Indian Journal of Biochemistry & Biophysics
Vol. 25, December 1988, pp. 560-565

Membrane Modifying Activity of Four Peptide Components of Antiamoebin, a Microheterogeneous Fungal Antibiotic

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Received 17 *October* 1988

Antiamoebin, a microheterogeneous fungal antibiotic containing a high proportion of α-aminoisobutyric acid (Aib), has been separated into four distinct peptide fractions by HPLC on a reverse phase C_{18} column. Using a chlorotetracycline fluorescence assay in sealed unilamellar vesicles, the four fractions have been shown to induce Ca^{2+} flux across lipid bilayer membranes. These fractions also act as mitochondrial uncouplers. Differences in the membrane modifying activities of the various peptide fractions are observable at low peptide concentrations. The results establish that incorporation of antiamoebin into lipid bilayers can modify the permeability properties of membranes. Circular dichroism studies of three peptide fractions establish gross similarities in conformation. The CD spectra are appreciably different from those reported for related peptide antibiotics like alamethicin.

The antiamoebins are peptide antibiotics, rich in α-aminoisobutyric acid, isolated from cultures of *Emericellopsis poonensis Thirum., Emericellopsis synnematicola Mathur and Thirum.* and *Cephalosporium pimprina Thirum*[1,2]. These peptides have considerable similarity in sequence to α-aminoisobutyric acid (Aib) containing membrane channel forming peptides of which alamethicin is the best studied example[3-6]. Fig. 1 lists a few representative sequences of membrane modifying peptides containing Aib residues. A characteristic feature of this class of peptides is the extensive microheterogeneity of the natural products, which often contain several closely related sequences[7-10]. Membrane pore forming abilities have been widely demonstrated for the larger peptides (19-20 residues) like alamethicins[4,5], suzukacillins[11] and trichotoxins[12]. However, there is no well documented report on the ionophoric properties of the shorter peptides (15-16 residues) like antiamoebins[2,13], emerimicins[14] and zervamicins[15]. Unpublished reports on the channel forming properties of these sequences have been referred to as footnotes in references 2, 14 and 15. Further, the absence of stable, well characterized conductance states has been noted for antiamoebin in black lipid membrane systems[16]. In this report, we describe the fractionation on reverse phase HPLC of antiamoebins into four distinct peptide fractions and demonstrate that these peptides facilitate translocation of Ca^{2+} ions across liposomal membranes. Further, all the four fractions function as uncouplers of oxidative phosphorylation in rat liver mitochondria. Preliminary circular dichroism (CD) spectra of three fractions are also presented.

Alamethicin I : Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

Suzukacillin : Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Aib-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol

Trichotoxin A-50 : Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Ala-Aib-Pro-Leu-Aib-D-Iva-Gln-Valol

Zervamicin IIA : Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol

Antiamoebin I : Ac-Phe-Aib-Aib-Aib-D-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-D-Iva-Hyp-Aib-Pro-Phol

Fig. 1—Sequences of some Aib containing membrane modifying peptides [The sequences shown correspond to a single major component]

Materials and Methods

Antiamoebin (isolate A) was the kind gift of Dr N. Narasimhachari, Medical College of Virginia, Virginia Commonwealth University and was originally isolated at Hindustan Antibiotics, Pune, India, as described earlier[1]. A second sample (isolate B) was obtained as gift from Dr K.S. Gopalakrishnan. Purification of antiamoebin (isolate A) was effected by reverse phase high performance liquid chromatography on a Lichrosorb RP-18 column (4 × 250 mm, 10 μm particle size) using an LKB HPLC system. For

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Abbreviations used: Aib, α-aminoisobutyric acid; CTC, chlorotetracycline; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether) N,N'-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulphonic acid.

each run, 1 mg of the peptide in 20 µl methanol was injected and gradient elution (65-85% MeOH-H_2O in 20 min, 85-95% MeOH-H_2O in 5 min) was used, with a flow rate of 0.8 ml min^{-1}. Peptides were detected at 226 nm. Purification of 50 mg of the peptide was achieved by repetitive injections and collection of fractions using a Superrac Fraction Collector. The individual peak fractions were reanalyzed for purity after each fractionation. After the first cycle of chromatography, Fr IB and Fr IIIB were contaminated with large amounts of Fr II. Fr II and Fr IVB were largely pure. Fr IB and Fr IIIB were rechromatographed to yield pure peptide fractions (Fig. 2).

Analytical HPLC comparisons of different antiamoebin isolates were also carried out on a Shimadzu LC-6A two pump system using a Shimpac 6 × 150 mm, C_{18} (5 µm) column using the following conditions. For each run, 20 µl of 1 mg ml^{-1} in methanol was injected. A gradient elution (65-75% MeOH-H_2O for 15 min, 75-85% MeOH-H_2O for 35 min, 85-95% MeOH-H_2O for 5 min and 95% MeOH-H_2O for 5 min) was used, with flow rate of 0.8 ml min^{-1}. UV detection was at 226 nm or 254 nm.

Small unilamellar liposomes were prepared from egg yolk lecithin according to published procedures[17]. The lecithin suspension in 5 m*M* HEPES - 100 m*M* NaCl (*p*H 7.0) was sonicated (Branson B-12 sonifier), under nitrogen at 40°C in 15 sec intervals, until optical clarity was achieved. The suspension was then centrifuged at 100,000*g* at 4°C for one hour. Two third of the supernatant was used in the transport experiments.

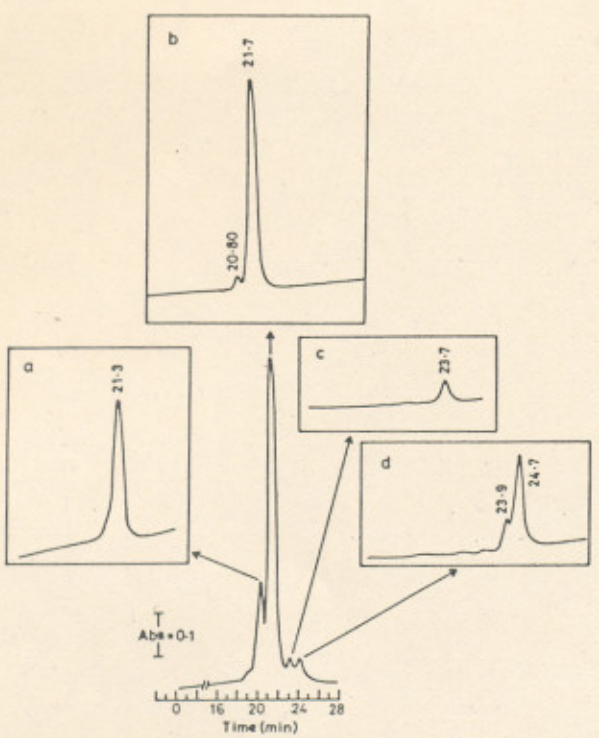

Fig. 2—HPLC profile for antiamoebin (1 mg peptide in 20 µl MeOH) [Conditions are as described under Materials and Methods. The peaks are named fractions IB, II, IIIB and IVB in the order of increasing retention times. The profiles shown in the boxes a-d are obtained after the first cycle of chromatography for Fr II and Fr IVB and the second cycle for Fr IB and Fr IIIB]

Fluorescence measurements were made on a Perkin Elmer MPF 44A fluorescence spectrometer. The Ca^{2+} transport assays using chlorotetracycline (CTC) as a probe were carried out as described earlier[18,19]. Assays were performed in a medium identical to that used in the preparation of the liposomes at 26°C. Peptide stock solutions were prepared in ethanol and concentrations were adjusted using absorbance values at 226 nm. The final alcohol concentration in the assay medium did not exceed 0.5% (v/v).

Mitochondria were isolated from rat liver by standard procedures[20]. Respiration rates were determined on a Hansatech oxygen electrode as described earlier[19,21]. The assay medium (2 ml) contained D(−)mannitol (220 m*M*), sucrose (70 m*M*), HEPES (2 m*M*), EGTA (0.5 m*M*), $MgCl_2$ (2.5 m*M*) and KH_2PO_4 (2.5 m*M*), *p*H 7.4.

Circular dichroism spectra were recorded on a JASCO J-500A spectropolarimeter linked to a data processor DP-501N. Sample concentrations were maintained at 2 mg ml^{-1} and a cell of 0.1 mm path length was used to record the spectra.

Results and Discussion

Fig. 2 shows an HPLC profile of antiamoebin (isolate A). Four components are clearly visible, while a fifth, minor, fast moving component is seen as a shoulder. In earlier studies of antiamoebin, two components labelled as antiamoebin I (98%) and II (2%) were identified by counter current distribution[2,13]. Subsequently on-line liquid chromatography, fast atom bombardment mass spectrometry of amino terminal nonapeptides of an antiamoebin hydrolysate, has suggested the existence of at least five sequence analogs[22]. The NMR spectra (^1H and ^{13}C) of fraction II (Fr II), obtained in the present study, have been shown to be in complete agreement with the composition suggested for antiamoebin I and a complete assignment of the ^1H NMR spectrum has been reported[23]. In this study, Fr IB, II, IIIB and IVB have been separated by HPLC. Fig. 2 (insets a-d) shows the HPLC profiles of purified fractions. Fr II and IVB showed minor cross-contamination with Fr IB and IIIB, respectively. A detailed comparison of the 270 MHz ^1H NMR spectra of Fr IB and Fr IIIB with that of Fr II suggests the following amino acid replacements:

Fr IB (Iva → Aib at position 5 or 12) and Fr IIIB (Hyp → Pro at position 10 or 13).

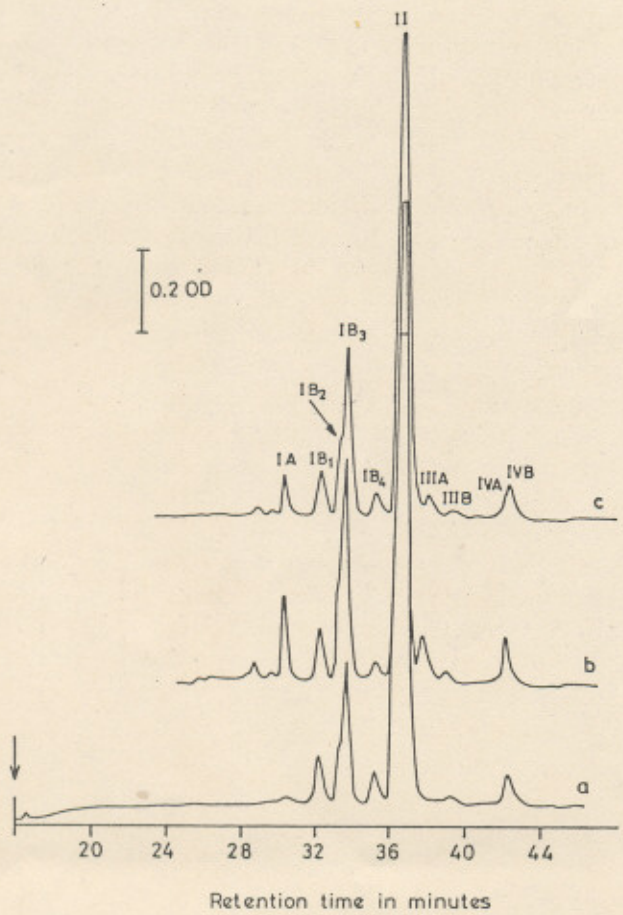

Fig. 3—Analytical HPLC profile of antiamoebin isolates A, B and an equimolar mixture of A & B [Peptide sample (20 µl; 1 mg ml^{-1}) was injected in each run. Detection was at 226 nm and gradient conditions were as described under Materials and Methods]

A careful HPLC comparison of a second antiamoebin sample (isolate B) revealed further heterogeneity (Fig. 3). Although Fr II was the major component in both isolates, the presence of distinct peptide components Fr IA, Fr IIIA and Fr IVA can be discerned in isolate B. Further, under conditions of higher resolution, Fr IB clearly shows the presence of additional components. These features are illustrated in the HPLC profile of a mixture of the two antiamoebin isolates. A distinctive feature of the antiamoebin Fr II sequence is the presence of N-terminal phenylalanine (Phe) and C-terminal phenylalaninol (Phol) residues. In order to establish the presence of both these residues in the various fractions, HPLC analysis was carried out with 254 nm detection corresponding to the Phe/Phol absorption band. Fig. 4 establishes that both antiamoebin isolates exhibit exactly similar HPLC profiles at both the detection wave-lengths (226 nm in Fig. 3 and 254 nm in Fig. 4), suggesting the presence of both aromatic residues in all the peptide components of antiamoebin.

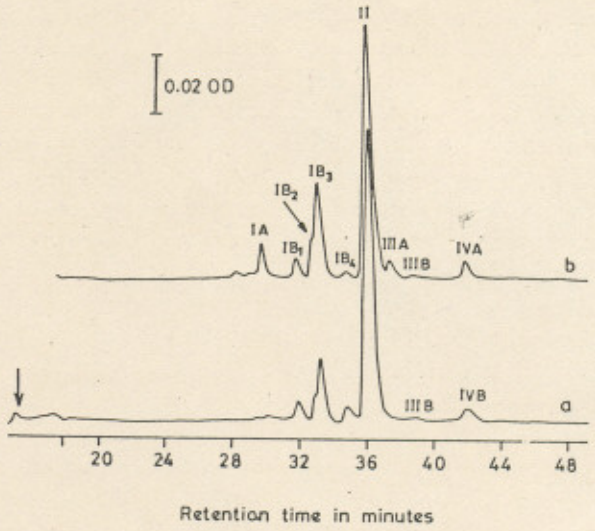

Fig. 4—Analytical HPLC profile of antiamoebin isolates A & B, detected at 254 nm [All other conditions were identical to those described for Fig. 3]

The ability of the various antiamoebin fractions from isolate A to facilitate transport of Ca^{2+} across lipid bilayer membranes was established using a CTC fluorescence assay in unilamellar egg phosphatidylcholine liposomes[18,19]. This technique utilizes the ability of CTC to permeate even those lipid membranes, which remain impermeant to cations in the absence of ionophores. Extravesicular CTC leads to a large basal fluorescence necessitating extensive background suppression. A critical analysis of this method has been published[24]. Fig. 5 illustrates the effect of addition of peptides on the fluorescence of CTC internalized in these liposomes. At low peptide concentrations (~ 4.7 µ*M*), the ability of the various fractions to induce Ca^{2+} influx into liposomes follows the order Fr IVB ~ Fr II ⩾ Fr IIIB > Fr IB. At higher peptide concentrations (~ 13.3 µ*M*), these differences between the various fractions are minimized (Fig. 5). Fig. 6 shows the steady-state fluorescence spectra of CTC in the liposomes and compares the intensity changes due to Ca^{2+} leakage with that induced by Fr II. The intensity changes observed on complete permeation of Ca^{2+} into liposomes by sonication are also shown. The Ca^{2+} transporting abilities of the four fractions are compared in Table 1, using these controls for quantitation. The results clearly establish that Fr IVB and Fr II are significantly more efficient in effecting Ca^{2+} flux across lipid membranes at low peptide concentrations.

The four peptide fractions also act as effective uncouplers of oxidative phosphorylation in rat liver mitochondria (Fig. 7). The influence of these fractions on the mitochondrial O_2 consumption rates are com-

pared in Table 1. The order of uncoupling activity, as measured by the decrease in the respiratory control index (RCI),is shown to be Fr IVB ~ Fr II > Fr IIIB > Fr IB. Mitochondrial uncoupling by the peptide channel formers, alamethicin and gramicidin A, has been suggested to occur by dissipation of transmembrane proton gradients[25,26]. Indeed, such channels have been suggested to contain structured "ice-like" water, which can function as a particularly effective proton conductor[25]. The similarities in the sequences of the major fraction of antiamoebin (Fr II) and alamethicin suggest that a similar mechanism could also be considered in the present case. It may be noted that the mitochondrial O_2 consumption assay has been carried out in a medium containing the Ca^{2+} chelator, EGTA. Thus it is unlikely that Ca^{2+} transport is involved in the uncoupling activity. Detergent-like activity resulting in nonspecific membrane damage is also unlikely at the low concentrations of peptide used[19].

It is of interest to note that the same relative order of membrane modifying activity is observed in both the

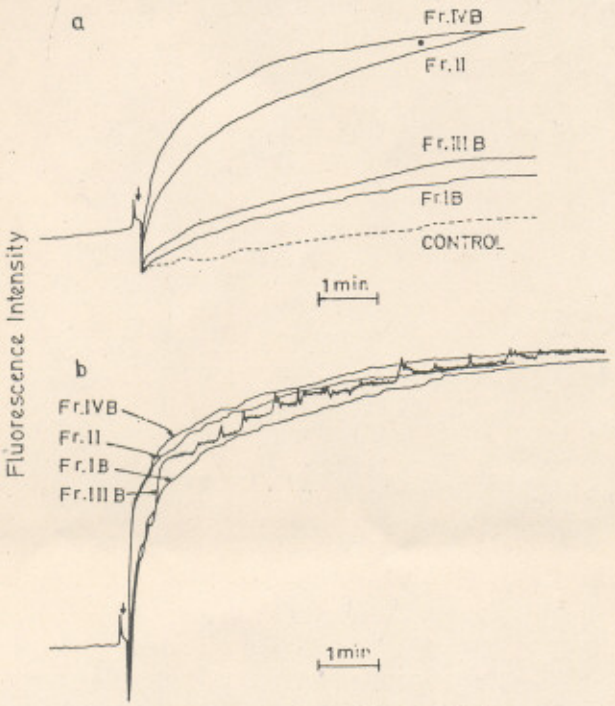

Fig. 5—Time-dependent changes in CTC-Ca^{2+} fluorescence in response to peptide addition [An increase in fluorescence corresponds to an influx of Ca^{2+} into the liposomes. Lipid was 0.35 m*M*. Peptides were added to a final A_{226} of 0.02 (a) corresponding to a concentration of ~ 4.4 μ*M* and 0.06 (b) corresponding to a concentration of ~ 12.6 μ*M*. The arrow shows the point of peptide addition]

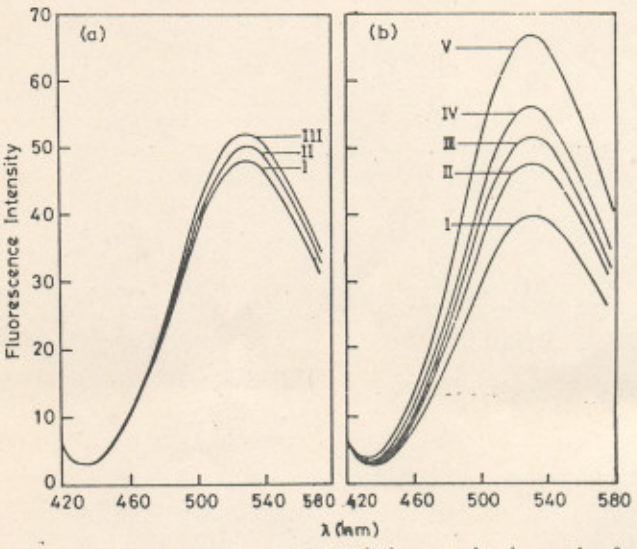

Fig. 6—Fluorescence spectra recorded at varying intervals after the addition of (a) Ca^{2+} to liposome-CTC mixtures [(I), 10 sec; (II), 5 min; (III), 15 min after Ca_{2+} addition] and (b) Fr II to liposome-CTC-Ca^{2+} mixtures [(I), 0 sec; (II), 10 sec; (III), 5 min; (IV), 15 min; (V), after sonication. Fr II was added to a final A_{226} of 0.02 corresponding to a peptide concentration of ~ 4.4 μ*M*. Spectra recorded after sonication represents limiting (100%) enhancement of CTC fluorescence]

Table 1—Mitochondrial Uncoupling and Ca^{2+} Transport Activity of Antiamoebin Fractions

Fraction	Mitochondrial oxygen consumption rate (ng atoms min^{-1})*			Decrease(%) in RCI†	Enhancement in F_{530}‡
	State 3	State 4	After peptide addition		
Fr. IB	204	72	96	25.00	48.3
Fr. II	192	66	270	75.5	60.5
Fr. IIIB	198	72	132	45.4	34.7
Fr. IVB	186	84	288	70.8	74.2

* Mitochondrial concentration in assay mixture was 0.34 mg protein ml^{-1}. Peptides were added such that the absorbance at 226 nm (A_{226}) was 0.009 in the assay mixture, which corresponds to a peptide concentration of ~ 1.9 μ*M*.

† Respiratory control index (RCI) is defined as the rate of oxygen consumption during state 3/rate of oxygen consumption during state 4.

‡ Fluorescence at 530 nm (F_{530}) was measured 15 min after peptide addition. Values are expressed as percent of limiting fluorescence enhancement at 530 nm, which is the enhancement obtained following the sonication of the suspension containing liposome, CTC and Ca^{2+}. Phospholipid concentration was 0.35 m*M*. Peptides were added such that the final A_{226} in the assay mixture was 0.02, corresponding to a concentration of ~ 4.4 μ*M*. The F_{530} enhancement at 15 min as a result of passive leakage of Ca^{2+} amounted to 26.9% of the limiting enhancement.

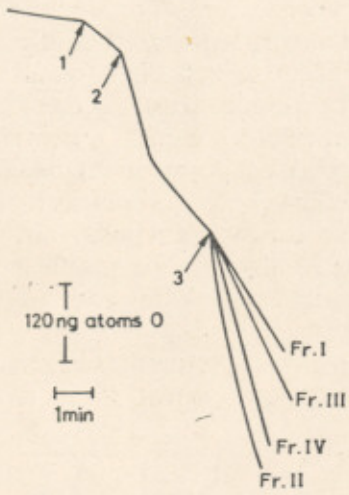

Fig. 7—Effect of antiamoebin fractions on state 4 respiration in rat liver mitochondria [Peptide concentrations are adjusted to a final A_{226} of 0.009, corresponding to a concentration of ~ 1.9 μM. Points 1, 2 and 3 indicate additions of succinate (7.5 mM), ADP (150 μM) and peptide respectively. Mitochondrial concentration, 0.34 mg protein ml^{-1}]

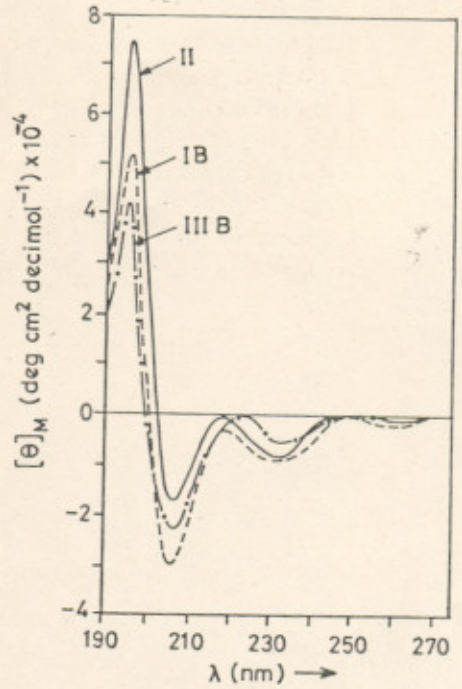

Fig. 8—Circular dichroism spectra of antiamoebins IB, II and IIIB in methanol [A 2 mg ml^{-1} sample was used to record the spectrum using a cell of 0.1 mm path length. All spectra are baseline subtracted]

Ca^{2+} transport and mitochondrial uncoupling assays at low peptide concentrations. A similar correspondence between the two assay systems has been demonstrated for synthetic peptide fragments of alamethicin[19]. This observation suggests that insertion of antiamoebin into lipid membranes followed by pore formation is likely to be the primary event in both sets of experiments. The differences observed between the various fractions, at low concentrations, may reflect differences in the lipid phase aggregation of these peptides to form functional pores[3,27]. At high concentrations, pore formation is presumably essentially complete in all cases, resulting in similar activities for all fractions.

Gross conformational differences between peptides of similar sequence can be probed by CD. Fig. 8 compares the CD spectra of antiamoebin fractions IB, II and IIIB. All the three fractions exhibit CD spectra characterized by a weak negative band at 235 nm, a second stronger negative band at 207 nm and a strong positive band at 195 nm. Although the exciton split π-π^* components at 195 and 207 nm are characteristic of α-helical polypeptides, the diminished magnitudes of the ellipticity values and the weak, long wavelength negative band are quite different from the CD patterns for classical helical structures[28]. Indeed the "nonhelical" character of the antiamoebin CD spectrum has been noted earlier[16]. However, NMR studies of Fr II have favoured a highly folded, helical conformation in organic solvents, stabilized by a large number of intramolecular 4 → 1 hydrogen bonds[23]. Further, the crystal structure of a synthetic 16 residue zervamicin analogue peptide, which possesses a similar disposition of Pro residues at the C-terminal, has demonstrated a helical conformation in the solid state[29]. While the antiamoebins undoubtedly adopt largely helical structures in solution, the unique nature of the CD spectra possibly reflect deviations from ideal helical disposition of the peptide chromophores. The similarities in the CD spectra of the three antiamoebin fractions in Fig. 8 suggest that there are no profound conformational differences. Nevertheless, appreciable differences are observed in membrane modifying activities, suggesting that subtle structural effects modulate the observed effects on lipid membranes. Although minor sequence variations may have little effect on backbone conformation, the precise positioning of sidechains on helix surfaces can greatly influence helix association, a feature necessary for membrane modifying activity[27,30]. Indeed, recent studies of the aggregation of hydrophobic helical peptides in the solid state have demonstrated that sequence variations can dramatically influence helix packing in crystals[31-34].

The present study clearly establishes that the antiamoebins can enhance cation permeability across lipid bilayer membranes. A detailed rationalization of the differences in the activities of the various fractions must await a complete characterization of the molecular structures of these peptides and their aggregated

forms. Nevertheless, it is pertinent to note that relatively minor sequence changes[22] can result in subtle differences in membrane modifying activity. Indeed, different conductance properties have been reported for two polypeptide fractions isolated from natural alamethicin sample[35].

Acknowledgements

One of us (MKD) was the recipient of a fellowship from the Indian Council of Medical Research and another (KK) of a research associateship from the Department of Biotechnology. This research was supported by a grant from the Department of Science and Technology, Government of India.

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