

Sequencing and synthesis of pardaxin, a polypeptide from the Red Sea Moses sole with ionophore activity

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Pardaxin, an amphipathic polypeptide secreted by the Red Sea flatfish *Pardachirus marmoratus* whose sequence is NH₂-G-F-F-A-L-I-P-K-I-I-S-S-P-L-F-K-T-L-L-S-A-V-G-S-A-L-S-S-G-G-Q-E, was synthesized by the solid-phase method. The structure was verified by sequencing. The synthetic polypeptide changed the resistance of lipid bilayers by forming pores. At 10⁻⁷–10⁻⁸ M, the synthetic pardaxin increased the frequency of the spontaneous release of quanta of acetylcholine at the neuromuscular junction by up to 100-fold, resembling the native product. Synthetic pardaxin seems to be a suitable tool for investigating the molecular structures underlying channel selectivity.

Pardaxin; Presynaptic activity; Neuromuscular junction; Planar lipid bilayer; Pore activity

1. INTRODUCTION

The Red Sea Moses sole flatfish (*Pardachirus marmoratus*) produces an ichthyotoxic secretion which repels aquatic organisms [1] and affects the in vitro activities of different physiological preparations [2]. The main neurotoxic protein component of this secretion has been isolated, characterized and named pardaxin [3,4].

The toxic effects of pardaxin have been attributed to its interference with the ionic transport of the osmoregulatory system in epithelium [5] and its presynaptic activity [6]. On a molecular level,

pardaxin forms voltage-dependent, ion-permeable channels [7,8] at concentrations below 10⁻⁷ M and cytotoxicity at higher concentrations, 10⁻⁴–10⁻⁷ M [4,8]. Since the pardaxin peptide seems to be a suitable tool for investigating the molecular properties underlying channel selectivity and voltage dependence [8,9], we have examined the primary structure of this peptide. The biological activity of the pardaxin synthesized by the solid-phase method appeared to be indistinguishable from that of the native product.

2. EXPERIMENTAL

2.1. Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids (Birmingham, AL). Dimethyl sulfoxide, trifluoroacetic acid propanol and *n*-decane were obtained from Aldrich (Milwaukee, WI). PAM resins for solid-phase peptide synthesis were purchased from Applied Biosystems (Foster City, CA) and Boc amino acids from Peninsula Lab (Belmont, CA). All other chemicals were of the purest grade commercially available.

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Abbreviations: TFA, trifluoroacetic acid; HOBT, hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; PBS, phosphate-buffered saline; PX, pardaxin; HPLC, high-performance liquid chromatography; MEPP, miniature end plate potential; PE, phosphatidylethanolamine

2.2. Synthesis, HPLC purification and sequencing pardaxin peptides

Pardaxin and two other peptides composed of 26 and 10 amino acids from the amino-terminal were synthesized by the solid-phase method on PAM resins using HOBT active esters (prepared fresh before each step) by mixing Boc amino acid, DCC and HOBT at a molar ratio of 1:1:1 at 0°C. The coupling reaction proceeded for 0.5 h at 0°C followed by two additional hours shaking at room temperature [10]. After cleavage from the resin using liquid hydrofluoric acid and preliminary extraction, the respective synthetic peptides were purified to a very high degree of homogeneity (96–99%) by reverse-phase HPLC on either an analytical or semipreparative ultropac, μ -Bondapak C18 column (Millipore Corp., Bedford, MA) or Vydac protein C4 column (The Separation Group, Hesperia, CA). The flow rate was 0.9 ml/min or 3 ml/min, respectively, and detection was by absorbance at 210 nm. Amino acid analyses of the synthetic peptides were performed using a Beckman model 6300 amino acid analyzer with ninhydrin detection. The reverse-phase HPLC analyses and separations were performed on a Gilson HPLC system composed of a model 811 dynamic mixer, model 802B manometric module, two model 303 pumps, and model 116 variable wavelength absorbance detector and a NEC model PC-8023A-C recorder, all driven by an Apple IIC computer.

Pardaxin I and II obtained from chromatofocusing [4] and the synthetic product were sequenced on an Applied Biosystems 440A sequencer and the PTH-amino acids analyzed on an Applied Biosystems 120A chromatography unit [11].

2.3. Lipid bilayer experiments

Bilayers of PE were formed at the tip of micropipettes by the double-dip method [12]. The solutions in the micropipettes and bath (100 mM KCl) were connected by silver/silver chloride electrodes to the input of a List EPC7 amplifier in the voltage-clamp mode. The clamp potential and the current were recorded on a Gould chart recorder.

2.4. Electrophysiological procedures

All experiments were performed on the sartorius nerve-muscle preparation from frog (*Rana temporaria*) with normal frog Ringer's solution containing (mM): 116 NaCl; 2 KCl; 1.8 CaCl₂; 2-*N*-hydroxylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.2) as previously described [6].

3. RESULTS

3.1. Pardaxin sequence and secondary structure predictions

Pardaxin was sequenced and found to be composed of 33 common amino acids (table 1). A high degree of similarity to the previously identified ten residues at the NH₂-terminal [4] is evident. Arginine, tyrosine, tryptophan, methionine and cysteines were absent. Pardaxin was identical to the *P. pavonius* toxin P2 recently isolated from

another species of *Pardachirus* flatfish [13]. Although the sequence of the second pardaxin pore forming polypeptide [4] was identical to that of pardaxin (table 1), these two polypeptides show physico-chemical differences [4] that may reflect an aggregation process during purification.

The sequence of pardaxin was analyzed by the program of Garnier et al. [14] to predict its possible secondary structure. The simplest interpretation of the results (table 1) was a helical region extending from residues 1–7 followed by a section of discontinuous extended sheet (residues 8–25). The remaining carboxy-terminal region was predicted not to have a regular secondary structure. Interestingly, the middle portion of the molecule (residues 6–28) was predicted to have a very high amphipathic character (amphipathic score 59.1) using the method of Margalit et al. [15].

The hydrophobic moment plot of pardaxin residue values (table 1) based on the consensus hydrophobicity scale of Eisenberg et al. [16] indicated that most of the points fell in or near the surface region but those for its highly hydrophilic carboxy-terminal lay in the globular region [16]. This property is typical for surface-seeking, amphiphilic proteins which have large helical hydrophobic moments, such as melittin, *Staphylococcus aureus* δ -toxin and other cytolysins [16].

3.2. Synthesis of pardaxin peptides

The syntheses of pardaxin and the two shorter fragments were verified by amino acid analyses and sequencing (not shown) and the presence of only one major peak on HPLC analyses. The native pardaxin isolated by liquid chromatography [4] gave two peaks on the C-4 reverse-phase column, which eluted at 38 and 56% acetonitrile (fig.1A). The sequences of the materials in the two peaks were identical (table 1), suggesting them to be physical conformers.

Surprisingly, synthetic pardaxin gave a single peak on HPLC (fig.1B) which was identical to the 56% acetonitrile peak of the native toxin (fig.1A). To verify a possible chemical interaction between the 38 and 56% peaks, samples of the native 38% protein peak and the synthetic 56% peak were mixed and incubated for 24 h at room temperature. The mixture showed two peaks on the C-4 column

Table 1

The amino acid sequence of pardaxin from the flatfish *Pardachirus marmoratus* and the empirical parameters of folding and hydrophobicity

Residue number	Amino acid residue ^a	Yield ^b	Secondary structure ^c	Hydrophobicity index		Hydrophobic moment ^f
				A ^d	B ^e	
1-NH ₂	Gly	1593	H	3.54	-1.51	0.18
2	Phe	362	H	4.71	-1.51	0.18
3	Phe	312	H	6.00	-0.60	0.29
4	Ala	409	H	6.41	-0.48	0.38
5	Leu	292		5.91	-0.70	0.45
6	Ile	267		6.17	-0.35	0.47
7	Pro	266	T	6.42	1.98	0.50
8	Lys	216	T	6.04	1.98	0.43
9	Ile	201	T	5.38	-0.80	0.40
10	Ile	242		4.51	-0.91	0.34
11	Ser	113		5.38	-0.11	0.42
12	Ser	120	C	6.32	-0.23	0.33
13	Pro	74		5.12	-0.58	0.37
14	Leu	41	T	4.38	-0.88	0.23
15	Phe	67		5.04	-0.53	0.37
16	Lys	21		5.70	-0.20	0.37
17	Thr	18		5.80	-0.95	0.47
18	Leu	73		5.41	-0.88	0.44
19	Leu	96		5.62	-0.53	0.46
20	Ser	46	C	6.12	-0.31	0.46
21	Ala	55	H	6.10	-0.66	0.43
22	Val	34		5.82	-0.53	0.36
23	Gly	39		5.82	-0.23	0.37
24	Ser	21		5.82	-0.18	0.29
25	Ala	16		5.44	-0.23	0.24
26	Leu	20	C	4.71	-0.15	0.18
27	Ser	-	C	4.71	0.18	0.26
28	Ser	-	C	4.78	0.63	0.17
29	Ser	33	C	4.02	0.58	0.13
30	Gly	11	C	3.00	0.53	0.12
31	Gly	11	C	2.48	0.53	0.17
32	Gly	8	C	1.97	0.53	0.15
33-COOH	Glu	3	C	1.45	0.50	0.17

^a The line between residues 6 and 28 represents the amphipathic segment of pardaxin determined by the method of Margalit et al. [15] which also predicted an amphipathic score of 59.1 and a range of angles between 80 and 100° in the segment with 23 predicted blocks

^b Yields (pmol) of the PTH-amino acid from each cycle of the Edman degradation

^c Computerized secondary structure predictions based on the method of Garnier et al. [14]. H, helix; T, β -turn; C, coil; E, extended

^d Hydrophobicity values calculated with a computerized program based on the method of Kyte and Doolittle [21]. Pardaxin average molecule hydrophobicity is 4.88

^e Hydrophobicity values calculated with a computerized program based on the method of Hopp and Woods [22]. Pardaxin average molecule hydrophobicity is -0.31

^f The hydrophobic moment was calculated as described by Eisenberg [10]

(fig.1C) which suggested that the 56% peak of the native toxin cannot be explained only by an aggregation process. This is also supported by the resistance of the 56% peak to chaotropic agents (not shown). The anomalous behaviour of the

native pardaxin on the HPLC-C-4 or C-18 [4] columns could be attributed either to the removal of the aminoglycosteroids during the purification process [4] or to an irreversible oligomerization process which requires further characterization.

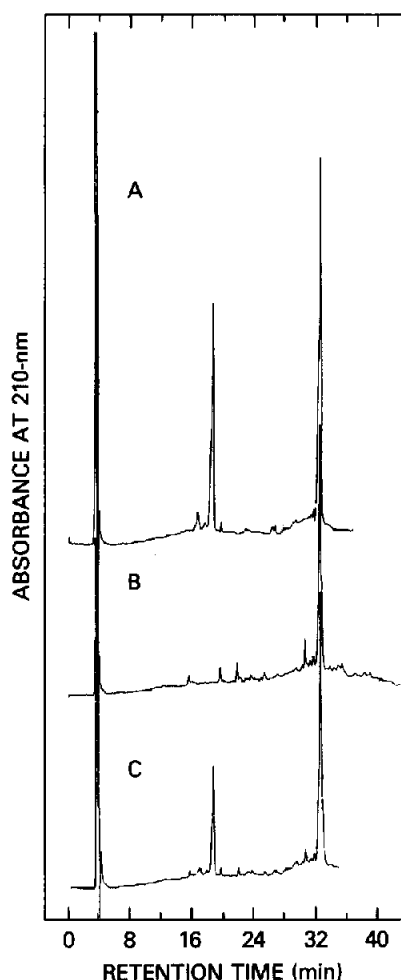


Fig.1. HPLC profiles of native and synthetic pardaxins on a reverse-phase C-4 column. (A) Native pardaxin after chromatofocusing; (B) synthetic pardaxin after HF cleavage; (C) mixture of native and synthetic pardaxin. Column: Vydac protein C-4 4.6/250 mm; elution conditions: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 15% B to 80% B in 50 min. Absorbance at 210 nm.

3.3. Effects of synthetic pardaxin on electrical properties of planar lipid bilayers

After the bilayer was found to be stable, pardaxin was added to one side, and the potential set at 20 mV. The currents across the bilayer in the voltage-clamp mode usually showed single-channel events within 10–20 min (fig.2). The channels produced by the synthetic pardaxin were found to be similar to those produced by the native one (fig.2). However, the synthetic compound was more po-

tent. The estimated minimum concentration of synthetic pardaxin required to produce channels (25 ng/ml) was about one tenth that necessary for the natural toxin. A synthetic 26 amino acid fragment (lacking the 7 amino acids at the carboxy-terminal) produced channels but in concentrations (100 μ g/ml) more than one thousand times that required for the 33 residue synthetic peptide. The hydrophobic amino-terminal decapeptide had no effect up to a concentration of 1.75 mg/ml.

3.4. The presynaptic effects of synthetic pardaxins

The presynaptic effects of the synthetic pardaxin at the neuromuscular junction were the same as those of the native toxin [6] (fig.3); it increased the frequency of the spontaneous release of transmitter quanta as well as the quantal content of the evoked end plate potentials (not given) and produced a progressive loss of resting potential (fig.3). In contrast the pardaxin-derived 26 and 10 peptides were without effect (lower part of fig.3; note that the addition of lanthanum ions (1 mM) caused a high-frequency burst of MEPPs before the frequency fell to zero).

4. DISCUSSION

In this report we have shown that the chromatographic and biological properties of synthetic pardaxin are similar or identical to those of the natural product isolated from the skin secretion of *P. marmoratus* flatfish [4]. The identity of the natural and synthetic pardaxin with regard to pore and presynaptic activity further suggests that the broad spectrum of pharmacological activities of native pardaxin [4,7] reflects intrinsic properties of this peptide and cannot be attributed to the potent aminoglycosideroids found in the *P. marmoratus* secretion [17]. The synthetic pardaxin was found to elute with retention times on HPLC identical to one of the peaks of the native peptide supporting the identity of the peptide sequences of the natural and synthetic compounds. However, the presence of two peaks, with identical primary composition, but different in chromatographic mobilities which might possibly represent physical conformers, requires further investigation. The sequence of pardaxin is unique when compared to the common polypeptide channel forming ionophores such as gramicidin [18], alamethicin

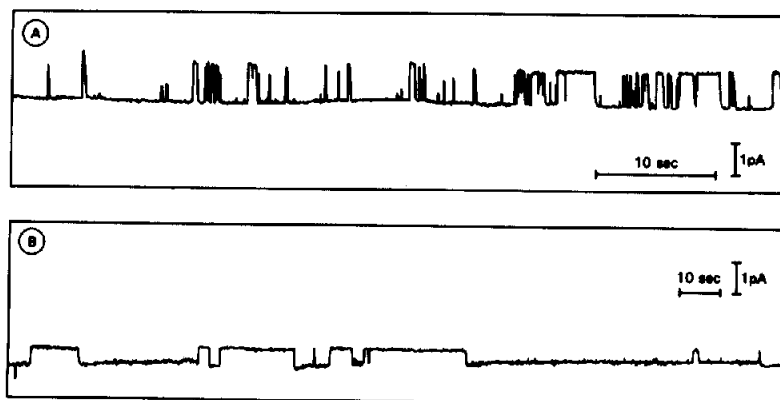


Fig.2. Single-channel recordings of native (A) and synthetic (B) pardaxin pores. Monolayers of phosphatidylethanolamine were prepared at the air-water interface. Addition of the synthetic or the native pardaxin to the bath resulted, after about 30 min, in pore formation as measured by the current fluctuations represented in A at 100 mV and B at 80 mV positive potentials. The single-channel conductance estimated from the amplitudes of the current steps divided by the applied voltage were in the range of 10 pS.

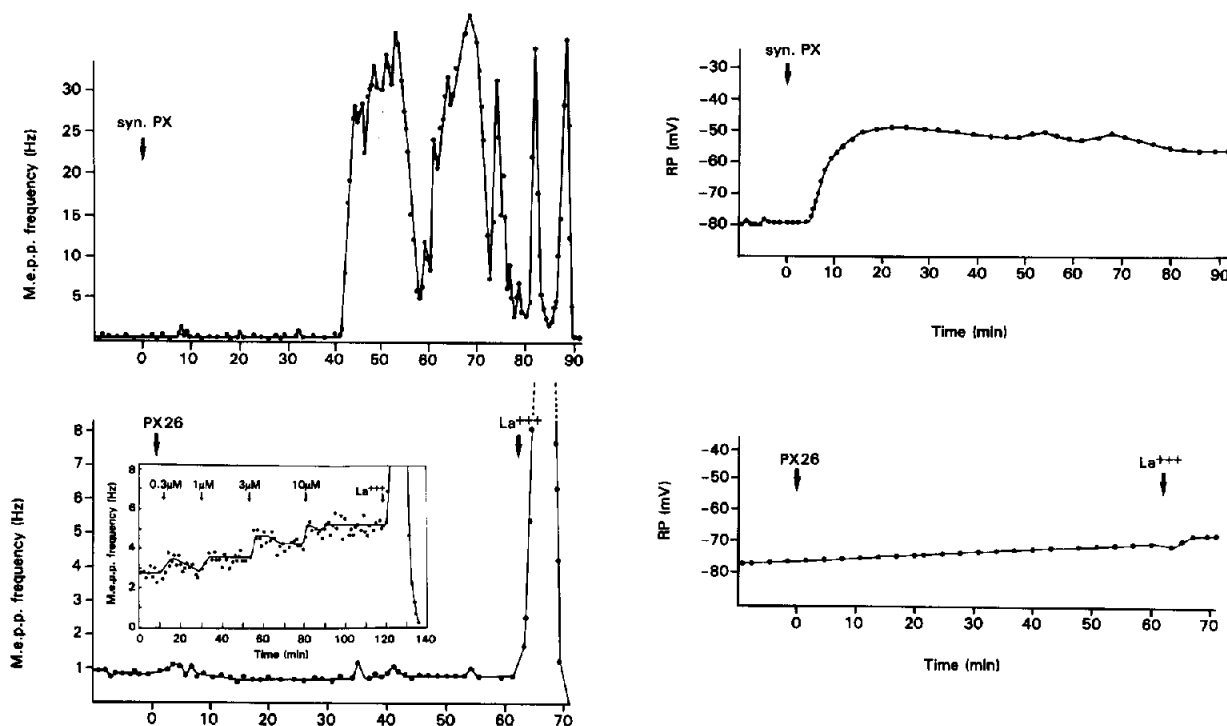


Fig.3. The effects of synthetic pardaxin and derived shorter segments on the release of acetylcholine at a single end-plate in the sartorius muscle of frog. The muscle was equilibrated in low-calcium Ringer's solution at room temperature. At time zero, the synthetic pardaxin ($2 \mu\text{g/ml}$) or derived peptides were added to the bath. The abscissa in all the graphs represents time in min. Ordinates: (left) the frequency of the MEPPs in Hz; (right) the resting potential (RP) in mV. Syn.PX, synthetic pardaxin; PX26, synthetic pardaxin fragment of 26 residues from the amino terminal; inset, consecutive additions of micromolar concentrations of the pardaxin derived decapeptide.

[19] or melittin [20]. The pardaxin peptide can be configured as an amphiphilic α -helix which spans a lipid bilayer [7,9]. Furthermore, models of the pardaxin pores support an antiparallel oligomer of several helical pardaxin monomers [7]. The dramatic loss of pore activity of the pardaxin peptide shortened to 26 or 10 residues in length is compatible with a mechanism of action that requires the peptide both to span the lipid bilayer of a membrane as an α -helical structure and to have a hydrophilic carboxy-terminal [7] supposed to be involved in selective ion binding and transfer. The availability of an active synthetic pardaxin peptide should facilitate future biophysical studies on pardaxin pore organization and function.

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