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Binding of the lipophilic cation tetraphenylphosphonium to phosphatidylcholine membranes

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The binding of the lipophilic cation tetraphenylphosphonium to bilayer membranes of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was measured in the concentration range of 5 mM to 0.5 M TPP^+Cl^-. By combining deuterium magnetic resonance with ultraviolet absorption spectroscopy the amount of membrane-bound and free TPP^+Cl^- could be determined over the whole concentration range measured. After taking into account surface potential effects by means of the Gouy-Chapman theory the binding isotherm could be quantitatively explained by the Langmuir adsorption isotherm. Each TPP^+ membrane binding site was found to comprise $n = 8–9$ lipids and the TPP^+ binding constant was determined as $K = 21\ M^{-1}$ at 25°C and 0.1 M NaCl.

Introduction

Lipophilic ions such as the positively charged tetraphenylphosphonium ion (TPP^+) are used as probes to measure the membrane potential across biological membranes [1]. The ability of the aromatic groups to delocalize and shield the electric charge and to increase lipid solubility facilitates the transport of these ions across the hydrophobic barrier [2,3]. Energetic considerations suggest that the potential energy function of positively charged lipophilic ions has a pronounced minimum near the membrane surface which, in turn, would imply a specific accumulation of these ions at the lipid-water interface [4].

The binding of TPP^+ to the membrane surface is not well characterized. Pickar and Benz [5] studied the transport of TPP^+ in planar bilayers by conductivity and relaxation techniques but could only derive the product βk_i where β is a partition coefficient and k_i a rate constant. Cafiso and Hubbell [6] investigated the binding of a spin-labeled phosphonium ion to sonicated vesicles of egg phosphatidylcholine for spin label concentrations up to 10^{-4} M. At these low concentrations the binding of the lipophilic ion to the membrane phase was sufficiently well described by a simple partition equilibrium with a partition coefficient of about 50–100, demonstrating an accumulation of the spin label probe in the membrane phase [6].

The present study was directed towards a more detailed elucidation of the binding mechanism of TPP^+. This required the measurement of the TPP^+ binding isotherm over a larger concentration range in order to differentiate between various binding models. As a membrane system we have used coarse dispersions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine deuterated at the α-choline segment [7]. POPC is a common, naturally occurring lipid; it is found, for example, in egg

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Abbreviations: TPP^+, tetraphenylphosphonium; $TPAs^+$, tetraphenylarsonium; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

yolk lecithin where it accounts for about 70% of the total lipid [8].

The binding of TPP^+ to POPC membranes was measured with deuterium magnetic resonance following essentially the same route as developed for Ca^{2+} binding [9]. The method is based on the observation that the binding of metal ions and hydrophobic ions induces a small conformational change at the lipid polar group. For membranes composed of [α-$^2H_2$]POPC this effect is reflected in a decrease of the quadrupole splitting of the deuterated choline segment [9–12]. Quantitatively, the observed variation of the quadrupole splitting was found to be linearly related to the amount of membrane bound Ca^{2+} or TPP^+. This could be demonstrated for TPP^+ by measuring the amount of bound TPP^+ directly with ultraviolet absorption spectroscopy, at least for TPP^+ concentrations up to 50 mM. With this calibration, the amount of bound TPP^+ could be determined from the ^{2}H-NMR spectra under conditions where ultraviolet spectroscopy was technically no longer feasible, i.e. in the concentration range up to 0.5 M TPP^+. Using this information, it was then possible to calculate the membrane surface charge density and also the membrane surface potential, the latter by application of the Gouy-Chapman theory [13]. Knowledge of the amount of bound TPP^+, of free TPP^+ at the plane of ion binding, and of the TPP^+ equilibrium concentration in bulk solution finally allowed a quantitative analysis of the binding isotherm.

Material and Methods

The ultraviolet absorption spectrum of an aqueous solution of tetraphenylphosphonium chloride is typical for phenyl groups with three resolved absorption bands at 262 nm, 268 nm, and 275 nm. The most intense band at 268 nm was used for concentration determinations. The absorbance varied linearly with the TPP^+ concentration (measured up to $2 \cdot 10^{-4}$ M) and the extinction coefficient at 268 nm (25°C) was determined as $\epsilon_{268} = 4543\ M^{-1} \cdot cm^{-1}$. The flat part in the spectrum between 290 nm and 350 nm was used for baseline corrections in samples with light scattering due to lipid turbidity.

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was selectively deuterated at the α-segment of the choline moiety as described previously [7]. All ^{2}H-NMR studies were performed with coarse lipid dispersions and the experimental conditions were essentially the same as detailed elsewhere [7,9].

Results

Fig. 1 shows a series of ^{2}H-NMR spectra recorded at different TPP^+ bulk concentrations. The figure indicates that the residual quadrupole splitting, i.e. the separation of the most intense peaks in the spectrum, decreases continuously with increasing TPP^+ concentration.

Next, the amount of TPP^+ bound to the membrane surface was determined as follows. Samples were prepared with 20 mg [α-$^2H_2$]POPC and 400 μl solution containing TPP^+Cl^- (5–50 mM) and 0.1 M NaCl. Equilibrium was attained by several freeze-thaw cycles [9]. The suspension was centrifuged and the clear supernatant removed with a pipet. A suitable concentration for ultraviolet spectroscopy was achieved by mixing 10 μl supernatant with 1 ml distilled water in a 1 cm quartz cuvette. The mean of at least five measurements per sample was used for TPP^+ determination. The difference between the TPP^+ content of the starting solution and the supernatant yielded the amount of TPP^+ bound to the POPC membrane. This parameter is denoted X_b in the following. Numerical results are summarized in Table I. The pellets of the above experiments were used immediately for ^{2}H-NMR measurements. A plot of the quadrupole splitting $\Delta\nu_\alpha$ versus X_b is shown in Fig. 2. The figure demonstrates a linear rela-

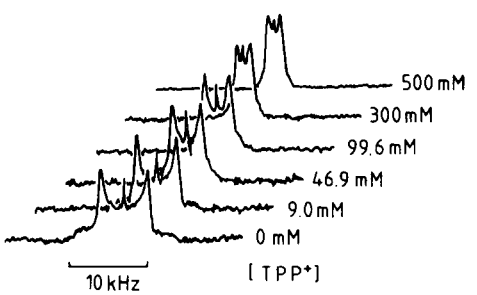

Fig. 1. ^{2}H-NMR spectra of coarse dispersions of POPC bilayers at various concentrations of TPP^+ Cl^- plus 0.1 M NaCl. The lipid was deuterated at the α-segment of the choline moiety ($(CH_3)_3NCH_2C^2H_2OP$–). Measuring temperature 25°C.

TABLE I

BINDING OF TETRAPHENYLPHOSPHONIUM (TPP^+) TO BILAYERS OF [α-$^2H_2$]POPC IN THE PRESENCE OF 0.1 M NaCl AT 25°C

C_{eq} (mM)	X_b [b] (mmol/mol)	$\Delta\nu_\alpha$ (kHz)	σ [e] (mC/m^2)	ψ_0 [f] (mV)	C_M [g] (mM)
0	0	5.80	0	0	0
4.39	9.3 [c]	5.47	2.2	3.0	3.9
9.00	15.0 [c]	5.08	3.5	4.7	7.5
18.3	26.1 [c]	4.49	6.1	7.7	13.5
46.9	53.0 [c]	3.61	11.0	12.4	28.9
99.6	65.0 [d]	3.01	15.4	14.8	56
300	96.4 [d]	1.70	22.7	15.3	165
500	97.4 [d]	1.66	22.9	12.7	304
500	102.1 [d]	1.46	24.1	13.4	297
7.93 [a]	31.4 [c]	4.44	7.4	3.2	7.0

[a] Measurement with 1 M NaCl.
[b] X_b denotes the amount of TPP^+ (in millimoles) which is bound to 1 mol of POPC.
[c] Calculated from the ultraviolet absorption spectrum of TPP^+.
[d] Calculated from the ^{2}H-NMR quadrupole splittings using the relationship $\Delta\nu_\alpha = 5.76 - 42.1\ X_b$.
[e] $\sigma = 0.235\ X_b$ (assuming a surface area of $S = 68$ Å^2 per POPC molecule).
[f] Calculated with the Gouy-Chapman theory.
[g] Concentration of TPP^+ ions in solution at the membrane-water interface; $C_M = C_{eq} \exp[-F_0\psi_0/RT]$.

tionship between the quadrupole splitting and the amount of bound TPP^+. Linear regression analysis yields

$$\Delta\nu_\alpha = 5.76 - 42.1\ X_b \qquad (1)$$

($\Delta\nu_\alpha$ in kHz, X_b in mol TPP^+/mol POPC, 25°C, 0.1 M NaCl).

Fig. 2 is based on a data set of TPP^+ concentrations up to 50 mM. For larger concentrations the ultraviolet absorption method was no longer applicable since the concentration difference between the starting solution and the supernatant became too small to yield reliable results. Under these conditions, X_b was determined exclusively from the ^{2}H-NMR spectra using the calibration Eqn. 1. These data for X_b are also summarized in Table I.

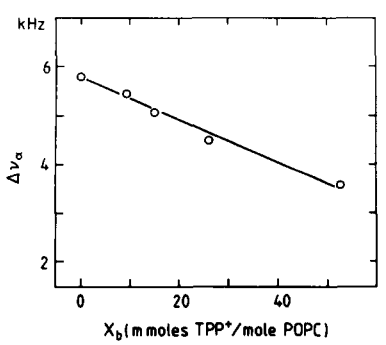

Fig. 2. Quadrupole splitting of the α-segment of POPC bilayers as a function of bound TPP^+. Measuring temperature 25°C. All data were obtained in the presence of 0.1 M NaCl.

The assumption of a linear relationship between the quadrupole splitting, $\Delta\nu_\alpha$, and the amount of bound TPP^+, X_b, is a critical step in the analysis and needs further justification. Which evidence do we have that an extrapolation of Eqn. 1 to TPP^+ concentrations > 50 mM is indeed valid? Only indirect arguments can be provided. (1) In binding studies with the positively charged local anesthetic dibucaine the quadrupole splitting $\Delta\nu_\alpha$ changed linearly with the amount of bound dibucaine in the range of 5.8 kHz ($X_b = 0$) to −4.8 kHz (X_b = 0.5 mole dibucaine/mole POPC) (Allegrini, P. and Seelig, J., unpublished data). Thus a linear relation was obtained for an even larger range of quadrupole splittings as encountered in the present study. (2) A similar calibration was possible for La^{3+}. In the experimentally accessible range the [α-$^2H_2$]POPC quadrupole splitting decreased linearly from 6 kHz to 1.7 kHz as the amount of bound LaI^{3+} increased from zero to $X_b = 0.08$ (mole La^{3+}/mole POPC). Thus cations of quite different

chemical structure give rise to linear effects between $\Delta\nu_\alpha$ and X_b. (3) Finally, TPP^+ was replaced by its analogue tetraphenylarsonium, $TPAs^+$, in order to measure the phosphorus spectrum of the POPC membrane. This was not possible in the TPP^+ studies because of the intense signal of TPP^+ itself. At $TPAs^+$ concentrations up to the saturation limit (approx. 0.3 M) the ^{31}P-NMR spectra of the POPC bilayer had the shape characteristic of a liquid crystalline bilayer with relatively little change of the chemical shielding anisotropy. At the same time, the deuterium quadrupole splitting behaved similarly as observed for TPP^+. The rather small change in the chemical shift anisotropy hence provides experimental evidence for a smooth change in the head group conformation. No additional head group conformation are induced at high $TPAs^+$ concentrations, which is a prerequisite for the extrapolation of Eqn. 1 to high concentrations.

Discussion

If X_b is the surface concentration of bound TPP^+ (mole TPP^+/mole POPC), S the surface area per POPC molecule, and e_0 the charge of an electron, then the surface charge density σ is given by

$$\sigma = e_0 X_b / S \tag{2}$$

In the following it is assumed that the surface charge is distributed homogeneously in the plane of the membrane. For POPC a surface area of $S = 68 \cdot 10^{-20}$ m^2 appears to be most probable [9] yielding

$$\sigma = 0.235\ X_b \tag{3}$$

(with σ in C $\cdot$ m^{-2}). The surface charge density σ creates a surface potential ψ_0 which may be calculated by means of the Gouy-Chapman theory [13]

$$\sigma = \left[2000\ \epsilon_r \epsilon_0 RT \sum_i C_{i,eq}(e^{-z_i F_0 \psi_0 / RT} - 1)\right]^{1/2} \tag{4}$$

($\epsilon_r = 78$, dielectric constant of water; ϵ_0 permittivity of free space; R gas constant; F_0 Faraday constant; $C_{i,eq}$ concentration of the ith electrolyte in the bulk phase (in moles per liter); z_i the signed charge of the ith species).

Due to the surface potential the free TPP^+ concentration (C_M) at the plane of ion binding is smaller than at the equilibrium concentration far away from the membrane. C_M can be calculated via the Boltzmann equation

$$C_M = C_{eq} \exp[-F_0 \psi_0 / (RT)] \tag{5}$$

The numerical results for σ, ψ_0, and C_M are also included in Table I. It may be noted that the surface potential remains rather small ($\psi_0 \leqslant 15$ mV) at all TPP^+ concentrations. Therefore, C_M deviates from C_{eq} by at most a factor of 2. A critical discussion of the assumptions involved in the Gouy-Chapman theory may be found elsewhere [2,13].

A plot of the deuterium quadrupole splitting versus the TPP^+ equililibrium concentration is shown in Fig. 3, suggesting a monotonic approach to a limiting adsorption. For a quantitative discussion we assume that each lipid head group constitutes a potential contact site and that binding of one TPP^+ excludes n lipids from further binding, i.e. one binding site is made up from n lipid contacts. Let us denote with $\{C_L^\circ\}$, $\{C_L\}$, and $\{C_{ML}\}$ the surface concentrations of the total

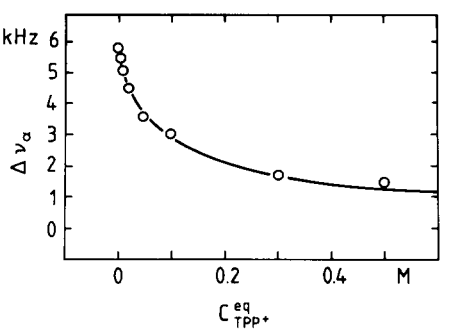

Fig. 3. Binding of TPP^+ to POPC bilayers. The figure represents the binding isotherm as measured by the variation of the quadrupole splitting of the α-segment with the equilibrium TPP^+Cl^- concentration (plus 0.1 M NaCl, 25°C). The solid line was calculated by combining the Langmuir adsorption isotherm Eqn. 9 (with $K = 22$ M^{-1} and $n = 8.0$) with the Gouy-Chapman theory (to correct for repulsion of TPP^+ at the plane of ion binding).

lipid, the free lipid, and the TPP^+ complex, respectively:

$$\{C_L^o\} = \{C_L\} + n\{C_{ML}\} \quad (6)$$

which may be expressed in mole fractions as

$$1 = X_L + nX_{ML} \quad (7)$$

X_{ML} is identical with the experimental parameters X_b introduced above.

$$X_{ML} = X_b \quad (8)$$

The simplest model to describe the binding of TPP^+ is to assume a chemical equilibrium according to

$$K = n\{C_{ML}\}/[\{C_L\}C_M] \quad (9)$$

$\{C_L\}/n$ is supposed to be the surface concentration of free binding sites. By means of Eqns. 6–8 the law of mass action may be written as

$$K = nX_b/[C_M(1 - nX_b)] \quad (10)$$

For convenience in testing data Eqn. 10 may finally be cast in the form

$$X_b/C_M = (K/n)(1 - nX_b) \quad (11)$$

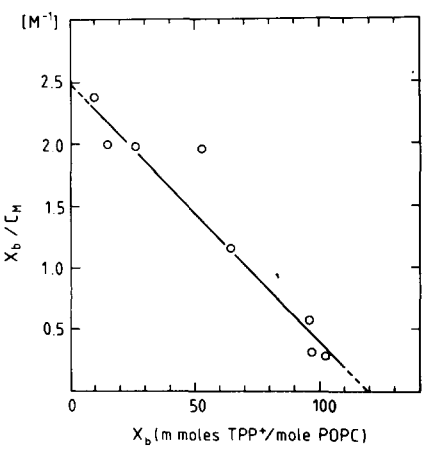

Fig. 4. Analysis of the TPP^+ binding data in terms of the Langmuir adsorption isotherm according to Eqn. 11, after correcting for TPP^+ repulsion at the plane of ion binding; numerical data taken from Table I.

If the model is correct a plot of (X_b/C_M) versus X_b should yield a straight line. Fig. 4 demonstrates that this is indeed borne out by the experimental data of Table I. From the intercepts with the x and y axis one finds $n = 8.3$ as the number of lipids per TTP^+ binding site and an association constant of $K = 21\ M^{-1}$ (25°C, 0.1 M NaCl).

The above model is based on the assumption of independent and non-overlapping binding sites, resulting in the Langmuir adsorption isotherm. The critical step in the model is the calculation of the concentration of free binding sites. Here the surface concentration of free contact sites (free lipids) was simply divided by n, the number of contacts covered by one TPP^+ molecule. This procedure is quite inadequate in many situations with interacting and overlapping binding sites as has been demonstrated theoretically for large ligands [14–16]. It may also be noted that the binding of metal ions to POPC membranes requires a more complicated binding model [9].

Quite often the Langmuir adsorption isotherm is written as

$$X_b = AC_M/(K_D + C_M) \quad (12)$$

Here A is the limiting degree of binding at high concentrations of C_M and K_d is the dissociation constant of the complex. These new parameters are related to those of Eqn. 9 according to

$$A = n^{-1} \quad K_d = K^{-1} \quad (13)$$

yielding numerical values of $A = 0.12$ (mole TPP^+/mole POPC) and $K_d = 48$ mM for the binding of TPP^+ to POPC.

We are now in a position to compare the pure lipid membrane with a biological membrane. The binding of TPP^+ to membrane vesicles from *Halobacterium halobium* was recently studied with ion selective electrodes in the concentration range of 10 μM to 1 mM TPP^+ [17]. The TPP^+ binding was quantitatively accounted for by Eqn. 12 (without correcting for surface potential effects). The dissociation constant was determined as $K_d =$ 19.5 mM and the maximum amount of TPP^+ binding as $A = 6.08 \cdot 10^{-8}$ (mole TPP^+/mg protein). The *Halobacterium halobium* membranes contain proteins and lipids and as a rough esti-

mate we assume a protein-to-lipid ratio of 3:1 (mg/mg). If all TPP^+ binds to the lipid components the maximum binding as referred to the lipid would be $18.2 \cdot 10^{-8}$ (mole TPP^+/mg lipid). Assuming an average molecular weight of 780 per lipid the maximum binding on a lipid molar basis is 0.14 (mole TPP^+/mole membrane lipid). Both the maximum binding and the dissociation constant agree broadly with those of the well-defined POPC model membrane suggesting that TPP^+ binding to *Halobacterium halobium* membranes may occur predominantly at the lipid patches, though other interpretations cannot be excluded.

Finally, the present results can be related to the partition coefficient β as defined in conductivity measurements [3,4]. Since one molecule TPP^+ covers 8.3 lipids the surface concentration of binding sites is $A^* \simeq 3 \cdot 10^{-11}$ mol $\cdot$ cm^{-2} which together with the measured binding constant $K = 21 \cdot 10^3$ cm$^3 \cdot$ mol^{-1} yields the partition coefficient of $\beta = A^*K = 6 \cdot 10^{-7}$ cm. This values is 4–5 orders of magnitude smaller than the β-value of negatively charged tetraphenylborate ($2 \cdot 10^{-2}$ cm) and may explain the much lower conductivity of TPP^+ compared to tetraphenylborate [3].

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