Pure & Appl. Chem., Vol. 62, No. 7, pp. 1417–1420, 1990.
Printed in Great Britain.

Structural chemistry and membrane modifying activity of the fungal polypeptides zervamicins, antiamoebins and efrapeptins

K. Krishna, M. Sukumar and P. Balaram

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

Abstract - The fungal polypeptides zervamicins, antiamoebins and efrapeptins have been fractionated into several polypeptide components by HPLC. A zervamicin fraction lacking tryptophan has been characterized and shown to possess an N-terminal leucine residue. The conformations of zervamicin IIA and a synthetic analog in solution are compared with those determined for the related peptide, antiamoebin. The results are consistent with a completely helical structure for the apolar analog of zervamicin in chloroform, with partial unfolding in dimethylsulfoxide. A similar conformation has been determined for natural zervamicin IIB. A synthetic analog of efrapeptin forms a continuous helix in apolar solvents while, partial unfolding is seen in polar solvents. Natural zervamicin is an effective uncoupler of mitochondrial oxidative phosphorylation. Significant differences in membrane modifying activity are noted for the natural peptide and the synthetic apolar analog of zervamicin.

Peptides of fungal origin containing a high proportion of α-aminoisobutyric acid (Aib) have been the focus of intensive investigations (ref. 1). Alamethicin, a 20- residue peptide produced by trichoderma viride, forms voltage gated transmembrane channels in phospholipid bilayer membranes (ref. 2) and is the best studied member of this class of molecules (ref. 3-6). A feature of the alamethicin sequence (Fig. 1) is the presence of a preponderence of hydrophobic amino acids, with the central Gln (7) residue and the C-terminal tripeptide containing the only polar moieties. In contrast, the shorter

Alamethicin I (II)	:	Ac-Aib-Pro-Aib-Ala-Aib-Ala(Aib)-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol.
Antiamoebin I (II)	:	Ac-Phe-Aib-Aib-Aib-D-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-D-Iva-Hyp-(Pro)-Aib-Pro-Phol.
Zervamicin IIA (IIB)	:	Ac-Trp-Ile-Gln-Aib(Iva)-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol.
Efrapeptin D	:	Ac-Pip-Aib-Pip-Aib-Aib-Leu- β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-Aib-X.

Fig. 1. Sequences of some Aib containing antibiotics

peptide antibiotics, antiamoebins (ref. 7) and zervamicins (ref. 8), contain several residues with polar functional groups (Thr, Hyp) and also have a cluster of Pro/Hyp residues at the C-terminal end of the molecule. The efrapeptins (from tolypocladium inflatum) are poorly characterized members of the Aib containing group of peptide antibiotics. The partial sequence of a major polypeptide component of the efrapeptins (Fig. 1) has been reported (ref. 9), but the nature of the C-terminal basic residue remains to be established. The efrapeptins also contain the unusual amino acids L-pipecolic acid (Pip) and β-alanine (β-Ala). Furthermore, while alamethicin, zervamicins and antiamoebins function as mitochondrial uncouplers of varying efficiencies (ref. 10, 11), the efrapeptins are potent inhibitors of mitochondrial ATP-synthase (ref. 12). Structural investigations of Aib containing synthetic model peptides have provided definitive evidence for the role of Aib in promoting helical backbone folding (ref. 13-15). This has led to channel models for

alamethicin composed of clusters of largely hydrophobic helices in lipid bilayers (ref. 1, 3)., with voltage gating suggested to arise from interaction of the macroscopic helix dipole with the applied electric field (ref. 16, 17). This report summarises some studies on antiamoebins, zervamicins and efrapeptins.

PEPTIDE ISOLATION ANF PURIFICATION

Zervamicins were isolated from cultures of emericellopsis salmosynnemata (strain CMI 58330) (ref. 18). An HPLC profile of a mixture of "zervamicins" is shown in Fig. 2. Peaks at 23-26 min. arise from acidic zervamicin components, which have been previously identified as possessing a single Glu residue at position 3 (ref. 8). The component at ~26 min.

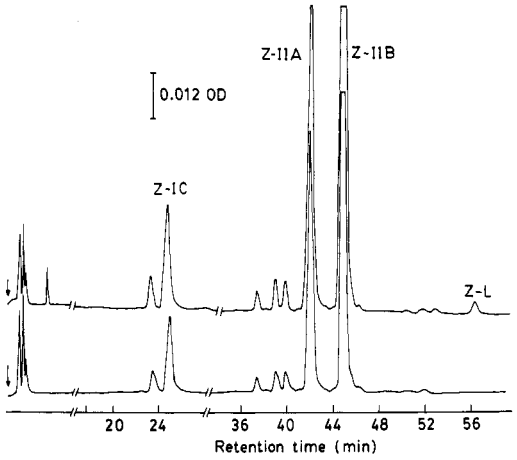

Fig. 2. HPLC profile of a mixture of zervamicins. C18, 5 μ, 6 x 150 mm column. Gradient of methanol-water, 65-75% methanol in 15 min., 75-78% in 12 min., 78-81% in 60 min. Detection 280 nm (bottom) and 226 nm (top).

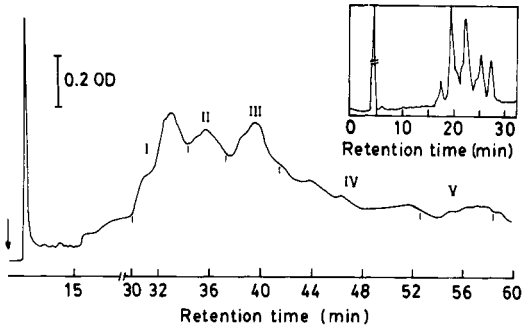

Fig. 3. HPLC profile of a mixture of efrapeptins. C8, 10 μ, 4 x 250 mm column. Gradient of methanol-water-0.1% TFA, 60-70% methanol in 15 min., 70-75% in 10 min., 75-80% in 15 min. (inset) C4, 10 μ, 4.6 x 250 mm column. Gradient of methanol-water-0.1% TFA, 65-75% methanol in 30 min., 75-95% in 10 min. Detection 226 nm.

corresponds to the peptide zervamicin IC (ZIC). Peaks between 36-52 min. are all neutral peptides with the major components corresponding to the previously sequenced peptides, zervamicins IIA (ZIIA) and IIB (ZIIB), respectively. The peak at 57.4 min. is a peptide which lacks the amino terminal Trp residue, characteristic of the zervamicins sequenced earlier (ref. 8). The absence of Trp is clearly suggested by a comparison of the HPLC profiles obtained by detection at 226 nm and 280 nm (Fig. 2). This peptide (Z-L), has been shown to possess a Leu residue in place of Trp at the amino terminus and a tentative sequence is Ac-Leu-Ile-Gln-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol. Supporting evidence for this assignment comes from the observation of a nuclear Overhauser effect (NOE) between the amino terminal blocking acetyl methyl protons and the NH proton of a Leu residue. The molecular mass, 1764 daltons, (FAB mass spectrum) is also consistent with this sequence assignment. Efrapeptins were isolated from cultures of tolypocladium inflatum niveum (strain IMI 202309) (ref. 19). The crude peptide mixture, inhibited oxidative phosphorylation in rat liver mitochondria. Fig. 3. shows an HPLC profile of the peptide mixture. The peaks were extremely broad and overlapping under a wide variety of conditions. Preliminary fractionation into components I-IV (Fig. 3) and analysis by mass spectrometry revealed that I has a major component with a molecular mass of 1619 and a minor component of 1605 daltons. Fractions II, III and IV have components with molecular masses of 1633, 1647, 1661 and 1675, respectively. The strongly hydrophobic and basic nature of the peptide limited further HPLC purification under the conditions used. The inset to Fig. 3 shows an HPLC profile obtained on a less hydrophobic C4 column. Work in progress is directed towards elucidating the sequence relationships of the efrapeptin components. Antiamoebins, from emericellopsis poonensis Thirum, were obtained as described elsewhere (ref. 11).

STRUCTURAL CHEMISTRY

The Pro/Hyp rich C-terminal tail in the antiamoebins and zervamicins should lead to an interruption of the continuous intramolecular hydrogen bonding pattern expected in helical Aib peptide structures (ref. 13-15). In the case of efrapeptins the presence of pipecolic acid (Pip), a proline homolog, and β-alanine (β -Ala) should lead to deviations from ideal helical conformations. An apolar synthetic analog of zervamicin IIA (Boc-Trp-Ile-Ala-Aib-Ile-Val-Aib-Leu-Aib-Pro-Ala-Aib-Pro-Aib-Pro-Phe-OMe) has been studied by ^{1}H-NMR. Analysis of temperature dependence and solvent perturbation of NH chemical shifts in DMSO and chloroform solutions suggested that a large number of amide NH groups are inacessible to solvent. In chloroform, with the exception of Trp (1) and Ile (2) NH groups, all the other NH groups are solvent shielded consistent with a completely helical conformation for the synthetic peptide. This is further supported by the observation of N_iH <-> $N_{i+1}H$ NOEs throughout the sequence, in chloroform. A partial unfolding of the structure at the amino terminus is observed in the strongly solvating medium DMSO, with the NH groups of Ala (3), Aib (4) and Ile (5) also being exposed to solvent. Interresidue NOEs of the type C^{α}_iH <-> $N_{i+1}H$, observed for residues 1 to 4, suggest an extended conformation for this portion of the molecule, in DMSO. X-ray diffraction studies of crystals obtained from methanol-water mixtures revealed a completely helical structure in the solid-state [Fig. 4] with all the prolines being comfortably accomodated into a 3_{10} helical structure, which has been termed as the " β-bend ribbon" (ref. 20). Natural zervamicin IIA has also been examined in detail by 2D NMR at 270 MHz and 500 MHz, in DMSO and methanol. Evidence for helical folding of residues 5 to 16 is obtained, with a partially unfolded amino terminus.

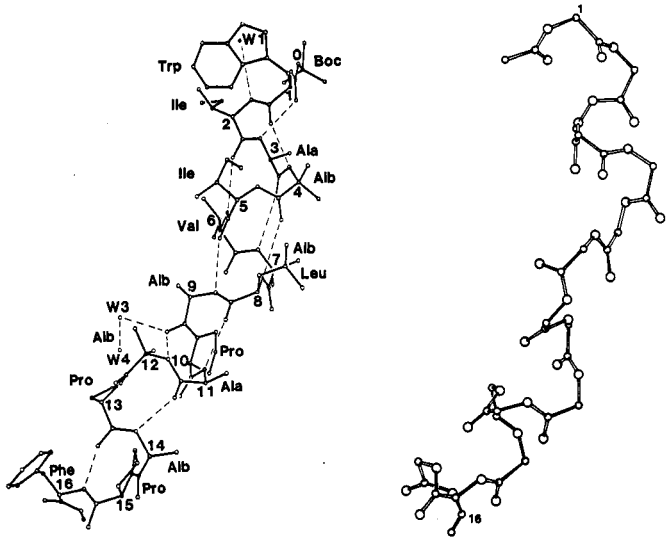

Fig. 4. (left) Molecular conformation of a synthetic apolar zervamicin analog, in crystals (ref. 20). (right) Backbone conformation of antiamoebin I derived from NMR data (ref. 11).

An NMR study of the major component of antiamoebin has indeed, also provided firm evidence for a largely helical conformation. NOE evidence supports a Type II β-turn at the N-terminus [Phe(1)-Aib(2)], a segment of left-handed helix followed by a Type II β-turn at Leu-(7)-Aib(8) and a right handed helix for residues 9 to 16 (ref. 11). A backbone conformation generated using these NMR derived parameters bears a remarkable resemblence to the crystal structure of the synthetic zervamicin analog (Fig. 4), although the precise torsional angles used are different. However, the Pro/Hyp rich C-terminus structures are almost identical. Structural information on the efrapeptins has been derived indirectly, using a synthetic 16-residue analog peptide sequence (Boc-Pro-Aib-Pro-Aib-Aib-Leu- β-Ala-Gly-Aib-Aib-Pro-Aib-Gly-Leu-Aib-Aib-OMe) in which the following replacements have been made; Pip to Pro, Iva to Aib. The unidentified C-terminal residue is absent. NMR analysis of this peptide in chloroform and DMSO solutions provides clear evidence for the solvent inaccessibility of all the amide protons except Aib (2) NH in $CDCl_3$. In DMSO, Gly (8), Aib (9) and Aib (15) NH groups are solvent exposed, in addition to Aib (2) NH. The chloroform structure is clearly a continuous helix in which both β-Ala (7) and Pro (11) are accomodated in the helix. The additional CH_2 group in β-Ala is presumably accomodated within an eleven or fourteen membered hydrogen bonded ring as opposed to the ten or thirteen membered rings in ideal 3_{10}- or α-helical structures, respectively.

The fragility of the structure in the vicinity of the central Leu- β -Ala-Gly region is evident in DMSO, where the Gly (8) and Gly (9) NH groups are solvent exposed. NMR studies on the hexapeptide Boc-L-Pip-Aib-Gly-Leu-Aib-Aib-OMe, (residues 12-16 of efrapeptin) support a helical conformation similar to that observed for the analog peptide with Pro in the place of Pip. Crystal structures of model pipecolyl peptides suggest that the conformational properties of Pip residues are fairly similar to those of proline (ref. 21).

MEMBRANE MODIFYING ACTIVITY

Both antiamoebin and zervamicin exhibit mitochondrial uncoupling activity suggesting their incorporation into the inner mitochondrial membrane. The synthetic, apolar zervamicin analog is appreciably less effective as an uncoupler. Similar behaviour was also observed in studies of peptide induced Ca^{2+} fluxes across liposomal membranes. An interesting feature of the zervamicins, particularly the synthetic analog is the tendency to cause liposome aggregation as evidenced by a dramatic increase in Rayleigh scattering on addition of peptide to liposomes. Single channel conductance activity in planar bilayers has also been observed for zervamicins IIA, IC and Z-L as well as the synthetic analog peptide. The life times of the channels formed by the synthetic apolar analog are dramatically shortened compared to those for the polar peptides (M.S.P. Sansom, personal communication). This is consistent with the involvement of polar side-chain functionalities in stabilizing channels formed by helix association in phospholipid bilayers.

Acknowledgements We are grateful to R. Rani and S. Agarwalla for the results of efrapeptin purifications and Professors B. Gibson, S. Iwasaki and M. S. P. Sansom for communicating mass spectral and electrochemical results.

REFERENCES

1. M.K. Mathew and P. Balaram, Mol. Cell. Biochem., 50, 47-64 (1983).
2. P. Mueller and D.O. Rudin, Nature, Lond., 217, 713-719 (1968).
3. R. Nagaraj and P. Balaram, Acc. Chem. Res., 14, 356-362 (1981).
4. R.O. Fox. Jr. and F.M. Richards, Nature, 300, 325-330 (1982).
5. G. Menestrina, K-P. Voges, G. Jung and G. Boheim, J. Membrane Biol. 93, 111-132 (1986).
6. J.E. Hall, I. Vodyanoy, T.M. Balasubramanian and G.R. Marshall Biophys. J. 45, 233-247 (1984).
7. R.C. Pandey, H. Meng, J.C. Cook, Jr., K.L. Rinehart, Jr., J. Am. Chem. Soc. 99 5203-5205 (1977).
8. K.L. Rinehart, Jr., L.A. Gaudioso, M.L. Moore, R.C. Pandey, J.C. Cook, Jr., M. Barber, R.D. Sedgwick, R.S. Bordoli, A.N. Tyler and B.N. Green J. Am. Chem. Soc. 103, 6517-6520 (1981).
9. D.A. Bullough, G.G. Jackson, P.J.F. Hendersen, F.H. Cottee, R.B. Beechey and P.E. Linnet, Biochem. Internat. 4, 543-549 (1982).
10. M.K. Mathew, R. Nagaraj and P. Balaram Biochem. Biophys. Res. Commun. 98, 548-555 (1981).
11. M.K. Das, S. Raghothama and P. Balaram, Biochemistry, 25, 7110-7117 (1986).
12. R.L. Cross and W.E. Kohlbrenner, J. Biol. Chem. 253, 4865-4873 (1978).
13. B.V.V. Prasad and P. Balaram, CRC Crit. Rev. Biochem. 16, 307-348 (1984).
14. C. Toniolo and E. Benedetti, ISI Atlas of Science: Biochemistry, 1, 225-230 (1988).
15. K. Uma and P. Balaram, Ind. J. Chem. 28B, 705-710 (1989).
16. M.K. Mathew and P. Balaram, FEBS Lett. 157, 1-5 (1983).
17. G. Boheim, W. Hanke and G. Jung, Biophys. Struct. Mech. 9, 181-191 (1983).
18. A.D. Argoudelis and L.E. Johnson, J. Antibiot. 27, 274-282 (1974).
19. P.E. Linnett and R.B. Beechey, Meth. Enzymol. 55, 472-518 (1979).
20. I.L. Karle, J. Flippen-Anderson, M. Sukumar and P. Balaram, Proc. Natl. Acad. Sci., USA 84, 5087-5091 (1987).
21. C. Toniolo, R. Bardi, A.M. Piazzesi, M. Crisma, P. Balaram, M. Sukumar and P.K.C. Paul, "Peptides 1988" (G. Jung and E. Bayer, Eds.) 477-479, Walter de Gruyter, Berlin. New York (1989).