# pH- and Ionic Strength-Dependent Fusion of Phospholipid Vesicles Induced by Pardaxin Analogues or by Mixtures of Charge-Reversed Peptides†

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ABSTRACT: The fusogenic properties of the neurotoxin pardaxin and eight of its analogues with small unilamellar vesicles (SUV), composed of egg phosphatidylcholine and phosphatidylserine (PC/PS), were investigated. Fusion was demonstrated by a lipid-mixing assay and by an increase in vesicle size as revealed by electron microscopy. The lipid-mixing assay was performed at either neutral (pH 6.8) or acidic (pH 4.5) conditions, in solutions containing either high or low salt concentrations. A low level of fusion could be induced at neutral pH only by pardaxin derivatives with amino groups at both the peptide's backbone and N-terminus. However, a marked enhancement in the fusogenic activity occurred when amino groups were present also in the C-terminus. Pardaxin analogues in which amino groups were substituted by carboxylic groups induced elevated levels of fusion only at high salt concentrations where enhancement of aggregation occurs, and acidic pH, which increased $\alpha$-helicity. The influence of mutual interactions between pardaxin's analogues possessing complementary charges on the lipid-mixing process was also studied. At neutral pH and high salt, an inactive acidic analogue increased the fusogenic activity of a complementary-charged basic peptide. However, such mutual interactions at low salt concentrations reduced the fusogenic activity of the pardaxin analogues. Analogues containing D-amino acids were not fusogenic, thus demonstrating the structural specificity of these observations. The results indicate that the charge, $\alpha$-helical structure, and aggregation of peptide monomers play an important role in the fusogenic ability of polypeptides.

Membrane fusion is a fundamental phenomenon that occurs in many biological processes. A variety of peptides and proteins participate in fusion processes. The well characterized viral fusion proteins have a stretch of approximately 20 hydrophobic amino acids, at either their amino terminus or internally, which is assumed to interact with target membranes, thereby inducing fusion (Gething et al., 1986; Lear & DeGrado 1987). Other known fusogens are basic peptides, such as poly(Lys), poly(His), and poly(Arg) (Gad, 1983; Walter et al., 1986; Bondeson & Sundler, 1990), and amphiphilic basic peptides, such as an albumin fragment (Garcia et al., 1984), melittin (Morgan et al., 1984; Murata et al., 1987), and gramicidin S (Eytan et al., 1988; Tournois et al., 1990). A common characteristic for these peptide fusogens is their apparent ability to destabilize bilayers. This destabilization can be accomplished by hydrophobic or electrostatic interactions, separately or combined. Studies with these peptides suggested that an appropriate orientation of the hydrophobic moiety and the presence of cationic charge in the peptide is sufficient to render them fusogenic. Although synthetic peptides may only partially mimic the complexity of the fusogenic properties of an intact viral protein, and small unilamellar vesicles (SUV)[1] may not be an appropriate model for biological membrane fusion, studies performed with them will probably advance understanding of the basic requirements of membrane fusion (Wilschut, 1990). However, despite extensive studies, the molecular mechanisms of membrane fusion are still unclear.

Pardaxin, a neurotoxic peptide, at low concentrations forms single channels in lipid bilayers, whereas at higher concentrations cytolysis is induced (Moran et al., 1984; Lazarovici et al., 1986; Shai et al., 1988). Recently, our detailed study on the interaction of pardaxin with lipid bilayers (Shai et al., 1990, 1991; Rapaport & Shai, 1991, 1992; Pouny & Shai, 1992) provided information on the structure, orientation, and aggregation state of pardaxin bound to phospholipid vesicles.

Herein, the properties of pardaxin and its analogues, some of which were recently synthesized by us (Shai et al., 1990, 1991; Pouny & Shai, 1992), to induce fusion of phospholipid membranes was evaluated. The contribution of the peptide's charges, $\alpha$-helical conformation, D-amino acid incorporation, and mutual interactions between charge-reversed analogues to peptide-induced, pH- and ionic strength-dependent fusion process was examined. Fusion was demonstrated by a lipid-mixing assay and by an increase in vesicle size verified by electron microscopy.

The importance of the charge of peptides, the $\alpha$-helical structure and monomer aggregation in the pH- and ionic strength-dependent fusion process is demonstrated. Moreover, mutual interactions between charge-reversed analogues either inhibit or enhance lipid mixing, depending upon the ionic strength of the environment. These results are discussed in light of the proposed mechanism for peptide-induced fusion.

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl; PAM, phenylacetamidomethyl; PC, egg phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; Rh, 5-(and 6-)carboxytetramethylrhodamine; Flu, 5-(and 6-)carboxyfluorescein; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid; HIV, human immunodeficiency virus.

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## EXPERIMENTAL PROCEDURES

*Materials.* Butyloxycarbonyl-Glu(*O*-benzyl)phenylacetamidomethyl (PAM) resin was purchased from Applied Biosystems (Foster City, CA). Butyloxycarbonyl amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis were obtained from Sigma. Egg phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (sodium salt-grade I) were purchased from Lipid Products (South Nutfield, U.K.). [5-(and 6-)carboxytetramethylrhodamine]phosphatidylethanolamine (Rh-PE), 7-nitrobenz-2-oxa-1,3-diazole-4-phosphatidylethanolamine (NBD-PE), 5-(and 6-)carboxyfluorescein (Flu) succinimidyl ester, and 5-(and 6-)carboxytetramethylrhodamine (Rh) succinimidyl ester were purchased from Molecular Probes. Cholesterol (extra pure) purchased from Merck (Darmstadt, Germany) was recrystallized twice from ethanol. All other reagents were of analytical grade. Buffers were prepared using double glass-distilled water.

*Synthesis, Fluorescent Labeling, Modification, and Purification of Peptides.* The peptides were synthesized by a solid-phase method on PAM-amino acid resin (0.15 mequiv) (Merrifield et al., 1982), as previously described (Shai et al., 1990, 1991; Pouny & Shai, 1992). Coupling was carried out with freshly prepared hydroxybenzotriazole active esters of butyloxycarbonyl amino acids. Fluorescently labeled analogues were prepared as described elsewhere (Rapaport & Shai, 1992). The synthetic peptides were purified to a chromatographic homogeneity of >95% by reverse-phase HPLC on a $C_4$ column using a linear gradient of 25–80% acetonitrile in 0.1% TFA, in 40 min. The peptides were subjected to amino acid analysis in order to confirm their composition.

*Preparation of Small Unilamellar Vesicles (SUV) and Their Visualization by Electron Microscopy.* SUV were prepared by sonication from PC/PS (1:1 w/w) alone or with the required amount either of a mixture of Rh-PE and NBD-PE (1% molar content each) or of Rh-PE and NBD-PE (0.1% molar content each). Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1 v/v), such that the mixture contained 10% (w/w) cholesterol. The solvents were evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were resuspended in the appropriate buffer by vortex mixing. The resulting lipid dispersion was sonicated (~10 min) in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., New York) until turbidity had cleared. The lipid concentration of the solution was then determined by phosphorus analysis (Barlett, 1959). For visualization, a drop containing vesicles was deposited onto a carbon-coated grid and negatively stained with uranyl acetate. The grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Vesicles thus formed are small unilamellar vesicles (Papahadjopoulos & Miller, 1967).

*Peptide-Induced Lipid Mixing.* Lipid mixing of SUV was measured using a fluorescence probe dilution assay, based on resonance energy transfer measurements (RET) (Struck et al., 1981). PC/PS SUV containing 1 mol % each of NBD-PE (energy donor) and Rh-PE (energy acceptor) were prepared in 25 mM HEPES, containing either 50 mM (low ionic strength) or 400 mM (high ionic strength) $Na_2SO_4$, at pH 6.8 or 4.5. The salt concentrations inside and outside the liposomes were kept identical. A mixture of labeled and unlabeled SUV (ratio of 9:84, final concentration 93 $\mu$M) was suspended in 1 mL of the buffer at room temperature, and a small volume of peptide in dimethyl sulfoxide was added at time zero. The increase in NBD fluorescence at 530 nm, with the excitation wavelength set at 467 nm, was monitored using Perkin Elmer LS-5 spectrofluorimeter. The fluorescence intensity before the addition of the peptide was referred to as 0% of mixing, and fluorescence intensity of freshly prepared solution of lipid vesicles (93 $\mu$M) containing 0.1 mol % of both probes was referred to as 100% lipid mixing.

*Fluorescence Studies.* The fluorescence emission spectra of fluorescently labeled pardaxin analogues were taken at room temperature and pH 6.8 in solutions of either high or low ionic strengths using an SLM-8000 spectrofluorometer. The concentrations of peptides (1.7 $\mu$M) were similar to those in the lipid mixing assay. The excitation wavelengths were set at 490 and 544 nm for the carboxyfluoresceine and tetramethylrhodamine derivatives, respectively.

## RESULTS

Pardaxin and eight of its analogues and derivatives (Table I), some of which have been previously characterized (Shai et al., 1990, 1991; Pouny & Shai, 1992), were synthesized and their ability to induce lipid mixing examined using SUV. These analogues include those in which the charge was modified either at the C-terminal or through the molecule and those containing D-amino acids at selected positions along the peptide backbone. The two fluorescently labeled pardaxin analogues, Flu-$Glu_2$-Par and C-Rh-Par (Rapaport & Shai, 1992), were used to study pardaxin's aggregation state in solutions of low or high ionic strength.

*Lipid Mixing Induced by Peptides.* The induction of intervesicular lipid mixing by pardaxin and its analogues was tested using PC/PS SUV and a lipid-mixing assay (Struck et al., 1981). In this assay fluorescence resonance energy transfer (RET) is measured between a fluorescence donor (NBD-PE) and a fluorescence acceptor (Rh-PE). These two fluorophores are not exchanged between phospholipid vesicles, even when the vesicles are aggregated (Düzgünes et al., 1987). The ability of the peptides to induce lipid mixing was elucidated by monitoring the fluorescence increase, measured at 530 nm, until a plateau was observed, which usually occurred 30 min after the addition of the peptides. This level was taken as the maximal activity attainable for each of the tested analogues. Increases in NBD-PE fluorescence always paralleled concomitant decreases in Rh-PE fluorescence. The potencies of the different peptides as inducers of lipid mixing under indicated various conditions were determined (Table II). Only C-diamino-Par induced significant lipid mixing at neutral pH. The acidic peptides, $Glu_2$-Par, Suc-Par, and Suc-$Glu_2$-Par, were inactive at low salt concentrations at both pHs (4.5 and 6.8). However, these acidic peptides induced high levels (70–88%) of lipid-mixing activity at high salt concentrations at pH 4.5. Among the analogues tested containing D-amino acids, only C-diamino-D-$Leu_2$-Par exhibited some (~5%), albeit low, lipid-mixing activity. The other two D-amino acid analogues tested, D-$Leu_2$-Par and Suc-D-$Leu_2$-Par, were inactive under all the condition tested.

The dose–response of the most active analogue, C-diamino-Par, at neutral pH and low salt concentration was examined. In separate experiments, each concentration of peptide was added to a fixed amount of vesicles. Over the range of the peptide/lipid molar ratios tested, 0.0045–0.055, the extent of intermembrane mixing was linearly proportional to the concentration of the added peptide (Figure 1).

*Kinetics of the Lipid Mixing.* The time course of fluorescence recovery can provide information about the rate of lipid mixing. Typical profiles of fluorescence recovery at either low (panel A) or high (panel B) ionic strength, as a function of time, for different pardaxin analogues, are depicted in

Table I: Amino Acid Sequences of Pardaxin and Its Analogues[a]

| peptide no. | peptide designation | peptide sequence | α-helix content[b] in liposomes (%) pH 6.8 | pH 4.5 |
|---|---|---|---|---|
| 1 | pardaxin | GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE | 49 | 35 |
| 2 | C-diamino-Par | GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE($NHCH_2CH_2NH_2$)($NHCH_2CH_2NH_2$) | 31 | nd[c] |
| 3 | C-dihydroxy-Par | GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE($NHCH_2CH_2OH$)($NHCH_2CH_2OH$) | 45 | nd |
| 4 | $Glu_2$-Par | GFFALIP**E**IISSPLF**E**TLLSAVGSALSSSGGQE | 27 | 44 |
| 5 | Suc-$Glu_2$-Par | Suc-GFFALIP**E**IISSPLF**E**TLLSAVGSALSSSGGQE | 12 | 44 |
| 6 | Suc-Par | Suc-GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE | 14 | 44 |
| 7 | D-$Leu_2$-Par | GFFA(D)LIPKIISSPLFKTL(D)LSAVGSALSSSGGQE | 6 | 6 |
| 8 | C-diamino-D-$Leu_2$-Par | GFFA(D)LIPKIISSPLFKTL(D)LSAVGSALSSSGGQE($NHCH_2CH_2NH_2$)($NHCH_2CH_2NH_2$) | nd | nd |
| 9 | Suc-D-$Leu_2$-Par | Suc-GFFA(D)LIPKIISSPLFKTL(D)LSAVGSALSSSGGQE | nd | nd |
| 10 | Flu-$Glu_2$-Par | **Flu**-GFFALIP**E**IISSPLF**E**TLLSAVGSALSSSGGQE | nd | nd |
| 11 | C-Rh-Par | GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE($NHCH_2CH_2NH$-**Rh**)($NHCH_2CH_2NH_2$) | nd | nd |

[a] Substituted residues are marked in bold type. Suc designates a succinate group. [b] Taken from Shai et al. (1991). [c] nd, not determined.

Table II: Ability of Pardaxin and Its Analogues To Induce Lipid Mixing[a]

| peptide designation | low salt pH 6.8 | low salt pH 4.5 | high salt pH 6.8 | high salt pH 4.5 |
|---|---|---|---|---|
| pardaxin | 8 | 4 | 7 | 25 |
| C-diamino-Par | 35 | 36 | 20 | 30 |
| C-dihydroxy-Par | 6 | 3 | 11 | 12 |
| $Glu_2$-Par | 0 | 0 | 0 | 70 |
| Suc-$Glu_2$-Par | 0 | 0 | 0 | 70 |
| Suc-Par | 0 | 2 | 3 | 88 |
| D-$Leu_2$-Par | 0 | 0 | 0 | 0 |
| C-diamino-D-$Leu_2$-Par | 5 | 0 | 0 | 0 |
| Suc-D-$Leu_2$-Par | 0 | 0 | 0 | 0 |

[a] Experiments were performed using small unilamellar vesicles (SUV) composed of PS/PC (1:1 w/w). Numbers represent the percentage of lipid mixing after 40 min, as compared to a 1:10 dilution (SD ± 10%).

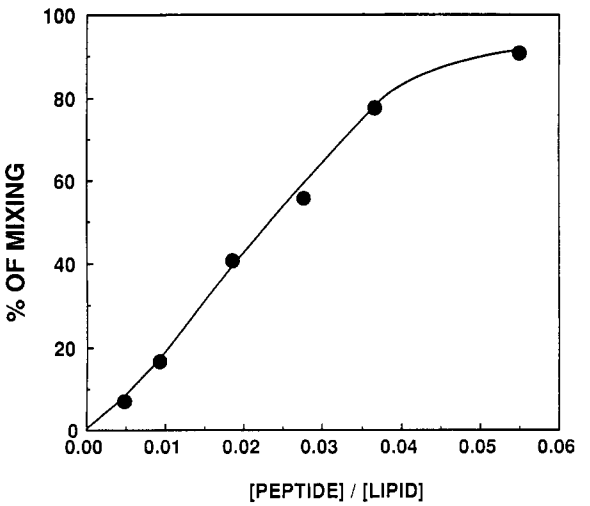


FIGURE 1: Dose dependence of lipid mixing of PC/PS (1:1) SUV induced by C-diamino-Par. Each concentration of C-diamino-Par was added to a mixture of SUV (9 μM phospholipid concentration) containing 1 mol % each of NBD-PE and Rh-PE, and unlabeled SUV (84 μM phospholipid concentration), in 25 mM HEPES and 50 mM $Na_2SO_4$, pH 6.8, at room temperature. The increase of the fluorescence intensity of NBD-PE was measured at 20 min after the addition of the peptide and is plotted versus the peptide/lipid molar ratio.

Figures 2 (neutral, pH 6.8) and 3 (acidic, pH 4.5). Inactive analogues are not shown. The kinetics of lipid mixing induced by C-diamino-Par at either neutral or acidic pHs and low ionic strength is fast, and the maximal activity is observed within few minutes (Figures 2 and 3). However, at high ionic strength the kinetics is slow. The shape of the profile (Figure 3B) obtained for the acidic analogue, Suc-Par, at pH 4.5 and high ionic strength, the only condition in which it shows activity, is different from those of the others. The weak activity exhibited by this peptide shortly after its addition increases sharply only after long incubation period.

*Fusion Induced by Mixtures of Analogues.* The effect of mutual interaction between two charge-reversed pardaxin analogues on the lipid-mixing process was only examined at pH 6.8. This condition was selected since at pH 4.5 and high salt both the positively and negatively charged analogues are active, which would make the interpretation of data difficult. The time course profiles of lipid mixing at pH 6.8 and low salt of C-diamino-Par alone and of mixtures containing equimolar concentrations of C-diamino-Par and either Suc-$Glu_2$-Par, Suc-Par (overlapping lines) or Suc-D-$Leu_2$-Par were determined (Figure 4A). The addition of acidic peptides, which were inactive by themselves, caused ~30% reduction in the activity of C-diamino-Par but only when the analogues contained only L-amino acids. No reduction in the lipid-mixing activity of C-diamino-Par was observed when Suc-D-$Leu_2$-Par was added, thus indicating the specificity of this mutual interaction. Similar results were obtained irrespective of whether the acidic peptides were premixed with C-diamino-Par or added to the lipid mixtures prior to the addition of C-diamino-Par. However, when acidic pardaxin analogues were added to lipid mixtures at high ionic strength containing either C-diamino-Par (Figure 4B) or C-dihydroxy-Par (Figure 5), up to a 4.5-fold enhancement of the mixing process was observed. As with lipid mixing at low ionic strengths, enhancement of lipid mixing was only observed with pardaxin analogues containing only L-amino acids and not with Suc-D-$Leu_2$-Par, which again indicated the structural specificity

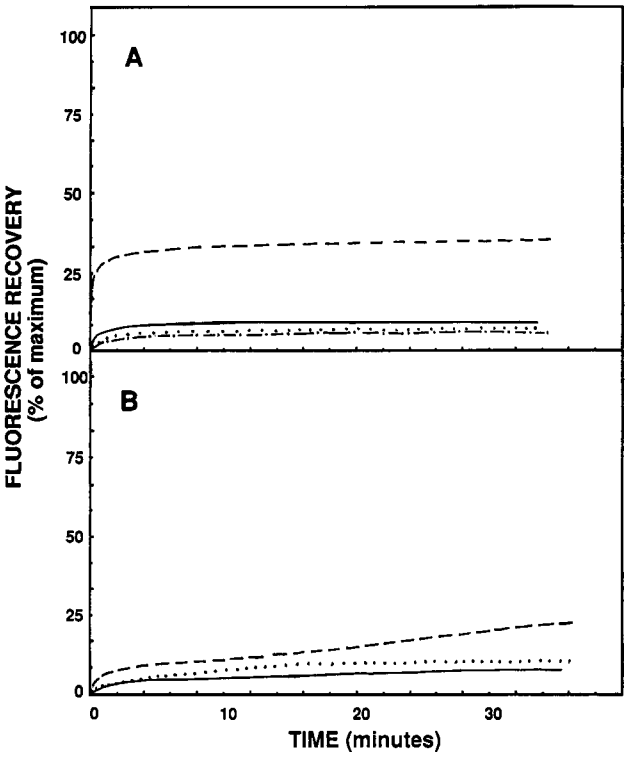


FIGURE 2: Time course of lipid mixing of PC/PS (1:1) SUV induced by pardaxin analogues at low and high ionic strength and pH 6.8. Peptides (1.7 $\mu$M) were added to mixtures of SUV (9 $\mu$M phospholipid concentration) containing 1 mol % each of NBD-PE and Rh-PE, and unlabeled SUV (84 $\mu$M phospholipid concentration). The increase in NBD-PE fluorescence at room temperature due to the addition of the peptides was monitored and plotted versus time. The peptide/lipid molar ratio was 0.018. (Panel A) 25 mM HEPES, 50 mM $Na_2SO_4$; (panel B) 25 mM HEPES, 400 mM $Na_2SO_4$. Peptides' designations: (—) pardaxin; (– – –) C-diamino-Par; (···) C-dihydroxy-Par; (- · -) C-diamino-D-$Leu_2$-Par.

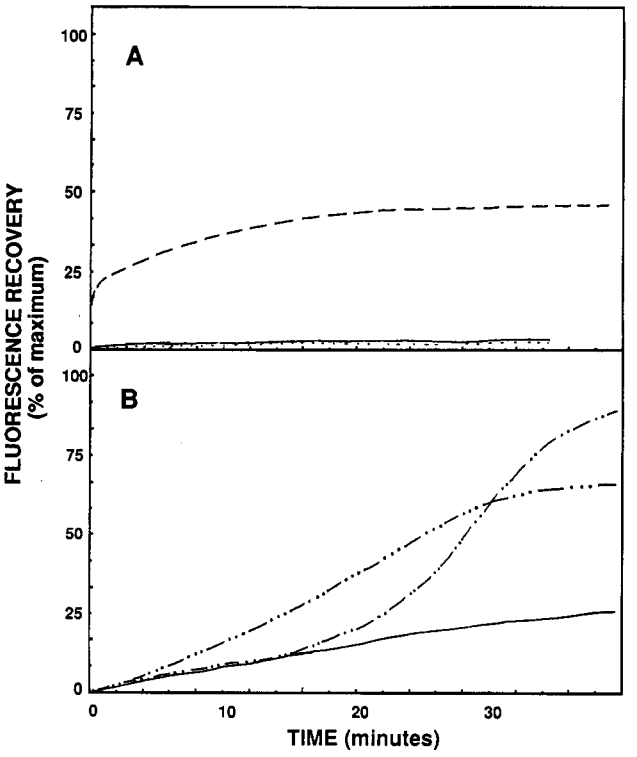


FIGURE 3: Time course of lipid mixing of PC/PS (1:1) SUV induced by pardaxin analogues at low and high ionic strength at pH 4.5. Peptides (1.7 $\mu$M) were added to mixtures of SUV (9 $\mu$M phospholipid concentration) containing 1 mol % each of NBD-PE and Rh-PE, and unlabeled SUV (84 $\mu$M phospholipid concentration). The increase in NBD-PE fluorescence at room temperature due to the addition of the peptides was monitored and plotted versus time. The peptide/lipid molar ratio was 0.018. (Panel A) 25 mM HEPES, 50 mM $Na_2SO_4$; (panel B) 25 mM HEPES, 400 mM $Na_2SO_4$. Peptides' designations: (—) pardaxin; (– – –) C-diamino-Par; (···) C-dihydroxy-Par; (– ··· –) Suc-$Glu_2$-Par; (– ·· –) Suc-Par.

of the interaction. Similar results were obtained irrespective of the order in which the analogues were added (Figures 4B and 5). When C-diamino-Par was replaced by pardaxin in the mixtures, only a slight effect occurred, even though pardaxin only differs from C-diamino-Par or C-dihydroxy-Par by two negative charges in its C-terminal amino acid. This further corroborated the specificity of the mutual interactions. The time course of lipid mixing induced by pairs of peptides (Figures 4B and 5) also revealed a quick kinetics when both peptides are present as compared to the active peptide alone (C-diamino-Par or C-dihydroxy-Par, respectively).

*Electron Microscopy.* Vesicles (490 $\mu$M), negatively stained, were visualized with an electron microscope before and after addition of pardaxin analogues (8.9 $\mu$M), which gives the same peptide/lipid molar ratio (0.018) used in the lipid-mixing experiments. Figure 6 shows a representative micrographs of vesicles taken at pH 4.5 and high ionic strength, without peptide (panel A), with $Glu_2$-Par (panel B), and with C-diamino-Par (panel C). The micrographs demonstrate that the observed lipid mixing appears concurrently with a size increase of a portion of the vesicles.

*Fluorescence Studies.* To study the self-aggregation state of negatively ($Glu_2$-Par) and positively (C-diamino-Par) charged pardaxin analogues at low or high ionic strength, the fluorescence intensities of probes covalently attached to either the N- or the C-terminal amino acids of the peptides (1.7 $\mu$M) at pH 6.8 were measured. $Glu_2$-Par was labeled at its N-terminal amino acid with Flu to generate Flu-$Glu_2$-Par, whose two termini are negatively charged. C-diamino-Par was labeled at its C-terminal amino acid with Rh to generate C-Rh-Par, whose C-terminal retained a positive charge. The emission spectra of Flu-$Glu_2$-Par and C-Rh-Par at low and high ionic strengths were determined. Figure 7 shows as an example the emission spectra of Flu-$Glu_2$-Par at low ionic strength (continuous line) and at high ionic strength (dashed line). Both labeled analogues showed decrease in their emission intensities at high ionic strengths, as compared to low ionic strengths. This observed reduction in emission intensity at high ionic strength is probably due to self-quenching of the probes caused by self-aggregation of the peptides, since the emission spectra of control molecules, Rh-$NHCH_2CH_2$-OH and Flu-$NHCH_2CH_2$-OH, was not affected by ionic strengths (data not shown).

## DISCUSSION

Fusion of phospholipid membranes is thought to involve three steps: vesicle aggregation, membrane destabilization, and merging of membranes and (aqueous) contents (Lucy, 1982; Blumenthal, 1987). Pardaxin and some of its analogues induced fusion of PC/PS SUV (Table II). This peptide-induced vesicle fusion was demonstrated by a probe-dilution assay for lipid mixing and by an increase in the average size of liposomes (Figure 6). The properties of pardaxin analogues involved in their induction of fusion are discussed in relation to the currently proposed mechanism of peptide-induced fusion.

*Vesicle Aggregation.* Pardaxin analogues interact with membranes by first adsorbing to the membrane's surface, penetrating the membrane, and then aggregating within (Rapaport & Shai, 1991, 1992; Pouny & Shai, 1992).

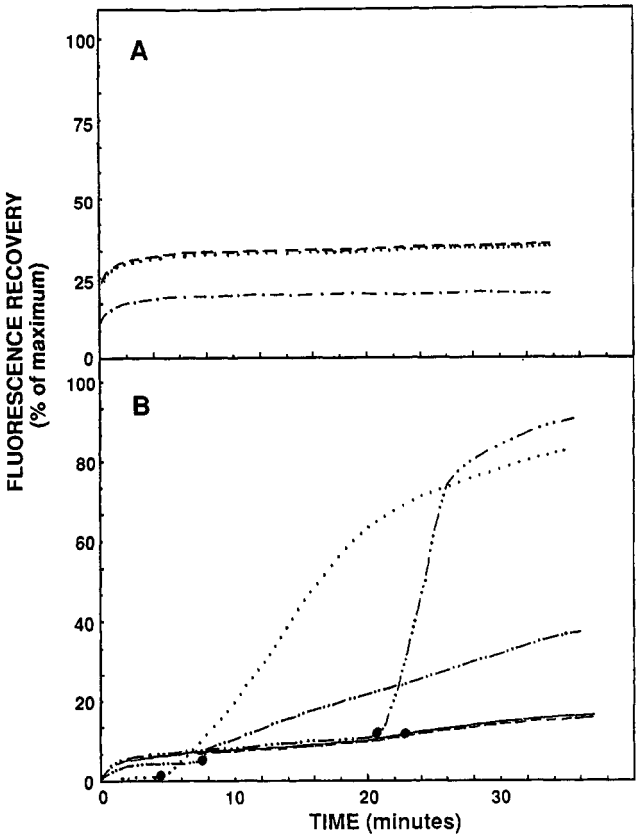


FIGURE 4: Time course of lipid mixing of PC/PS (1:1) SUV induced by mutual interaction between C-diamino-Par and acidic analogues. Peptides (1.7 $\mu$M of each) were added to a mixture of SUV (9 $\mu$M phospholipid concentration) containing 1 mol % each of NBD-PE and Rh-PE, and unlabeled SUV (84 $\mu$M phospholipid concentration), at pH 6.8 and room temperature. The resulting increase in NBD-PE fluorescence was monitored at 530 nm and plotted versus time. (Panel A) 25 mM HEPES, 50 mM $Na_2SO_4$: (– – –) C-diamino-Par; (– · –) a mixture of C-diamino-Par and Suc- Par or Suc-$Glu_2$-Par; (···) a mixture of C-diamino-Par and Suc- D-$Leu_2$-Par. (Panel B) 25 mM HEPES, 400 mM $Na_2SO_4$. (—) C-diamino-Par alone (1.7 $\mu$M); each one of the other curves depicts the profile generated when the addition of one peptide (1.7 $\mu$M) followed by the addition of a second one (1.7 $\mu$M) at a time designated by a filled circle: (– ··· –) first C-diamino-Par followed by Suc-$Glu_2$-Par; (···) $Glu_2$-Par followed by C-diamino-Par; (– – –) C-diamino-Par followed by Suc-D-$Leu_2$-Par; (– ·· –) Par followed by C-diamino-Par.

Therefore, as was postulated for gramicidin-induced fusion (Tournois et al., 1990), pardaxin analogues may influence the forces between interacting bilayers, thereby enhancing aggregation of vesicles. Indeed, native pardaxin induces vesicle aggregation (Lazarovici et al., 1988). The N-terminal of pardaxin is believed to become inserted within the lipid bilayer, thus anchoring the peptide to the membrane, while the C-terminal lies on the surface (Rapaport & Shai, 1991). A positively charged C-terminal may attract other negatively charged phosphate groups located on other liposomes, which can lead to apposition, followed by fusion. Thus, the positive charge at the C-terminal of C-diamino-Par could be responsible for high fusogenic activity (Figure 2). At neutral pH, basic, but not acidic, derivatives induced vesicle fusion. Thus, the interaction between the cation groups of the basic peptides and the head groups of the acidic liposome is more essential to the induction of fusion than the hydrophobic interaction between the amphipathic peptides and lipids. The ionic strength dependence of the fusion process at neutral pH is modest for aqueous Columbic interactions. A similar effect of salt concentration was observed when a peptide segment of HIV gp41 interacted with lipid bilayers (Rafalski et al., 1990).

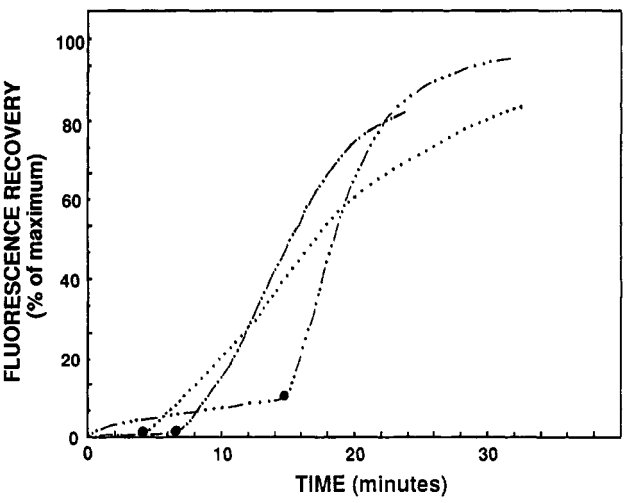


FIGURE 5: Time course of lipid mixing of PC/PS (1:1) Suv induced by mutual interaction between C-dihydroxy-Par and acidic analogues. Peptides (1.7 $\mu$M of each) were added to a mixture of SUV (9 $\mu$M phospholipid concentration) containing 1 mol % each of NBD-PE and Rh-PE, and unlabeled SUV (84 $\mu$M phospholipid concentration), at pH 6.8 and room temperature in 25 mM HEPES and 400 mM $Na_2SO_4$. The resulting increase in NBD-PE fluorescence was monitored at 530 nm and plotted versus time. Each curve depicts the profile generated by the addition of one peptide (1.7 $\mu$M) followed by the addition of a second one (1.7 $\mu$M) at a time designated by a filled circle: (– ··· –) C-dihydroxy-Par followed by Suc-$Glu_2$-Par; (– ·· –) Suc-$Glu_2$-Par followed by C-dihydroxy-Par; (···) $Glu_2$-Par followed by C-dihydroxy-Par.

*Membrane Destabilization, Peptides' α-Helicity, and Aggregation within the Membrane.* After vesicle aggregation the next step in the proposed fusion mechanism is membrane destabilization. Pardaxin and some of its derivatives perturb the lipid packing of vesicles and cause leakage of their contents (Shai et al., 1990, 1991). A causal relationship between peptide-induced SUV fusion and leakage of aqueous liposome contents was proposed by Wharton et al. (1988). However, destabilization of the lipid packing alone is not sufficient to cause fusion. Apparently the ability of a peptide to adopt α-helical structure and to self-aggregate within the membrane is also required for fusion. Peptides that contain acidic or D-amino acids, and whose vesicle incorporation and membrane destabilization efficiencies are comparable to those of pardaxin, but have low level of α-helical structure at neutral pH (Rapaport & Shai, 1991; Pouny & Shai, 1992) do not cause fusion (Table II). The one exception is C-diamino-D-$Leu_2$-Par, which could induce low levels of PC/PS SUV fusion (~5%), probably the result of pure electrostatic interactions like the fusion induced by polylysine or polyarginine. A correlation between the α-helical content of a peptide and its membrane-destabilizing and fusogenic properties was also noted with the model peptide GALA (Parente et al., 1988) and segments of the influenza virus HA-2 protein (Lear & DeGrado, 1987; Wharton et al., 1988, Rafalski et al., 1991) as well as model amphipathic peptides (Lee et al., 1992). This correlation is consistent with the view that helical structure allows the peptides' amide groups to form internal hydrogen bonds, a necessary energetic condition for their insertion into the apolar membrane interior (Roseman, 1988).

Using fluorescently labeled peptides, it was demonstrated that neutral or positively charged pardaxin analogues tend to self-aggregate efficiently within PC vesicles, while an acidic analogue ($Glu_2$-Par) aggregated to a much lower extent and D-$Leu_2$-Par does not aggregated at all (Rapaport & Shai, 1991; Pouny & Shai, 1992). The difference in this self-aggregation between pardaxin and its acidic analogues is

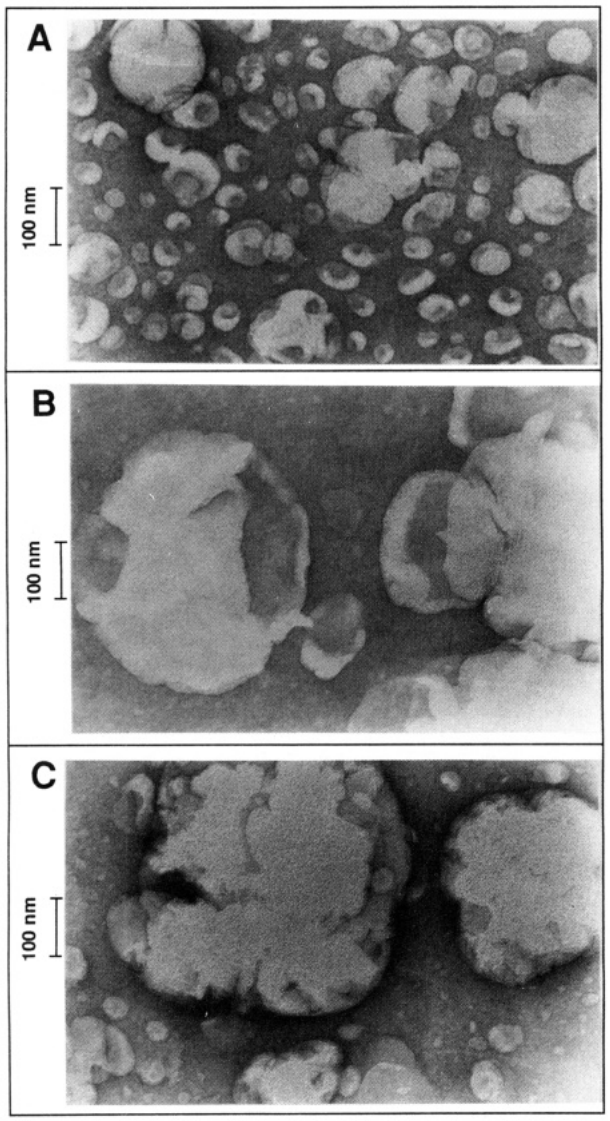


FIGURE 6: Electron micrographs of negatively stained liposomes. (A) PC/PS SUV alone; (B) PC/PS SUV incubated with $Glu_2$-Par for 10 min; (C) PC/PS SUV incubated with C-diamino-Par for 10 min. The bar represents 100 nm.

expected to be even bigger in the PC/PS SUV system used herein, since the negative charge of the PS head groups can compensate, at least partially, for the Columbic repulsion among positively charged pardaxin monomers. Self-aggregation of gramicidin monomers (Tournois et al., 1990), as well as oligomerization of a mutant of HIV-1 gp 41 protein (Freed et al., 1992) and a peptide segment of the influenza virus HA-2 fusion protein (Wharton et al., 1988), has also been implicated in their mediation of fusion. However, $\alpha$-helical structure and aggregation are not sufficient to mediate fusion, since native pardaxin and other peptides, capable of forming high aggregates (Rapaport & Shai, 1991, 1992), mediate little if any fusion (Table II), suggesting that an appropriate charge is also important for peptide-induced fusion of negatively charged liposomes.

*pH Dependence Peptide-Induced Lipid Mixing.* In acidic solutions of high ionic strength, acidic peptides induce higher amounts of fusion than basic peptides (Table II; Figure 3). This behavior might be due to high salt concentrations' screening of charges on both peptides and liposomes and the acidic pH's protonation of the carboxylate groups of the peptide, resulting in their neutralization. An enhanced hydrophobicity and screening of charges should reduce Columbic repulsion and increase hydrophobic interactions

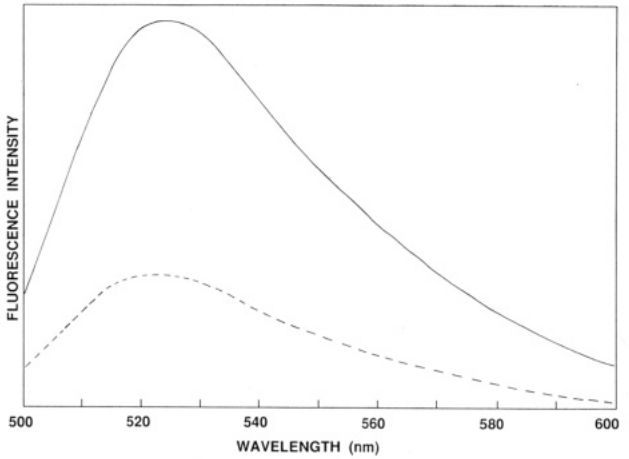


FIGURE 7: Effect of ionic strengths on the emission spectra of fluorescently labeled pardaxin analogue. Flu-Glu2-Par (1.7 $\mu$M) was incubated at pH 6.8 in 25 mM HEPES containing 50 (continuous line) or 400 (dotted line) mM $Na_2SO_4$ at room temperature. The excitation wavelength was set at 490 nm.

among peptide monomers, thus enhancing self-aggregation of peptides, a prerequisite for fusion. pH-dependent behavior has been demonstrated for a variety of fusogenic peptides, including the model peptide GALA (Parente et al., 1988), modified HA-2 fusion peptide (Wharton et al., 1988; Rafalski et al., 1991; Murata et al., 1992), and melittin derivatives (Murata et al., 1987). The differences in the kinetics of peptide-induced fusion under neutral and acidic conditions can be explained by a mechanism proposed for fusion induced by viral proteins. This kinetic model views the overall fusion reaction as a sequence of a second-order process of membrane adhesion, the rate-limiting step, followed by the first-order fusion itself (Nir et al., 1982; Bentz et al., 1983, 1988). At neutral pH, basic pardaxin analogues can cross-link negatively charged vesicles by electrostatic interactions and charge neutralization, thus inducing fast apposition. However, at acidic pH, a dehydration potential must be overcome by hydrophobic interactions. Thus the kinetic of apposition is slower because under these conditions the cross-linking capacity of the peptides is slower. At both neutral and acidic pH, once sufficient vesicle aggregation occurs, due to cross-linking of vesicles with peptides, fusion is rapidly induced.

*Effects of Mutual Interaction between Charge-Reversed Pardaxin Analogues on Vesicle Fusion.* When inactive acidic peptides (Suc-Par, $Glu_2$-Par, or Suc-$Glu_2$-Par) were mixed with active peptides (C-diamino-Par or C-dihydroxy-Par) at neutral pH, depending on the ionic strength, opposite effects were observed (Figures 4 and 5). A low ionic strength, these mixtures of analogues inhibited the fusion process (Figure 4A), while at high ionic strength they enhanced both the kinetics and magnitude of fusion (Figures 4B and 5). These mutual interactions are structurally specific, since analogues containing D-amino acids did not affect the fusogenic potential of other analogues. The difference in high and low ionic strengths may be due to the different types of interactions involved in the fusion process in these two ionic strengths. At neutral pH and low ionic strength the fusion is predominantly induced by charge attraction between positive peptides and negative vesicles. Since mutual interactions are assumed to neutralize the peptide (C-diamino-Par or C-dihydroxy-Par) charges, these interactions would also reduce the electrostatic interaction between the peptides and vesicles and thus reduce fusion. However, at high ionic strengths, peptide charges are heavily screened, which facilitates the formation of large aggregates, as revealed in the quenching experiments (Figure 7), and hydrophobic interactions are important for inducing

fusion. Under these conditions, vesicle charges are also screened, and high levels of vesicle aggregation occur due to cross-linking of vesicles by the electrically neutralized hydrophobic peptide aggregates. The mutual interaction of an equimolar mixture of an anionic peptide and its charge-reversed cationic analogue, which were inactive by themselves, was previously shown to induce fusion of egg zwitterionic PC large unilamellar vesicles (Murata et al., 1991). However, in contrast to our findings with pardaxin-induced fusion of negatively charged liposomes, this enhancement of fusion was only observed at low ionic strengths, while high ionic strengths abolished the effect. High salt concentration is probably inducing a higher screening effect on the aggregation of the currently used negatively charged liposomes as compared to its effect on zwitterionic ones. A difference in the aggregational state of the peptides in the high/low ionic strength solutions may also be responsible for this differences, although the information on the peptides' state of aggregation in both systems is incomplete.

The concentration dependence fusion induced by the most potent analogue, C-diamino-Par, also provides information about the fusion mechanism (Figure 1). For a given concentration of C-diamino-Par, lipid mixing does not proceed to completion but rather approaches a limit, which increases with the peptide to lipid ratio. This suggests that the peptide incorporation is not homogeneous and that only a limited number of vesicles incorporated sufficient peptide molecules to induce fusion. This is further supported by electron micrographs showing heterogeneous populations of large fused SUV vesicles and small nonfused ones. Similar behavior was suggested for the fusion induced by gramicidin (Tournois et al., 1990) and by amphiphilic model peptides (Murata et al., 1991).

In summary, the results of the present study reveal that appropriate charge, $\alpha$-helical structure, and incidence of self-aggregation of a peptide, both in solution and within the membrane, play an important role in the fusogenic ability of peptides. Moreover, the importance of hydrophobic versus electrostatic interactions in fusion may vary depending on the pH and ionic strengths of the media and on the charge of the target vesicles.

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