposomes was studied by monitoring lipid mixing. This was classically determined by monitoring the decrease in resonance energy transfer (RET) between the fluorescently labeled lipids *N*-Rh-PE and *N*-NBD-PE, present in the same vesicles, as estimated by an increase in the *N*-NBD-PE signal due to the fusion with unlabeled liposomes present in excess (nine-fold). This probe dilution assay was chosen for its relative insensitivity to the aggregation of vesicles [31]. As shown in Fig. 1C, the addition of peptide **1** to a mixture of the two vesicles populations *which both contained a thiol-reactive lipid derivative* provoked a lipid mixing indicative of fusion. The extent of fusion was dependent on the composition of the liposomes; thus for the negatively charged vesicles PC/PG/Chol/DPPE-PEG$_3$-Mal (65/20/50/15), a 24% increase in the fluorescence of *N*-NBD was observed within the time frame used whereas for the neutral vesicles PC/Chol/BOP-PEG$_4$-AcBr (85/50/15) this increase was 38%. These results, which point to the occurrence of a fusion triggered by peptide **1**, correlate well with the observations made above that leakage of the vesicles is more pronounced in the zwitterionic vesicles as compared to the negatively charged ones. Using the present assay we also found that covalent coupling of **1** to the surface of the vesicles was a prerequisite for observing a lipid mixing indicative of the vesicle fusion. Thus, in control experiments addition of **1** to liposomes that were devoid of thiol-reactive (phospho)-lipid anchors did not lead to measurable increases of *N*-NBD fluorescence (not shown). Interestingly, comparison of Fig. 1A and C shows a fairly good correlation between leakage and fusion. This conclusion was substantiated by the observation that liposomes containing the thiol-reactive derivative DPPE-AcBr, which have been shown above to undergo a very limited aggregation and fusion when reacted with **1**,

did not fuse according to the lipid mixing assay (not shown).

In contrast to the other liposomes, with the stealth vesicles (PC/PG/Chol/DPPE-PEG_{45}-Mal; 70/20/50/10), after 1 h in the presence of the peptides we observed only 3% lipid mixing (not shown) which correlates well with their lack of leakage (see above). According to Basañez et al. [40], the extent of lipid mixing is reduced up to 40-fold in the presence of 6% of PEG_{45} chains at the surface of liposomes. Moreover, the fusion between the vesicles is completely suppressed.

We next assessed the fusion with the same lipid mixing assay using this time an excess of *unlabeled liposomes that were devoid of thiol-reactive lipid derivatives*. Under these conditions, addition of peptide **1** to a 1:9 mixture of labeled PC/PG/Chol/DPPE-PEG_3-Mal (65/20/50/15) and unlabeled PC/PG/Chol (80/20/50) liposomes did no lead to an observable increase of *N*-NBD fluorescence (not shown). This result, which could be correlated with a much reduced aggregation and leakage, is of special interest and indicates that most probably the fusion triggered by **1** occurs preferentially between liposome populations that carry the peptide at their surface. In the present experimental setting this residual fusion remains silent because it does not result in a probe dilution, and this despite the presence of a ninefold excess of putative 'target' vesicles that carry no peptide. It therefore seems probable that a specific interaction of **1** with the bilayers of the vesicles, to which these peptides are covalently bound, is responsible for these liposomes to become fusion-competent.

Altogether, these results indicate that the aggregation of the vesicles that is triggered by the peptide **1** results, in the non-stealth liposomes, into a fusion which in this particular case is also accompanied by a leakage of their content. At present it is difficult to conclude whether fusion and vesicle destabilization resulting in leakage are in competition, if the fusion triggered by these peptides is leaky, or if the observed leakiness results from the destabilization of bigger vesicles resulting from multiple rounds of fusion, etc. To answer these questions we next studied the fusion events by monitoring liposome content mixing. This study was also useful to confirm that the lipid mixing observed above was due to true fusion and not to artifacts such as probe exchange or membrane hemifusion between the outer layers of the vesicles. The fusion assay was performed according to a classical approach using with two types of liposome populations, one containing the probe ANTS and the other the quencher DPX [32]. Formation of the ANTS/DPX complex in the lumen of the fused vesicles results in the quenching of the ANTS fluorescence whereas the fluorescence that is measured results from the addition of the fluorescence of non-fused liposomal ANTS and free ANTS resulting from the leakage of either the ANTS/DPX complex and encapsulated ANTS. On addition of peptide **1** to these liposomes, which both contain a thiol-reactive derivative, an immediate reduction of about 10% of the fluorescence was measurable (Fig. 5), which is indicative of the occurrence of a limited fusion event leading to sealed vesicles. This first phase is followed by a much slower decrease in fluorescence whose low magnitude compared to the one expected by the lipid mixing assay (see above) most probably underlines the leakiness of this fusion event.

3.6. Membrane active properties of peptide **2**

The same types of experiments as described above for peptide **1** were performed in parallel with the shorter α-MSH analog **2**. Altogether the results were qualitatively similar but peptide **2** is less active than **1** in promoting the aggregation and leakage of the vesicles. There was, however, one noticeable difference: liposomes containing DPPE-AcBr, the thiol-reactive derivative with a very short spacer, were much more resistant to aggregation and leakage (i.e. no leakage was observed) on coupling to peptide **2** than to **1**. It seems therefore that the length and the local mobility of the peptide at the surface of the vesicles could represent crucial factors in their destabilizing interaction with the membranes. Similarly the extent of lipid mixing in the fusion assay was less pronounced with **2** than with **1** (e.g. with the neutral liposomes the coupling of peptide **2** resulted in a 10% quenching relief compared to 38% with **1**).

3.7. Interaction of α-MSH analogs with membranes

Membrane fusion triggered by small peptides has

been documented in which hydrophobic interactions are important elements in the mechanisms involved. To determine whether **1** and **2** interact with the bilayers we have studied the changes in the fluorescence emission peak (λ_{max}) of their single tryptophan residue when the peptides were conjugated to the liposomes. The emission maximum shifts to lower wavelength (blue shift) as the polarity of the environment decreases. Thus, if the tryptophan is shielded from the solvent by insertion into the lipid bilayer, the resulting blue shift could be used to follow the degree of insertion. Besides, an increase in tryptophan fluorescence anisotropy could also be related to a reduced mobility of the tryptophan residue interacting with the membrane.

The results given in Table 3 indicate that blue shifts of the fluorescence emission spectra of the tryptophan residue of **1** and **2** could be observed with vesicles containing the BOP-PEG_4-AcBr or DPPE-PEG_3-Mal anchors; these shifts are accompanied, in liposomes that do not undergo aggregation (i.e. [ligand] = 5 mol%), by an increase of the tryptophan fluorescence anisotropy, indicating a restriction in the mobility of the peptides. Especially the most important blue shift observed with the negatively charged vesicles (about 10 nm) is well correlated with the higher fluorescence anisotropy value. These data indicate that after covalent binding to the liposomes, the tryptophan of the peptides migrates from a highly polar (the buffer) to a less polar environment, with more restricted movements. However, this blue shift is still far from the one obtained for a tryptophan deeply buried in the hydrophobic acyl chain region of the bilayer ($\Delta\lambda$ = 20–30 nm) [41]. A 10 nm blue shift clearly indicates that the average environment is less polar than the bulk aqueous phase, but significantly less hydrophobic than the deep acyl chain region of the bilayer. In fact, both $\Delta\lambda_{max}$ and r stand comparison with values obtained with the model derivative tryptophan octyl ester for which the probable depth of penetration of the indole ring was shown to be approximately 10 Å [42,43]. In the case of zwitterionic vesicle-coupled peptides, the increase of both blue shift and fluorescence anisotropy is less pronounced, but nevertheless perceptible. These small variations could be explained by some sticking and/or partial sinking of the peptide at the membrane interface. In contrast, vesicles that carry a short spacer arm (AcBr) or in contrast a very long one (PEG_{45}) do not show any blue shift; moreover, although an increase of r was measurable for the conjugated peptides, the difference with the free peptides was not as large even as it was in the neutral vesicles. This slight change in r is more probably due to the loss of degree of freedom due to covalent binding rather than to some interaction with the bilayer interface. These results on the incidence of the spacer arm lengths correlate well with those obtained with the aggregation, leakage and fusion studies (see above).

To further probe the location of the tryptophan residue of **1** and **2** on the bilayers, we also studied the quenching of the fluorescence of this residue by hydrophilic agents such as KI. Quenching experiments can reveal accessibility of fluorophores to quenchers, and iodide ion is considered to have access only to tryptophans exposed to the solvent. For these experiments, in order to avoid the screening of the conjugated peptides, we prepared vesicles (PC/PG/Chol/BOP-PEG_4-AcBr; 75/20/50/5) that contain lower proportions of the thiol-reactive anchor and thus avoid their aggregation. As shown in Fig. 6, the quenching of the tryptophan fluorescence by KI is lower when the peptides are coupled to the vesicles, suggesting that in this latter case this residue after being fully exposed becomes less accessible to the aqueous environment presumably because of its masking by a (at least partial) penetration into the membrane. Moreover, the difference of accessibility for each peptide as probed by iodide quenching is fully coherent with the location of the tryptophan residues as inferred by blue shift and fluorescence anisotropy measurements.

4. Discussion

In this work, we have shown that chemical coupling of short positively charged superpotent α-MSH analogs, of seven and 13 residues (Table 1) to the surface of preformed zwitterionic (PC/Chol) or negatively charged (PC/PG/Chol) liposomes results in a rapid and extensive aggregation of the vesicles. These phenomena, which were accompanied by membrane destabilization (leakage) and fusion, were only observed when a covalent linkage had occurred to the

interacting bilayers. Thus, free peptides **1** or **2**, even at a 20-fold molar excess compared to the concentration of bound peptides, were unable to cause similar effects. In agreement with this point, kinetic studies of the conjugation of the peptides to the thiol-reactive groups present at the surface of the liposomes indicated a good correlation between this reaction and the observed aggregation/leakage of the vesicles. The presently described membrane-active effects of melanotropin peptides such as **1** and **2** has never been documented before. It therefore seems likely that in the absence of a strong electrostatic attraction of these peptides to the vesicles, the covalent coupling to the outer layer of the bilayer provides a high local concentration of **1** and **2** which might result in a particularly important membrane-perturbing effect. Such a dramatic increase in membrane-destabilizing activity of a small peptide when coupled to hydrophobic anchors has been observed before, see e.g. [44].

Several observations are of importance to better understand the effects of melanotropin peptides on membranes. When the thiol-reactive anchors amounted to 15 mol% of total phospholipids, the aggregation of the liposomes caused by peptides **1** and **2** was correlated with a leakage of their inner aqueous contents. These two effects were fairly insensitive to the global charge of the vesicles, to the nature of their phospholipid acyl chains and to a range of pH typically known to regulate the activity of pH-sensitive fusogenic peptides [45]. Among the different liposomes studied, those prepared with zwitterionic lipids were, however, the most prone to leakage. It is therefore possible that, in contrast to the charged vesicles which are somewhat less sensitive to aggregation because of charge/charge repulsion, the neutral ones can undergo more extensive vesicle–vesicle interactions and destabilization. Lipid mixing and content mixing assays were used in conjunction to obtain rates of membrane destabilization and fusion; we have thus established that the aggregation/leakage observed with **1** and **2** were correlated with the fusion of the vesicles. It seems therefore that the aggregation triggered by covalent coupling of the peptides is indicative of a profound perturbation of the membrane structure leading to the bilayer fusion; this latter process, which is accompanied by leakage of the aqueous content of the vesicles, is therefore not as 'tight' as the fusion observed for example by the group of Hoekstra. These authors have extensively studied the fusion of vesicles triggered by the covalent coupling, via a disulfide bond, to the surface of preformed PC liposomes of a 11 amino acid amphipathic negatively charged peptide [46–50]. In their case, however, the fusion occurred only between liposomes carrying the peptide and positively charged 'target' vesicles with which they interact first because of charge complementarity. In our study the aggregation/fusion is not correlated with electrostatic interactions between the vesicles and, strikingly, the fusion is only observed between liposomes that carry the peptides **1** or **2**. Thus, in the lipid mixing fusion assay no decrease of RET could be observed when *N*-NBD-PE/*N*-Rh-PE-labeled vesicles, which were conjugated to **1**, were mixed at a 1:9 ratio with unlabelled vesicles (i.e. devoid of the fluorescent labels) that did not carry the peptide. It seems therefore that for the vesicles to undergo aggregation/fusion both partners must experience a modification of their bilayers by the conjugation of the peptide. Although the results of the ANTS/DPX content mixing assay are in favor of true vesicle fusion and not just a mixing of external leaflets, the release of the vesicles' internal contents observed during these events somewhat limits the conclusions that can be drawn. We certainly underestimate the extent of fusion triggered by **1** or **2** and presently it is difficult to delineate whether the leakage is due to non-tight fusion events or to a lack of stability of large vesicles resulting from multiple rounds of fusion.

A second set of observations relates to the dependence of the aggregation/leakage/fusion on the density of ligands **1** and **2** conjugated to the surface of the vesicles and on the length of the spacer arms that separate these peptides from the membrane bilayers. A threshold was observed and at a density of less than 5 mol% of peptide vs. total phospholipid, little vesicle destabilization was measurable under our experimental conditions (Fig. 3). This indicates that a relatively high concentration of peptide must be present on the liposome surface to become membrane-active. This destabilization was also found to be much dependent on the spacer arm length. Thus, a very short linker such as in DPPE-AcBr mostly abolishes the phenomenon with peptide **2** and limits the effectiveness of the longer peptide **1**. It seems

therefore that both peptides **1** and **2** need a minimal freedom of motion and orientation at the surface of the bilayers to engage in their membrane-destabilizing effects. At this level it should also be noted that the shorter peptide **2**, which contains the minimal 'message sequence' of the melanotropin peptides, is somewhat less active than the analog **1** in triggering the aggregation/fusion of the vesicles. In sharp contrast with all the foregoing results, when **1** or **2** were conjugated to the surface of liposomes via a very long spacer such as PEG_{45} no leakage/fusion could be observed but aggregation was still measurable. This uncoupling of aggregation and leakage in this special case bears out our previous conclusions that the leakage of the vesicles results from fusion events. The absence of fusion of vesicles that contain DPPE-PEG_{45}-Mal by coupling the peptides is not surprising since the PEG layer is well known to hinder such events in sterically stabilized vesicles [51,52]. However, the persistence of an aggregation of our DPPE-PEG_{45}-Mal-containing liposomes is more puzzling, revealing either a penetration/perturbation of the bilayers to which the peptides are conjugated and/or the bridging of the vesicles via peptide–peptide interactions.

The mechanisms by which peptides **1** and **2** are able to trigger the aggregation/fusion of the liposomes to which they become covalently attached are at the present level of our study more difficult to decipher. It should be noted that the liposome systems used here are not especially fusogenic, i.e. they contain limited proportions – or no – of anionic phospholipids and are devoid of PE. Both peptides are water-soluble and bear respectively +2 and +1 charges, at least at neutral pH values, and their mean hydrophobicities estimated with the Eisenberg consensus scale [53] are about −0.15 and −0.2. These values are somewhat lower than the average hydrophobicity of e.g. viral N-terminal fusion peptides [50]. The melanotropin peptides **1** and **2** are also characterized by the presence of bulky hydrophobic amino acids which confer to them a certain degree of amphipathy but from a structural viewpoint they are too short to cross a membrane. The three-dimensional structure of the melanotropin hormones, including the analogs used here, have been determined either by molecular dynamics or by NMR. A consensus structure was established characterized by the presence of a β-turn-like motif in the middle of the α-MSH molecule that involves precisely the residues His^6-L/D-Phe^7-Arg^8-Trp^9 that are necessary for the receptor binding and biological activity ('message sequence') [54,55]. This conformation is even more pronounced with the D-Phe^7 analogs [56]. An interesting feature which emerges is that this domain forms a continuous hydrophobic surface and it has been suggested that it interacts with hydrophobic counterparts on the MC1R. Arg^8 is also believed to point its positively charged side chain towards ionized Asp and Glu residues belonging to the transmembrane domains 2 and 3 of the receptor which have been shown by site-directed mutagenesis to be important for its function [7].

As mentioned in Section 1 the interaction of α-MSH and its analogs with membranes has already been studied [10–14]. With negatively charged vesicles the peptide was found to interact first electrostatically, this step being followed by a subsequent penetration of the peptide into the hydrophobic core of the bilayer. The depth of this penetration is still a matter of debate and the interpretations depend on the biophysical techniques used and on the peptide structure, e.g. [NDP]-α-MSH was found to perturb the membranes more than α-MSH itself [13]. The spontaneous partitioning of peptides such as **1** and **2** into the bilayers of liposomes once they are covalently linked to their surface – provided that the spacer arm is long enough – has been clearly borne out by our present studies. Indeed, the conjugation step circumvents the necessity of electrostatic interactions to bring the peptides in close contact with the membranes and permits the study of interactions with bilayers of different composition. The Trp residues of **1** and **2**, which belong to the β-turn motif and the active domain of the hormones, were conveniently used as fluorescent probes of their interaction with the membranes. The shift to a shorter wavelength of their emission maxima clearly indicated that these peptides insert in the more hydrophobic acyl chain region of the lipid bilayers. Interestingly, this effect was as pronounced in **1** and **2**, suggesting that in the longer peptide the Trp residue is buried to a similar extent despite the difference in potency between both peptides in triggering the aggregation/leakage/fusion of the liposomes. This partitioning into the membranes was confirmed by a

change in fluorescence anisotropy of the anchored peptides and by the study on the changes of Trp fluorescence quenching by soluble, aqueous-phase I^- quencher. Altogether, it is very likely that the covalent binding of **1** and **2** to the surface of the liposomes results in their insertion into the bilayers. The three-dimensional model that was proposed for the mode of interaction of the melanotropin hormones with their receptor could also be extended to the insertion of the peptides **1** and **2** into membranes; thus the β-turn domain could insert its hydrophobic residues – which include Trp^9 – within the hydrophobic core of the bilayers whereas Arg^8 could position its guanidinium as a counter-ion of a surface phosphate headgroup. It should be noted that, as proposed by Pascutti et al. [57], this particular conformation could be stabilized by the membrane's low dielectric constant environment.

The mechanism by which the insertion of **1** and **2** is translated into an alteration of the physical properties of membranes resulting in the aggregation and fusion of the liposomes that carry these peptides remains at the moment a matter of speculation. A vast literature is devoted to the mode of action of fusogenic peptides which encompass a large variety of structures and conformations, see e.g. [18]. In line with the preferred β-structure of **1** and **2**, peptides adopting β-sheet or β-hairpin conformations have been found to display fusogenic activity [58–60]. Moreover, Pécheur et al. have shown that for their 11-mer peptides a helical structure is not sufficient to observe fusion; in contrast, segregation of hydrophilic and hydrophobic amino acids seems to be more important [47]. Therefore, the asymmetric distribution of hydrophilic and hydrophobic amino acids in peptides **1** and **2**, which is thought to play a key role in conferring membrane-destabilizing and, eventually, fusion properties to peptides [15,61], could be an important factor.

In conclusion, the membrane-active properties of the α-MSH analogs we have uncovered are of interest from a membrane biophysical viewpoint. Peptides **1** and **2** represent additional examples of peptides too small to span a membrane that become fusogenic, provided they are covalently bound to membranes [44,46–50]. Our work also extends the earlier observations on the interaction of melanotropins with liposomal and natural membranes by showing the existence of membrane perturbations much larger than anticipated. Such effects could be relevant to the biological mode of action of these peptides. Indeed, in the 1980s Schwyzer et al. [62,63] have postulated that α-MSH by interaction with the lipid phase might trigger structural modifications in the membrane-spanning domains of their receptor and thus enhance the peptide–receptor interaction and activity. Finally, because of their properties, these α-MSH analogs could also be of interest in the design for example of gene/drug delivery constructs and fulfill a dual mission: cell targeting and endosomal compartment destabilization [20].

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