Biochimica et Biophysica Acta 813 (1985) 307–312
Elsevier

BBA 72488

Binding of hematoporphyrin derivative to membranes. Expression of porphyrin heterogeneity and effects of cholesterol studied in large unilamellar liposomes

Smadar Cohen and Rimona Margalit *

Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978 (Israel)

(Received September 2nd, 1984)

Key words: Hematoporphyrin-membrane interaction; Liposome; Binding equilibrium; Cholesterol; Porphyrin aggregation

The binding of hematoporphyrin derivative to membrane was studied at the molecular level, employing fluorimetric techniques and using liposomes (large unilamellar) to model biological membranes. Two specific issues were probed: (a) the effect of increasing the porphyrin-liposome incubation period (37°C, neutral pH, in the dark) from 2 h up to 24 h, with liposomes composed of PC or PC/cholesterol 7:3 (molar ratio); (b) the effects of membrane lipid composition, in terms of mol% cholesterol in PC/cholesterol liposomes, on the porphyrin-membrane binding equilibrium, for a long incubation period (16 h). The data were processed and found to be in good agreement with the following proposed model: With time ($t > 2$ h), the porphyrin fractions into three components, two of them binding to membrane with high and low affinities, respectively, the time effect reaching a plateau at 16 h. Linkage was observed between the slow process and changes in the available pools of each fraction. At sufficiently long incubations, the magnitude of each of the two binding fractions was found to be (roughly) 30%, independent of the membrane lipid composition. On the other hand, the magnitude of the binding constants was found to depend on the lipid composition, that of the high-affinity fraction decreasing from 9000 M^{-1} for 0% cholesterol, to 3000 M^{-1} for 40% cholesterol, then increasing back to 5000 M^{-1} upon further increase in cholesterol (to 50% mol) and that of the low-affinity fraction going from 1000 M^{-1}, to 100 M^{-1}, to 300 M^{-1} for similar lipid compositions. The origin of the time effect, in terms of porphyrin-specific processes, and the biological relevance of the present findings are discussed.

Introduction

In a previous communication [1] we have reported our molecular level studies on the binding of hematoporphyrin and hematoporphyrin derivative to liposomes (large unilamellar) composed of PC only. Emphasis was on the relationship between porphyrin aggregation in the aqueous phase and porphyrin-membrane binding. We have found that, regardless of the initial porphyrin aggregation state within the range of monomer to dimer dominance, only monomers are bound to the liposomes at equilibrium, residing within the lipid domains of the particle.

In the present communication we report our continued studies on porphyrin-membrane interactions, probing the effects of membrane lipid composition. We have focused on the binding of hematoporphyrin derivative to liposomes composed of PC and cholesterol, varying in their cholesterol concentration over the 0–50 mol% range.

Hematoporphyrin derivative is known to be a mixture of several porphyrin species (see, for ex-

* To whom correspondence should be addressed.
Abbreviation: PC, phosphatidylcholine.

ample, Refs. 2–12), yet under our previous experimental conditions the system behaved as if it were a 'single species' [1], implying that no substantial perturbation of the initial balance among the different components of hematoporphyrin derivative has taken place under those conditions. Therefore the magnitude determined for the binding constant [1] should be a weighted average of the contributions of several membrane-binding species within hematoporphyrin derivative. To force the system into expressing the heterogeneity of hematoporphyrin derivative, we decided to increase the length of the incubation period, in stages, up to 24 h. The rationale for this direction was based on preliminary experiments we have conducted, and on studies of cellular systems [10,13], showing differences in hematoporphyrin derivative binding for different periods of incubation. The effects (if at all) of time were tested not only on liposomes composed of PC, but also on those containing cholesterol, to assess whether time effects are restricted to a given lipid composition.

Thus, two specific questions were addressed in this study:

(i) Is there an effect of the length of the incubation period on the porphyrin-liposome binding equilibrium, and of what nature.

(ii) Is there an effect of cholesterol on the porphyrin-liposome binding equilibrium, and is it cholesterol-concentration dependent.

Materials and Methods

Materials

Hematoporphyrin derivative was purchased from Porphyrin Products (Logan, UT, U.S.A.), and is prepared there according to Lipson [14]. We have verified its heterogeneity by chromatographic procedures. Cholesterol and egg PC (type VII-E) were purchased from Sigma Chemical Co. Triton X-100 was purchased from Serva Feinbiochemica, Heidelberg. All other reagents were of analytical grade.

The hematoporphyrin derivative was treated with alkali in the course of solution preparation. The routine of solution preparation and the protection of all porphyrin-containing systems were as previously described [1].

Phosphate-buffered saline comprised 3.3 mM KH_2PO_4/3.3 mM Na_2HPO_4/4.7 mM KCl/143 mM NaCl, adjusted to pH 7.2.

Fluorescence spectra were recorded on a Perkin-Elmer fluorimeter model MPF-44B. Absorption spectra were recorded on a Cary 118.

Methods

The hematoporphyrin derivative concentration of all reaction mixtures was $5 \cdot 10^{-8}$ M (mass of hematoporphyrin derivative was taken as 650 Da, according to Ref. 4). The liposomes were large unilamellar of the reverse-phase evaporation type, prepared according to the method of Szoka et al. [15] from PC only or from PC/cholesterol mixtures varying in their molar ratios up to 1 : 1. The experimental procedures employed to study the binding of porphyrins to membranes were similar to those previously described [1]. Briefly, a series of reaction mixtures was prepared, buffered by phosphate-buffered saline, containing the constant hematoporphyrin derivative concentration listed above and increasing concentrations of liposomes, in the 0.1–2.0 mg lipid/ml range. Reaction mixtures were incubated for desired periods, at 37°C, in the dark. Separation of liposomes was effected by ultracentrifugation. Fluorimetry was used for porphyrin determinations. Absorbance would not be sensitive enough for the low porphyrin concentrations needed to be determined ($5 \cdot 10^{-8}$), even with cells giving an optical path of 10 cm. To determine porphyrin, the samples were dissolved in 10% Triton X-100, in methanol, and the fluorescence was read at 627 vs. a calibration curve, obtained under the same conditions. Quantitative determination of cholesterol was carried out according to Ref. 16, in the final liposome preparation used for the binding studies.

Results

Hematoporphyrin derivative binding to liposomes. Effects of increasing the incubation period.

Increasing the length of the incubation period clearly affected the binding (as will be detailed below), the effect taking approximately 16 h to reach a plateau. Data for selected periods (the points), presented in Figs. 1 and 2, illustrate even prior to any analysis two major features of the effect of time: (a) the effect is not restricted lipo-

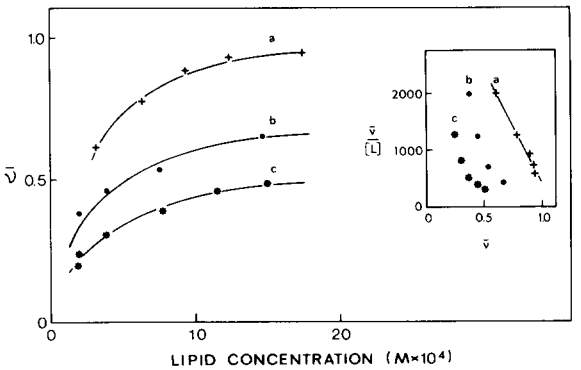

Fig. 1. Langmuir isotherm of hematoporphyrin derivative binding to PC liposomes for the following periods of incubation: (a) 2 h; (b) 4 h. (c) 19 h. Incubations were in the dark, at 37°C and neutral pH. Points are experimental, solid curve are theoretical expectations drawn according to Eqn. 1 in the text, for the respective parameters listed in Table I. Inset: Scatchard plots of the same data. The solid curve is theoretical, drawn according to linear regression analysis.

somes composed of PC only; (b) for incubation periods of more than 2 h, saturation is observed with $\nu_{max} < 1$, in contrast to $t = 2$ h, where $\nu_{max} = 1$ is observed. This is even more pronounced when the data are plotted according to Scatchard [17] (see inset to Fig. 1). For $t = 2$ h a single linear plot intersecting the abscissa at a value close to 1 can be drawn through all the data points, fitting the model for one type of site [1]. In contrast, for $t = 4$ h, and moreso for $t = 19$ h, the data points do not fit a single linear plot, their pattern implying the existence of more than one type of binding site [17,18]. As will be shown in the Discussion section, we have made use of this implication in the interpretation of the data.

Hematoporphyrin derivative binding to PC / cholesterol liposomes: effects of lipid composition

To assess the effects of cholesterol, we have studied the binding of hematoporphyrin derivative to PC/cholesterol liposomes, varying the cholesterol concentration from 0 to 50% mol. To dissociate the effect of the lipid composition from any interference from the effect of time, we have settled on a single, sufficiently long, incubation period of 16 h.

Typical results (the points) are illustrated in Fig. 3. The observation that the data for the different systems (different in terms of percent cholesterol) do not coincide, and the trend in the spread of the data, even though the same incubation period was used for all systems, is already an indication that there is an effect of cholesterol, related to its concentration. Also, and similar to the case for 0% cholesterol (i.e., 100% PC), saturation is observed at $\nu_{max} < 1$. In general, the transition from no cholesterol to different levels of it does not show any qualitative discontinuity, but rather, continuous quantitative differences.

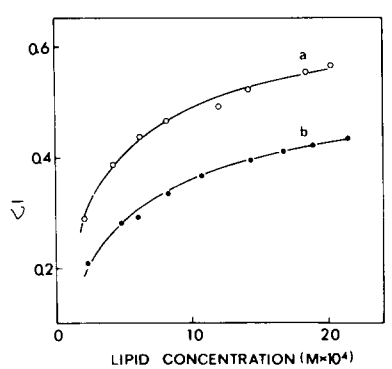

Fig. 2. Langmuir isotherms for hematoporphyrin derivative binding to PC/cholesterol (molar ratio, 7:3) liposomes. Incubation conditions as in Fig. 1 above, periods: (a) 9 h; (b) 16 h. Points are experimental, solid curves are theoretical expectations, drawn according to Eqn. 1 in the text, for the respective parameters listed in Table I.

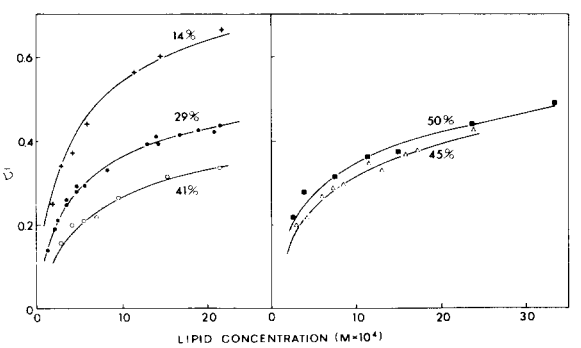

Fig. 3. Langmuir isotherms for hematoporphyrin derivative binding to PC/cholesterol liposomes varying in their cholesterol concentrations, as indicated on the figure. Incubation conditions as those listed in Fig. 1 above, for 16 h. Points are experimental. Solid curves are theoretical expectations drawn according to Eqn. 1 in the text, for the respective parameters listed in Table II.

Analysis of the binding isotherms

The following issues should (and can) be taken into account upon modeling the binding of hematoporphyrin derivative to liposomes (with or without cholesterol). (a) Hematoporphyrin derivative is a mixture of several porphyrin species, major among those identified are hematoporphyrin, HVD (hydroxyethylvinyldeuteroporphyrin) and PP [9] and several types of aggregate. (b) The binding data obtained for incubation periods of $t > 2$ h indicate the existence of more than one type of binding site. (c) Size consideration makes it unlikely that a single porphyrin molecule will bind to more than one liposome.

Considering these issues, we propose that at sufficiently long incubation periods the heterogeneity of hematoporphyrin derivative comes into expression, in terms of its effects on membrane binding, as follows: The hematoporphyrin derivative fractions into three components, two of them with membrane-binding capacities but differing in their affinity for the membrane, the third fraction not binding (or with a binding constant too low to be detected) under our experimental conditions. The absence of distinct steps in the binding isotherms (see Figs. 1–3), is an indication that the differences in the membrane affinities of the two binding fractions are less than several orders of magnitude. Still, since one will obviously have a binding constant of higher magnitude than the other, we will distinguish between the two binding fractions by denoting them as the high- and low-affinity species, bearing in mind that these are relative terms.

Defining the fractions of the high-affinity, low-affinity and non-bonding species, per mole hematoporphyrin derivative as f_1, f_2 and f_3, the expression for a 'two types of site, one type of ligand' model, for the present case, can take the following form:

$$\bar{\nu} = f_1 \frac{K_1[L]}{1 + K_1[L]} + f_2 \frac{K_2[L]}{1 + K_2[L]} \quad (1)$$

Where K_1 and K_2 are the binding constants for the high- and low-affinity components, respectively.

We have, accordingly, processed the data for all the experimental systems. The solid curves drawn in Fig. 1–3 are the theoretical expectations according to Eqn. 1, for the magnitudes of f_1, f_2, K_1 and K_2 listed in Tables I and II. As seen, good agreement between experimental data and theoretical expectations has been obtained for each case, supporting the model proposed. Also, as predicted above, the difference between the magnitudes of the high- and low-affinity constants are moderate, roughly one order of magnitude for a given membrane preparation. In the Discussion we will make use of these binding parameters in our attempt to assess the issues defined in the Introduction, namely the effects of time and of the cholesterol.

TABLE I

BINDING OF HEMATOPORPHYRIN DERIVATIVE TO LIPOSOMES: EFFECTS OF TIME

Lipid composition (PC/cholesterol molar ratios)	Incubation period (h)	f_1	f_2	K_1 (M^{-1})	K_2 (M^{-1})
1:0	2	1.1		3900	
1:0	4	0.39	0.42	9000	1500
1:0	19	0.26	0.40	9000	1000
7:3	9	0.52	0.21	5500	300
7:3	16	0.32	0.24	5000	700

TABLE II

BINDING OF HEMATOPORPHYRIN DERIVATIVE TO PHOSPHATIDYLCHOLINE/CHOLESTEROL LIPOSOMES: EFFECTS OF CHOLESTEROL CONCENTRATION

All data are for long incubations (16 h).

Cholesterol (mol%)	f_1	f_2	K_1 (M^{-1})	K_2 (M^{-1})
0	0.26	0.40	9000	1000
14	0.34	0.47	9000	1000
29	0.32	0.24	5000	700
41	0.31	0.36	3000	100
45	0.31	0.43	3500	200
50	0.32	0.35	5000	300

Discussion

Effects of time

Concentrating, first, on the liposomes containing PC only, the data (recall Table I) show that with time the hematoporphyrin derivative-liposome system is released from the 'single species'

behavior into expressing the heterogeneity of hematoporphyrin derivative. The binding constant suggested to be a weighted average (see Introduction) splitting, indeed, into two constants with higher and lower magnitudes.

A simple interpretation of the nature of the time effect would be if the binding process itself would be extremely slow, taking many hours to reach equilibrium. However, this would not fit with the known fast partitioning of porphyrins from aqueous into organic and detergent phases [1,7,10,19–21]. Nor with our observations (unpublished data) and those of others [12,13,22,23] using incubations of 10, 30 and even 1 min and showing porphyrin binding to membranes to be a fast process. Another observation which would be difficult to reconcile with slow membrane-binding kinetics is seen in Table I for both types of lipid composition. The time-course of the changes in the magnitudes of the binding constants is faster than that of the changes in the magnitudes of the fractions. On the basis of the above we argue that the time effect cannot be due to slow kinetics of porphyrin-membrane binding. Yet some slow process is obviously taking place. Clues to the nature of the slow process can be found in the properties of hematoporphyrin derivative itself. Thus, we have found that freshly prepared hematoporphyrin derivative solutions (even as low in concentration as those used here) devoid of any membrane particles undergo changes in their state as the solution is allowed to age, on the time-scale used in the present study [25]. Further support in attributing the slow change to hematoporphyrin derivative itself, rather than involving the membranes, can be found in our data (recall Table I) showing that the slow changes are in the size of the available pools of the high- and low-affinity species and not in the membrane-binding process, and also in the data on the cholesterol effect, which will be discussed in the following section.

We therefore propose that the observed effect of time stems from slow aging processes of the hematoporphyrin derivative itself, independent of the membrane particles. Thus at different periods, thermodynamics of different porphyrin-liposome systems are studied.

The major findings of this part of our studies, namely the existence of the time-effect, the time-scale and the nature of the effect, are similar to observations on hematoporphyrin derivative binding and retention in vitro [10,13,23] and in vivo [4,8,9,24]. We suggest this gives biological relevance to our molecular level studies and insight into the molecular events taking place when hematoporphyrin derivative is introduced into biological systems in vitro and in vivo.

The effect of membrane cholesterol concentration

Scanning the parameters listed in Table II, several features of the effect of cholesterol become apparent.

(a) Similar to the case of 0% cholesterol, for all cholesterol-containing systems there is one high-affinity and one low-affinity species, with roughly an order of magnitude between the respective binding constants.

(b) For each type of binding species there is a definite trend in the dependence of the binding constant on the cholesterol level: a decrease in the magnitude of the constant with the increase in cholesterol from 0 to 40 mol% and a mild reversal of this trend upon additional increase in cholesterol.

(c) In contrast (and it should be emphasized again that only long incubations are discussed here), the magnitues of f_1 and f_2 are quite similar among the different PC/cholesterol systems, indicating independence of the cholesterol level. The latter is in agreement with the findings of the former section, therefore constituting additional experimental support for the conclusion that the fractionation observed is a property of hematoporphyrin derivative, independent of the membrane particles in the system.

On the other hand, the trends in the magnitudes of each type of binding constant with the increase in the cholesterol concentration seem to reflect properties of the membrane in terms of its lipid composition. Each PC/cholesterol preparation behaving as a distinct type of ligand. Our data show that a change in a single parameter among the many components of a biological membrane, namely the cholesterol level, suffices to cause differences of up to two orders or magnitude in the porphyrin-membrane binding constant (see the high-affinity at 14 mol% cholesterol vs. the low-affinity at 41 mol% cholesterol). This suggests

that the cholesterol content of a membrane should be considered among the factors influencing the membrane binding and retention of hematoporphyrin derivative in vivo.

Acknowledgment

This investigation was supported by PHS grant 5 R01 CA33739-02 awarded to R.M. by the National Cancer Institute, DHHS, U.S.A.

References

1 Margalit, R., and Cohen, S. (1983) Biochim. Biophys. Acta 736, 163–170
2 Kessel, D. (1981) Cancer Res. 41, 1318–1323
3 Kessel, D. (1982) Cancer Res. 42, 1703–1706
4 Berenbaum, M.C., Bonnet, R. and Scourides, P.A. (1982) Br. J. Cancer 45, 571–581
5 Moan, J. and Sommer, S. (1981) Photobiochem. Photobiophys. 3, 93–103
6 Moan, J. Christensen, T. and Sommer, S. (1981) Cancer Lett. 15, 161–166
7 Kessel, D. and Rossi, E. (1982) Photochem. Photobiol. 35, 37–41
8 Dougherty, T.J. (1983) Photochem. Photobiol. 38, 377–379
9 Dougherty, T.J., Boyle, D.G. Weishaupt, K.R., Potter, W.R., Bellnier, D.A. and Wityk, K.E. (1983) in Porphyrin Photosensitization (Kessel D. and Dougherty, T.J., eds.), pp. 3–25, Plenum Press, New York
10 Kessel, D. and Chou, T.-H. (1983) in Porphyrin in Photosensitization (Kessel, D. and Dougherty, T.J., eds.), pp. 115–127, Plenum Press, New York
11 Moan, J., Sandberg, S., Christensen, T. and Elander, S. (1983) in Porphyrin Photosensitization (Kessel, D. and Dougherty, T.J. eds.), pp. 165–179, Plenum Press, New York
12 Goyal, G.C., Blum, A. and Grossweiner, L.I. (1983) Cancer Res. 43, 5326–5830
13 Kessel, D. and Chou, T.-H (1983) Cancer Res. 43, 1994–1999
14 Lipson, R., Baldes, E. and Olsen, A. (1961) J. Natl. Canc. Inst. 26, 1–8
15 Szoka, F.C. and Papahadjoupoulos, D. (1980) Annu. Rev. Bioenerg. 9, 467–508
16 Zlatkis, A. and Zak, B. (1968) Anal. Biochem. 29, 143
17 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
18 Feldman, H.A. (1972) Anal. Biochem. 48, 317–338
19 Kessel, D. (1977) Biochemistry 16, 3443–3449
20 Margalit, R., Cohen, S. and Shaklai, N. (1983) Biochem. J. 209, 547–552
21 Margalit, R. and Rotenberg, M. (1984) Biochem. J. 218, 445–450
22 Kessel, D. and Kohn, K.I. (1980) Cancer Res. 40, 303–307
23 Christensen, T., Sandquist, T., Feren, K., Wakvik, H. and Moan, J. (1983) Br. J. Cancer 48, 35–43
24 Bugelski, P.J., Porter, C.W. and Dougherty, T.J. (1981) Cancer Res. 41, 4606–4612
25 Margalit, R., Rotenberg, M. and Cohen, S. (1984) Porphyrin Photosensitization Workshop, Abstract