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Characterization of 9-aminoacridine interaction with chromatophore membranes and modelling of the probe response to artificially induced transmembrane ΔpH values

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Abstract

We analyze the adsorption of the fluorescent monoamine 9-aminoacridine to the membrane phase of photosynthetic chromatophores, in the physiological interval of pH values ranging from 5.5 to 8.5 and at ionic strengths of 0.005 and 0.150 M. The interaction of the probe with the membrane phase is described with S-shaped isotherms of the Hill type and is modulated by electrostatic effects as modelled with the Gouy-Chapman-Boltzman theory. This description is consistent with different values of the surface charge density of the chromatophore membranes decreasing from about $1.3 \cdot 10^{-3}$ to about $0.5 \cdot 10^{-3}$ $e^-/\text{Å}^2$, on changing the pH from 8.5/7.5 to 6.5/5.5, respectively. Furthermore we show that, when the free concentrations of the probe in the inner and outer vesicle compartments are computed from the adsorbing isotherms at the proper pH values, the model considering the equilibrium distribution of the neutral monoamine following the onset of a ΔpH is sufficient to describe the dependence of the artificially induced transmembrane ΔpH values on the observed quenching of the probe fluorescence.

Keywords: Adsorption isotherm; Probe–membrane interaction; Fluorescent probe; 9-Aminoacridine; Transmembrane pH difference; Bacterial chromatophore

1. Introduction

Over 20 years ago fluorescent amines, such as the monoamine 9-aminoacridine, were introduced as probes for ΔpH determination in acidic-inside biological vesicular systems [1]. Since then these molecules have been extensively used in studies aimed to describing kinetic and thermodynamic aspects of energy conservation in energy coupling membranes and in co-reconstituted systems (for reviews, see [2–5]). In these experiments, the quenching of the probe fluorescence is commonly related to the extent of the transmembrane ΔpH generated by the activity of the light- and/or ATP-dependent proton pumps. This notion is mainly based on the observation that the fluorescence is restored to its original level when ΔpH is dissipated by effectors such as uncoupling agents, weak acids, ionophores and specific inhibitors of proton pumps.

The model for ΔpH determination is based on the assumptions that the neutral form of the amine is the only permeant species through the vesicles membrane and that the fluorescence intensity is proportional to the concentration only in the outer compartment [1]. The following equation, describing the monocation/neutral molecule equilibria of a monoamine behaving ideally in the presence of a transmembrane pH difference, is used to evaluate the transmembrane ΔpH:

$$pH_i = -\log\left(C_i/C_0\left(10^{-pH_o} + 10^{-pK_a}\right) - 10^{-pK_a}\right) \quad (1)$$

where pH_i, pH_o and pK_a are the values of the pH in the inner and outer compartments and that of the pK_a of the monocation-neutral molecule equilibrium, respectively. C_i and C_0, are the concentrations of the probe free in the inner and outer aqueous compartments. Assuming that the only fluorescent form of the amine is that free in the aqueous compartment, C_i and C_0 can be directly related to

Abbreviations: 9AA, 9-aminoacridine; ACMA, 9-amino-2-chloro-6-methoxyacridine; BChl, bacteriochlorophyll; Tricine, *N*-tris-(hydromethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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the quenching (Q, expressed as the fraction of the total fluorescence) of the probe fluorescence ($C_i = n_i/V_i = Qn_TV_i$; $C_o = n_o/V_o = (1 - Q)n_T/V_o$ with n_T = total amount of probe). For strong monoamines, such as 9AA [6], whose $pK_a \gg pH_o$ and pH_i, Eq. (1) simplifies to [1]:

$$\Delta pH = \log Q/(1 - Q) + \log V_o/V_i \quad (2)$$

The model was tested in our and other laboratories, mainly using 9AA [7–14]. It was concluded that 9AA behaves non-ideally, especially when it concentrates within the inner compartment of a vesicle system. By imposing artificially ΔpH values of known extent both in chromatophores and in liposomes [9,10], it was observed that the linear dependence of ΔpH on $\log Q/(1 - Q)$ expected for 9AA was verified for ΔpH values ranging from about 1 to 2.5 units, and that the inner volume estimated by extrapolating at ΔpH = 0 was much larger than the actual one (up to 60-times when compared to the value of 10 μl per BChl micromoles, as determined with EPR techniques [15]). This was considered as indicative of probe–membrane interactions, and therefore used in the evaluation of light induced ΔpH values according to Eq. (2) to account for binding of the molecules to the membrane phase.

Different interpretations of the membrane-probe interaction have been proposed. Hydrophobic partition of the dye between the aqueous buffer and the membrane lipids, in chloroplasts, influenced by the light induced electrical potential difference at the interface and/or across the membrane has been considered [11,12]. In addition, formation of dimers and multimers on the membrane, due to electrostatic interaction of the cation with the charged groups at the membrane surface may be responsible both for fluorescence quenching and for ΔpH overestimation [13,14]. At low ionic strength, when screening of surface charges is particularly reduced, the extent of the interaction is also used to evaluate the surface charge density in membranes from different sources [16]. In negatively charged phosphatidylserine vesicles, the quenching of 9AA fluorescence is explained in terms of ΔpH-induced excimer formation of the excited molecules at the membrane and this is considered a drawback for using this probe as a ΔpH indicator [14,17,18].

From the above considerations, a major question arises as to whether any fluorescent amine which interacts with the membrane phase can reliably be used to quantitate transmembrane ΔpH.

Recently we have shown that a 9-aminoacridine derivative, 9-amino-6-chloro-2-methoxyacridine (ACMA), despite its large interaction with the membrane phase, can be used to quantitate ΔpH values of low extent in chromatophore membranes, provided that the equilibrium concentrations of the probe in the aqueous phases is determined from the proper binding isotherm [19].

In this paper we investigate, with a microdialysis apparatus, the thermodynamic aspects of 9AA interaction with chromatophores from photosynthetic bacteria and show that after correcting for binding, similarly to what found for ACMA, Eq. (1) is sufficient to model the dependence of artificially imposed ΔpH values of known extent on the observed quenching of the probe fluorescence.

2. Materials and methods

2.1. Materials

Inside-out vesicle membranes (chromatophores) were obtained by mechanical rupture of a green strain of the photosynthetic bacterium *Rhodobacter capsulatus*, harvested in the late logarithmic phase of growth, and the concentrations of endogenous Ca and Mg ions in the vesicle populations were spectroscopically detected after complexation with cresolphthalein complexone and Titan yellow, respectively [20]. The determination of phospholipid, protein and bacteriochlorophyll contents was done according to the procedure already described [21].

9AA was purchased from Fluka Chemie (Buchs, Switzerland). Valinomycin, oligomycin, nigericin, FCCP and Tricine were obtained from Sigma (St. Louis, MO). All other chemicals, including Na_2HPO_4 and NaH_2PO_4, were reagent grade.

2.2. Spectroscopic determination of 9AA concentrations

Static fluorescence of 9AA was measured using a Jasco Model FP-550 spectrofluorimeter with excitation and emission wavelengths set at 400 and 450 nm, respectively [8,9]. Absorbance spectra were detected using a Jasco 7850 double beam spectrophotometer. Absorbance and emission intensities of 9AA were calibrated in terms of the concentration of the probe. 9AA was dissolved in ethanol at a final concentration of 2 mM and as such added to a Na_2HPO_4-NaH_2PO_4 buffer at pH ranging from 5.5 to 8.5. For the experiments of microdialysis, the probe was dissolved directly in the phosphate buffer. No difference of the 9AA spectroscopic properties was noticed at a given final concentration of the probe.

The absorbance and emission intensities of 9AA in aqueous buffers were strictly proportional to the concentration in the ranges 2–150 and 0.1–50 μM, respectively (d = 1 cm). This was also verified when the ionic strength of the solution was changed by 0.005 to 0.25 M (by adding Mg ions to a sodium tricine buffer or by changing the phosphate buffer concentration), or when the pH was increased from 5.5 to 8.5. An extinction coefficient of 6.1 ± 0.2 mM^{-1} cm^{-1} was determined for 9AA in the aqueous buffer solution.

2.3. Measurements of 9AA adsorption at the membrane / solution interface

Interactions of the probe with the chromatophore membranes were detected using the Teflon module of a rotatory

equilibrium microvolume dialyzer (well size 0.25 ml, Hoefer, San Francisco, Ca) equipped with a 6000–8000 Da cutoff dialysis membrane and a water cooling trough, as previously described [22]. Temperature was kept at 27 ± 1° C. During dialysis, mixing into the sample chamber was provided by a 1 mm Teflon bead.

The standard assay medium for dialysis contained a Na_2HPO_4-NaH_2PO_4 buffer solution at ionic strengths of 0.005 (low) and 0.15 (high) M and pH ranging from 5.5 to 8.5. Dark adapted chromatophores, suspended in the same dialysis buffer, were added in the sample chamber at a final bacteriochlorophyll concentration of 20 μM. This corresponds to about 50 nmol of phospholipid and 50 μg of proteins.

Initially (at $t = 0$), dark adapted chromatophores are in equilibrium with a known total concentration of the probe present in both the dialysis compartments. As a consequence of 9AA adsorption on the chromatophore membranes, a probe concentration difference is established through the dialysis membrane with a consequent coupled diffusional flow vector of 9AA molecules towards the compartment with chromatophores. Its magnitude decreases in time (t) till a final equilibrium distribution of the probe between the aqueous and chromatophore membrane phases is established at $t = \infty$. As demonstrated elsewhere [22], the dependence of the concentration $C(t)$ of the probe in the compartment free of chromatophores as a function of the dialysis time t is:

$$C(t) = C(0)\exp(-t/\tau) + C(\infty)(1 - \exp(1 - t/\tau)) \quad (3)$$

where $C(0)$ and $C(\infty)$ are the initial and final free equilibrium concentrations of the probe in the aqueous phase; τ is a kinetic parameter whose value in the presence of adsorbing membranes in the second compartment of the dialysis well is dependent on the concentration of the probe relative to the membranes and ranges from τ_M (the time constant in the absence of adsorbing vesicles) to $2\tau_M$ [22]. Under the experimental conditions described above τ_M was 3900 ± 500 s. $C(t)$ was determined by measuring 9AA fluorescence in the compartment free of chromatophores and using proper calibration curves. $C(\infty)$ and τ were computed as fitting parameters to Eq. (3). Adsorption of the probe to the dialysis apparatus was detected by washing the microdialyzer well with the dialysis buffer containing 50 mM $MgCl_2$. The amount of probe recovered after a period of time equal to $3 \times \tau$ was dependent on the ionic strength of the dialysis experiment (it was $\leq 5\%$ or $\leq 20\%$ of that initial concentration, at high and low ionic strength, respectively). Routinely 9AA diffusion through the dialysis membrane was detected for about $3 \times \tau$ and the amount of probe recovered after washing was included in the mass balance to its total concentration. Each probe concentration was determined in triplicate and the average value was used for fitting the data to the above model. The experimental error was within 10%.

2.4. Calibration of the quenching of 9AA fluorescence in response to known transmembrane ΔpH values

ΔpH values were artificially imposed on chromatophore membranes with a procedure already described [10,19]. Chromatophores, corresponding to a BChl concentration of 20 μM, were equilibrated in the dark for about 2 h at the imposed pH value by keeping a high ionic strength. These conditions are the same routinely used to determine ΔpH values sustained by the activity of the proton pumps. The quenching of 9AA fluorescence was recorded when the pH transition was induced. The pH was changed by acidic (5.5) to progressively alkaline values (up to a final value of 8.5), using the proper amount of phosphate salt. In this way the pH value is changed while keeping constant the ionic strength of the medium. In some experiments, different initially imposed pH (ranging from 5.5 to 8.5) were brought to a final alkaline value of 8.5. Since the proton pumping activity is usually detected at a pH of 8.5, the last experimental condition should be similar to that occurring when 9AA redistributes between the outer and inner compartments, following the ΔpH generation. In either case, no significant difference of the Q value for an imposed ΔpH value was detected. The initial and final pH values were determined using a Radiometer PH M 62 pH meter.

Photophosphorylation and ATPase activity in the dark which depend on the 9AA-BChl molar ratio, were measured as previously described [20].

2.5. Theoretical evaluation of ΔpH from the quenching of 9AA fluorescence and curve fitting

As previously detailed [19], an iterative procedure implemented in a computer program was used to calculate free 9AA concentrations from the adsorption isotherms at the different ΔpH values. Briefly, for a given value of the free probe concentration, the routine program re-evaluates the total concentration of 9AA from Eq. (5), after calculating the corresponding surface concentration σS as the numerical solution of Eq. (6) with the root-finding routine of Newton-Raphson [23]. The external free concentration of the probe at a given Q value is evaluated with Eq. (8) and its surface density is again computed from the adsorption isotherm by considering the ratio between the external and the total adsorbing area. Finally, from above and from the mass balance of Eq. (4), in combination with Eq. (6), C_i is computed considering the ratio of the internal to the total adsorbing surface, and V_i equal to 10 μl $BChl^{-1}$ [15].

Analysis of the results was done with an Olivetti 486 personal computer equipped with a Hewlett-Packard laserjet III printer. Curve fitting was performed using a non-linear least square minimization routine based on a gradient-

expansion algorithm [24]. Parameters were calculated therefrom and their standard deviation was obtained as the square root of the diagonal elements of the error matrix [24]. Programs were written in QBASIC.

3. Results

3.1. Adsorption of 9AA on the chromatophore membranes

The distribution of 9AA between the aqueous and the membrane phases can be described for simplicity by a three step model in which the permeation of the probe through the membrane comprises adsorption at the external water/membrane interface, diffusion to the opposite internal face and desorption into the osmotic inner compartment of the vesicles (V_i) [25]. Considering the mass balance to the total number of 9AA moles ($n^T_{(9AA)}$), the following equation can be derived:

$$n^T_{(9AA)} = (C_o V_o + \sigma_o S_o) + (C_i V_i + \sigma_i S_i) \quad (4)$$

where C_o and C_i are the probe concentration in the inner (V_i) and outer (V_o) compartments at equilibrium with and dependent on the corresponding surface densities (σ_i and σ_o). S_i and S_o are the inner and outer adsorbing surfaces of the chromatophore membrane.

In the dark, at $\Delta pH = 0$, a symmetrical distribution of the probe in the inner and outer compartments ($C_o = C_i$) and symmetrical surface densities of the adsorbed molecules ($\sigma_i = \sigma_e = \sigma$) can be assumed. Under these conditions and considering that $V_i \ll V_o$, Eq. (4) simplifies to:

$$n^T_{(9AA)} = C_o V_o + \sigma(S_i + S_o) = C_o V_o + \sigma S \quad (5)$$

where $C_o = C(\infty)$ (as evaluated from Eq. (2)), and $S = S_i + S_o$ is the total adsorbing surface.

According to this hypothesis, the results of the microdialysis experiments are used for a quantitative evaluation of the adsorbing isotherm. Binding of 9AA to dark adapted chromatophores is documented in Fig. 1A–D. Adsorption of 9AA is affected by the pH value (in the explored range from 5.5 to 8.5) and by the ionic strength of the dialysis buffer. The data are best fitted least χ^2 to sigmoid functions (of the Hill type [26]):

$$C_{o\cdot i} = K_H(\sigma S/(\sigma_{max} S - \sigma S)) \times \exp[\sigma S/(\sigma_{max} S - \sigma S) + A\sigma S] \quad (6)$$

where $C_{o\cdot i}$ is the equilibrium concentration of the probe free in the bulk aqueous buffer at $t(\infty)$ (as evaluated from Eq. (2)); K_H, $\sigma_{max} S$ and A are the parameters of the binding isotherm [26] and σS is the amount of probe

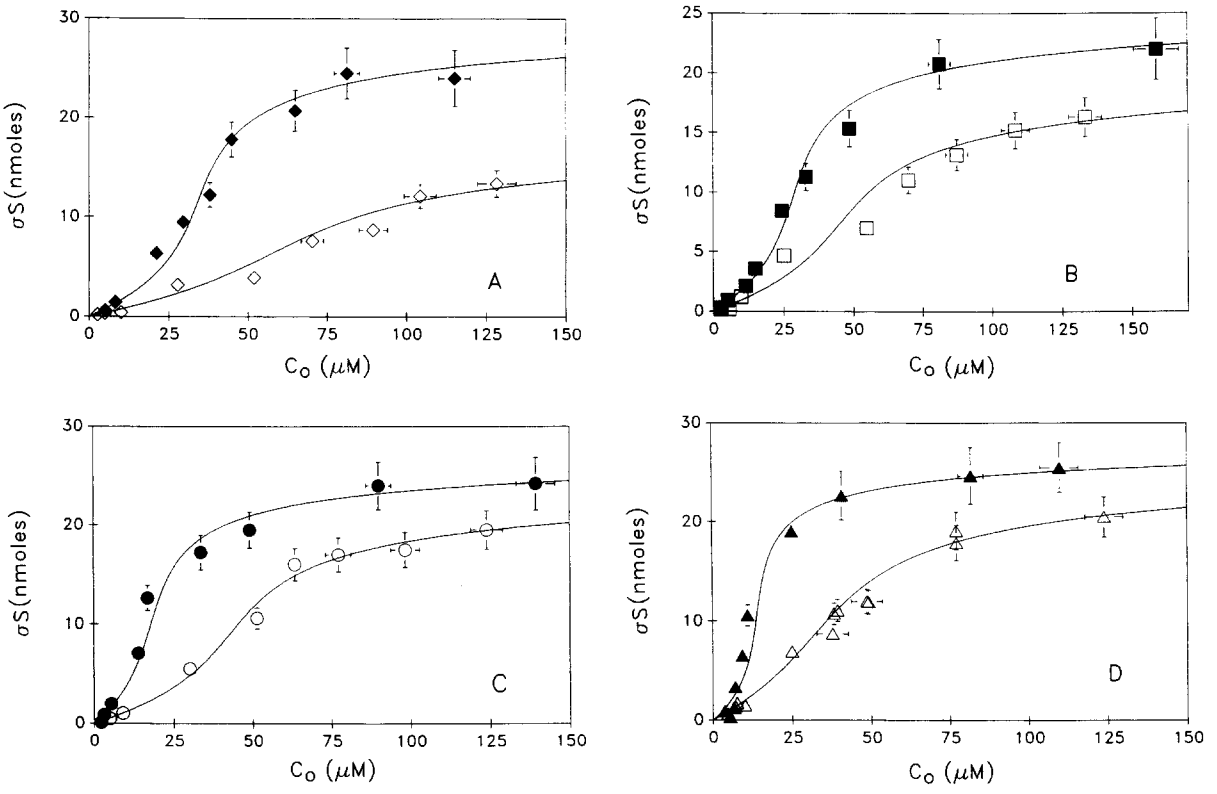

Fig. 1. (A–D) Adsorption of 9-aminoacridine onto dark-adapted chromatophore membranes. The number of 9AA nanomoles bound to the membrane phase (σS) is plotted as a function of the equilibrium concentration of the probe free in the aqueous buffer (C_o). Adsorption is determined (as described in Materials and methods) at the following pH values: 5.5 (A, diamonds), 6.5 (B, squares), 7.5 (C, circles) and 8.5 (D, triangles). The ionic strength is set at 0.005 and 0.15 M (filled and open symbols, respectively in each figure).

Table 1
Parameters of 9AA adsorption on chromatophore vesicles as a function of pH and ionic strength

pH	I (M)	K_H (nmol)	$\sigma_{max}S$ (nmol)	A (nmol^{-1})
8.5	0.150	187±2	33.1±0.3	−0.11±0.01
	0.005	115±1	33.0±0.3	−0.17±0.01
7.5	0.150	291±3	30.5±0.3	−0.16±0.01
	0.005	121±1	33.0±0.3	−0.15±0.01
6.5	0.150	300±3	30.0±0.3	−0.19±0.01
	0.005	208±2	30.9±0.3	−0.17±0.01
5.5	0.150	440±4	30.0±0.3	−0.14±0.01
	0.005	260±2	35.0±0.3	−0.14±0.01

I, ionic strength; the parameters of the adsorption isotherm (Eq. (6)) K_H, $\sigma_{max}S$ and A are respectively the adsorption constant, the saturation surface density of the probe (σ_{max}) multiplied by the total adsorption surface area (S) and a fitting parameter (A). According to the Hill model [26], σ_{max} and A are related to the co-area (a') and co-pressure (b') in the van der Waals two-dimensional equation: $\sigma_{max} = 1/b'$ and $A = 2a'/k_BTS$ [19].

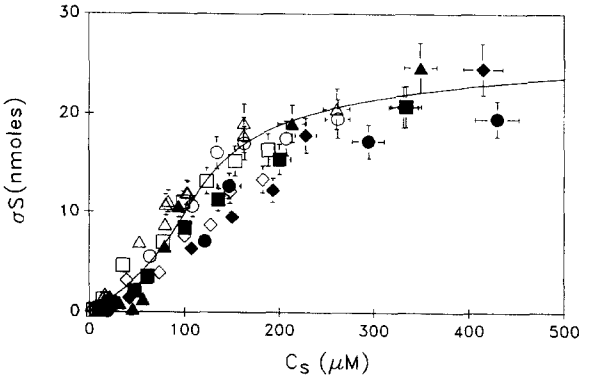

Fig. 2. Adsorption of 9-aminoacridine onto dark adapted chromatophore membranes after correcting for the electrostatic effects. 9AA concentrations at the membrane surface (C_s) are evaluated from the Boltzmann distribution (Eq. (7)), assuming surface charge densities for the chromatophore membrane of $1.25 \cdot 10^{-3}$ and $0.5 \cdot 10^{-3}$ $e^-/Å^2$ at pH 8.5, 7.5 and 6.5, 5.5, respectively. The values of the parameters of the fitting isotherm (Eq. (6)) are: $K_H = 627 \pm 6$ nmoles, $\sigma_{max}S = 34.4 \pm 0.1$ nmol and $A = -0.134 \pm 0.001$ nmol^{-1}). Symbols are as in Fig. 1A–D.

adsorbed onto the membrane (detected for convenience as number of nanomoles adsorbed on the membrane phase). As reported in Table 1, at a fixed pH value, the parameters of the binding isotherm are particularly affected by the value of the ionic strength, indicating that 9AA is undergoing electrostatic effects in its interaction with the chromatophore membrane. This is further supported when the data are analyzed in terms of the Gouy-Chapman model for the diffusive double layer and the Boltzman distribution [27]:

$$C_s = C_o \exp(-z_i e\psi_s/k_BT) \quad (7)$$

where C_s is the probe concentration in equilibrium with the membrane phase at the surface of the membrane. ψ_s is the surface potential of chromatophores evaluated by means of the Grahame equation [28] and z_i, e, k_B and T are respectively the charge number, the electronic charge, the Boltzmann constant and the absolute temperature. On considering the electrostatic effects, the dependence of the adsorbed 9AA on C_s can be described by single isotherm (see Fig. 2), provided that at least two different values of the chromatophore surface charge densities are assumed ($1.25 \cdot 10^{-3}$ $e^-/Å^2$ at pH 7.5 and 8.5, and $0.5 \cdot 10^{-3}$ $e^-/Å^2$ at pH 5.5 and 6.5) in order to evaluate ψ_s by means of the Grahame equation.

3.2. 9AA response to artificially induced ΔpH values

Experiments previously described have shown that, on establishing an artificial ΔpH across membrane vesicles, a quenching of 9AA acridine fluorescence can be detected [9,10]. Its amplitude increases at increasing ΔpH value (Fig. 3). Assuming that the only fluorescent form of the probe is that free in the external bulk aqueous buffer (C_o), the extent (Q) of the quenching of 9AA fluorescence can be directly related to the amount of probe free in the external aqueous compartment (C'_o):

$$C'_o = C_o - QC_o \quad (8)$$

From this, by considering the adsorption isotherm (Eq. (6)) and from the mass balance (Eq. (4)), the nanomoles of probe entering the inner chromatophore phase can be computed. Subsequently, the internal free concentration (C_i) of the probe is calculated, considering that the area of

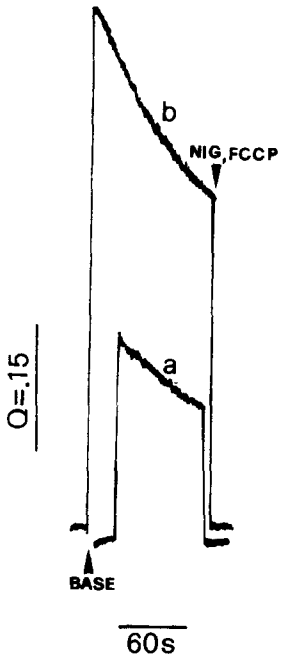

Fig. 3. Response of 9AA fluorescence to artificially induced transmembrane ΔpH values. The experimental conditions are described in Materials and methods. Curve a: ΔpH = 0.89±0.05; curve b: ΔpH = 2.54±0.05. The initial pH value was 5.55±0.05 units. Nigericin (NIG) or FCCP are added at a final concentration of 2 μM.

the inner desorbing surface is one third of that of the adsorbing one and assuming for V_i its measured value [15]. The inner pH value (pH_i) is then evaluated from Eq. (1).

In our model the set of adsorption isotherms determined at high ionic strength are used to evaluate C_i and C'_o at the different ΔpH values. The theoretical dependence of ΔpH on Q is computed by assuming an external pH value of 8.5 and considering the inner phase at progressively acidic pH values (8.5, 7.5, 6.5, and 5.5). According to this model, at pH 8.5 and at $\Delta pH = 0$, when the concentration of 9AA and BChl are respectively 5 and 20 μM, 82% of the probe is free in the aqueous phase, after equilibrating with the membrane phase at high ionic strength. The isotherm used is the one shown in Fig. 1D (open symbols). At ΔpH values values of 1, 2 and 3, when the Q values are 0.125, 0.55 and 0.85, the amount of free 9AA in the solution (outer and inner volumes) is 75.70, 33.53 and 10.94% of the total amount, the remainder being adsorbed on the total adsorbing surface of chromatophores. According to the binding isotherms, however, most of the probe entering the inner phase ($n_i = C_i V_i + \sigma_i S_i$), is bound to the inner surface. It is computed that $\sigma_i S_i$ is 99.29, 98.94 and 87.99% of n_i at ΔpH equal to 1, 2 and 3 units, respectively. The computed C_i values, in turn, are 50.53, 227.58 and 692.52 μM at the different ΔpH values.

The overlapping of the theoretical and experimental data points is evident in Fig. 4. It is obtained in the different ΔpH intervals with the approximation that the isotherm parameters can describe adsorption also for sets of pH values centered around the pH at which the isotherm was evaluated. Deviations between the theoretical and the experimental data in this model may be noticed when the isotherm parameters assumed for the inner adsorption are the ones measured at a pH value at least 2 units different from the experimental one. This can be explained considering that the isotherms are determined in the 5.5–8.5 range with a stepwise increase of one pH unit. A finer tuning of the isotherm parameters as a function of the pH values is however hampered by the standard deviation associated with the results of the microdialysis experiments (see Table 1).

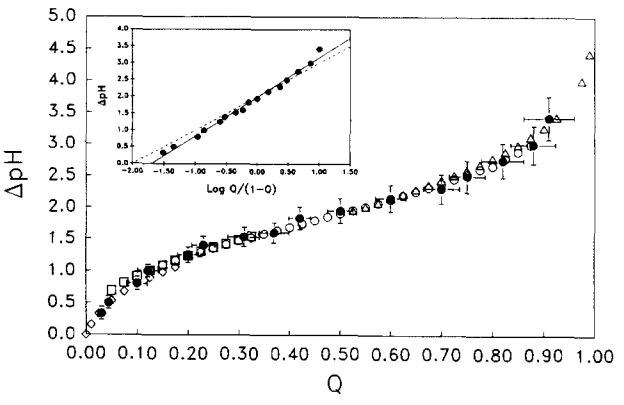

Fig. 4. Dependence of ΔpH on the quenching of 9-aminoacridine. The experimental data (filled circles with standard deviation) and the theoretical values obtained from the model are compared. The theoretical values are represented as open diamonds, squares, circles and triangles, depending on the isotherm used at high ionic strength to describe probe adsorption at a different inner pH value (symbols are the same used in Fig. 1A–D at the corresponding pH vlaues). The data are shown as a function of the quenching (Q) of 9AA fluorescence, and, in the inset, the experimental ones as a function of log $Q/(1-Q)$. In this case, fitting is computed with linear regression of all the points (solid line) and by assuming the ideal behavior predicted by Eq. (2) (dashed line).

Consistently with the experimental procedure for the calibration curves outlined in Materials and methods, free concentrations of the probe at a given ΔpH can be also computed by keeping the pH value at pH 5.5 in the inner phase and progressively bringing it alkaline in the outer surface. The superimposition of theoretical and experimental data points, however, is not significantly different from that indicated in Fig. 4 (data not shown).

Solution to Eq. (6) can be found only numerically and no simple analytical function relating ΔpH and Q can be derived. Curve fitting of the theoretical and experimental data (not shown for clarity of representation of the different sets of theoretical points) is obtained with the empirical curve:

$$\Delta pH = AQ/(B-Q)\exp[Q/(B-Q) + CQ] \qquad (9)$$

where A, B, and C are three fitting parameters, that can be used to relate any quenching of fluorescence observed to the corresponding ΔpH value. With our data the value of A, B and C are 15.64 ± 0.68, 1.51 ± 0.03 and -3.77 ± 0.11, respectively.

For comparison with the model of Eq. (2), the experimental data are plotted also as a function of $\log Q/(1-Q)$ (as shown in the inset). The slope of the linear regression of the data (solid line) is about 20% higher than that expected from the model of Eq. (2) (dashed line), indicating a non ideal behavior of 9AA. By extrapolating at $\Delta pH = 0$ the dashed and the solid lines, the inner volume of chromatophores is found to be 50- and 100-fold greater than the experimental one, respectively. These apparent V_i values can be used in Eq. (2) to empirically correct the probe–membrane interaction, as previously suggested [9,10]. However, only considering as the empirical calibration curve the linear regression to the experimental data, ΔpH values evaluated according to Eq. (2) are equal to those computed with Eq. (9) up to about 3 ΔpH units. Above this limit, extrapolation to higher pH values is realistic only using Eq. (9) and not the linear regression.

4. Discussion

A major result of this investigation is to quantitate the binding of 9AA to the chromatophore membranes. According to our results this fluorescent probe can be used to

determine the transmembrane ΔpH sustained by the physiological activity of the proton pumps in spite of its interaction with the membrane phase. This is brought out by comparing the experimental with the theoretical data which indicate that the model of Eq. (1) is sufficient to evaluate ΔpH provided that the probe membrane interaction is included in the computation.

The probe-membrane equilibrium is mostly modulated by the ionic strength of the solution and to a less extent by its pH value. Differently from previous experiments where the binding of ACMA was characterized only at pH 8.5 and at ionic strengths ≥ 0.150 M [19], electrostatic interactions of 9AA with the membrane are brought out by changing the ionic strength by a factor of 30 and by investigating adsorption in a range of pH values. Considering the high value of the probe pK_a ($pK_a = 9.99$ for 9AA), at the physiological pH values tested, the monocation is the predominant species and the concentration ratio of the neutral molecule to the monocation increases by 3 orders of magnitude on changing the pH from 8.5 to 5.5. The dependence between the free and membrane-bound probe is modelled with S-shaped isotherms, particularly suited to describe multi-molecular adsorption on non-ideal and non-localized monolayers, when lateral attraction within the monolayers becomes significant [27]. This type of isotherm was also used to describe adsorption of ACMA on chromatophores [19], suggesting that binding of fluorescent amines to membranes occurs via strong intermolecular interactions as generally indicated by S-shaped isotherms [29]. The results are consistent with what found by other authors in chloroplasts, where probe–membrane interaction was similarly described with S-type curves [12].

As for ACMA [19], saturation is obtained at a molecular ratio of the probe to phospholipid of 0.5 (corresponding to about one/two molecule/s of bound probe per nm^2, namely one/two molecule/s per lipid polar head surface area. These figures are evaluated on assuming an average total adsorbing surface area of $3.2 \cdot 10^{-3}$ m^2 per BChl nanomole [30] and a molar ratio of about 800 between the lipid and the reaction centre [21]. The extreme high value of saturation, which is pH and ionic strength independent (Table 1), is approx. 5-fold lower than that observed for the hydrophobic anion tetraphenylboron in chromatophores [22] and indicates stacking of the probe at the membrane/solution interface, where the probe concentration is related with the Boltzman factor to that in the bulk.

The high saturation density also suggests that 9AA interacts with the membrane phase in its neutral form. This is consistent with the assumption that only the neutral amine re-distributes through the membrane following a ΔpH generation [1]. In order to be consistent with the above remarks, a minimum model of 9AA interaction with the membrane phase would therefore include that: (i) electrostatic effects are dominant at the interphases, as the fitting of the data with the Grahame model indicates, and that (ii) the acid-base equilibria should be shifted at the membrane surface toward the neutral form, which hydrophobically interacts with the bilayer. The monocation is more stable in the bulk polar medium than at the interface, where it is repelled by the image forces and possibly by the inside positive dipole potential present in the boundary region [31]. A similar pK_a shift of neutral molecule-ion equilibrium was recently documented for *p*-nitrophenol and its anion at the air/water interface [32].

Modelling of the 9AA-membrane interaction with the Gouy-Chapman-Boltzman theory allows an estimation of the surface charge density of the chromatophores membranes, which in the range of alkaline pH values (7.5–8.5) is in agreement with a similar value previously obtained with fast spectroscopy combined with potentiometry [33]. A less negative value at more acidic pH values is however consistent with possible phospholipid [34] or protein group pK values at the membrane surface [35] and with a previous indication that in photosynthetic membranes the surface charge distribution is negligible around pH 5 [36].

Our model for evaluating ΔpH from the quenching of 9AA fluorescence mainly relies on Eq. (1), as previously discussed [1,9,10]. The basic assumptions are: (i) the only relevant fluorescent form of the amine is that free in the aqueous environment; (ii) the free amine behaves ideally and only its neutral form diffuses freely through the membrane; (iii) the adsorption of the probe on the membrane can be described by Eq. (6).

As to (i), it was shown before that the spectral characteristics of absorption and fluorescence emission of 9AA were not altered by the interaction with chromatophores except for the attenuation registered in the presence of the membranes (which could be corrected for by considering inner filter effects) and on following the formation of a protonic gradient [10]. Moreover, it must be noticed that we did not register (data not shown and Ref. [10]) any fluorescence emission of possible excimers, documented in a region centered around 550 nm for 9AA in water (at concentrations > 1 mM) [37] and in phosphatidylserine vesicles, at a lipid-9AA ratio of about 42 [14,18]. According to our data any dye molecule associated with the membrane phase is hardly fluorescent in the region centered around 550 nm.

Regarding point (ii) this is the general assumption at the basis of the use of amines in detecting transmembrane ΔpH [1]. It was measured before that, in contrast to the 9AA neutral form, the partition coefficient of the monocation was zero in a variety of hydrophobic solvents [38] and in our experiments the extremely high value of saturation is pointing to the same conclusions.

The use of the Hill isotherm (iii) is based on the coincidence between the best fit of our experimental data and this equation. In addition, in order to simulate the empirical calibration curve, we need to assume that the inner adsorbing surface is about one third of the external adsorbing one. As previously noticed [19], this ratio is higher than that expected for a spherical shell model and is

consistent with the asymmetric distribution of lipid molecules detected in these membranes [21].

As indicated by the binding isotherm, about 20% of the dye is associated with the membrane in the absence of any ΔpH. Any possible overestimation of adsorption due to dark Donnan potentials are neglected (under our experimental conditions the dark Donnan potential may be evaluated to be equivalent to a $\Delta pH < 0.1$ already at the low value of ionic strength [39]. According to our model, further binding is promoted by ΔpH formation and produces a negligible mass displacement from the outer to the inner aqueous compartment of the vesicles. Indeed, the probe entering the inner phase remains largely bound to the inner surface. The total adsorbing membrane surface is therefore the relevant parameter in this model, although its explicit value is not necessary in the computation (σS is evaluated from the mass balance equation and it is expressed in terms of probe nanomoles). At a constant adsorbing area, a 3-fold change of the V_i value produces a ΔpH change of only 0.05 units. This would then compensate for possible over- or under-estimations of the V_i value as determined with EPR [40].

In conclusion, the procedure here outlined, can be extended to any membrane system, provided that adsorption of the probe is similarly characterized. In this case, the empirical calibration curve (Eq. (9)) is sufficient to correct for the membrane probe interactions and can be used to quantitate ΔpH from the quenching of the probe fluorescence.

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