Biochimica et Biophysica Acta 860 (1986) 125–130
Elsevier

BBA 73228

A fluorescence and microcalorimetric study of the interaction between lasalocid A and phospholipid vesicles

Francisco J. Aranda and Juan C. Gómez-Fernández *

Departamento de Bioquímica, Facultad de Veterinaria, Universidad de Murcia, Espinardo, 30071 Murcia (Spain)

(Received February 3rd, 1986)
(Revised manuscript received May 1st, 1986)

Key words: Ionophore–membrane interaction; Lasalocid A; Phospholipid vesicle; Fluorescence; Differential scanning calorimetry

(1) The binding of lasalocid A to dipalmitoylphosphatidylcholine (DPPC) vesicles was studied following changes in the intrinsic fluorescence of this ionophore. The binding calculations indicated a dissociation constant of 6.98 ± 1.5 μM at 48°C, i.e., above the transition temperature (T_c) of the pure phospholipid, with a number of binding sites of 1 per 22 ± 2.5 molecules of phospholipid, while at 23°C, i.e., below the T_c of the pure phospholipid, the dissociation constant was 9.15 ± 0.24 μM and the number of binding sites was 1 per each 29 ± 1.6 molecules of DPPC. (2) Changes in the temperature induced changes in fluorescence intensity of lasalocid A mainly upon phase changes, indicating a progressive decrease in the transition temperature accompanied by a broadening of the transition as lasalocid A concentration was increased. (3) Fluorescence quenching experiments with *N*-methylnicotinamide showed a certain accessibility of the fluorophoric group of the ionophore to the aqueous quencher. (4) Differential scanning calorimetry showed that increasing concentrations of lasalocid A drastically modified the thermotropic profile. At concentrations higher than 5 mol%, a second peak appeared, possibly due to a lateral phase segregation of lasalocid A trapping some phospholipid molecules. (5) The results are interpreted in terms of limited solubility of lasalocid A in the phospholipid vesicles, this solubility being higher in fluid than in rigid phospholipid. Lateral segregation seems to occur with formation of more than one phase. At least the salicylic acid residue of the ionophore appears to be located near the polar head group of the phospholipid.

Introduction

Lasalocid A (X537A, lasalocid) is a carboxylic ionophore which has been shown to bind alkali metal ions, alkaline earth metal ions [1,2], rare earth metal ions [3,4] and transition metal ions. It also binds primary and secondary ammonium ions [1].

The molecular structures of metal-free lasalocid and its metals salts were early determined by means of X-ray crystallography [5–8]. The use of lasalocid A in experimental biology and technology directed attention to the cation complexation process. Structural and kinetics studies of several metals and biogenic amine complexes of lasalocid A in nonpolar solvents were carried out by means of proton NMR [9–11] and circular dichroism [12]. The conformational dynamics of the ionophore has been studied in solvents of different grade of polarity by means of circular dichroism [13].

Lasalocid A has been used for studying the role of calcium in physiological systems [14,15], and

* To whom correspondence should be addressed.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; T_c, gel-to-liquid-crystal phase-transition temperature.

the calcium-transport mechanism by the ionophore through phospholipid membranes has been studied [16].

However, lesser attention has been paid to the interaction of lasalocid A with the surrounding phospholipids. The use of phospholipid vesicles is a good approaching method to study this type of interaction. A more detailed understanding of the interaction of lasalocid A complexes and phospholipid bilayers would lead to an enhancement of the ionophore's utility as a research tool as well as to provide some useful information in connection with the subject of membrane interactions with small hydrophobic molecules.

It has been shown that the intrinsic fluorescence of the ionophore is due to a salicylic acid residue which contributes to the complexing centre [17], and that the binding of the ionophore to membranes can be followed by changes in its fluorescence signal [18]. Its fluorescence has a polarity dependence, so that there is an increasing quantum yield with decreasing solvent polarity [18].

We use in this paper the fluorescence of lasalocid A as a convenient method for examining its interaction with phospholipid bilayers. We also use a well-known and no perturbing technique as differential scanning calorimetry (DSC) to determine the influence of lasalocid A on the phospholipid thermotropic phase transition, which is another way of studying the interaction between lasalocid A and the phospholipid bilayers.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Fluka, Buchs, Switzerland. Lasalocid A sodium salt and *N*-methylnicotinamide were from Sigma, Poole, Dorset, U.K. All other reagents were of analytical grade. Twice distilled and deionized water was used in all the experiments.

Phospholipid mixtures were prepared by combination of chloroform solutions containing appropriate amounts of phospholipid and lasalocid A (and the adecuate amounts of *N*-methylnicotinamide when necessary) giving a chloroform solution with a final volume of 50–200 μl in a small test tube. After drying under a nitrogen steam, the sample was then further dessicated under vacuum during 2–3 h to remove the last traces of the solvent. The phospholipid mixtures were resuspended at 75 mM for the calorimetric measurements and, unless otherwise indicated, at 0.5 mM for the fluorescence measurements in a buffer containing 50 mM KCl/5 mM Tris (pH 7.3). Multilamellar vesicles were formed by carefully mixing using a brench vibrator, and keeping the temperature at 50–55°C (above the main T_c transition temperature of DPPC). Mixing was continued until an homogeneous and uniform suspension was obtained. At the pH of study (7.3) this involves the singly negatively charged form of lasalocid A complexated with the monovalent cations of the buffer [16].

Steady-state fluorescence measurements were done using a Perkin-Elmer MPF 44B spectrofluorimeter. The excitation and emission wavelengths used were 310 and 415 nm, respectively.

Samples for differential scanning calorimetry measurements, 15 μl, were sealed in small aluminium pans and scanned in a Perkin-Elmer DSC-4 instrument, using a reference pan containing the buffer. The heating and cooling rates were 4 K/min. The range of temperatures studied was from 25 to 55°C. Peak areas were measured by weighing paper cut-outs of the peaks. The instrument was calibrated using indium as a standard.

Results

Fluorescence measurements

Binding of lasalocid A to DPPC vesicles . Fig. 1a shows the uncorrected fluorescence emission spectra of lasalocid A (5 μM) in the presence of increasing concentrations of DPPC vesicles ranging from 0 to 500 μM at 48°C, i.e., above the T_c of pure DPPC. The enhancement of fluorescence emission that occurs at increasing concentrations of phospholipid is taken to indicate an association between the ionophore and the DPPC bilayers. It was reported before [18] that the fluorescence of the ionophore has a polarity dependence with increasing quantum yield as the solvent polarity decreases. Fig. 1b shows the plot of relative fluorescence intensity vs. DPPC concentrations for the same samples at 48°C, and the inset of Fig. 1b

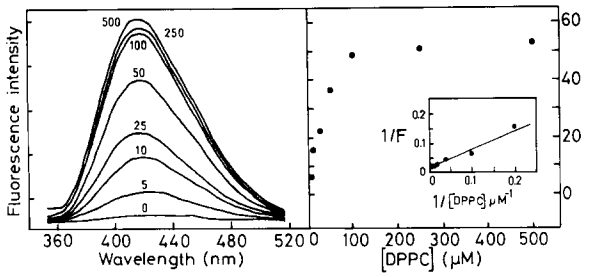

Fig. 1. (a) Uncorrected emission spectra for lasalocid A in the presence of increasing concentrations of DPPC, ranging from 0 to 500 μM (μM DPPC concentrations appear on the curves), at 48°C. Each experiment corrsponds to a different vesicle preparation. Binding experiments were carried out in a buffer containing 50 mM KCl and 5 mM Tris (pH 7.3) and with a lasalocid A concentration of 5 μM. (b) Plot of lasalocid A fluorescence vs. increasing concentrations of DPPC at 48°C. The inset of Fig. 1b shows the double reciprocal plot of fluorescence, $1/F$, vs. the total DPPC concentration in the vesicle suspension, in μM^{-1}.

shows the reciprocal of the fluorescence enhancement against the reciprocal of the DPPC concentration at this temperature. A straight line was obtained from the double reciprocal plot which was extrapolated to calculate the y-intercept, and from this the lasalocid A fluorescence enhancement, when all the ionophore present in these samples (5 μM) is bound to the DPPC vesicles, was estimated [19]. In a similar way, the lasalocid A fluorescence enhancement when all the ionophore is bound to the DPPC vesicles at 23°C, was estimated (data not shown). A Scatchard analysis [20] was performed on the binding of lasalocid A (ranging from 0 to 125 μM) to DPPC vesicles (500 μM) at 48 and 23°C, i.e., above and below the T_c of the pure DPPC. The results are plotted in Fig. 2, where ν represents the concentration of lasalocid A bound to DPPC vesicles vs. the concentration of DPPC vesicles and c represents the concentration of lasalocid A free. The results show that at 48°C, i.e., above the T_c of pure DPPC, the number of binding sites is $n = (45.2 \pm 5) \cdot 10^{-3}$ (μM/μM), which means that approx. one molecule of lasalocid A binds per 22 ± 2.5 DPPC molecules, with a dissociation constant (K_d) of 6.98 ± 1.5 μM. At 23°C, i.e., below T_c of pure DPPC, K_d was found to be equal to 9.15 ± 0.24 μM with a maximum binding of 1.29 ± 1.6 lasalocid/DPPC molar ratio.

The influence of temperature on fluorescence intensity. Fig. 3 shows changes in the fluorescence intensity of increasing concentrations of lasalocid A intercalated into bilayers of DPPC, as a function of temperature. Samples were heated from 25 to 50°C. It was observed that fluorescence intensity decreased uniformly as temperature increased until reaching a temperature at which a sharp fluorescence enhancement occurred, and again the fluorescence intensity decreased as the temperature increased. The temperature at which the fluorescence intensity enhancement took place was clearly dependent on the lasalocid A concentration: this temperature decreased as the lasalocid A concentration increased, and ranged from approx. 41°C for the sample with the least lasalocid (a DPPC/lasalocid A molar ratio of 100 : 1) to about 33°C for the sample with more intercalated lasalocid A (a DPPC/lasalocid A molar ratio of 2 : 1). Therefore as the lasalocid A concentration is increased the transition temperature remarkably goes down with respect to T_c of the pure DPPC (41°C). It is worthwhile to observe that not only the fluorescence enhancement temperature is lower, but also this enhancement is larger when the lasalocid A concentration is increased.

Quenching of lasalocid A fluorescence by N-methylnicotinamide. Stern-volmer plots for the quenching of lasalocid A fluorescence by *N*-methylnicotinamide (Fig. 4) show that both lasalocid A in aqueous solution and lasalocid A incorporated

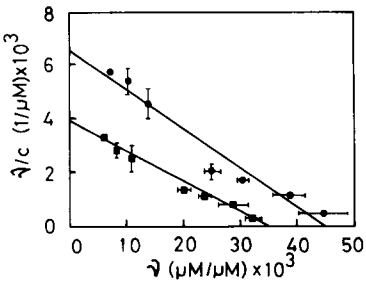

Fig. 2. Scatchard plot for the binding of lasalocid A (0–125 μM) to DPPC vesicles (500 μM), where ν = [lasalocid A]$_{bound}$/[DPPC], and c = [lasalocid A]$_{free}$. Each point represents the average of three different experiments and the bars represent the standard deviation. ●, 48°C; ■, 23°C.

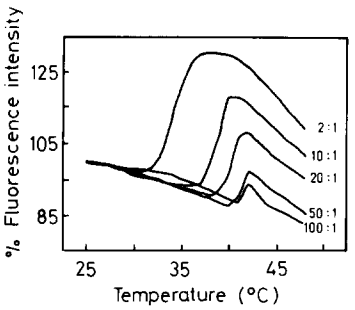

Fig. 3. Fluorescence intensity of increasing concentrations of lasalocid A intercalated into DPPC bilayers (0.5 mM) vs. temperature. Molar DPPC/lasalocid A ratios are indicated on the curves.

into DPPC bilayers are quenched by this agent. The quenching constants for the ionophore (i.e. the slope of the Stern-Volmer plot) were 0.8 and 0.6 M^{-1} for the DPPC bilayer-bound lasalocid A at 23 and 48°C, respectively (i.e., above and below T_c of the phospholipid); for these temperatures the constants are a factor of respectively 7 and 9.5 lower than that of the lasalocid A in aqueous solution (5.7 M^{-1}).

DSC measurements

Fig. 5 shows the heating and colling DSC

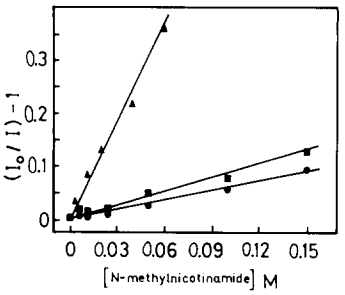

Fig. 4. Stern-Volmer plot for the quenching of the lasalocid A fluorescence by *N*-methylnicotinamide. Samples for quenching experiments were prepared mixing chloroform solutions of DPPC, lasalocid A and *N*-methylnicotinamide, and vesicles were formed as indicated in the section of Methods. Each point of the plot corresponds to a different vesicle preparation. DPPC and lasalocid A concentrations were 0.5 mM and 5 μM, respectively. ▲, quenching of lasalocid A fluorescence in aqueous solution; quenching of lasalocid A fluorescence intercalated in DPPC bilayers at 23°C (■) and 48°C (●).

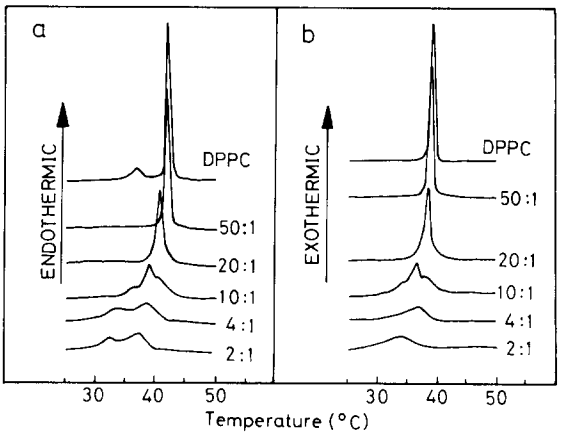

Fig. 5. DSC calorimetric profile for the thermotropic-gel-to-liquid-crystal phase transition for pure DPPC and DPPC/lasalocid A systems. DPPC concentration was 75 mM and the molar DPPC/lasalocid A ratios are indicated on the curves. (a) Heating experiments; (b) cooling experiments.

calorimetric profile of the thermotropic gel-to-liquid crystalline transition of DPPC and those of DPPC:lasalocid A mixtures with increasing concentrations of ionophore. Heating curves (Fig. 5a) revealed that the presence of increasing concentrations of lasalocid A produced a blurring of the pretransition and a broadening plus decreasing in height of the main transition peak (molar DPPC/lasalocid ratios of 50:1 and 20:1 samples). With increasing proportion of lasalocid A in the bilayer the profile of the thermotropic transition becomes more complex, giving rise to a new peak at temperatures lower than the T_c of pure DPPC, while the main transition peak progressively decreased (in the DPPC/lasalocid molar ratio of 10:1 a shoulder is also present at a temperature higher than T_c). The cooling curves (Fig. 5b) were symmetrical with the corresponding heating ones, except by the normal hysteresis and by the observation that in the samples most concentrated in lasalocid A (molar DPPC/lasalocid ratios of 4:1 and 2:1) only one peak appeared at intermediate temperature at which the two heating curves peaks were localized.

Fig. 6 shows the values of ΔH for the thermotropic transition of DPPC vs. the molar lasalocid A/DPPC ratios. For pure DPPC ΔH was estimated to be of 35.6 kJ/mol. It can be observed that the increasing concentrations of lasalocid into

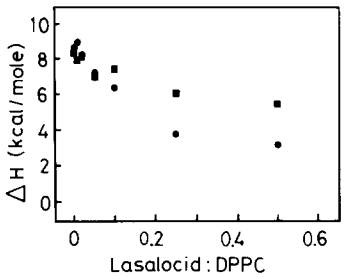

Fig. 6. ΔH for the gel-to-liquid-crystal phase transition for systems containing different molar lasalocid A/DPPC ratios. ■, heating experiments; ●, cooling experiments.

the bilayer have the effect of decreasing ΔH of the DPPC transition being the value of ΔH for the most concentrated sample (molar lasalocid A/DPPC ratio of 0.5) of 23.0 and 13.8 kJ/mol for the heating and cooling experiments, respectively.

Discussion

The intrinsic fluorescence properties of lasalocid A may be exploited to gain information about its location in phospholipid membranes.

The experiments reported above allow the calculation of the binding parameters of the ionophore to DPPC liposomes, showing that the binding is slightly favoured when the membrane is fluid, since above T_c the K_d value is lower and the n value is higher than below T_c.

The presence of lasalocid A may perturb the phospholipid phase transition as seen both in changes in fluorescence intensity with temperature and DSC experiments. Both types of technique closely agree in showing that T_c decreases as more lasalocid A is included in the membrane so that it is as low as 32°C (fluorescence) or 31°C (DSC) for a DPPC/lasalocid A molar ratio of 2.1. DSC is somehow more precise than fluorescence intensity measurements, since it detects different transitions that probably arise from different phases.

This phase separation is suggested by the appearance of two peaks in the transition profile, at DPPC/lasalocid A molar ratios lower than 20 : 1. It has to be remembered here that as concluded above from changes upon fluorescence binding measurements, the maximum molar ratio that could be achieved is 22 : 1 DPPC/lasalocid. These results probably mean that lasalocid A in excess of this concentration segregates in the plane of the membrane, possibly trapping some phospholipids and hence generating the peak of the calorimetric profile that melts at lower temperature. Similar results obtained from DSC experiments made on mixtures of cannabinoic drugs and DPPC were given a similar explanation [21]. On the other hand the peak with a higher melting temperature probably arises from the effect of the lasalocid A dispersed on the bulk phospholipid, so that the transition of this phase also occurs at temperatures lower than the one corresponding to pure DPPC. According to the examples shown by Jain and Wu [22], a shift in the temperature of the transition peak may be interpreted as an interaction of the molecule with the polar head group of the phospholipid.

Nevertheless, the effects seen in the calorimetric experiments obtained at very high phospholipid/lasalocid A ratios ($\geq 20:1$) might not be particularly relevant to the normal interactions of lasalocid A with membranes, since lasalocid A is active in bilayers at very low concentrations.

The phase transition produces an increase in the intensity of the intrinsic fluorescence of lasalocid A and this may be due, at least partially, to the higher solubility of lasalocid A in fluid than in rigid DPPC, as discussed above, although an increase in the lasalocid A quantum yield during the transition interval of temperatures may be not excluded.

The fact that the amplitude of the fluorescence change at the main transition markedly increases as the ionophore-to-lipid ratio increases (Fig. 3) might be attributed in principle, to either a self-quenching of fluorescence or to an enhancement of fluorescence at high local concentrations of ionophore that begin to come into play as the level of ionophore in the bilayers increases. However, we favor the first possibility, since we observed self-quenching of fluorescence in ethanolic solutions of lasalocid A when comparing the fluorescence intensity of solutions with lasalocid A concentrations ranging between 0.36 and 23 mM (results not shown).

Finally, the fluorescence quenching experiments indicate that at least the fluorophoric ring of lasalocid A is situated at the aqueous interface

of the outer monolayer, since this quencher may not penetrate into the hydrophobic core of phospholipid vesicles [23]. The fact that a higher value for K_Q was found for the quenching experiments performed below T_c for those above T_c may reflect a change in localization of the ionophore being more hidden to *N*-methylnicotinamide when the phospholipid is fluid.

In conclusion, our fluorescence and DSC experiments indicate a limited solubility of lasalocid A in DPPC vesicles, forming separate phases at very high concentrations, and that the localization of lasalocid A in the membrane allows the salicylic acid residue to be situated near the interface of the outer half of the bilayer with the aqueous environment, with the lasalocid A molecule somehow interacting with the polar head group of DPPC.

Acknowledgement

This work was financially supported by grant no. 3401(83)01 from CAICYT (Spain).

References

1 Pressman, B.C. (1972) in The Role of Membranes in Metabolic Regulation (Melman, M.A. and Hanson, R.W., eds.), pp. 149–157, Academic Press, New York
2 Degani, H., Friedman, H.L., Navon, G. and Kosower, E. (1973) J. Chem. Soc. Chem. Commun. 431–432
3 Pressman, B.C. (1973) Fed. Proc. Fed. Am. Soc. Exp. Biol. 32, 1968–1703
4 Fernández, M.S., Celis, C.H. and Muntal, M. (1973) Biochim. Biophys. Acta 323, 600–605
5 Bissell, E.C. and Paul, I.C. (1972) J. Chem. Soc. Chem. Commun., 967–968
6 Maier, C. and Paul, I.C. (1971) Chem. Commun, 181–182
7 Schmidt, P.G., Wang, A.H.J. and Paul, I.C. (1974) J. Am. Chem. Soc. 96, 6189–6191
8 Johnson, S.M., Herrin, J., Lin, S.J. and Paul, I.C. (1970) Chem. Commun., 72–73
9 Patel, D.J. and Shen, C. (1976) Proc. Natl. Acad. Sci. USA 73, 1786–1790
10 Shen, C. and Patel, D.J. (1976) Proc. Natl. Acad. Sci. USA 73, 4277–4281
11 Shen, C. and Patel, D.J. (1976) Proc. Natl. Acad. Sci. USA 74, 4734–4738
12 Degani, H. and Friedman, H.L. (1974) Biochemistry 13, 5022–5032
13 Painter, G.R., Pollack, R. and Pressman, B.C. (1982) Biochemistry 21, 5613–5620
14 Entman, M.L., Gillette, P.C., Wallick, E.T., Pressman, B.C. and Schwartz, A. (1972) Biochem. Biophys. Res. Commun. 48, 847–853
15 Caswell, A.H. and Pressman, B.C. (1972) Biochem. Biophys. Res. Commun. 49, 292–298
16 Haynes, D.H., Chin, V.C.K. and Watson, B. (1980) Arch. Biochem. Biophys. 203, 73–89
17 Johnson, S.M., Herrin, J., Lin, S.H. and Paul, I.C. (1970) J. AM. SOC. 92, 4428–4435
18 Haynes, D.H. and Pressman, B.C. (1974) J. Membrane Biol. 16, 195–205
19 Brocklehurst, J.R., Freedman, R.B., Hanckock, D.J. and Radda, G.K. (1970) Biochem. J. 116, 721–731
20 Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672
21 Bach, D., Raz, A. and Goldman, R. (1976) Biochim. Biophys. Acta 436, 889–894
22 Jain, M.K. and Wu, N.M. (1977) J. Membrane Biol. 34, 157–201
23 Kauffman, R.F., Chapman, C.J. and Pfeiffer, O.R. (1983) Biochemistry 22, 3985–3992