Biochimica et Biophysica Acta, 1153 (1993) 170–174

BBAMEM 76155

Kinetics of incorporation of porphyrins into small unilamellar vesicles

Christine Vever-Bizet and Daniel Brault *

Laboratoire de Biophysique, INSERM U. 201, CNRS U.A. 481, Muséum National d'Histoire Naturelle, 43 Rue Cuvier, 75231, Paris Cedex 05 (France)

(Received 21 June 1993)

Key words: Porphyrin; Liposome; Incorporation; Kinetics; Equilibrium; Fluorescence

The kinetics of hematoporphyrin or deuteroporphyrin incorporation in egg phosphatidylcholine small unilamellar vesicles have been investigated by fluorescence stopped-flow measurements. The processes can be described by a fast equilibrium. The on-rate constant is nearly diffusion controlled regardless of the compound used and the pH. The affinity of these porphyrins for the vesicles is merely governed by the exit rate which depends on the structure of the porphyrin and on its charge determined by pH.

Introduction

A new therapy of tumors is being developed which is based on the accumulation within tumors of lipophilic photosensitizers, in particular molecules belonging to the class of porphyrins. Under light irradiation, these photosensitizers are able to generate a very toxic short lived species, singlet oxygen, which reacts with surrounding biomolecules resulting in specific damages [1,2]. At the cellular level, internal membranes, in particular mitochondria, are the main targets and the efficiency of photosensitizers is related to their intracellular incorporation [3]. Photosensitizers are generally administered as aqueous solutions, but in some cases, liposomes or lipoproteins have been used as delivery systems, a protocol which was found to increase the specificity of hydrophobic drugs for tumors [4]. Thus, it appears that interactions of photosensitizers with lipidic systems are key events in photodynamic therapy.

Using egg phosphatidylcholine small unilamellar vesicles as membrane models, we previously showed that the partition of dicarboxylic porphyrins between the bulk aqueous phase and the hydrophobic vesicle bilayer is dominated both by the presence of polar chains on the porphyrin cycle and by the charge of the propionic chains which depends on pH [5,6]. These physicochemical data gave the first support to the hypothesis that pH may control, to some extent, the selective uptake of porphyrins by tumors which are more acidic than normal tissues [7]. In vivo experiments [8] as well as studies on model systems [9] further confirmed the validity of this hypothesis. The problem of porphyrin localization within the bilayer was also adressed [10,11].

We now report on the first study of porphyrin binding to small unilamellar vesicles on a milliseconds time scale which permits further insight into the mechanism of the interactions of porphyrins with membranes and transport systems. It also illustrates important features of the dynamics of solubilizates in organized assemblies [12].

Materials and Methods

Small unilamellar vesicles (SUV) of egg phosphatidylcholine (EPC, type VIIE, Sigma, St. Louis, MO) were prepared as described elsewhere [5] by sonication of a suspension of the phospholipids in aqueous solutions containing sodium phosphate (20 mM), sodium phthalate (20 mM) and NaCl (0.15 M). Sonication was performed under argon in a ice bath using a sonifier (Branson model B15, Danbury, CT) until clear solutions were obtained (about 15–20 min). The stock vesicle solution was diluted to the desired lipid concentration with buffer at appropriate pH and allowed to equilibrate for 2 h at room temperature.

Hematoporphyrin (HP) and deuteroporphyrin (DP), the structures of which are shown in Fig. 1, were prepared and purified as described elsewhere [13]. Their purity, as checked by HPLC [14], was at least

* Corresponding author. Fax: +33 1 40793705.

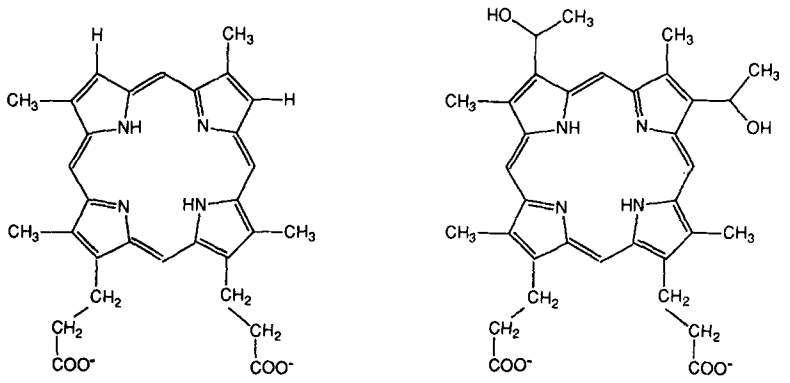

Fig. 1. Structures of deuteroporphyrin (DP, left) and hematoporphyrin (HP, right).

99%. Stock solutions of the porphyrins in tetrahydrofuran were diluted with aqueous solutions buffered to the desired pH. The final solutions contained less than 0.3% of tetrahydrofuran.

The vesicle and porphyrin solutions were mixed using a Durrum-Gibson stopped-flow apparatus (Palo Alto, CA) with a dead time of about 3 ms. The porphyrin concentration after mixing was $1 \cdot 10^{-7}$ M and the lipid concentration ranged between $2 \cdot 10^{-6}$ and $4 \cdot 10^{-5}$ M. The temperature was 22°C. The excitation light provided by a 75 W short arc xenon lamp was passed through a monochromator set at around 400 nm with a bandwidth of 6 nm. The fluorescence emission was collected above 610 nm using a low cut filter (Schott OG 610, Mainz, Germany) which reduces the contribution of the non-incorporated porphyrin. The signal was recorded using a digital oscilloscope (Nicolet model 3091, Madison, WI) and then, fed to a computer (Hewlett-Packard model 9816, Fort Collins, CO) to be stored and analyzed using standard routines.

Conventional emission fluorescence spectra were recorded using a SPEX instrument (Edison, NJ) at a temperature of 22°C. The excitation was set at 400 nm.

Results

In a first step, the partition, at equilibrium, of the porphyrin between the vesicle lipidic phase and the water medium was determined by static fluorescence measurements at some selected pH between 7.5 and 5.5. The gradual addition of preformed vesicles to DP or HP solutions in this pH range led to emission fluorescence changes very similar to those already published (see Fig. 2 in Ref. 5). They indicated incorporation of the porphyrin into the lipidic phase of the vesicles. These changes were analyzed according to the method described by Brault et al. [5] to yield apparent overall affinity constants (K_{stat}) which are summarized in Table I. The aggregation of DP at the lower pH, even at the low porphyrin concentration used, made it difficult to determine an accurate value for the affinity constant at pH 5.5. The aggregation was much lower with HP. For the sake of convenience the affinity constants refer to phospholipid concentrations.

The incorporation of porphyrins into vesicles is accompanied by red shift and increase in fluorescence emission. These changes were detected in stopped flow experiments. The rise of fluorescence emission ob-

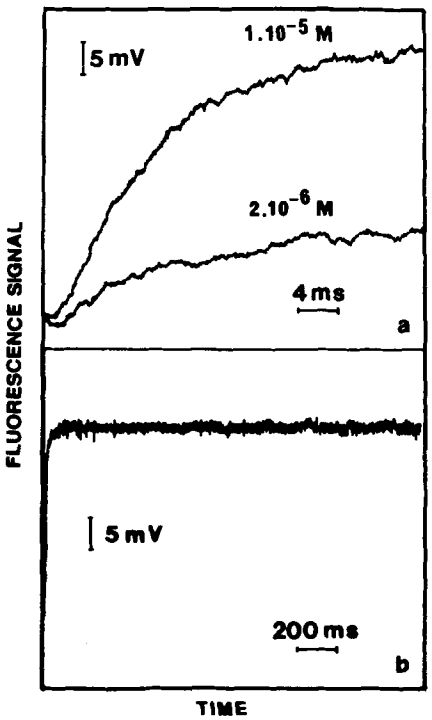

Fig. 2. Fluorescence signal recorded after rapid mixing of deuteroporphyrin with EPC vesicles at pH 7.5. Top: initial fast process observed at two phospholipid concentrations; the amplitude of the signal is shown to depend on the lipid concentration. Bottom: longer time scale, no more change is observed (the lipid concentration was $1 \cdot 10^{-5}$ M).

served when the porphyrin and vesicle solutions were mixed occurred within a few tens of milliseconds. The signal was found to be monoexponential and its amplitude increased with the phospholipid concentration. After the initial change, no more evolution was observed over seconds. An exemple is given in Fig. 2 for DP. This behavior strongly suggests that the process involves a fast equilibrium which is displaced to the right (as shown below) when the vesicle concentration is increased. In most cases, the concentration in phospholipid highly exceeds that in porphyrin and no more than a dozen of molecules are incorporated within a vesicle. It is thus believed that the interaction of a porphyrin molecule with a vesicle does not depend on the presence of other incorporated molecules. In these conditions, and if exchange of porphyrins between the outer and inner layers of the vesicle does not occur in the time scale investigated, then, the process can be described by a single equilibrium:

$$P_{AQ} + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} P_L$$

P_{AQ} stands for a porphyrin molecule in the aqueous bulk solution, L for a vesicle and P_L for the porphyrin incorporated into the vesicle. Pseudo-first order kinetics should be observed with an observed reaction rate constant:

$$K_{obs} = k_{on}[L] + k_{off} \quad (1)$$

where k_{on} and k_{off} correspond to the rate constants for the entrance and exit processes, respectively [12]. An equilibrium constant, K_{kin}, can be derived from these kinetic constants:

$$K_{kin} = k_{on} / k_{off} \quad (2)$$

In agreement with the proposed scheme, in all cases, the fluorescence signal was found to be monoexponential. The values of k_{obs} derived from these signals were plotted versus the phospholipid concentration. As expected from Eqn. 1, and whatever the pH was, linear plots with non-zero intercept were found with correlation coefficients better than 0.99. An example is given in Fig. 3. The values of k_{on} and k_{off} derived from such plots, along those of K_{kin} calculated from Eqn. 2, are reported in Table I. Interestingly, the constant calculated from the kinetic data, K_{kin}, was found to be close to the constant, K_{stat}, determined at equilibrium outlining the validity of the proposed scheme.

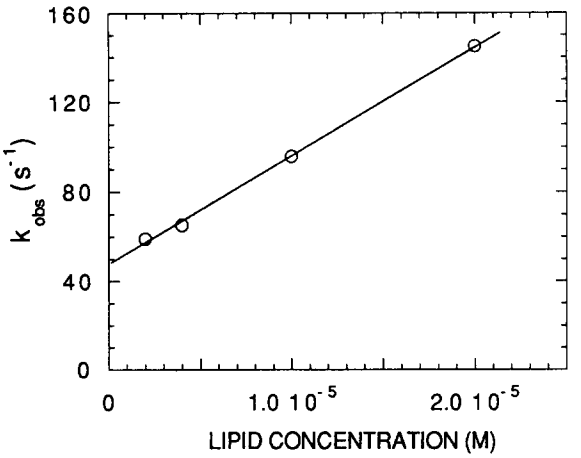

Fig. 3. Dependence of the observed pseudo-first order rate constant on the phospholipid concentration. Experiments were performed with deuteroporphyrin at pH 7.5.

Discussion

In aqueous solutions, the pK values of the carboxylic groups of HP and DP have been previously determined to be 5.45, 4.95 and 6.0, 5.4, respectively [5]. Thus, in the pH range investigated, the porphyrins in the bulk aqueous solution exist as their dianionic forms at the highest pH (two carboxylic groups deprotonated) and are partly neutralized at the lower pH value. It can be noted that no or little protonation of the imino-nitrogen occurs in this pH range [16]. It is remarkable that the reaction rate constants for the incorporation of the porphyrin (k_{on}, second column in Table I) are almost the same between pH 7.5 and 5.5,

TABLE I

pH	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_{kin} (M^{-1})	K_{stat} (M^{-1})	k_{on} ($Ves^{-1}s^{-1}$)
Deuteroporphyrin					
5.5	$4.3 \cdot 10^6$	≤ 2	–	–	$(1.1–1.5) \cdot 10^{10}$
6.5	$4.6 \cdot 10^6$	18.2	$2.5 \cdot 10^5$	$2.1 \cdot 10^5$	$(1.1–1.6) \cdot 10^{10}$
7.5	$4.9 \cdot 10^6$	48	$1.0 \cdot 10^5$	$1.2 \cdot 10^5$	$(1.2–1.7) \cdot 10^{10}$
Hematoporphyrin					
5.5	$2.3 \cdot 10^6$	43	$5.4 \cdot 10^4$	$7.6 \cdot 10^4$	$(5.6–8.0) \cdot 10^9$
6.5	$\sim 4 \cdot 10^6$	~ 100	$\sim 4 \cdot 10^4$	$2.7 \cdot 10^4$	$(1.0–1.4) \cdot 10^{10}$

i.e., independent of the ionization state of the carboxylic chains of the porphyrin. Also, the values obtained for DP and HP were found to be very similar.

Further insight into the mechanism can be obtained by considering rate constants relative to the vesicle concentration. These values can be calculated by multiplying the above k_{on} values by the number of phospholipid molecules in each vesicle. According to Huang [17] or Johnson [18], sonicated EPC vesicles have a mean radius ranging between 10.5 and 12 nm and are made of 2500–3500 phospholipid molecules. Values of k_{on}, expressed in $Ves^{-1}\,s^{-1}$ units, have been computed according to these figures and are reported in the last column of Table I.

An upper limit for the rate constant of incorporation can be calculated assuming that the interaction between the porphyrin and the vesicles is diffusion controlled. Considering vesicles with a radius of R_v, and neglecting the size of the porphyrin, this limit would be [19]:

$$k_d = 4\pi R_v D N_A \qquad (3)$$

where D is the sum of the diffusion coefficients of the porphyrin and the vesicles, and N_A the Avogadro number. The diffusion coefficient of EPC vesicles has been determined to be $2 \cdot 10^{-11}$ $m^2\,s^{-1}$ [20]. No value is available in the litterature for the diffusion coefficient of DP or HP in the pH range investigated. A diffusion coefficient of $1.3 \cdot 10^{-10}$ $m^2\,s^{-1}$ has been reported for a related porphyrin, protoporphyrin [21]. This value was derived from electrochemical measurements performed at high pH and high ionic strength, however. It should correspond to strongly solvated charged species and can be considered as a lower limit. Alternatively, the diffusion coefficient of the porphyrin in water can be approximated using the Stokes-Einstein equation

$$D = kT/6\pi\eta R$$

where k is the Boltzman constant, T the temperature and η the viscosity of water [19]. If we grossly assimilate the porphyrin to a sphere with a radius of 0.64 nm, i.e., the distance between the center of the macrocycle and peripheral methyl groups [22], a value of $3.56 \cdot 10^{-10}$ $m^2\,s^{-1}$ can be obtained for the diffusion coefficient. The diffusion limited rate constant for the interaction of porphyrins with EPC vesicles can thus be computed to range between $1.1 \cdot 10^{10}$ $M^{-1}\,s^{-1}$ and $3.4 \cdot 10^{10}$ $M^{-1}\,s^{-1}$ depending on the values retained.

Clearly, the experimental values, k_{on} ($Ves^{-1}\,s^{-1}$), are very near the diffusion limit regardless of the charge of the molecule and the nature of the side-chains. On the other hand, the rate constant for the exit process (k_{off}) was dependent both on the pH and on the nature of the porphyrin. This effect accounts, alone, for the observed changes in affinity. In agreement with the scheme reported elsewhere [10,11], these results suggest that, depending on the charge of the propionic groups and the polarity of the side-chains, the porphyrin is more or less buried in the lipidic bilayer. These findings, in agreement with previous data on the exchange of solubilized molecules or labeled fatty acids between vesicles [12,23,24] give direct evidence that fast, diffusion controlled, equilibria prevail in such systems. Noteworthy, the affinity for the vesicles is mainly governed by the exit process.

The rapidity of the processes outlined in the present study should also be kept in mind when using liposomes to transport photosensitizers. Exchange of the photosensitizer between the liposomes and serum proteins and redistribution among serum proteins [25] could be very fast, even in the case of hydrophobic compounds.

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