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Temperature-dependent modes for the binding of the polyene antibiotic amphotericin B to human erythrocyte membranes. A circular dichroism study

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The interaction of amphotericin B with isolated human erythrocyte ghosts was monitored by circular dichroism at 37°C and 15°C. Although different, these spectra were not concentration dependent over a concentration range covering the inducement of K^+ leakage and hemolysis, which suggests the existence of only one bound amphotericin B species. At 15°C, the spectra indicate that amphotericin B is complexed with membrane cholesterol; the complex formation is saturable but not cooperative. At 37°C new spectra are observed, and their existence is conditioned by the presence of membrane proteins. The binding is cooperative but not saturable. The amphotericin B right side-out vesicles complexation is temperature as well as ionic strength dependent: at high ionic strength it is the same as with ghosts, with the same temperature dependence. At low ionic strength it is characteristic of an interaction with cholesterol, regardless of temperature. In the large unilamellar vesicles reconstituted from the total lipid extracts of erythrocyte membranes, amphotericin B is complexed with cholesterol, regardless of temperature and ionic strength. These results indicate that there are two different modes of amphotericin B complexation with erythrocyte membranes, reversible one in the other, depending on the molecular organization of the membrane and the presence of membrane proteins.

Introduction

Amphotericin B, a heptaene macrolide antibiotic used clinically in the treatment of systemic mycosis, is toxic to cells containing sterols in their membranes. It is generally assumed that it interacts with cell membranes but its molecular mechanism is not well understood [1,2].

A large number of recent studies on amphotericin B have been devoted to the action of this drug on animal cells as it can strikingly augment both humoral and cell immunity [3] and as it is also highly effective in combination therapy for tumors [4]. Studies on erythrocytes as models of animal cells deserve special attention for several reasons. First, since the properties of erythrocyte membranes are relatively well understood, erythrocytes would seem a logical model for the study of the interaction of amphotericin B with cell membranes. Second, erythrocytes are often used to test the toxicity of polyene antibiotics on animal cells and it is necessary to check the validity of this control. Finally, sensitivity to the immunostimulant effects of amphotericin B in mice has been shown to be genetically controlled: some inbred strains show vigorous responses to ampho-

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tericin B, while other strains show little or no response [5]. Interestingly, the erythrocytes of highly responsive mice were not very sensitive to the amphotericin B-induced hemolysis while the reverse was true for less responsive mice [6]. The study of the interaction between amphotericin B and erythrocytes is therefore useful for investigating the origin of the specificity of immune responses to the antibiotic.

Several studies have yielded information on the interaction of amphotericin B with erythrocytes from a functional point of view [1,2] but nothing is known at the molecular level. No direct information is yet available on the extent of drug binding to erythrocytes, although indirect results have been obtained by considering the extent of hemolysis as a function of erythrocyte and amphotericin B concentration [7].

Generally speaking, little is known about the binding of amphotericin B to cells, partly because the free drug is only slightly soluble in water. Therefore it is difficult to separate non-bound amphotericin B from cells, both sedimenting at the same rate when centrifuged. Optical spectroscopy could provide a good tool for studying the binding in so far as the absorption wavelengths of amphotericin B are different from those of cell components (which is not the case with erythrocytes due to the presence of hemoglobin) and are sensitive to binding. Indeed electronic absorption [8–10] and circular dichroism [11,12] have both been used to measure amphotericin B binding to phospholipid vesicles. Unfortunately these methods cannot be used to study cells since cells scatter light. One way to overcome this limitation is to use membrane ghosts or vesicles instead of whole cells. Indeed light scattering from membrane ghosts is much weaker than that of cells and does not keep to record spectra of exogen bound molecules (see, for instance, Refs. 18 and 19). As for the case of vesicles their light scattering is still weaker, its exact extent depending on the size of the vesicles and they have often been used as model membranes for spectroscopic studies.

Consequently, we present in this paper a study by circular dichroism of the interaction between amphotericin B and membranes of human red blood cells, right side-out vesicles, and large unilamellar vesicles prepared from total lipid extract of erythrocytes.

Materials and Methods

Amphotericin B, a generous gift of Squibb, was dissolved in dimethyl sulfoxide (1 mg in 100 μl) from Merck and then in buffer to give $2 \cdot 10^{-4}$ M stock solution. The stock solution was used in the four hours following its preparation. The sealed ghosts and the sealed right side-out vesicles were prepared by the method of Steck [13]. Human erythrocytes were isolated from freshly drawn blood by centrifugation at $1500 \times g$ for 10 min and removal of serum and buffy coat; they were then washed three times in isotonic phosphate-buffered saline (150 mM NaCl/5 mM sodium phosphate (pH 7.4)). Hemolysis was carried out by diluting 1 volume of the erythrocyte pellet in 50 volumes of 5 mM sodium phosphate buffer (pH 7.4) on ice. The membranes were collected by centrifugation at $16000 \times g$ for 10 min and then were washed once in 5 mM sodium phosphate buffer and incubated for 1 h at 37°C in phosphate-buffered saline.

Sealed ghosts were separated from unsealed membranes by centrifugation for 1 h at $35000 \times g$ of the membrane suspension which had been deposited on the surface of a solution containing 43% sucrose, 25 mM NaCl and 5 mM sodium phosphate (pH 7.4) [14].

For the preparation of right-side-out vesicles erythrocytes were lysed and the membranes were resuspended in 0.5 mM sodium phosphate (pH 7.4) and incubated on ice for 60–90 min. $MgSO_4$ was added to the suspension to give a final concentration of 0.1 mM $MgSO_4$ and the suspension was immediately centrifuged at $25000 \times g$ for 30 min. The pellet was resuspended in a buffer consisting of 0.5 mM sodium phosphate/0.1 mM $MgSO_4$ (pH 7.4) and then homogeneised by three passages through a No. 27 gauge needle. Sealed right side-out vesicles were isolated by centrifugation of the membrane preparation on Dextran T-110 of density 1.03 g/ml and by partition between Dextran T-500 and polyethylene glycol 6000 (for details, see Ref. 13).

In order to reconstitute large unilamellar vesicles, total lipids were extracted from mem-

branes by a mixture of chloroform and methanol [15] and vesicles were prepared by the reverse phase evaporation method [16].

The colorimetric ammonium ferrothiocyanate determination of membrane phospholipids [17] was performed as follows. After counting red blood cells by light microscopy, a known number of cells were hemolysed and therefore a known number of ghosts was obtained.

A standard curve was obtained by plotting the number of ghosts versus absorbance derived from the colorimetric determination of the phospholipids in this number of ghosts. The ghost concentrations of unknown samples were then colorimetrically determined and expressed as number of ghosts per ml.

The membranes were used within three days after preparation. After 2 h incubation of the membrane suspension with the desired amount of amphotericin B, in a glass tube and at the chosen temperature, the suspension was poured into a quartz cuvette which had been preincubated at the same temperature. The path-lengths of the cuvette used were 2 cm, 1 cm, 0.5 cm and 0.2 cm.

CD spectra were recorded with a Jobin-Yvon Mark III dichrograph equipped with a thermostat. After having transferred the cuvette into the spectrophotometer we waited until the signal stabilized (approx. 5 min). The signal stability was also tested after spectrum recording. In the spectra reported in Figs. 1 and 3 the background due to the ghosts scattering has been subtracted. This background was not affected by the addition of amphotericin B and became progressively negligible as the amphotericin B/ghost ratio increased. The amphotericin B spectra depended neither on the cuvette path-length (varied for this control between 0.05 and 2 cm) nor on the distance between the sample cell and the detector (varied between 0 and 10 cm from the end-window of the photomultiplier tube).

For binding experiments, amphotericin B at different concentrations was incubated for 2 h at the chosen temperature, either with the membrane suspension, or with only the buffer. After incubation, the contents of each tube were poured in the quartz cuvette and the intensity of the excitonic doublet centered at 345 nm, which is characteristic of free amphotericin B aggregated in buffer, was measured. Since at this wavelength amphotericin B complexed with membrane has no detectable CD, the intensity of the excitonic doublet corresponded to the concentration of free amphotericin B in buffer and could be used to calculate the amount of antibiotic complexed with membranes.

Results

The CD spectra of amphotericin B in phosphate buffer saline is concentration dependent [11]. Below $2 \cdot 10^{-7}$ M, three positive bands are observed at 409, 386 and 366 nm. As the concentration increases, this spectrum is progressively superseded by one consisting of an intense doublet centered at 344 nm and negative bands at 423, 393 and 368 nm. These characteristics are poorly temperature dependent.

In the presence of ghosts or vesicles, these spectra decreased or even disappeared for an amphotericin B/lipid ratio sufficiently low. The exact position of the CD bands of the new spectra

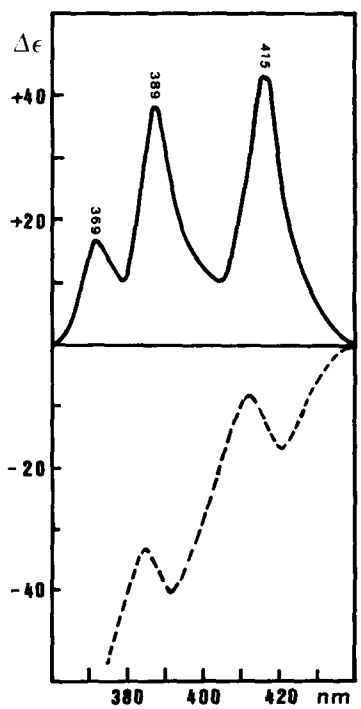

Fig. 1. Circular dichroism spectra at 37°C of 10^{-6} M amphotericin B free (– – –) and in the presence of erythrocyte ghosts ($2 \cdot 10^{8}$ ghosts/ml) (———) or right side-out vesicles both at high ionic strength (150 mM KCl). Cell path length 0.5 cm.

depended on the temperature and the ionic strength.

We shall consider first the spectra of amphotericin B at concentrations ranging from $4 \cdot 10^{-7}$ M to $4 \cdot 10^{-6}$ M in the presence of $2 \cdot 10^{8}$ ghosts/ml or vesicles, that is amphotericin B/phospholipid ratios ranging from $5 \cdot 10^{-3}$ to $5 \cdot 10^{-2}$ and amphotericin B/cholesterol ratios ranging from $6.4 \cdot 10^{-3}$ to $6.4 \cdot 10^{-2}$. Within this concentration range we observed the same spectra.

At 37°C with ghosts and with right-side-out vesicles at high ionic strength (150 mM KCl) we observed three positive bands at 415, 389, 369 nm with $\Delta\varepsilon$ around 43, 38 and 16, respectively (Fig. 1). With either right-side-out vesicles at low ionic strength (0.5 mM sodium phosphate) or vesicles reconstituted from the total lipid extract of erythrocyte membranes (regardless of ionic strength), we observed (Fig. 2) spectra with two positive bands at 422 and 396 ($\Delta\varepsilon = 53$ and 48). A third positive band was observed at 384 nm with right side out vesicles and at 380 nm with total lipid extract vesicles, with $\Delta\varepsilon$ around 20. In both cases, three weak negative bands or troughs were observed at 414, 389 and 369 nm. A shoulder was always observed around 408 nm.

At 15°C with ghosts (at high ionic strength) we observed positive bands at 422 and 396 nm with $\Delta\varepsilon$ around 46 and 34 and negative bands at 414 and 389 nm ($\Delta\varepsilon = 12$ and 27, respectively) (Fig. 3).

At 15°C with right-side-out vesicles or with vesicles reconstituted from the total lipid extract of erythrocyte membranes we observed the spectra represented on Fig. 2 regardless of ionic strength.

At amphotericin B concentrations higher than $4 \cdot 10^{-6}$ M, that is amphotericin B/cholesterol ratios higher than $6.4 \cdot 10^{-2}$, the spectra observed as the concentration of antibiotic increased were progressively modified compared to those previously described, apparently because an increasing amount of amphotericin B remained free.

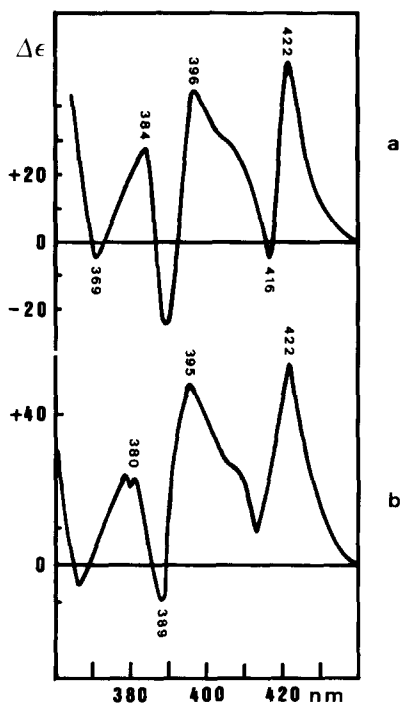

Fig. 2. Circular dichroism spectra of 10^{-6} M amphotericin in the presence of: (a) right side-out vesicles from erythrocyte membrane at 37°C and low ionic strength (0.5 mM sodium phosphate) or at 15°C regardless of ionic strength; (b) large unilamellar vesicles resonstituted from membrane total lipid extracts at 15°C or 37°C and regardless of ionic strength.

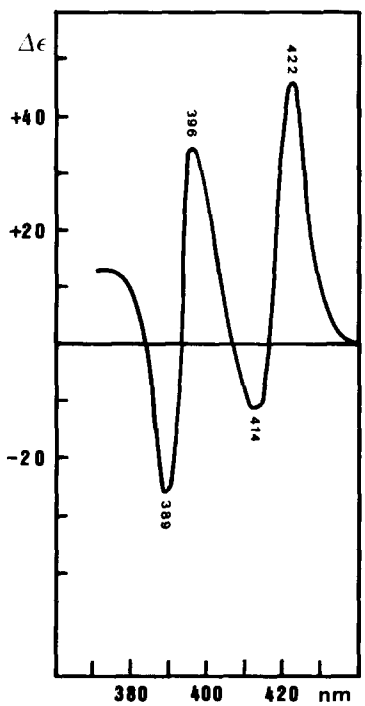

Fig. 3. Circular dichroism spectra at 15°C of 10^{-6} M amphotericin B in the presence of erythrocyte ghosts ($2 \cdot 10^{8}$ ghosts/ml) at high ionic strength (150 mM KCl). Cell path length 0.5 cm.

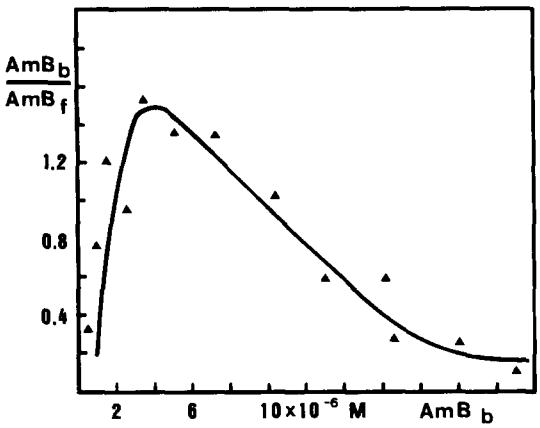

Fig. 4. Scatchard plot of binding of amphotericin B to erythrocyte ghosts at 37°C and high ionic strength (150 mM KCl). AmB_b and AmB_f are the molar concentrations of bound and free amphotericin B. Concentration of ghosts $2 \cdot 10^8$/ml. Concentrations of amphotericin B between 10^{-6} and 10^{-4} M.

Therefore, between 450 and 370 nm, negative bands appeared with progressively increasing intensity, corresponding to free amphotericin B. Similarly around 345 nm, where no amphotericin B CD was observed at low antibiotic/lipid ratio, an intense dichroic doublet progressively appeared corresponding to free, aggregated amphotericin B. We could therefore take advantage (as described in the experimental part) of the absence of CD around 350 nm in the bound amphotericin B spectrum, to determine the amount of free amphotericin B as monitored by the intense doublet and then to draw a Scatchard plot of the data.

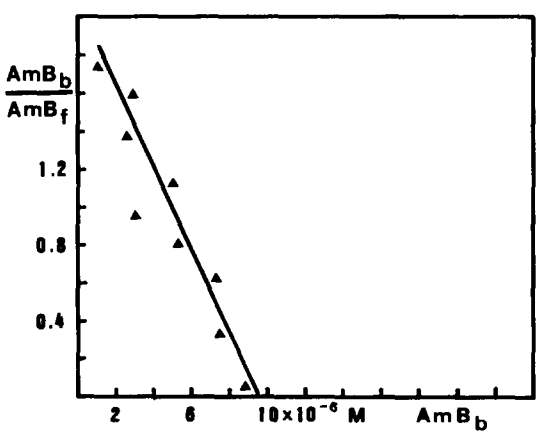

Fig. 5. Scatchard plot of binding of amphotericin B to erythrocyte ghosts at 15°C and high ionic strength (150 mM KCl). AmB_b and AmB_f are the molar concentrations of bound and free amphotericin B. Concentration of ghosts $2 \cdot 10^8$/ml. Concentration of amphotericin B between 10^{-6} and 10^{-4} M.

The results obtained at 37°C and high ionic strength (150 mM KCl) could not be represented by a straight line (Fig. 4). It appeared that for an antibiotic/cholesterol ratio between $1.6 \cdot 10^{-2}$ and $1.3 \cdot 10^{-1}$ inclusive, the interaction was cooperative: the bound/free amphotericin B ratio started at 0.4 for an antibiotic/cholesterol ratio of $1.6 \cdot 10^{-2}$, increased and reached a maximum value of 1.4, corresponding to a partition coefficient $P = 1.4 \cdot 10^4$. The Hill constant of the binding was $n = 2.1$. For higher amphotericin B doses, the bind/free ratio decreases and reaches a plateau for amphotericin B/cholesterol $= 9.6 \cdot 10^{-1}$, corresponding to partition coefficient $P = 0.2 \cdot 10^4$.

At 15°C and high ionic strength (150 mM KCl); the Scatchard plot gave a straight line intercepting the axis of abscissas for a bound amphotericin B/cholesterol ratio around 0.10, that is one amphotericin B molecule for ten cholesterol molecules (Fig. 5).

When the temperature was decreased from 37°C to 15°C, the spectrum of the bound species observed at 37°C was transformed into that observed at 15°C. Reheating at 37°C restored the initial spectrum.

Discussion

Circular dichroism of chlortetracycline [18] or anion-transport inhibitors [19] was already used to monitor their interaction with erythrocyte ghosts. As for polyene antibiotics, changes induced in the shape of their CD spectra by addition of erythrocyte ghosts were already noted with lucensomycin [20], amphotericin B [21], filipin [22] and candicidin [23]. In the present study we have analyzed the changes induced in the CD of amphotericin B by addition of erythrocyte ghosts according to two different approaches. The first one, performed in conditions similar to those used with chlortetracycline and anion-transport inhibitors, consisted in an analysis of the new spectrum appearing when the drug binds to the membranes. The second approach consisted in measuring the amount of amphotericin B remaining free in the presence of membranes, as measured by the amplitude of the CD signal in a region where the bound antibiotic does not give any signal.

1. Binding

(a) At 37°C, in the presence of $2 \cdot 10^8$ ghosts/ml, for an amphotericin B concentration ranging from $4 \cdot 10^{-7}$ to $4 \cdot 10^{-6}$ M, we observed only one CD spectrum, corresponding to one species of bound amphotericin B. The concentrations of amphotericin B required to induce K^+ leakage and hemolysis in phosphate-buffered saline are included in this range [30]. In fact, these effects do not occur at the same concentration of amphotericin B. K^+ leaks out at a lower antibiotic concentration than that which causes hemolysis and there is good evidence that it leaks through an amphotericin B-membrane complex. The observed hemolysis is actually a consequence of the membrane permeabilisation by the antibiotic and occurs by a colloid-osmotic mechanism [24,25]. Lipid peroxidation by amphotericin B also has a role in the mechanism of hemolysis [26]: peroxidation makes the membrane more fragile, makes the cells more sensitive to osmotic shock. The possibility of a detergent mechanism has also been raised. Our results indicate that only one species of bound amphotericin B is detected within the concentration range covering both K^+ leakage and hemolysis in agreement with the colloid-osmotic mechanism. As the relationship between lipid peroxidation and hemolysis has been demonstrated, we think that either the amount of antibiotic responsible for this effect is not sufficient to be detected by CD or the conformation adopted by the antibotic molecule to achieve it, is the same as that adopted for forming pores through the membranes. Our results rule out the possibility of a detergent mechanism, at least for ratios of amphotericin B to cholesterol of around $6 \cdot 10^{-2}$. Of course, at higher concentration, amphotericin B might act by a detergent mechanism, but it would not be possible to observe by CD a new conformational species since at these ratios the corresponding spectrum would be hidden by that of free amphotericin B.

At 37°C, the cooperative character of the amphotericin B-membrane association clearly indicates that the antibiotic interacts with membrane by perturbing its structure and so increasing its affinity for the next antibiotic molecules. It means that the first bound antibiotic molecules cause a dose-dependent structural modification of the membrane which is responsible for the observed increase of the partition coefficient. Having reached its maximum value, the bound/free ratio decreases. Thus, for the doses corresponding to this part of the curve, membrane affinity for amphotericin B decreases and reaches a saturation value corresponding to a bound/free ratio of about 0.2. Therefore, it is clear that the membrane affinity for amphotericin B is modulated by the antibiotic itself, and whether the affinity increases or decreases is dose dependent.

Another interesting feature of amphotericin B binding to erythrocyte ghost membranes is its non-saturability: even for high antibiotic doses (corresponding to bound antibiotic/phospholipid ratio of about 0.3) the curve reaches a plateau at a bound/free ratio of 0.2, indicating that the antibiotic is distributed between membranes and water with a partition constant of $0.2 \cdot 10^4$.

The questions raised are how so much amphotericin B is able to bind membrane without any detectable saturation, and whether the membrane is disrupted and fragmented by the antibiotic. To answer these questions we tested the detergent effect of amphotericin B on intact erythrocytes suspended in isotonic buffer containing 110 mM KCl, 10 mM NaCl and 60 mM sucrose (pH 7.4), 5 mM sodium phosphate. These conditions would exclude the possibility of colloïd-osmotic lysis. At the maximal antibiotic dose studied, i.e. 10 amphotericin B molecules per molecule of phospholipid, we observed only 10% of the hemolysis. This indicates that even if this small degree of hemolysis results from a detergent effect, this mechanism is relatively unimportant. Therefore, we think that in spite of the high antibiotic doses used, the ghost membranes may be locally deformed but are neither disrupted nor solubilized by amphotericin B as would be the case with a detergent. That no significant membrane damage was observed in images of freeze-fractured membranes of either eyrthrocytes or ghosts treated with high concentrations of amphotericin B (10^{-4} M) would confirm this hypothesis [31].

Our choice of temperature range was guided by the physiological interest of 37°C; furthermore, since some structural changes occur in the membrane at about 20°C [27,28,29], we thought it of interest to study the amphotericin B binding below this temperature.

(b) At 15°C the spectroscopic characteristics of bound amphotericin B are different from those at 37°C: if a single species is observed at both temperatures, its CD bands are not located at the same wavelengths. The binding is also different in so far as the bound/free ratio decreases linearly and saturation corresponds to about one amphotericin B molecule bound for 10 cholesterol molecules. This binding curve can be well described by Scatchard formalism as the binding of ligand to a set of sites, independent and limited in number. The intersection of the straight-line with the abscissa indicates that there are $2 \cdot 10^7$ binding sites per ghosts, corresponding to 10 cholesterol molecules per site (if we assume that amphotericin B is bound to cholesterol).

Strom et al. [7] studied the binding of amphotericin B to bovine erythrocytes at room temperature by considering the relationship between the amount of antibiotic required to produce 50% hemolysis and the erythrocyte concentration. These authors found heterogeneous distribution of association constants. However, comparison among mammalian species is not straightforward because permeability inducement differs among them [32].

2. Nature of the bound species

The wavelengths of the amphotericin B CD bands, as well as their signs, appeared to be very sensitive to the conformation of the antibiotic and may be considered as a 'finger print' of the interaction [2]. It is therefore possible to compare the spectra presently described to those obtained both with binary systems of amphotericin B and cholesterol [33] or lipoproteins and serum albumin [34], and with amphotericin B in the presence of phospholipid vesicles [11,35].

The spectra obtained in the presence of ghosts at 15°C and high ionic strength, in the presence of right-side-out vesicles at 37°C and low ionic strength or 15°C regardless of ionic strength and in the presence of reconstituted lipidic vesicles, regardless of temperature and ionic strength, are closely similar to and resemble the spectra of amphotericin B complexed either with cholesterol in water [33], or with 50% cholesterol-containing dipalmitoylphosphatidylcholine vesicles [11], or with lipoproteins [34]. It would therefore seem that for these different membrane preparations derived from erythrocyte membrane, amphotericin B is complexed with membrane cholesterol. Then, assuming that all membrane cholesterol interacts with membrane bound amphotericin B, the intersection of the straight line of the Scatchard plot with abscissa for amphotericin B binding to ghosts at 15°C (Fig. 5) gives statistically one amphotericin B molecule for ten cholesterol molecules. This would be equivalent to the stoichiometry of the amphotericin B-cholesterol complex.

The CD spectrum of amphotericin B complexed either with ghosts or right-side-out vesicles at 37°C and high ionic strength consists of three positive bands that are at the same wavelengths as those of absorption. It is strikingly similar to that of amphotericin B in the presence of human serum albumin in water [34] but does not resemble the spectra obtained in water in the presence of cholesterol and already described [33]. No resemblance can either be found with the spectra obtained in the presence of pure phospholipid vesicles [11] or of cholesterol-containing vesicles [11,35]. The close similarity observed with the spectrum obtained in the presence of serum albumin is not sufficient to ascertain the existence of an amphotericin B-protein complex: an amphotericin B-cholesterol complex, with a conformation different from those already characterized by CD [33], could as well exist because the CD spectra of the aggregates of amphotericin B-cholesterol complexes obtained in water, strongly depends on the exact conditions of mixing. In any case the antibiotic is not in a monomeric state as shown by the high intensity of the CD signal ($\Delta\varepsilon_{416} \approx 40$) as compared to that observed with monomers in water, in DMSO or in phospholipid bilayers ($10 < \Delta\varepsilon < 15$) [11,12].

To resume, we observed two types of CD spectra of amphotericin B complexed with erythrocyte membranes: the first one (Fig. 1), indicates perhaps the interaction of amphotericin B with protein. The second one (Figs. 2 and 3) corresponds to amphotericin B complexed with cholesterol.

For the vesicles reconstituted from the total lipid extracts of the erythrocyte membranes, amphotericin B is always complexed with cholesterol regardless of temperature and ionic strength. We never observed the spectrum, that we think to be possibly characteristic of antibiotic-

protein interaction in ghosts and right-side-out vesicles. This is in favor of the hypothesis of amphotericin B–protein interaction in ghosts and right-side-out vesicles since the lipidic vesicles lacking proteins, never present this kind of complexation.

For ghost and right-side-out vesicles, both containing the membrane proteins, the mode of amphotericin B-membrane complexation is determined by temperature and ionic strength of the medium.

For ghosts at 37°C and high ionic strength, amphotericin B seems to interact with some membrane proteins. The cooling at 15°C induces the complexation with cholesterol, and reheating to 37°C reinduces the initial spectrum. Many authors have claimed that erythrocyte membranes change structurally at about 20°C [27–29]; such changes could be responsible for the difference in amphotericin B–membrane interaction above and below 20°C. Since it was shown that above −5°C there is neither any lipid phase transition nor phase separation in cholesterol-rich or cholesterol-poor domains [36], the mechanism of the change that occurs at about 20°C remains unknown.

In a buffer of the same low ionic strength as that used for right-side-out vesicles (0.5 mM sodium phosphate), fragmentation of ghosts occurs, leading to the formation of vesicles [13]. Therefore, a comparison of the spectra obtained at low and high ionic strength, similar to that done with vesicles, would not have been meaningful for the ghosts. However, we observed that the complexation of amphotericin B with ghosts in buffer containing 5 mM sodium phosphate only (the buffer used to hemolyse the red cells during the preparation of the ghosts) give simultaneously two types of complexes. The increase in ionic strength to 150 mM KCl restores the spectrum indicating amphotericin B–protein interaction.

With right-side-out vesicles at high ionic strength (the same that for the ghosts: 150 mM KCl) the amphotericin B–membrane complexes are the same as with ghosts as well as the temperature dependence and the reversibility of the change from one complex to the other. The principal difference in membrane organization between ghosts and right-side out vesicles is the loss of the native asymmetric distribution of phosphatidylethanolamine and phosphatidylserine (still present in the ghosts) during the formation of right-side-out vesicles, probably because of some perturbation of the proteic network during membrane fragmentation [37]. It seems then, that this difference has no influence on amphotericin B–membrane complexation.

For right-side-out vesicles at low ionic strength (0.5 mM sodium phosphate) amphotericin B is complexed with cholesterol regardless of temperature: cooling or heating has no effect on the complex formed. More, increase in ionic strength at 37°C abolishes the amphotericin B-cholesterol complexation and induces the interaction between amphotericin B and protein.

It is well known that the ionic strength can influence the membrane, in particular its interfacial electrical potential [38], the fluidity of its hydrophobic interior [38], the structure of the proteic network [39] and the cholesterol environment [40]. More, it has also been shown by electron microscopy that the changes induced in red cell membrane morphology by high amount of amphotericin B or nystatin are ionic strength dependent [31]. Our CD results also show such a dependence exclusively in the membranes containing proteins.

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