ELSEVIER

Biophysical Chemistry 77 (1999) 69–77

Biophysical Chemistry

Thermodynamic and spectroscopic features of the behavior of amphotericin B in aqueous medium

Diana Romanini[a], Gabriela Avalle[a], Bibiana Nerli[a], Guillermo Picó[a,b,*]

[a] *Department of Chemical Physics and IFISE (CONICET), Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Suipacha 570, 2000 Rosario, Argentina*
[b] *Institut de Chimie B6, Sart Tilman, University of Liege, Liege, Belgium*

Received 25 August 1998; received in revised form 31 December 1998; accepted 18 January 1999

Abstract

The interaction between amphotericin B molecules in aqueous medium solution was studied using absorption and circular dichroism approaches. The results showed that at concentrations below 1 μM of amphotericin B, an equilibrium between the monomer and aggregate occured with a constant of approximately 0.6×10^6 M^{-1}. The aggregate formation constant was dependent on the experimental conditions of the medium: its value increased at acidic pH values, while alkaline medium induced the equilibrium displacement to the monomer formation. Either neutral salts or chaotropic agents such as urea prevented the formation of the aggregate. The presence of net electrical charge on the amine and carboxyl groups plays a role in the thermodynamic stability of the aggregate. A hydrophobic effect was also found between the monomer form and the water molecules of neighbours. In the aggregate formation water molecules were released contributing to an increase in the entropic change.

Keywords: Amphotericin B; Polyene antibiotic; Absorption; Fluorescence; Circular dichroism

1. Introduction

Amphotericin B (AMB) is a potent polyene antibiotic used in patients with deep-seated mycotic infection produced by transplantations and the acquired immunodeficiency syndrome. AMB is the only polyene antibiotic which is administered intravenously, with the commercial name of Fungizone. Due to its low solubility, deoxycholic acid is added to avoid the precipitation by micelle formation [1]. It has been reported that the aggregation of amphotericin B in aqueous medium conditions its pharmacological activity, because of its capacity to form aggregates of high molecular weight which decrease its antifungal activity [2]. The optical properties that characterize the AMB solutions are due to the presence of seven conjugate double bonds in its molecule. Several studies

* Corresponding author. Fax: +54 341 4804598; e-mail: agraf@fbioyf.edu.ar

0301-4622/99/$ - see front matter
PII: S0301-4622(99)00009-5

have been carried out about the formation of micelles of AMB in aqueous medium [3], however, there is a discrepancy in the AMB concentration value at which the micelle formation of AMB begins. At present, there are no measurements about thermodynamic parameters associated to the aggregate formation. The aim of this work was to study the behavior of AMB in aqueous medium as a function of temperature, pH, neutral salts and serum albumin, and to determine thermodynamic parameters associated to the aggregation, in order to know the molecular mechanism by which the aggregation process of AMB is carried out.

2. Materials and methods

2.1. Chemicals

Amphotericin B (AMB) and human serum albumin (HSA) fatty acid free ($< 0.0005\%$) were purchased from Sigma Chem Co.

AMB was used without further purification, dissolved in pure dimethyl sulfoxide until a 6 mM solution, the solution was kept in the dark at -20°C. Its stability was tested measuring the absorption at 408 nm and it was used for 3 weeks. This solution was added to aqueous solution, the final concentration of dimethyl sulfoxide did not exceed 0.1% v/v. The following buffers were used: sodium acetate pH 4.0, sodium phosphate pH 7.4, and glycine pH 9.9. In all cases the ionic strength of the solution was 0.033 M.

2.2. Spectroscopical measurements

Absorption measurements were performed in a Perkin Elmer Lambda 2S spectrophotometer using a thermostatized cuvette of 1-cm pathlength with a slit of 0.5 nm. The monomer–aggregate equilibrium for the AMB was resolved by the following approximation. It is based on the fact that at 408 nm it is predominantly the monomer that absorbs. From the curve of absorbance at 408 nm vs. the total AMB concentration, the monomer molar extinction coefficient and the AMB concentration at which the monomer concentration is independent of the AMB total concentration (which corresponds to the AMB critical micellar concentration) were calculated. Then, the aggregate formation constant was determined assuming a multiple equilibrium model between monomer and micelles of all sizes, in which all the stepwise association constants have values of similar magnitude. The equilibrium association constant (K_A) was evaluated from Eq. (1) of Mukerjee and Ghosh [4].

$$\left(\frac{[\mathrm{AMB}]_M}{[\mathrm{AMB}]_T}\right)^{1/2} = 1 - K_A[\mathrm{AMB}]_M \quad (1)$$

where $[\mathrm{AMB}]_T$ is the total AMB concentration and $[\mathrm{AMB}]_M$ is the equilibrium monomer concentration. By plotting $([\mathrm{AMB}]_M/[\mathrm{AMB}]_T)^{1/2}$ vs. $[\mathrm{AMB}]_M$, a straight line was obtained and the association constant (K_A) was determined from its slope. The following thermodynamic parameters associated to the equilibrium studied were also calculated; the free energy ($\Delta G°$) was calculated from: $\Delta G° = -RT \ln K_A$, the enthalpic change ($\Delta H°$) was calculated through the van't Hoff equation: $\mathrm{d}\ln K_A/\mathrm{d}T = \Delta H°/RT^2$ assuming an independence of the $\Delta H°$ value of the temperature change (in the temperature range between 20 and 37°C), and the entropic change ($\Delta S°$), through the equation:

$$(-\Delta G° + \Delta H°)/T = \Delta S°.$$

Circular dichroism spectra (CD) were performed in a Jobin Yvon dichrograph Marck 6, using a thermostatized cuvette of 2-cm pathlength, the slit was varied in an automatic form between 0.2 and 0.7 nm. A repetitive scanning of four cycles was used. The results were expressed as ellipticity (θ) using Eq. (2)

$$\theta = \frac{2.303\Delta\mathrm{Abs}180}{4\pi} \quad (2)$$

where ΔAbs is the observed difference in absorbance for the left and right circular components of the incident light. From the θ vs. total AMB concentration curve, the aggregate–monomer equilibrium association constant was calculated using Eq. (3):

$$\theta_T = [\theta]_M[AMB]_M + [\theta]_A[AMB]_A \quad (3)$$

where $[AMB]_M$ and $[AMB]_A$ are the equilibrium AMB concentrations as monomer and aggregate forms, respectively, $[\theta]_A$ and $[\theta]_M$ are their molar ellipticities and θ_T is the observed ellipticity. By choosing an incident light wavelength at which $[\theta]_M$ is negligible, $[\theta]_A$ may be obtained from the slope of the last part of the θ_T vs. $[AMB]_T$ curve, and the $[AMB]_A$ can be calculated using Eq. (3). Therefore the $[AMP]_M$ can be obtained by $[AMB]_M = [AMP]_T - [AMP]_A$ and the equilibrium association constant (K_A) can be evaluated from Eq. (1).

Analysis of the data: all the data were stored in a computer and then processed with commercial software using a general multiparametric curve-fitting program for non-linear regression analysis.

3. Results

3.1. Electronic absorption spectra of AMB

At low concentrations (0.6 μM) and at pH 7.4 three defined peaks are observed at 408, 385 and 365 nm, while a wide band of minor intensity is observed at 340 nm. As the AMB total concentration increases the spectrum is progressively modified as it is shown in Fig. 1B. When the total AMB concentration was increased all the peaks enhanced their intensity (the 340-nm peak increased in a drastic way) without a shift in their position. This behavior may be explained by the existence of two main spectroscopic species of AMB, both, concentration-dependent: the monomeric which absorbs at 365, 385 and 408 nm, and the aggregate form of AMB with a maximum absorption peak at 340 nm [3]. Then, the absorption spectra change with total AMB concentration is due to a displacement of the equilibrium between these two spectroscopic species. The effect of pH on the equilibrium of the two species was analyzed between pH 4.3 and 9.9. Fig. 1A–C shows that at low pH, the AMB form which predominates is the aggregate form, while at basic pH the equilibrium is displaced to the monomer form. It was found that the position of the aggregate peak was dependent on the medium pH, varying from 329 to 346 nm when the pH raised from 4.3 to 9.9. On the other hand, the positions of the peaks at 365, 385 and 408 nm were independent of medium pH. Fig. 2 shows that the absorbance at 408 nm vs. the total concentration of the AMB curve reached a plateau whoose value was dependent on the medium pH. The same behavior was observed for all the monomer bands (data not shown), therefore we selected the absorption measurements at 408 nm to carry out all the calculations because no interference from other bands is possible. On the other hand, Fig. 2 shows a monotone increase of the absorbance at 340 nm when the AMB total concentration was increased, which suggested the formation of aggregates. The interconvertion between the monomeric and aggregate forms was reversible (data not shown).

The equilibrium association constant (K_A) in different medium conditions was calculated as it was described in Section 2, in all the cases linear plots were obtained as shown in Fig. 3. Table 1 shows the K_A values for the monomer–aggregate equilibrium obtained under different experimental conditions and the corresponding thermodynamic parameters were also obtained. The composition of the medium on the monomer–aggregate equilibrium such as the presence of 2 M urea and 0.3 M sodium salts of the halides: F^-, Cl^- and Br^- was tested. The urea displaced the equilibrium to the monomeric form and no aggregation constant was calculated. All the assayed sodium halide salts produced an important decrease in K_A, without a significative difference between them.

The thermodynamic parameters associated to the aggregate–monomer equilibrium were calculated as it was described in Section 2. The results obtained are shown in Table 1. It was found that the aggregate formation was associated to a little positive enthalpic change and a positive entropic change, which suggests that a hydrophobic effect is associated to the aggregate formation [5]. At acidic pH values, the thermodynamic parameters showed a significant decrease of the enthalpic

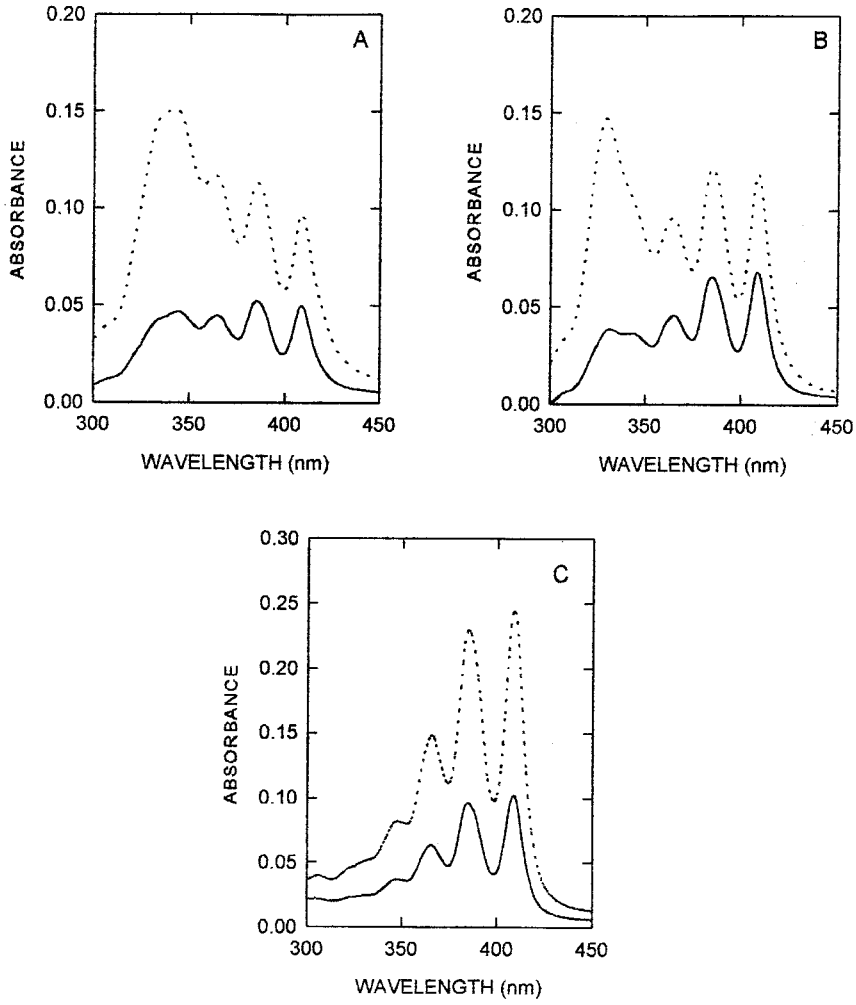


Fig. 1. Absorption spectra of AMB at 0.6 μM (——) and 2.1 μM (···); (A) pH 4.3; (B) pH 7.4; (C) pH 9.9. Temperature 20°C. Ionic strength 0.033.

change which became negative, and an important decrease of the entropic change.

3.2. Circular dichroism spectrum of AMB

Fig. 4 shows the CD spectra of AMB at pH 4.3, 7.4 and 9.9. An intense positive band at 328 nm can be observed. It has been reported [3] that this band is an indicator of the level of aggregation of the polyene antibiotic. On the other hand, some negative bands can be observed at 351 nm and others between 380 and 410 nm as it is shown in Fig. 4. We used the band at 328 nm because it is directly related with the aggregate form concentration. Moreover, the CD 328 and 351 nm bands increase their intensity without modifying their peak position when the pH varies from 9.9 to 4.3, which confirms the results obtained from the absorption spectrum and suggests that the acidic pH favors the stability of the aggregate. The effect of temperature on the CD spectrum of AMB is also shown in Fig. 4. A significant decrease in the CD bands was found when the temperature was increased, suggesting an enthalpic contribution in the aggregate formation.

Fig. 5 shows the dependence of the CD signal

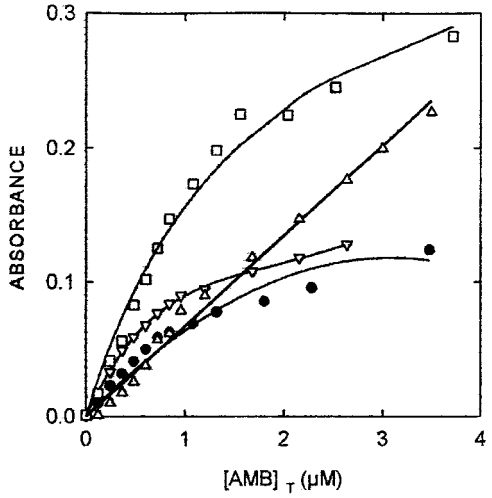


Fig. 2. Dependence of the absorption at 408 nm with the AMB concentration at different pH values: (●) pH 4.3; (□) pH 9.9; (▽) pH 7.4. Dependence of the absorption at 340 nm with AMB concentration (△) pH 7.4. Temperature 20°C.

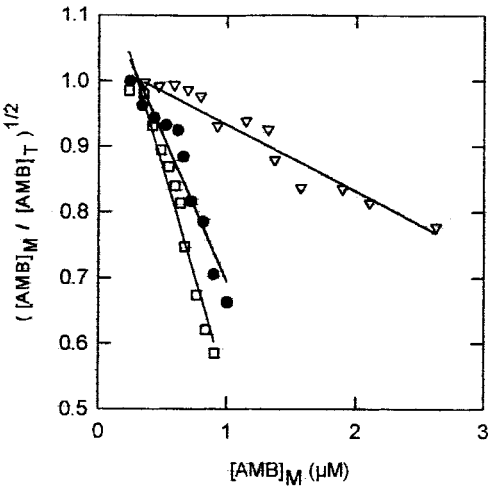


Fig. 3. Linear $([AMB]_M/[AMB]_T)^{1/2}$ vs. $[AMB]_M$ plots obtained at different pH values: (●) pH 4.3; (□) pH 7.4; (▽) pH 9.9. Temperature 20°C.

at 328 nm on the AMB total concentration. Table 2 shows the K_A values yielded under different experimental conditions obtained from the CD measurements. It can be seen that similar order values to those from the absorption measurements were obtained.

3.3. Polarity medium influence on the AMB aggregate formation

Table 3 shows the changes in the absorption spectrum of AMB in different ethanol–water solutions. The decrease of the microenvironment polarity by ethanol addition did not produce any significant change either in the absorption magnitude nor in the position of the aggregate form peak, however, an increase in the absorption at the 365-, 385- and 408-nm peaks (which correspond to the monomer form) was observed when the ethanol concentration varied from 0 to 100% v/v (data at 365 and at 385 nm are not shown). Moreover, the effect of human serum albumin

Table 1
Equilibrium constant values (K_A) and thermodynamic parameters associated to the aggregate-monomer equilibrium of AMB at different medium conditions calculated from absorption (408 nm) data

A				B	
pH	$K_A \times 10^{-5}$ (M^{-1})	$\Delta H°$ (Kcal/mol)	$\Delta S°$ (cal/°/mol)	Anions	$K_A \times 10^{-5}$ (M^{-1})
4.3	5.4 ± 0.5	-6.2 ± 1.8	5 ± 1	F^-	3.9 ± 0.4
7.4	6.6 ± 0.4	1.5 ± 3.1	31 ± 6	Cl^-	4.3 ± 0.4
9.9	1.0 ± 0.1	1.3 ± 0.3	27 ± 3	Br^-	4.2 ± 0.3

Temperature 20°C. (A) Ionic strength 0.04 M; (B) ionic strength 0.30 M, pH 7.4.

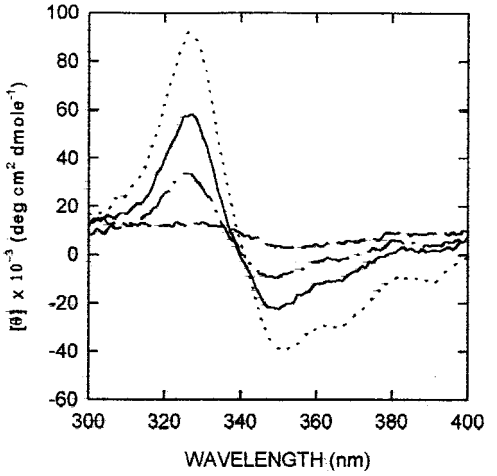


Fig. 4. Circular dichroism spectra of AMB (0.6 μM): (···) pH 4.3; (———) pH 7.4; (–·–·–) pH 9.9; temperature 20°C; (– – – –) pH 7.4; temperature 38°C.

Table 2
Equilibrium constant values (K_A) associated to the aggregate-monomer equilibrium of AMB at different medium conditions calculated from CD (328 nm) data

A	
pH	$K_A \times 10^{-5}$ (M^{-1})
4.3	11.2 ± 1.6
7.4	5.9 ± 0.8
9.9	13.60 ± 0.02

Temperature 20°C, ionic strength 0.04 M.

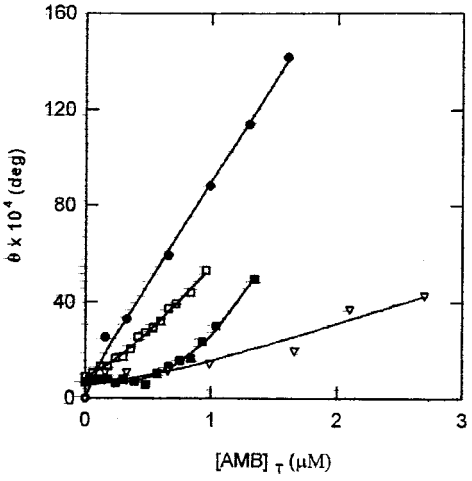


Fig. 5. Dependence on the CD band at 328 nm vs. the AMB concentration: (●) pH 4.3; (□) pH 7.4; (▽) pH 9.9; temperature 20°C and (■) and pH 7.4, 38°C.

was assayed on the basis of spectroscopical properties of the AMB, because a previous report has demonstrated that albumin binds AMB with high affinity [6]. Fig. 6A,B shows the modification in the absorption and CD spectra of AMB in the absence and presence of an increasing concentration of HSA. It can be observed that albumin increased the absorption band of AMB at 365, 385 and 408 nm, without modification of the peaks position, while a decrease of the band at 340 nm was observed which suggested the aggregate form decreased in concentration by its dissociation to the monomer form. No modification on the CD band of AMP was found, only a decrease of the band intensity at 328 and 351 nm was observed, which might be associated to a diminution of the aggregate concentration because its dissociation occurs when the AMB monomer binds to the HSA.

4. Discussion

Previous reports [7,8] have found that the absorption and CD spectra of AMB exhibit important changes as a function of the solvent medium. This is due to the perturbation of the $\pi^* \leftarrow \pi$ transition. In aqueous medium the absorption and CD spectra are also perturbed, which reflects excitonic interaction between the AMB molecules. It has been informed that AMB forms aggregates whoose size is estimated at 2000 molecules [7]. AMB is a rectangular ring molecule containing a segment of seven conjugated carbon–carbon double bonds, while a series of hydrophilic groups are aligned along the opposite side of the molecule.

Table 3
Spectral changes of AMB with the variation of the medium polarity

[AMB] (μM)	Ethanol concentration (% v/v)					
	0		50		100	
	Peak position	Absorbance	Peak position	Absorbance	Peak position	Absorbance
0.6	(*)	(*)	(*)	(*)	346.0	0.0161
	408.6	0.0301	408.4	0.0492	407.2	0.0560
3.0	345.6	0.0853	347.2	0.0716	346.0	0.0784
	408.6	0.1277	408.6	0.2502	407.4	0.2866

* No peak was detected in the proximity of 345 nm.

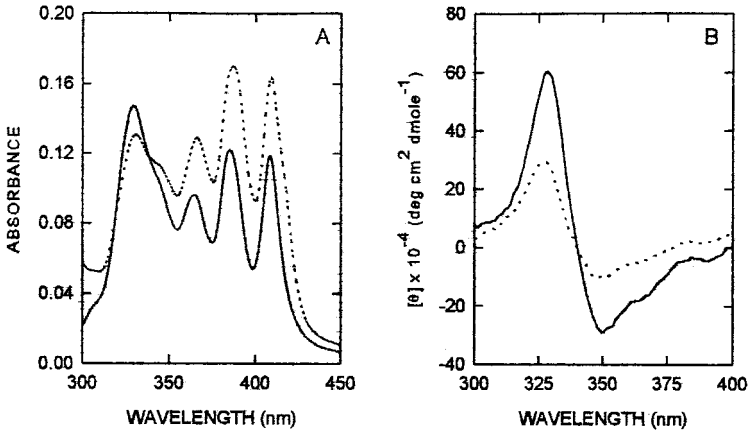
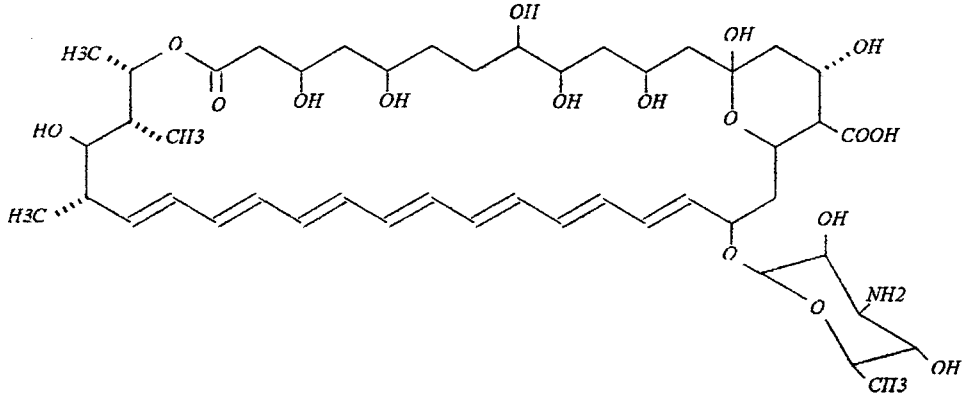


Fig. 6. Effect of HSA on the absorption (A) and CD (B) spectra of AMB, pH 7.4, temperature 20°C. AMB concentration 1 μM and HSA concentration 0 μM (——) and 10 μM (⋯ ⋯).

An amino sugar mycosamine is attached to the macrolide ring by a glycoside bond (see Fixed Graphic 1). Thus, the macrolide is amphiphile and also amphipatic due to the presence of a carboxyl and an amino group, with a pK_a of 5.7 and 10.0, respectively [5], both charged at physiological pH. This molecular character of AMB produces the formation of aggregates of AMB molecules in aqueous medium decreasing its solubility. The characteristic optical properties of the AMB due to the presence of seven conjugated double bonds in its molecule makes the study of its behavior in aqueous medium particularly appealing.

The absorption spectrum of the AMB at very low concentration shows the vibrational resolution of the polyene chromophores such as *cis*-parinaric and retinoic acids [9]. When the concentration increases, the observed band at 340 nm is assigned to a new ground state interaction between the molecules of the chromophore [3] due to the formation of aggregates.

Mazerski and Borowski [5] have proposed a multi-step model of polyene antibiotic self-association. For this model, in aqueous solution, the polyene antibiotic can exist in many different species with a molecular weight in the range of 1000 (monomer) to a few million (colloid micelles). Molecules of AMB monomer have a strong tendency to reduce the apolar surface exposed to water, and they interact between them forming aggregates. According to Mazerski and Borowski



Graphic 1.

[5] this first step is responsible for the spectroscopical changes observed. The formation of the aggregate produces an enhancement of the absorption band at 340 nm with a shift from 329 to 346 nm, which suggests an increase of the apolar character of the AMB environment. This interaction is evident at very low concentrations of AMB. The intensity of this band is enhanced in a continuous manner when the AMB concentration is increased above the micellar critical concentration. The existence of an isosbestic point near 350 nm and the fact that the different bands do not vary their position but they do change their intensity allow us to conclude that spectral changes reflect an equilibrium between two different forms, the monomer and aggregate form, respectively. Our electronic spectra of AMB at increasing concentrations of AMB showed an enhancement of the bands at 365, 385 and 408 nm reaching a plateau at high AMB concentration (see Fig. 2), while Bolard et al. [10] reported a decrease in the intensity of these bands at increasing AMB concentration. It can be pointed out, that no modification of the monomer bands position was found for the different medium conditions (temperature, pH, neutral salt, urea), which suggested that these medium conditions do not perturb the $\pi^* \leftarrow \pi$ transitions in AMB [9]. The modification of the intensity of the absorption band of AMB when it is bound to albumin can be explained on the basis of the simple theory of electronic spectra for polyene chromophores [11].

It has been reported that [12] the requirements for exciton interaction are strict, depending on the relative orientation and distances of the chromophores in the aggregate form. The aggregate formation was dependent on the medium conditions as it was demonstrated by the dependence of the band at 340 nm on the medium conditions.

Electronic absorption allows the detection of very little amounts of the AMB monomer, whereas the CD measurements do the same with the associated species amounts. We have performed the absorbance measurements at very low AMB concentration, in the range 0.03–1 μM, which allowed us to detect very small changes in the bands, while Bolard et al. [10] obtained the spectra at higher AMB concentration (0.1–100 μM). Under these experimental conditions, AMB is present as aggregates, this spectroscopic form of AMP has either a very high absorption or scattering of the light. Both phenomena induced an increase of the molar absorption of AMB at the 340-nm band.

We found a very low critical micellar concentration value (approx. 1 μM) which is in agreement with those obtained for other polyenic antibiotics such as nystatin and fillipin [2].

Previous results obtained by molecular modeling [5] indicate that aggregation driving-forces of AMB originate from hydrophobic interaction. A significant participation of electrostatic forces has also been reported between the terminal 35-OH groups and amino-sugar oxygen O-42 (see Fig. 7) as well as intramolecular hydrogen bonds between 43-OH and carboxylic groups of the AMB molecule, and between carboxyl groups and 15-OH. It has been postulated [5] that the geometry of this network determines the stability and therefore, the architecture of the aggregate. The chemical nature of the AMB molecule determines its poor solubility and its amphiphatic properties. An understanding of the structure and conformation of the antibiotic and its variability under different conditions such as pH, neutral salts, temperature, etc., is important for the proposition of both a molecular model for AMB behavior in aqueous solution and its interaction with protein which might produce a different geometry from the above mentioned by stabilizing the aggregate, or not. Our absorption and CD observations demonstrate that the formation of the aggregate is favored at acidic pH values, which confirms that the protonated form of the carboxylic group participates in the stabilization and formation of the aggregate form as postulated by Castanho et al. [12]. On the other hand, the presence of a positive net charge at the amino group, which occurs at a pH below 10 also stabilizes the aggregate. Moreover, the ionic strength increase induces the destabilization of the aggregate, confirming the participation of an electrostatic component. However, no significative differences on the K_A were observed between

the different sodium halide salts, which is in agreement with the basis of the Debye–Huckel theory [13]. Some studies have reported that urea induces modification of the entropic state of the water molecules, the so called 'hydrophobic effect' by the water–urea complex formation [14]. Our results from the absorption and CD measurements confirm that this water plays an important role in the AMB–AMB interaction because the urea presence inhibited the aggregate.

Finally, it was found that the polarity diminution of the AMB environment due to either its binding to albumin or the ethanol medium addition, induce dissociation of the aggregate form into the monomer one and produce an increase of the dipolar moment of the excited state transition of the AMB monomer.

Acknowledgements

This work was supported by a grant from Secretaría de Ciencia y Técnica (U.N.R.) and The Third World Academy of Sciences (Trieste). We thank Prof. C. Houssier, University of Liege (Liege, Belgium) for the use of the dicrograph and Mrs Robson for the language correction of the manuscript.

References

[1] S. Omura, H. Tanaka, Macrolide Antibiotics: Chemistry, Biology and Practice, Academic Press, N.Y., 1984, p. 351.
[2] R. Bittman, W. Chen, L. Blau, Biochemistry 13 (1974) 1374–1379.
[3] A.R. Balakrishnan, K.R.K. Easwaran, Biochim. Biophys. Acta 1148 (1993) 269–277.
[4] R. Hunter, Foundations of Colloid Science, vol. I, Oxford University Press, New York, 1989.
[5] J. Mazerski, E. Borowski, Biophys. Chem. 57 (1996) 205–217.
[6] D. Romanini, B. Farruggia, G. Picó, Biochem. Mol. Biol. Int. 44 (1998) 595–603.
[7] C. Ernst, J. Grange, H. Rinnert, G. Dupont, J. Lematre, Biopolymers 20 (1981) 1575–1588.
[8] C. Tanford, Physical Chemistry of Macromolecules, John Wiley, New York, 1961.
[9] C. Berde, B. Hudson, R. Simoni, L. Sklar, J. Biol. Chem. 254 (1979) 391–400.
[10] J. Bolard, M. Seigneuret, G. Boudet, Biochim. Biophys. Acta 599 (1980) 280–293.
[11] L. Sklar, B. Hudson, M. Petersen, J. Diamond, Biochemistry 16 (1977) 813–818.
[12] M. Castanho, A. Continho, M. Prieto, J. Biol. Chem. 267 (1992) 204–209.
[13] A. Adamson, Physical Chemistry of Surfaces, Mack Printing Co., Easton, PA, 1963.
[14] T. Sarma, J. Ahluwalia, J. Phys. Chem. 76 (1972) 1366–1369.