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Perturbation of the lipid bilayer of model membranes by synthetic signal peptides

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The interaction of synthetic peptides corresponding to the signal sequences of *Escherichia coli* alkaline phosphatase: Lys-Gln-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-Val-Thr-Lys-Ala-OCH_3, chicken lysozyme: Met-Lys-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys(Bzl)-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-OCH_2-C_6H_5 and variant of the chicken lysozyme signal sequence with a charged residue in the hydrophobic region: Lys-Leu-Leu-Ile-Ala-Leu-Val-Leu-Lys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-OCH_3 with model membranes of brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) have been investigated by 90° light scattering and fluorescence spectroscopy. Our results indicate that the association of signal peptides with model membranes results in extensive perturbation of the lipid bilayer so as to cause fusion of PS vesicles and aggregation of PC vesicles. The vesicles are also rendered permeable to hydrophilic molecules like carboxyfluorescein. The variant peptide with the lysine residue in the hydrophobic region also has the ability to perturb lipid bilayers of model membranes.

Introduction

Proteins destined for secretion in eukaryotes as well as for compartments like periplasmic space and outer membranes in prokaryotes like *Escherichia coli* are synthesized as precursors with amino-terminal extentions or signal sequences [1–3]. These sequences are generally 15–25 amino acids long and rich in hydrophobic amino acids [3]. While there is no primary structure homology, all signal sequences are characterized by a positively charged amino-terminal region followed by a stretch of hydrophobic amino acids. Gene fusion experiments and in vitro reconstitution studies have indicated that signal sequences may have all the information for proper localization of proteins in cells [4–7]. Detailed biochemical analysis of the process of protein export in eukaryotes has revealed how targeting of proteins destined for secretion to the endoplasmic reticulum, is accomplished [8,9]. A protein-RNA complex the 'signal recognition particle'interacts with the signal sequence of nascent secretory proteins and targets the complex of ribosome, mRNA and nascent chain to the endoplasmic reticulum [10]. It is conceivable that molecules like signal recognition particle may have a role in targeting precursor proteins to the inner membrane of *E. coli*. Once the targeting to the membrane site is achieved, it is not clear how proteins traverse the membrane barrier. Essentially two views for translocation of proteins across membranes exist [10–13]. One

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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school envisages direct partitioning of proteins into the lipid bilayer wherein translocation is initiated by interaction of the signal sequence with the lipid bilayer [11–13]. Another school favours translocation of protein through an aqueous channel formed by intrinsic membrane proteins [10]. However, experimental evidence presently available does not favour either model unequivocally.

A striking feature of all signal sequences is a stretch of 10–15 amino acids which are highly hydrophobic. The importance of the hydrophobic stretch for a signal sequence to be able to initiate export of proteins has been demonstrated in eukaryotes and prokaryotes [1]. Hence, it is conceivable that interaction of signal sequences with lipid components of membranes may have a role in the initiation of translocation of proteins across membranes. In an effort to understand how the association of signal sequences and their variants with the lipid component of membranes modulate the membrane bilayer structure, we have studied the interaction of synthetic peptides corresponding to signal sequences of chicken lysozyme, *E. coli* alkaline phosphatase and a variant of the chicken lysozyme signal sequence which has a lysine residue in the hydrophobic region (Fig. 1), with unilamellar lipid vesicles of phosphatidylserine (PS) and phosphatidylcholine (PC).

Experimental procedures

Synthesis of peptides

Peptides 1 was synthesized by solid-phase methods. The details of synthesis and characterization of the peptide has been described elsewhere [14]. Peptide 2 was synthesized by solution phase methods by a combination of stepwise and fragment condensations. Peptide 3 was synthesized as follows. The carboxy terminal fragment Leu-Val-Leu-Lys (ε-benzyloxy carbonyl) Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-CH_3 was synthesized by the solid-phase method [15] and fragment Boc-Lys (ε-Boc)-Leu-Leu-Ile-Ala-OH by solution phase methods. Both the fragments were coupled in solution (in dimethylformamide: condensing agent – dicyclohexylcarbodiimide/1-hydroxybenzotriazole) to yield the desired peptide. Peptides 2 and 3 were purified by column chromatography on silica gel followed by partition chromatography on Sephadex LH-20 [14]. The amino-acid composition of the purified peptides was determined after hydrolysis in 6 M HCl in vacuo on an LKB 4151 alpha plus amino-acid analyzer. The results of the analyses were as follows:

Peptide 2: Met 0.95 (1), Lys 1.00 (1), Ser 0.73 (1), Leu 7.2 (7), Ile 0.80 (1), Val 0.95 (1), Gly 1.13 (1), Ala 1.82 (2), Phe 0.91 (1), Pro 1.00 (1).

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1       Lys-Gln-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-
         1                  5                  10                  15

        Val-Thr-Lys-Ala-OMe
                   20

2       Met-Lys-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys(Bzl)-Phe-Leu-Pro-Leu-Ala-
         1                  5                  10                  15

        Ala-Leu-Gly-OBzl


3       Lys-Leu-Leu-Ile-Ala-Leu-Val-Leu-Lys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-
         1                  5                  10                  15

        Gly-OMe
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Fig. 1. Primary structures of synthetic signal peptides. Peptide 1 corresponds to the signal sequence of *E. coli* alkaline phosphatase and peptide 2 corresponds to the signal sequence of chicken lysozyme. The primary structures of the signal sequences were obtained from Ref. 3. Bzl, benzyl; OBzl, benzyl ester; OMe, methyl ester.

Peptide 3: Lys 1.95 (2), Leu 7.12 (7), Ile 0.89 (1), Ala 3.00 (3), Val 1.0 (1), Phe 1.10 (1), Pro 1.12 (1), Gly 1.00 (1).
Theoretical values are indicated in parentheses. Serine value is not corrected for loss during hydrolysis. Peptide 2 differs from the signal sequence of chicken lysozyme at position 2, where arginine has been replaced by Lysine. This change which is conservative was done primarily to facilitate synthesis. Lysine was introduced in peptide 3 to generate a 'variant' of 2 with a charged residue in the hydrophobic region.

90° light scattering

Scattering experiments were done on a Hitachi 650-10S fluorescence spectrophotometer with both excitation and emission monochromators set at 400 nm. Small unilamellar vesicles used for the light-scattering experiments were prepared by sonication (Branson B-50 sonifer) of an aqueous dispersion of egg PC, purified from egg yolk by the method of Singelton [16] and purified bovine brain PS [17] in Hepes buffer (pH 7.4)/100 mM NaCl.

Fusion of vesicles

The fusogenic properties of peptides 1–3 was studied by the Tb^{3+}-dipicolinate [18] and fluorescence self-quenching [19] assays.

Tb^{3+}-dipicolinate assay. Two populations of small unilamellar vesicles were prepared in (a) 15 mM $TbCl_3$ and 150 mM sodium citrate and (b) 150 mM dipicolinic acid sodium salt in Hepes buffer (5 mM) adjusted to a final pH of 7.4. Phospholipids (PS and PC) were dispersed aqueous medium and sonicated to clarity. Vesicles were separated from non-encapsulated material by gel filtration on Sephadex G-75. Elution buffer was 5 mM Hepes (pH 7.4)/100 mM NaCl/1 mM EDTA. Aliquots of vesicles containing trapped Tb^{3+} and dipicolinic acid were mixed and peptides were added from a stock solution in methanol. Fluorescence was monitored at 491 nm ($\lambda_{ex} = 276$ nm). Percentage maximum fluorescence of Tb^{3+} was determined as described by Wilschut et al. [18].

Fluorescence self-quenching assay. A stock solution of octadecyl Rhodamine B chloride (R-18) was prepared in ethanol. An aliquot from the stock solution (2 n moles) was added to 100 μg/ml of PS or PC vesicles in Hepes (pH 7.4)/100 mM NaCl. After incubation at 25° C, 15 μg/ml of lipid vesicles labelled with R-18 was mixed with 60 μg/ml of unlabelled lipid. The fluorescence scale was calibrated such that the residual fluorescence of lipid vesicles was taken as zero and the value obtained after addition of Triton X-100 as 100% (infinite dilution). Aliquots of peptides 1–3 were added to the lipid vesicles and rise in fluorescence at 580 nm ($\lambda_{ex} = 550$ nm) was noted as a function of time.

Release of vesicles of vesicle contents

The ability of peptides 1–3 to cause release of entrapped vesicle contents was checked by monitoring the fluorescence intensity of carboxyfluorescein [20–22] (Molecular Probes, OR, U.S.A.) encapsulated in PS and PC vesicles at high self-quench concentrations, on addition of peptides. Lipid film (PC or PS) was dispersed in aqueous medium containing 100 mM carboxyfluorescein and 5 mM Hepes (pH 7.4) and sonicated for 30 min. Vesicles were separated from non-encapsulated carboxyfluorescein by gel filtration on Sephadex G-75 (elution buffer was 5 mM Hepes (pH 7.4)/100 mM NaCl. Carboxyfluorescein was excited at 493 nm and the emission at 540 nm was monitored. Complete release of carboxyfluorescein was obtained by addition of Triton X-100 (0.1%, v/v). Peptide solutions (2 mM) in methanol were added in aliquots to lipid vesicles with trapped carboxyfluorescein. Control experiments were performed with neat methanol.

Lipid concentrations were estimated by the method of Stewart [23].

Results

The 90° light scattering profiles of small unilamellar vesicles of PS in presence of peptides 1–3 is shown in Fig. 2. A small increase is discernible upto a concentration of 4 μM (lipid : peptide molar ratio = 45). With increasing peptide concentration, there is a considerable increase in scatter intensity in the presence of 1. Relatively smaller increases in scatter are observed in the presence of peptides 2 and 3. No change in the scattering profile in the presence of smaller

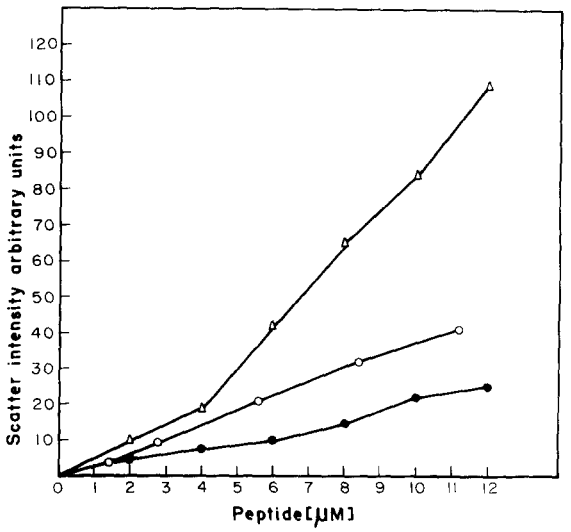

Fig. 2. 90° light-scattering profiles of PS vesicles (150 μM) in presence of peptides 1–3 as a function of peptide concentration. The scatter intensities represent steady-state values: peptide 1 (△); peptide 2 (●); peptide 3 (○).

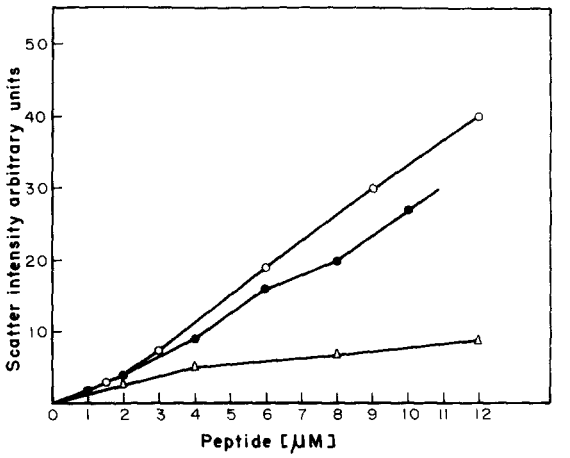

Fig. 3. 90° light-scattering profiles of PC vesicles (150 μM) in presence of peptides 1–3 as a function of peptide concentration. The scatter intensities represent steady-state values: peptide 1 (△); peptide 2 (●); peptide 3 (○).

fragments of 2, i.e., Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-OCH_3 and Leu-Val-Leu-Cys(Bzl)-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-OCH_3 was observed, although these peptides bind to lipid vesicles and are associated with the hydrophobic core of the lipid bilayer [24].

The scattering profiles of small unilamellar vesicles of PC in the presence of 1–3 are presented in Fig. 3. Peptides 2 and 3 cause a larger increase in scatter than 1. Thus 2 and 3 interact with both negatively charged and zwitterionic vesicles, whereas 1 interacts preferentially with negatively charged vesicles. Since the increase in scatter can be correlated to either aggregation or fusion of vesicles, it is evident that the association of 1–3 with PS and PC vesicles results in aggregation or fusion.

Fusogenic property of peptides 1–3

Fusion of lipid vesicles can be followed by Tb^{3+}-dipicolinate [18] and R-18 [19] assays. The Tb^{3+}-dipicolinate assay monitors mixing of aqueous compartments of vesicles that undergo fusion. In the R-18 assay, the relief in fluorescence quenching of the fluorophore in a mixture of vesicles, one with the fluorophore at self-quench concentrations and the other without the fluorophore is monitored. When the two populations of vesicles fuse, the surface density of the fluorophore decreases resulting in an increase in fluorescence. The R-18 assay thus monitors merging of the lipid bilayers undergoing fusion. When peptides 1–3 were added to PS vesicles with entrapped Tb^{3+} and dipicolinic acid (see Experimental procedures for details) only a small rise in fluorescence was detected. However, this observation does not rule out fusion of vesicles in the presence of peptides 1–3. For if the association of 1–3 with the lipid vesicels resulted in rapid release of trapped vesicle contents, then the Tb^{3+}-dipicolinate assay would not register fusion. Hence, the R-18 assay which monitors merging of the lipid bilayers undergoing fusion was employed to determine whether association of peptides 1–3 with PS and PC causes fusion.

Fig. 4 shows the development of R-18 fluorescence in PS vesicles containing R-18 and unlabelled PS vesicles in presence of 1 and 3 as a function of peptide concentration and time. At high lipid : peptide ratio, there is a rapid increase in fluorescence and no further increase is observed after the initial enhancement. However, at lower lipid : peptide ratio, a rapid rise in fluorescence intensity is followed by a gradual increase in fluorescence. At a lipid : peptide ratio of 10 : 1, a steady state value is observed after 50 min. The

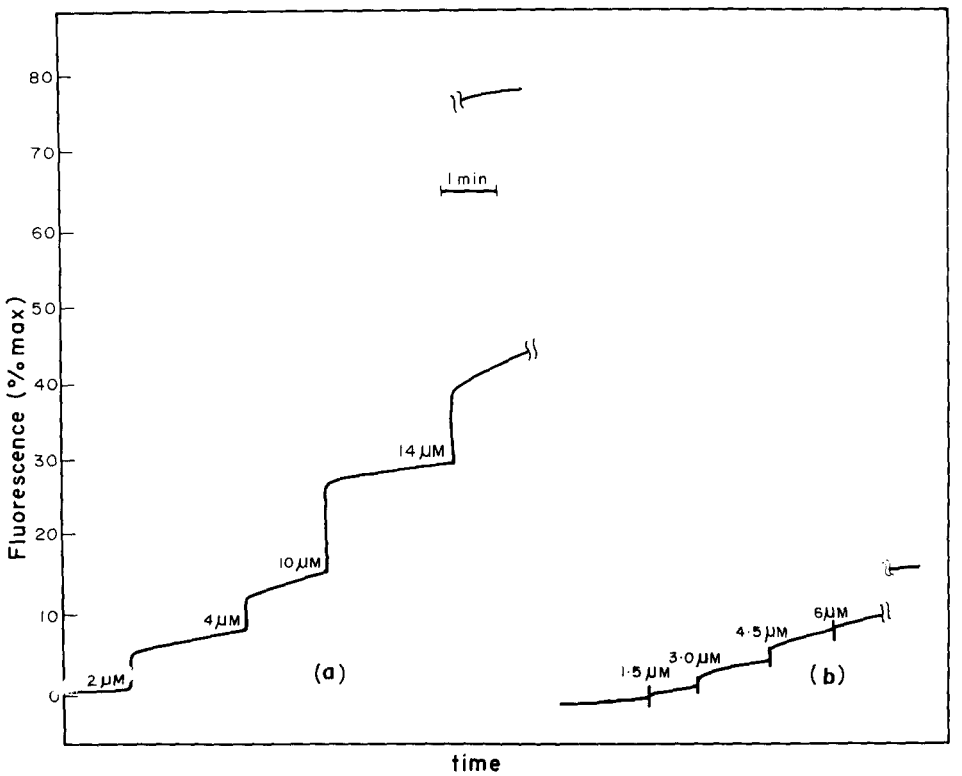

Fig. 4. Fusion of PS vesicles induced by peptides 1 and 3 monitored by the R-18 assay. Liposomes labelled with R-18 and unlabelled liposomes (final concentration of lipid = 130 μM, ratio of labelled to unlabelled lipid was 1 : 4) were suspended in 1 ml of Hepes buffer (pH 7.4)/100 mM NaCl. Increase in fluorescence was monitored as a function of time and peptide concentration. Initial concentration of peptides were 1 (2 μM), 3 (1.5 μM) (after addition at $t = 0$). (a) Peptide 1, (b) peptide 3.

extent of fusion is considerably less in the presence of 3. No rise in fluorescence was observed in the presence of 2, indicating that this peptide cause aggregation but not fusion. When similar experiments with R-18 were performed with PC vesicles, no increase in fluorescence was observed in the presence of peptides 1–3. Hence, the increase in the 90° light scattering of PC vesicles in the presence of 1–3 can be attributed to aggregation and not fusion.

Release of trapped vesicle contents

In order to determine whether association of peptides 1–3 with lipid vesicles results in release of vesicle contents, the fluorescence intensity of carboxyfluorescein entrapped in PS and PC small unilamellar vesicles was monitored. Dilution of carboxyfluorescein initially encapsulated at high self-quenching concentrations in the vesicles results in a large enhancement in fluorescence intensity. Since dilution of carboxyfluorescein will arise only if the vesicles are rendered leaky, this enhancement in fluorescence intensity would reflect intactness of the vesicles. Fig. 5 shows the release of carboxyfluorescein from PS vesicles in presence of peptides 1–3. It is evident that peptide 1 renders PS vesicles considerably more leaky than does 2 or 3.

Release of carboxyfluorescein from PC vesicles in presence of 1–3 is shown in Fig. 6. Release of carboxyfluorescein is observed only in the presence of 1 and 3. The initial rapid efflux of carboxyfluorescein immediately after addition of peptides observed in the case of PS vesicles is not observed with PC vesicles.

Discussion

We have investigated the interaction of synthetic peptides corresponding to signal sequences

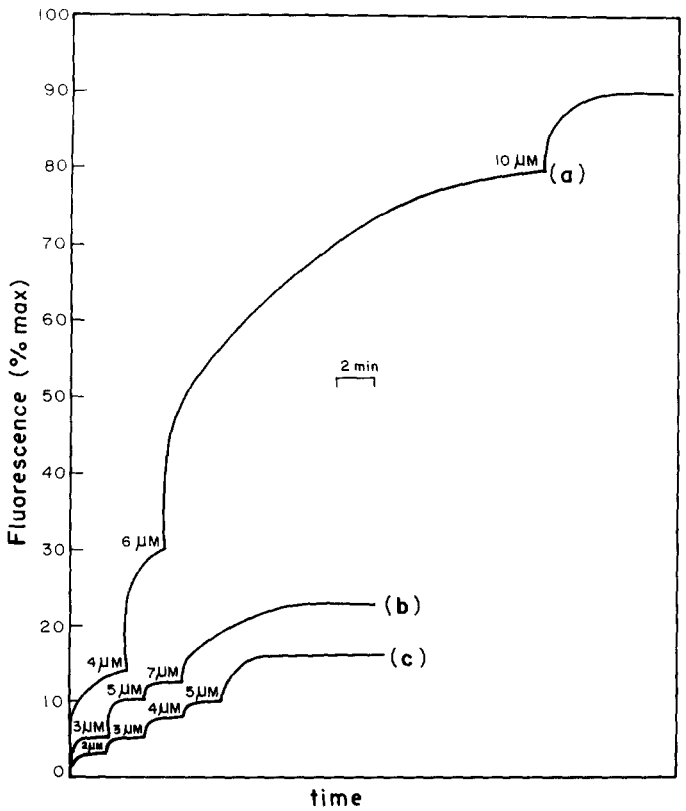

Fig. 5. Release of encapsulated carboxyfluorescein from PS vesicles in presence of peptides 1–3. Liposomes (150 μM) containing trapped carboxyfluorescein were suspended in 1 ml of Hepes buffer (pH 7.4)/100 mM NaCl. Peptides were added at $t = 0$. Initial concentrations of peptides were 1 (2 μM), 2 (1 μM), 3 (1.5 μM). Increase in fluorescence was monitored as a function of time and peptide concentration. (a) Peptide 1; (c) peptide 2; (b) peptide 3.

and a signal sequence 'variant' with a charged residue in the hydrophobic region, with model membranes of PS and PC. Our results indicate that association of the signal sequence of *E. coli* alkaline phosphatase with lipid vesicles leads to extensive perturbation of the lipid membrane so as to cause fusion of PS vesicles and release of trapped vesicle contents. The membrane-perturbing effects are less pronounced in the presence of the chicken lysozyme signal peptide. Preliminary observations indicate that the signal sequences of *E. coli* λ-receptor and M13 coat protein have the ability to cause aggregation of lipid vesicles [25,26]. Peptides 1 and 2 have a contiguous stretch of 10–13 hydrophobic amino acids. The only difference between peptide 1 and 2 in terms of the distribution of charged and hydrophobic amino acid is the presence of a positively charged amino acid, lysine, near the carboxy terminus. It is conceivable that the presence of two positive charges at the amino and carboxy termini separated by a stretch of hydrophobic amino acids is responsible for the lipid-bilayer-perturbing properties of peptide 1. An examination of the primary structures of a large number of signal sequences reveals the presence of charged amino acids in the carboxy-terminal region in several sequences. Thus, while all signal sequences can conceivably partition into the hydrophobic core of the membranes lipid bilayer, the extent to which they perturb lipid bilayers are likely to be different.

Genetic studies in *E. coli* [1] have indicated that introduction of charged residues in the hydrophobic region renders signal sequence non-functional (i.e., such mutant signal sequences are unable to initiate export of proteins). Since introduction of charged residues in the hydrophobic region would reduce the length of continuous stretch of

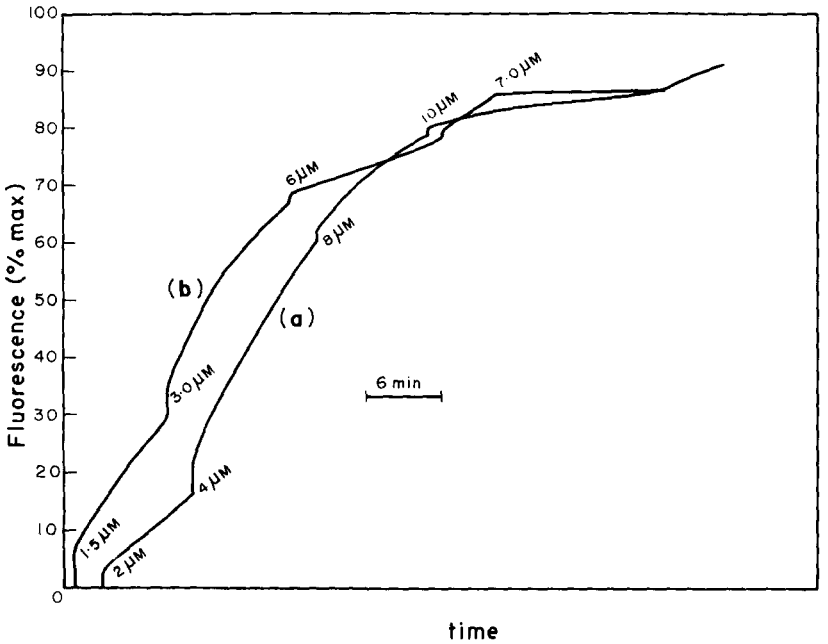

Fig. 6. Release of encapsulated carboxyfluorescein from PC vesicles in presence of peptides 1–3. Liposomes (150 μM) containing trapped carboxyfluorescein were suspended in 1 ml Hepes buffer (pH 7.4)/100 mM NaCl. Peptides were added at $t = 0$. Initial concentration of peptides were 1 (2 μM), 3 (1.5 μM). Increase in fluorescence was monitored as a function of time and peptide concentration. (a) Peptide 1; (b) peptide 3.

hydrophobic amino acids, it would be expected that such peptides do not interact with lipid component of membranes and thereby would be unable to initiate export of proteins. However, peptide 3, which has a lysine residue in the middle of the hydrophobic region, interacts with lipid vesicles and perturbs the lipid vesicles so as to cause the release of trapped molecules like carboxyfluorescein. Hence, it is unlikely that 'mutant' signal sequences with charged amino acids in the hydrophobic region are unable to initiate export of proteins due to their inability to interact with the lipid bilayer of membranes.

We have observed perturbation of the lipid-bilayer by signal peptides at fairly low lipid : peptide ratios. However, it is likely that local 'faults' are introduced in the lipid bilayer on partitioning of individual peptide chains into the lipid bilayer. When the peptide concentration in the lipid vesicles is increased, the 'defects' become extensive, resulting in aggregation, fusion and release of trapped contents.

Signal sequences are cleaved off precursor proteins by membrane-bound signal peptidases before the translocation of proteins across membranes is complete [2]. Attempts to isolated signal peptides from membranes have been unsuccessful [30,31] presumably due to rapid degradation by signal peptidases [32]. Hence we believe that it is the initial interaction of signal sequences with membranes that opens up a pathway for translocation of proteins. We postulate that signal sequences insert into the lipid bilayer of membranes after the targeting to the membrane site has occurred. The presence of the signal sequence results in the formation of 'local defects' in the lipid bilayer. Translocation of proteins can then conceivably occur through these defects. Alternatively, the perturbation of the lipid bilayer could result in the rearrangement of some membrane proteins so as to open up aqueous channels for translocation.

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