Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 998-1002

INTERACTION OF THE POLYENE ANTIBIOTIC AMPHOTERICIN B WITH PHOSPHOLIPID BILAYER MEMBRANES : A CIRCULAR DICHROISM STUDY

G. BOUDET and J. BOLARD

Département de Recherches Physiques
Laboratoire associé au C.N.R.S. nº 71
Université Pierre et Marie Curie
4, place Jussieu
75230 PARIS Cedex 05, FRANCE

Received April 4, 1979

SUMMARY

The circular dichroism of amphotericin B "Fungizone" has been used to study its interaction with lecithin, dimyristoyl and dipalmitoyl phosphadidylcholine vesicles with or without cholesterol. It appears that circular dichroism monitors species other than those monitored by electronic absorption. As a result of association, new spectra appear which are of opposite signs according to wether the vesicles are in the gel state or in the liquid crystalline state ; the cholesterol dependance of the rate of these changes is also different according to the state of the vesicles. It is concluded that the model proposed by Hsu Chen and Feingold for the gel state should not be rejected on the basis of data obtained in the liquid crystalline state.

The polyene antibiotic amphotericin B can interact with a variety of model and biological membranes. As with other polyene antibiotics the mode of interaction may be complex, depending on the presence or the absence of a sterol and on the sterol : amphotericin ratio. It has been shown that amphotericin B can interact with cholesterol in aqueous solution in a stereochemically and stoichiometrically defined manner (1,2,3) ; in lipid monomolecular layers (4) as well as bilayers (5), in presence of cholesterol, amphotericin B at a concentration lower than 10^{-5}M can create aqueous pores of specific size in the membrane, after having interacted with cholesterol (6,7,8). At higher concentration (10^{-4}M) it may cause vesicle destruction (9) even in the absence of cholesterol. A recent study (10) using gel permeation chromatography and NMR has confirmed and extended these results.

The characteristic optical properties of amphotericin B, due to the presence in the molecule of a system of 7 conjugated double bonds, make it an easy object of study in the presence of phospholipid vesicles. For instance several studies have been performed on its electronic spectra and

0006-291X/79/110998-05$01.00/0

recently the absorption band at 388 nm has been used to monitor its binding with dimyristoyl phosphatidylcholine vesicles (11). We report here results of circular dichroism (C.D.) studies on amphotericin B "Fungizone". C.D. is expected to be a better tool than electronic absorption since it is very much more sensitive to environmental modifications ; it has already been used by Bittman et al. (12) to investigate the interaction of filipin with lecithin vesicles but these authors did not carry out a detailed study.

MATERIALS AND METHODS

L-α - phosphatidylcholine from egg yolk, type V-E, L-α - phosphatidylcholine dipalmitoyl (DPPC) and L-α - phosphatidylcholine dimyristoyl (DMPC) were purchased from Sigma and used without further purification. Amphotericin B was obtained from Serva as the Squibb preparation, Fungizone, containing sodium deoxycholate.

Vesicle dispersions were prepared by dissolving weighed amounts of phosphatidylcholine and cholesterol in chloroform and then removing the solvent in vacuo. The appropriate buffer (tris - HCl pH 7.5) was added to give the desired concentration and the sample was quickly vortexed, then sonicated to clearness for about 5 minutes in a MSE 150 W Mk 2 sonicator, the sonication bath being maintained in each case above the transition temperature of the phospholipid. Amphotericin was added either directly from the aqueous stock-solution or after having added dimethyl formamide to the stock-solution (the final proportion in the sample being lower than 1%) in order to compare our results with those of other authors. The only differences between these two preparations occur in the intensity of the dichroic doublet at 330 nm.

All the experiments described below have been done with a final amphotericin B concentration of 1.25×10^{-5}M. We have chosen this concentration because it is of the same order of magnitude as that used in the NMR and permeability measurements and is well adapted to our C.D. experiments.

Circular dichroism was measured with a Jobin-Yvon dichrograph Mark III, carefully calibrated with a low-pressure Hg lamp.

RESULTS

Amphotericin B "Fungizone" is thought to be present in water as micelles (13). At a concentration of 10^{-4}M, i.e. above the critical micellar concentration (CMC), the absorption spectra exhibit bands at 419, 388 and 329 with corresponding negative dichroïc bands at 420 ($\Delta\varepsilon$ = - 40), 388 nm ($\Delta\varepsilon$ = - 72) and a very intense bisignated signal centered at 329 nm ($\Delta\varepsilon$ = - 650 ; + 700) attributed to an excitonic coupling in the aggregated species (13). At a concentration of 10^{-7}M, i.e. under the CMC, absorption bands are observed at 409, 384 and 365 nm ; we have observed only two weak positive D.C. bands at 409 and 384 nm.

Absorption spectra of 1.25×10^{-5}M amphotericin in the presence of phospholipid vesicles. In all the cases described below the bands observed around

TABLE I

	lecithin	DMPC	DPPC
		10° C	22° C
$T < T_c$		421 (+ 18) 394 (+ 10) 377 (+ 4)	421 (+ 16) 394 (+ 10) 377 (+ 4)
	22° C	36° C	47° C
$T > T_c$	419 (- 18) 392 (- 18) 375 (- 8)	419 (- 16) 392 (- 30) 374 (- 15)	419 (- 10) 392 (- 10) 374 (- 15)

Wavelength(nm) and intensities ($\Delta\varepsilon$) of the C.D. peaks of amphotericin B in the presence of phospholipid vesicles with cholesterol, below and above the phase transition temperature (T_c). The $\Delta\varepsilon$ indicated are corrected from the continuous positive background due to the differential dichroic scattering corresponding to the large size of the particles of the aggregated amphotericin.

409 and 384 nm in free amphotericin are immediately shifted to 415 and 388 nm and intensified as has been observed by Bittman and Chen (11, 12) in the case of lecithin and DMPC in the liquid crystalline state. On the other hand the band at 329 nm disappears more or less rapidly but at a rate unrelated to that of the shift at 415 nm.

C.D. of amphotericin B (Table I)

a) In the presence of 1.7 mM lecithin vesicles at room temperature, i.e. above the phase transition temperature.

In the presence of 0.1 mM cholesterol, the bisignated intense signal at 329 nm decreases rapidly and disappears totally in 3 or 4 hours. On the other hand, the intensity of the bands near 400 nm remains almost constant though their position is slightly shifted from 420 and 389 nm to 419 and 392 nm respectively. The disappearence of the bisignated signal allows a negative band at 375 nm to be observed. Without cholesterol we observe the same modifications but at a much slower rate.

b) In the presence of 1.7 mM DMPC or DPPC vesicles.

We have studied these interactions by C.D. in order to compare our results to those obtained by Chen and Bittman (11) by electronic absorption and by Hsu Chen and Feingold (14) by permeability measurements. These phospholipid present gel-liquid crystalline state transitions at 23ºC in the case of DMPC and 41ºC in the case of DPPC.

Above the phase transition temperature (at 36ºC with DMPC) : with or without cholesterol one observes the same type of C.D. spectra as with lecithin.

Below the phase transition temperature (at 10ºC with DMPC, at room temperature with DPPC). Without cholesterol the bisignated signal at 329 nm disappears totally in 1 hour. The initial C.D. signal between 350 and 450 nm is also rapidly modified and the negative bands are replaced by positive ones at 421 and 394 nm and a new one at 377 nm. With 0.1 mM cholesterol one observes the same modification. In addition, we note that if the temperature of the mixture is raised above the phase transition temperature, one immediately observes the reappearence of the bisignated band at 329 nm.

DISCUSSION

The very fast absorption changes observed near 410 and 388 nm are said (11) to arise from adsorption of amphotericin monomers onto the vesicles and not from amphotericin molecules in a channel configuration.

It is important to note that in our results the C.D. changes do not occur at the same rate as the absorption changes ; in addition, the new C.D. peaks are not at the same wavelength as the absorption peaks. We conclude that absorption and C.D. do not monitor the same species. This is not surprising since for instance it is also the case for free amphotericin in water : at a 10^{-6}M concentration the absorption spectrum (409, 384 nm) represents mainly the monomeric species (about 90% of the total amphotericin) while the C.D. spectrum (420, 388 nm) represents only the aggregated species.

The C.D. spectrum resulting from the interaction of amphotericin and lecithin or DMPC vesicles in the liquid crystalline state (i.e. in the conditions of the kinetic (11), permeability (8) and NMR measurements (10)), consists of three negative bands at 419, 392 and 375 nm and thus monitors the appearance of species which are neither free micellar nor free or adsorbed monomeric amphotericin. With DMPC and DPPC vesicles in the gel

state (i.e. in the conditions of the permeability measurements of Hsu Chen and Feingold) positive dichroic bands appear at a high rate, even without cholesterol. Thus these results parallel those obtained by Hsu Chen and Feingold and furthermore indicate that, if we relate the dichroic species to the permeability changes, these species are not the same below and above the phase transition temperature.

In these conditions it appears clearly that it is not possible to compare the conclusions obtained on the fluidity - permeability relation ships in the gel state, with conclusions obtained on the fluidity - monomeric amphotericin/phospholipid vesicles association relation ships in the liquid crystalline state. Thus, the latter results cannot be used to choose between the model proposed by Hsu Chen and Feingold and the model with transmembrane channels constituted of amphotericin-cholesterol complexes.

REFERENCES

1. Norman, A.W., Demel, R.A., De Kruyff, B., Geurts Van Kessel, W.S.M. and Van Deenen, L.L.M. (1972), Biochim. Biophys. Acta, 290, 1-14.
2. Schroeder, F., Holland, J.F., Bieber, L.L., (1972) Biochem. 11, 3105.
3. Bittman, R., Fischkoff, S.A. (1972) Proc. Natl. Acad. Sci. USA, 12, 3795-3799.
4. Demel, R.A., Crombag, F.J.L., Van Deenen, L.L.M., Kinsky, S.C. (1968) Biochim. Biophys. Acta, 150, 1.
5. Van Zutphen (1971), Biochim. Biophys. Acta 241, 310.
6. Finkelstein, A., Holz, R. (1973) in Membranes, Lipid bilayers and antibiotics, Eisenman ed. p. 377.
7. De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A., Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30-43.
8. Van Hoogevest, P., De Kruijff, B. (1978) Biochim. Biophys. Acta, 511, 397-407.
9. Sessa, G., Weissmann, G. (1967) Biochim. Biophys. Acta, 135, 416.
10. Gent, M.P.N., Prestegard, J.H. (1976) Biochem. Biophys. Acta, 426, 17-30.
11. Chen, W.C., Bittman, R. (1977) Biochem. 16, 4145-4149.
12. Bittman, R., Chen, W.C. and Anderson O.R. (1974) Biochem. 13, 1364-1370.
13. Rinnert, H., Thirion, C., Dupont, G. (1977) Biopolymers 16, 2419-2427.
14. Hsu Chen, C., Feingold, D.S. (1973) Biochem. Biophys. Res. Comm. 51, 972-978.