Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 384-390

CONFORMATIONAL CHANGE ON CALCIUM BINDING BY THE LIPOPEPTIDE ANTIBIOTIC AMPHOMYCIN. A C.D. AND MONOLAYER STUDY

J.H.LAKEY*, R.MAGET-DANA AND M.PTAK

Centre de Biophysique Moléculaire, CNRS and the Université d'Orléans, 1A, Avenue de la Recherche Scientifique, 45071 Orléans Cédex 2, France

Received November 12, 1987

The acidic linear lipopeptide amphomycin is a calcium dependent antibiotic which is thought to bind to carrier lipids such as dolichol monophosphate. The actual role of Ca^{++} is not definitely established and in this article we have examined the peptides interactions with a range of divalent cations. By CD we have shown that a conformational change is induced by Ca^{++}, Sr^{++} and Ba^{++} but not by Mg^{++}, Zn^{++}, Cd^{++} or Gd^{+++} . Monolayer studies show a decrease in molecular area and an increase in film stability when the subphase contains Ca^{++}. The ensemble of results provides preliminary evidence for the formation of a β hairpin structure on ion binding (Ka $(Ca^{++}) \simeq 2.4 \times 10^{3} M^{-1}$) which could enhance amphomycin's bilayer solubility.

Amphomycin is an acidic linear lipopeptide antibiotic (Fig 1) produced by Streptomyces canus (1,2) and its in vitro antibacterial activity, like that of A21978C (3), is highly dependent upon the ambient calcium concentration (4). Amphomycin inhibits cell wall biosynthesis in Gram-negative bacteria at the level of phospho-MurNAc-pentapeptide transferase (5), an enzyme which plays a role in the transfer of peptidoglycan precursors across the cytoplasmic membrane. In addition it blocks similar systems in eukaryotes and Bannerjee et al (6) showed that it inhibited dolichol phosphate glycosylation in calf brain microsomes. They suggested that amphomycin may form a calcium dependent complex with the various carrier lipids in these reactions. This would be analogous to the model for the action of bacitracin which employs a divalent cation in its binding to the carrier lipid's phosphate moiety (7). Here we investigate the interaction of amphomycin itself

*To whom correspondence should be sent, present address : European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, F.R.G.

0006-291X/88 $1.50

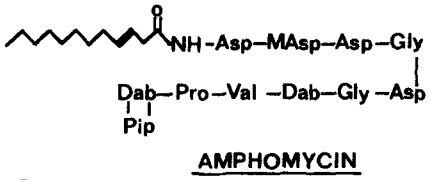
with divalent cations by the use of circular dichroism in aqueous solution and surface balance techniques at the air-water interface.

METHODS

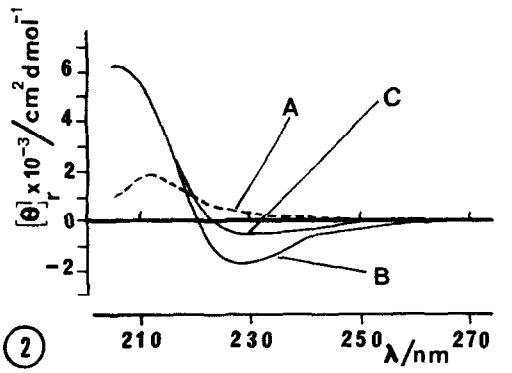
Sodium amphomycin, a gift from F Hyldig-Nielsen of H. Lundbeck Co. Copenhagen, was further purified by HPLC (Waters Inc.) on a C18 column with methanol/ water (75/25) as solvent. Circular dichroism measurements were carried out on unbuffered solutions in which the pH ranged from 6.0 to 7.0. Amphomycin concentrations (nominally 2 x 10^{-4}M) were determined by OD at 220 nm followed by correction for subsequent salt addition from concentrated stock solutions (volume changes < 5 %) ; samples were then equilibrated overnight. Spectra were recorded from samples of 1 mm path length at 25°C on a Jovin-Yvon IV autodichrograph. Each spectrum is the result of between 5 and 15 accumulated scans. Monolayer experiments were performed using a Langmuir film balance system previously described (8). For surface pressure - area isotherm measurements Amphomycin, dissolved in hexafluoro-isopropanol, was spread onto the subphase with a 50 µl Hamilton microsyringe. After evaporation of the solvent the monolayer was compressed at approximately 15 A^2 $molec^{-1}$ min^{-1}. The equilibrium spreading pressure (πe) was measured at constant area (20 cm^2) by depositing a small amounts of amphomycin on the aqueous surface until no further increase in surface pressure was observed (9).

RESULTS

Circular dichroism The lack of aromatic residues in amphomycin (Fig.1) results in a flat CD signal at wavelengths greater than 250 nm. Fig.2 (A) shows that the CD spectrum of sodium amphomycin in distilled water has only a weak positive ellipticity $[\theta]$ at 210 nm. On the addition of $MgCl_2$, $CdCl_2$ and $ZnCl_2$ at concentrations up to 50 mM, $GdCl_3$ (15 mM) or EDTA (1 mM) the



(1)



(2)

FIGURE 1 . Structure of Amphomycin. (after ref. 2) Dab = Diaminobutyric acid ; Pip = D - Pipecolic acid ; MAsp = Methyl aspartic acid.

FIGURE 2 . CD spectra of amphomycinin H_2O. Temperature 25°C path length 1 mn. A) Na-amphomycin B) Na-amphomycin + 10 mM $BaCl_2$ C) Na-amphomycin + 10 mM $CaCl_2$ $[\theta]$ is the molar ellipticity per peptide bond.

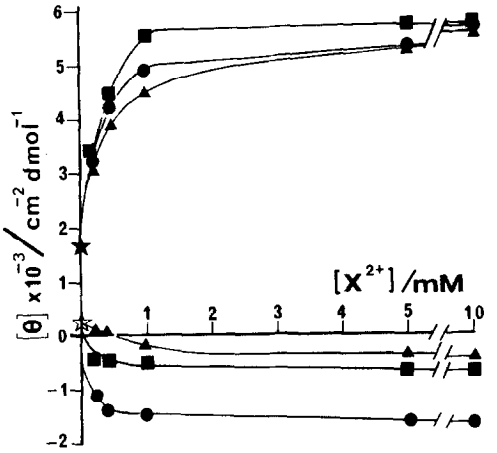


FIGURE 3. CD Spectral maxima [θ] as a function of divalent cation concentration [X]. Temperature 25°C path length 1 mm, ▲ $CaCl_2$; ■ $SrCl_2$; ● $BaCl_2$. Upper curves start from ★ and are [θ] at 210 nm Lower traces start from ☆ and are [θ] values at 228 nm. Na-amphomycin concentration = 2 x 10^{-4} M.

spectrum remained unchanged. However on adding $CaCl_2$ the spectrum becomes more convoluted with an increase in the positive band at 210 nm and the appearance of a negative extremum at 228 nm (Fig.2 (C)). This result clearly provides strong evidence for a binding of calcium ions by amphomycin and is reproduced by the next two Group II elements, Sr and Ba, with the magnitude of the negative band at 228 nm increasing in the order $Ca^{++} < Sr^{++} < Ba^{++}$. (Fig.2(B), Fig.3). The limiting positive values at 210 nm for all three ions are similar but their respective slope values reflect different binding affinities. If at this stage we assume that the increase in [θ] is proportional to the concentration of a 1:1 calcium amphomycin complex, we can calculate the association constant Ka (10) such that K(Ca)= 2.4 x $10^3 M^{-1}$, K(Sr)= 4.1 x $10^3 M^{-1}$ and K(Ba)= 3.3 x $10^3 M^{-1}$.

Monolayers Amphomycin is capable of forming stable monolayers. When crystals were placed on an aqueous surface, the amphomycin molecules spread spontaneously causing a rapid increase in the surface pressure which reached a maximum value π_m and then decreased to a final stable value defined as the equilibrium spreading pressure (π_e) Fig.4A. Figure 4B illustrates the various π values obtained with a range of cations in the subphase. Both the π_m and π_e values reached when amphomycin is spread on 1 M $CaCl_2$ are lower (22.5 and 14 mN m^{-1} respectively) than those recorded on an $MgCl_2$ (30 and 20 mN

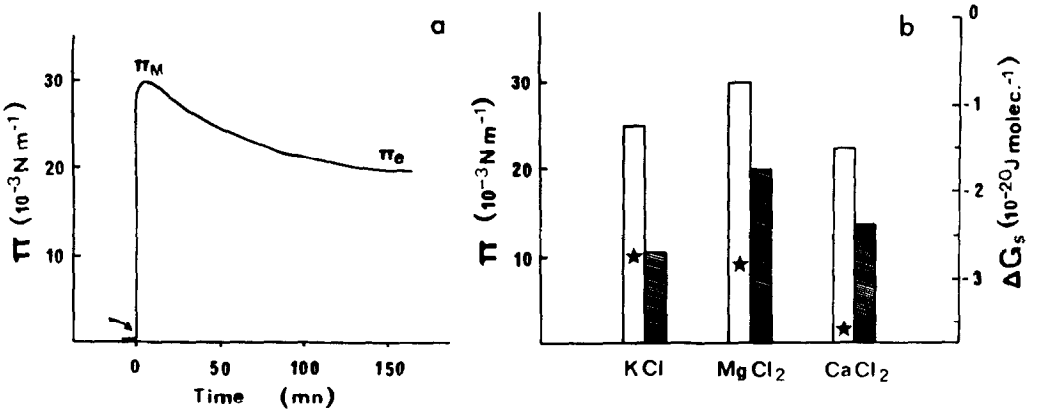


FIGURE 4 . Spreading of amphomycin molecules from a crystal at the air water interface. A) Variation of the surface pressure with time after crystal addition on 1 M $MgCl_2$. B) Comparison of some of the spreading parameters on various ionic subphases. Open bars : π_m, shaded bars = π_e ★= ΔG_s.

m^{-1}). However it is the reduction in the molecular free energy on spreading that is the true measure of a molecules tendency to form a monolayer. Hence we have determined the molecular area (Ae) of the lipopeptide at the pressure π_e from the surface pressure-area (π-A) curve (Fig. 5) and calculated the reduction in the molecular free energy (ΔGs) thus (9) :

$$\Delta Gs = -Ae.\pi_e \qquad (1)$$

The results presented in Fig.4B show that the systems free energy is reduced to a greater extent by calcium than by either of the two other cations. The shape of the π-A curve provides additional information in that the mean

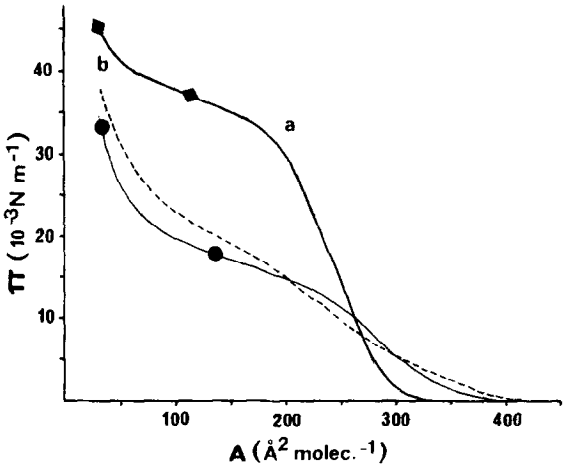


FIGURE 5 . Isotherm compression curves of amphomycin films on various ionic subphases ●— = 1 M KCl ; --- = 1 M $MgCl_2$; ◆— = 1 M $CaCl_2$. a and b indicate points at which the film compressibility was calculated (see text).

molecular area of amphomycin in the uncompressed state can be found at the lift-off of the curve (π=0). This shows that the change from a $MgCl_2$ to a $CaCl_2$ subphase causes a reduction in cross-sectional area from 420Å^2 to 350Å^2. All the π-A curves exhibit a transition region preceded by an initial steep rise in π and followed by a second steep increase. On 1 M $CaCl_2$ this region begins at 33 mN m^{-1} and ends at 40 mN m^{-1} whilst on $MgCl_2$ it runs from 16 to 25 mN m^{-1} (KCl values = 13 and 22.5 mN m^{-1} respectively). As these changes in the form of the curve are related to how easily the film is compressed we can quantify the changes better by calculating the compressibility of the film β :

$$\beta = 1/A * (dA/d\pi)T \qquad (2)$$

just before (point a) and just after (point b) the transition. This shows that for both KCl and $MgCl_2$ subphases the β values at a and b are comparable (βa = 23.5 N^{-1}m and βb = 38 N^{-1}m on KCl ; βa = 26.5 N^{-1}m and βb = 25.5 N^{-1}m on $MgCl_2$). This implies that the difference in the A values before and after the transition is related only to a difference in molecular orientation. On the other hand when the cation in the subphase is calcium the compressibility of the film is much higher after the transitional point than before it (βa = 12.5 N^{-1}m and βb = 83.5 N^{-1}m). This cannot be explained simply in terms of molecular orientation.

DISCUSSION

Amphomycin can be considered as a unique ionic amphiphile, the properties of which depend on the conformation of its charged peptide moiety. The presence of four Asp residues suggest the possibility of complex formation with cations and especially with divalent species such as Ca^{++}. The CD results presented here indicate that amphomycin interacts strongly and selectively with certain divalent cations. Of these strontium is bound most tightly, but it is calcium that has most biological significance. The monolayer free energy decreases, which result from more or less specific interfacial interactions between the film molecules and the subphase (11), also provide clear evidence that the peptide interacts more strongly with Ca^{++} than with

Mg^{++} or K^{+}. The form of the CD curves is similar to that of a type II β-turn (12) although the strength of the ellipticity is less than that recorded on known β-turn forming polypeptides (12). It is therefore possible that, on binding Ca^{++}, the linear peptide folds into a hairpin shape in which the two hydrophobic ends are brought together. Such a structure would (from molecular model building) have a maximum area of 370 $\AA^2$ which agrees well with the monolayer measurements. The Mg^{++} form, which should not be as sharply bent, displays, in fact, an area only possible in molecular terms by the opening out of the hairpin. From a theoretical standpoint the formation by amphomycin of a β structure is suggested by its Asp-Gly-Asp-Gly motif which it shares with known β hairpins (13) and some calcium binding loops of calmodulin (14). Such a change of conformation would turn amphomycin into a perfectly amphiphilic molecule with dimensions similar to that of membrane lipids. One sign of this would be an enhancement of its monolayer forming properties such as the large increase in the surface pressure observed in the π-A curves. This however could be simply a consequence of the stabilisation of the monolayer due to stronger interaction of the lipopeptide with calcium ions. We will also require more data to analyse the events surrounding the increase in compressibility of the Ca^{++} form at the end of the transition phase. Surface potential measurements of amphomycin films would be of direct use in this respect whilst in general terms a solution conformation may be obtained by ^{1}H-NMR. Nevertheless the results clearly show that the calcium dependent activity of amphomycin has a parallel in a similarly selective binding which results in a specific conformational change.

ACKNOWLEDGMENTS

The help and interest of Drs André Brack and Yves Trudelle is gratefully acknowledged. J.H.L thanks the Science and Engineering Research Council (U.K.) for the award of a NATO postdoctoral fellowship.

REFERENCES

1. Heineman, B., Kaplan, M.A., Muir, R.D. and Hooper, I.R. (1953) Antibiot. Chemother. 3, 1239-1242.

2. Bodansky, M., Sigler, G.F. and Bodansky, A. (1973) J.Am. Chem. Soc. **95**, 2352-2357.

3. Eliopolous, G.M., Thaubin, C., Gerson, B. and Moellering, R.C. (1985) Antimicrob. Agents Chemother. **27**, 357-362.

4. Matsui, M., Oka, Y. and Araki, T. (1963) J. Antibiotic. **A16**, 7-11.

5. Tanaka, H., Oiwa, R., Matsukura, S., Inokoshi, J. and Omura, S. (1982) J.Antibiot. **35** 1216-1221.

6. Banerjee, D.K., Scher, M.G. and Waechter, C.J. (1981) Biochemistry **20**, 1561-1568.

7. Stone, J.K. and Strominger, J.L. (1971) Proc. Natl. Acad. Sci. (U.S.A) **68**, 3223-3227.

8. Harnois, I., Maget-Dana, R. and Ptak, M.(1987) J. Colloid Interface Sci.(in press).

9. Gaines (1966) in "Insoluble monolayers at liquid-gas interfaces" Interscience, New York pp 136-203.

10. Privat, J-P., Delmotte, G., Mialonier, G., Bouchard, P. and Monsigny, M. (1974) Eur. J. Biochem. **47**, 5-14.

11. Fowkes, F.M. (1962) J. Phys. Chem. 66, 385-392.

12. Woody, R.W. (1974) in "Peptides, polypeptides and proteins" (Blout, E.R., Bovey, F.A, Goodman, M. and Lotan, N. Eds) Wiley-Interscience, New York.

13. Blundell, T.L., Sibanda, B.L., Sternberg, M.J.E. and Thornton, J.M. (1987) Nature 326, 347-352.

14. Cheung, W.Y. (1980) Science 207, 19-27.