Biochimica et Biophysica Acta, 984 (1989) 360–364
Elsevier

BBAMEM 74550

A synthetic peptide corresponding to the hydrophobic amino terminal region of pardaxin can perturb model membranes of phosphatidyl choline and serine

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(Received 1 February 1989)
(Revised manuscript received 16 June 1989)

Key words: Amino acid sequence, terminal; Synthetic peptide; Pardaxin; Model membrane; Phosphatidylcholine; Phosphatidylserine

Peptides corresponding to the amino terminal region of pardaxin from *Pardachirus pavoninus* (Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe) have been synthesized and their interaction with model membranes of phosphatidyl choline and serine studied by 90°C light scattering and fluorescence spectroscopy. The amino terminal 8-residue peptide and the protected 15-residue peptide cause only aggregation of lipid vesicles. The deprotected 15-residue peptide has the ability to cause aggregation and release of entrapped carboxyfluorescein with both phosphatidyl choline and serine lipid vesicles, like pardaxin. The membrane-perturbing ability of the amino terminal 15-residue peptide can be attributed to its ability to adopt an α-helical conformation which is amphiphilic in nature in a hydrophobic environment.

Introduction

Pardaxins are peptides of approx. 35 residues in length present in the defense secretions of the Red Sea Moses sole *Pardachirus marmoratus* [1] and Pacific Peacock sole *Pardachirus pavoninus* [2]. Pardaxins exhibit diverse pathological and pharmacological effects like smooth muscle contraction in guinea pig ileum [3], activation of acetyl/cholinesterase [4], viron destruction [5,6], and lysis of red blood cells [7]. These effects are presumed to arise from their ability to perturb membranes. Support for this assumption comes from the observation that pardaxins are highly surface active [2], induce aggregation of phosphatidyl serine vesicles [8], increase the permeability of planar lipid bilayers in a voltage dependent manner at low concentration [9] and cause release of entrapped hydrophilic molecules at high concentrations [8]. The membrane-modifying properties of pardaxins are thus similar to gramicidin A, alamethicin and melittin [10,11]. However, the primary structures, net charge and lengths of these peptides differ considerably. It is therefore desirable to evaluate the influence of peptide chain length and charge on the interaction of pardaxins with membranes. In this report we describe studies on the interaction of synthetic peptides corresponding to the amino terminal region (residues 1–15) of Pardaxin 1 from *P. pavoninus* (Fig. 1) with small unilamellar vesicles of phosphatidyl choline and phosphatidyl serine.

Experimental

Synthesis of peptides. Peptide corresponding to the first 15 residues of pardaxin 1 and other amino terminal fragments i.e. peptides P1–P6 (Fig. 1) were synthesized by solution phase methods. Dipeptide couplings were effected in dichloromethane (CH_2Cl_2) by dicyclohexylcarbodiimide (DCC). Tripeptides and longer peptides were synthesized in dimethylformamide with DCC and 1-hydroxybenzotriazole. The amino terminal (Boc = *t*-butyloxycarboxyl) and carboxy terminal (methyl esters) protecting groups were removed by treatment of peptides with 85% formic acid and NaOH, respectively. The side chain protecting groups were benzyloxycarbonyl(Z) for Lys and benzyl ether(Bzl) for Ser. The major fragments synthesized were Boc-Gly-Phe-Phe-Ala-OCH_3, Boc-Leu-Ile-Pro-Lys(Z)-OCH_3, Boc-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OMe. Boc-Gly-Phe-Phe-AlaOH was coupled to H_2N-Leu-Ile-Pro-Lys(Z)-OCH_3 to yield the amino terminal octapeptide of pardaxin.

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Pardaxin P1:

Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-
1 5 10 15
Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu
20 25 30

Synthetic peptides:

P1. Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-OCH_3

P2: Boc-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OCH_3

P3: Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OCH_3

P4: Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-OCH_3

P5: Ile-Ile-Ser-Ser-Pro-Leu-Phe-OCH_3

P6: Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-OCH_3

Fig. 1. Primary structures of pardaxin P1 (Ref. 2) and synthetic peptides corresponding to the 1–15 region of pardaxin P1.

Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-OH was coupled to H_2N-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OCH_3 to yield the amino terminal 15-residue fragment of pardaxin. All peptides were purified by column chromatography on silica-gel (230–400 mesh, Merck, F.R.G.) using mixtures of chloroform and methanol as eluants. The homogeneity of purified peptides were confirmed by fast performance liquid chromatography (FPLC) using pepRPC (reverse phase, C-18) column and quantitative amino acid analysis on an LKB 4151 Alpha Plus amino acid analyzer. The amino acid analyses of the synthetic peptides were as follows:

P4: Gly 1.01 (1), Phe 2.0 (2), Ala 1.08 (1), Leu 0.95 (1), Ile 0.9 (1), Pro 0.97 (1), Lys 1.0 (1)

P5: Ile 1.7 (2), Ser 1.57 (2), Pro 0.97 (1), Leu 1.06 (1), Phe 1.0 (1)

P6: Gly, 1.2 (1), Phe 3.0 (3), Ala 1.11 (1), Leu 1.8 (2), Ile 2.82 (3), Pro 2.0 (2), Ser 1.38 (2), Lys 1.11 (1)

Numbers in parenthesis indicate theoretical values. Ser values are not corrected for loss during hydrolysis. The protecting groups in the peptides were removed by treatment with trifluoroacetic acid/thioanisole/metacresol (5.0:0.4:0.4, v/v) at 40 °C [12].

Interaction of peptides with lipid vesicles. Small unilamellar vesicles of phosphatidyl choline (PC), purified from egg yolk [13], and phosphatidyl serine (PS) purified from bovine brain [14] were prepared by sonication (Branson B-50 sonifier) of an aqueous dispersion of lipid in 5 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) buffer (pH 7.4). The ability of peptides to cause aggregation of lipid vesicles was studied by 90 °C light scatter in a Hitachi 650-10S spectrofluorimeter with the excitation and emission monochromators set at 350 nm. Fusogenic properties of the peptides were examined by the Tb^{3+}-dipicolinic acid assay [15]. The membrane modifying properties of the peptides were further determined by monitoring the release of 6-carboxyfluorescein trapped in PC and PS vesicles [16]. Peptides were weighed accurately (5 mg) and stock solutions were prepared in methanol. Concentration of peptides was determined by quantitative amino acid analysis on LKB4151 Alpha Plus amino acid analyzer. Lipid was estimated by the method of Stewart [17]. All experiments were carried out at 25 °C.

Circular dichroism (CD) studies. CD spectra were recorded on a JASCO-J-20 spectropolarimeter in cells of path length 1 mm. The concentration of the peptide stock solutions were determined by quantitative amino acid analysis. For studies in micelles, sample was prepared as follows: an aliquot of peptide and the appropriate micelle forming compound in organic solvent were mixed and dried. The dried mixture was reconstituted in water with vortexing. Mean residue ellipticities were calculated using the formula

$$[\theta]_M = \frac{\theta \cdot 100 \cdot M \cdot \mathrm{deg} \cdot \mathrm{cm}^2 \cdot \mathrm{dmol}^{-1}}{l \times c}$$

where θ = observed reading in degrees, l = path length of cells in cm, c = concentration of peptide in g/l and M = mean residue weight.

Results and Discussion

The 90 °C light scattering profiles of PC and PS small unilamellar vesicles, in presence of peptides P1–P6, as a function of peptide concentration are shown in Figs. 2a and 2b. The protected peptides P1, P3 and the deprotected 15-residue peptide P6 cause a large increase in the scatter of PC vesicles. The increse in scatter of PC vesicles as a function of time in the presence of P6 (similar profiles were observed for P1 and P3) is shown as inset in Fig. 2a. A rapid increase in scatter on addition of peptide is observed. After the initial rapid rise, there is no further increase in scatter as a function of time, at all concentrations of the peptide. In the case of PS vesicles, the protected and deprotected 8 and 15 residue peptides P1, P3, P4 and P6 cause increase in scatter. The relative increase in scatter is much more in the presence of protected peptides, particularly with increasing peptide concentration. No precipitation or increase in scatter with peptides alone was observed. Hence the increase in scatter is a consequence of the association of peptides with lipid vesicles. Peptides P2, P5 and smaller tetrapeptide fragments had no effect on the scatter profiles of PC and PS vesicles.

Increase in light scatter can arise either due to aggregation or fusion of lipid vesicles. The fusogenic properties of peptides P1–P6 were investigated by the Tb^{3+}-dipicolinate assay. In this assay, two populations of vesicles with entrapped Tb^{3+} and DPA are mixed in medium containing EDTA. In the event the vesicles fuse, there would be mixing of the aqueous compartments of the two populations of vesicles resulting in Tb^{3+} binding to dipicolinic acid. Terbium ions have

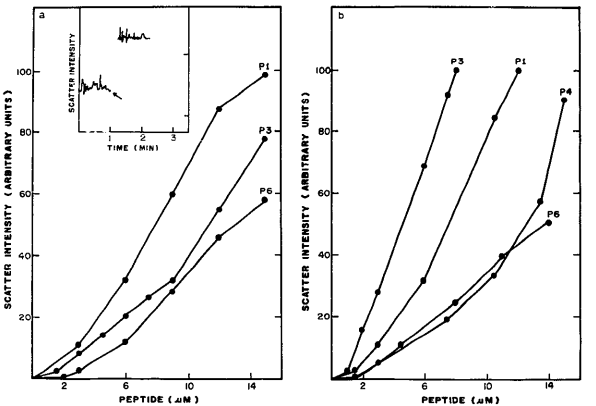

Fig. 2. 90° light scattering profiles of lipid vesicles in presence of peptides P1–P6. (a) Peptides added to PC vesicles (lipid = 150 μM). Inset: Representative scatter profile in presence of P6 as function of time. Peptide is added at the time indicated by an arrow. (b) Peptides added to PS vesicles (lipid = 150 μM). P1–P6 correspond to the synthetic peptides in Fig. 1.

very weak fluorescence but on binding to dipicolinic acid there is a considerable increase in fluorescence. However, when peptides P1–P6 were added to PC and PS vesicles with encapsulated Tb^{3+} and dipicolinic acid there was no increase in Tb^{3+} fluorescence. Hence the increase in scatter can be attributed to aggregation and not fusion.

In order to get further insight into the membrane-modifying properties of peptides P1–P6, their abilities to induce release of entrapped carboxyfluorescein (CF) from PC and PS vesicles was monitored. The release of CF (expressed as percentage of maximum release obtained by treatment with Triton X-100) from PC and PS vesicles, as a function of peptide concentration and time are shown in Figs. 3a and 3b. Only the deprotected 15-residue peptide P6 has the ability to cause release of entrapped CF. Release of CF to a small extent is observed with the protected peptide P3 only at a rela-

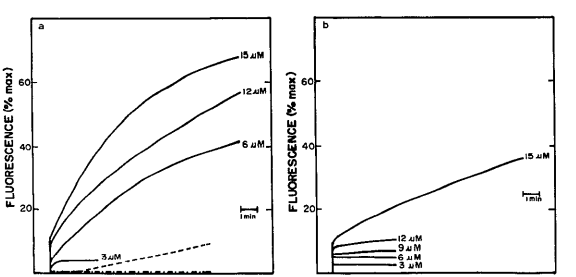

Fig. 3. Release of encapsulated carboxyfluorescein (CF) from PC and PS vesicles in presence of P1–P6. (a) PC vesicles (150 μM). (b) PS vesicles (150 μM). ———, P6, concentrations indicated correspond to different amounts of P6 added; – – –, P3 (15 μM); ·–·–·, P1, P2, P4, P5 (15 μM).

tively high concentration and only with PC vesicles. With PS vesicles, significant release of CF is observed at a concentration of 15 μM as against 6 μM in the case of PC vesicles. However, complete release of CF is not observed with PS and PC vesicles even at a lipid/peptide ratio of 10 : 1.

A comparison of the rates at which P6 causes aggregation of lipid vesicles and release of entrapped carboxyfluorescein (i.e. inset in Fig. 2a and Figs. 3a and 3b) indicates that the aggregation process is much more rapid than release of CF. Hence if the increase in 90 °C scatter was the result of fusion, an initial rapid rise in fluorescence due to mixing of aqueous components of the fused vesicles followed by a decrease in fluorescence due to leakage of liposomal contents and complexing of Tb^{3+} with EDTA present in the medium would have been observed in the Tb^{3+}/DPA assay. Since no rise in fluorescence is observed in the fusion assay, the increase in 90 °C scatter is clearly due to aggregation and not fusion.

In order to rationalize the membrane-modifying properties in terms of structure, the conformation of P6 in trifluoroethanol (TFE) and micelles of sodium dodecyl sulphate was determined by circular dichroism (CD) spectroscopy. Fig. 4 shows the CD spectra of P6 in TFE and SDS. Comparison of these spectra with the reference spectra of Greenfield and Fasman [18] indicates an α-helical content of $\approx$ 35% and random conformation $\approx$ 65% with no β-structure. The helical representation for P6 is shown in Fig. 5. An amphiphilic structure is clearly discernible. An average hydrophobicity of 0.62 and hydrophobic moment of 0.28 clearly favours strong membrane association for P6.

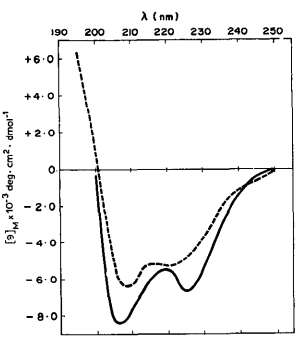

Fig. 4. Circular dichroism spectra of P6 in trifluoroethanol (– – –) and sodium dodecyl sulphate micelles (———). Peptide concentration = 0.1 mg/ml.

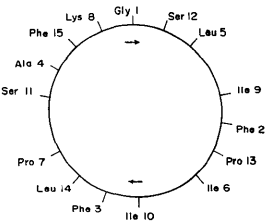

Fig. 5. Axial projection of the α-helical conformation of P6 as described by Schiffer and Edmundson [19]. Average hydrophobicity (0.62) and hydrophobic moment (0.28) are calculated by the methods of Eisenberg et al. [20] on a computer.

Thus, peptide P6 corresponding to the first 15 amino terminal residues of pardaxin has the ability to interact with PC and PS vesicles so as to cause aggregation and release of entrapped carboxyfluorescein. The membrane-modifying properties of P6 are more marked on zwitterionic PC vesicles. The membrane perturbing ability of P6 can be attributed to its ability to adopt an amphipathic helical conformation. Support for this argument comes from the observation that the peptide P6 in which the side chains are protected have no membrane-perturbing ability. While P6 interact with lipid vesicles in a manner similar to pardaxin isolated from *P. pavoninus* the lipid/peptide ratios at which the membrane-perturbing effects are manifested are different. The maximum lipid/peptide ratio at which the membrane-perturbing abilities is discernible in the presence of P6 is 50 : 1 whereas pardaxin induces aggregation at a ratio of 300 : 1 [8]. Likewise, P6 causes release of entrapped hydrophilic molecules like CF at a ratio of 50 : 1 and pardaxin at 200 : 1. Thus, at equivalent lipid concentration 4–7-times more P6 is needed to cause detectable release of entrapped hydrophilic molecules like CF and discernible aggregation of lipid vesicles respectively. These differences may stem from the presence of two lysine and two glutamic acid residues in pardaxin as compared to one lysine in P6 as a result of which electrostatic interactions between peptide molecules and with lipid vesicles would be more predominant in pardaxin.

The carboxy terminal region of pardaxin is comprised of relatively hydrophilic amino acids as compared to the amino terminal portion and is thus likely that the amino terminal region plays an important role in the membrane-modifying properties of the toxin. Since peptide P6 interacts with model membranes in a manner similar to pardaxin, it is conceivable that it also exhibits some of the pathological and pharmacological

effects shown by the toxin. Studies directed towards this end are currently in progress in our laboratory.

References

1 Lazarovici, P., Primor, N. and Loew, L.M. (1986) J. Biol. Chem. 261, 16704–16713.
2 Thompson, S.A., Tachibana, K., Nakanishi, K. and Kubota, I. (1986) Science 233, 341–343.
3 Primor, N. (1984) Br. J. Pharmacol. 82, 43–49.
4 Primor, N. and Lazarovici, P. (1981) Toxicon 19, 573–578.
5 Pal, R., Barenholz, Y. and Wagner, R.R. (1981) J. Biol. Chem. 256, 10209–10212.
6 Pal, R., Barenholz, Y. and Wagner, R.R. (1981) J. Virol. 39, 671–685.
7 Primor, N. and Zlotkin, E. (1975) Toxicon 13, 227–231.
8 Lelkes, P.I. and Lazarovici, P. (1988) FEBS Lett. 230, 131–136.
9 Loew, L.M., Benson, L., Lazarovici, P. and Rosenberg, I. (1985) Biochemistry 24, 2101–2104.
10 Nagaraj, R. and Balaram, P. (1981) Acc. Chem. Res. 14, 356–362.
11 Bernheimer, A.W. and Rudy, B. (1986) Biochim. Biophys. Acta 864, 123–141.
12 Bodanszky, M. and Bodanszky, A. (1984) Int. J. Pept. Protein Res. 23, 287–291.
13 Singelton, W.S., Gray, N.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53–56.
14 Lipid Biochemical Preparation (1980) (Bergelson, L.D., ed.) p. 180, Elsevier, Amsterdam.
15 Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
16 Weinstein, J.N., Yoshikami, S., Henkart, P., Blumerithal, R. and Hagin, W.A. (1977) Science 195, 489–491.
17 Stewart, J.C.M. (1980) Anal. Biochem. 104, 10–14.
18 Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4116.
19 Schiffer, M. and Edmundson, A.B. (1967) Biophys. J. 7, 121–135.
20 Eisenberg, D., Weiss, R.M. and Terwilliger, T.C. (1982) Nature 299, 371–374.