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9-AMINOACRIDINE FLUORESCENCE CHANGES AS A MEASURE OF SURFACE CHARGE DENSITY OF THE THYLAKOID MEMBRANE

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Summary

1. When suspended in a low cation-containing medium, chloroplast thylakoid membranes and carboxymethyl-cellulose particles quench the fluorescence from 9-aminoacridine (Searle, G.F.W. and Barber, J. (1978) Biochim. Biophys. Acta 502, 309—320).

2. Relief of this quenching is achieved by adding cations to the suspension medium with the order of effectiveness being $C^{3+} > C^{2+} > C^{+}$, indicating that the fluorescence acts as an indicator of the surface electrical potential.

3. Using the Gouy-Chapman theory, the differential effect of divalent (methyl viologen) and monovalent (K^{+}) cations has been used to calculate surface charge densities.

4. The calculations indicate that the surface charge density on the thylakoids significantly increases when cations are added to the low cation-containing medium. Under the same conditions the surface charge density of glutaraldehyde-fixed thylakoids and carboxymethyl-cellulose particles remained essentially constant.

5. It is argued that the 9-aminoacridine technique is able to probe localized areas on the membrane surface and that the variability of the surface charge density of untreated thylakoids may be due to redistribution of charges associated with membrane stacking as suggested by Barber and Chow (Barber, J. and Chow, W.S. (1979) FEBS Lett. 105, 5—10).

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Chl, chlorophyll.

Introduction

It has been shown in previous papers [1,2] that the fluorescence probe, 9-aminoacridine, can be used to study the properties of the diffuse layer adjacent to an electrically charged surface. The reason for this is that this compound carries a net positive charge at neutral pH and becomes poorly fluorescent when concentrated near a negatively charged surface. The exact reason for the fluorescence quenching is unknown and will only occur when the bulk solution does not contain significant levels of cations so that the surface potential is large. Addition of other cations, particularly multivalent cations, will decrease the surface potential and bring about a displacement of the acridine dye from the diffuse layer resulting in an overall increase of fluorescence of the suspension. In this paper our aim has been to use this property to estimate the surface charge density of the thylakoid membrane with the view to comparing this value with those obtained from other techniques [3—6].

Materials and Methods

Chloroplasts were isolated from peas using the procedure reported elsewhere [7]. Chlorophyll was determined by the method of Arnon [8]. For experiments the chloroplasts were subjected to osmotic shock during preparation and suspended in a medium containing 0.1 M sorbitol, 1 mM Hepes, 0.85 mM KOH and adjusted to pH 7.5 with HCl. Fluorescence from 9-aminoacridine was excited using light transmitted by a Balzer UG1 interference filter and detected at right angles to this beam by an EMI 9558B photomultiplier shielded by a Balzer B40 (498 nm) filter [1,2]. Salts were added as small aliquots from stock solutions. For calculations, the bulk concentrations of the salts added were estimated by spinning down the chloroplasts or carboxymethyl-cellulose particles and analysing the supernatant. Flame photometry was employed for K^+ determinations but when methyl viologen was used as a divalent cation, its concentration was determined spectroscopically at 393 nm by reducing it with sodium dithionite in the presence of 0.3% ammonia (w/w). Glutaraldehyde pretreatment of thylakoids was accomplished by treating membranes (0.5 mg Chl/ml) with 1% glutaraldehyde for 15 min at room temperature followed by washing with above low-salt medium.

Results

Although the addition of thylakoid membranes to a low-salt medium containing 9-aminoacridine caused a significant quenching of the fluorescence from the dye, it was usually necessary to add small quantities of EDTA (about 10—25 μM) to obtain the minimum fluorescence level. The necessity to do this had not been noted in previous work [1,2] and presumably is due to small quantities of residual divalent cations adsorbed on the membrane surface even under the low-ionic conditions used. As shown in Fig. 1 addition of salts to the medium brings about an increase in the 9-aminoacridine fluorescence and their effectiveness is related to the valency of the cation [1,2].

In principle it should be possible to use the type of data shown in Fig. 1

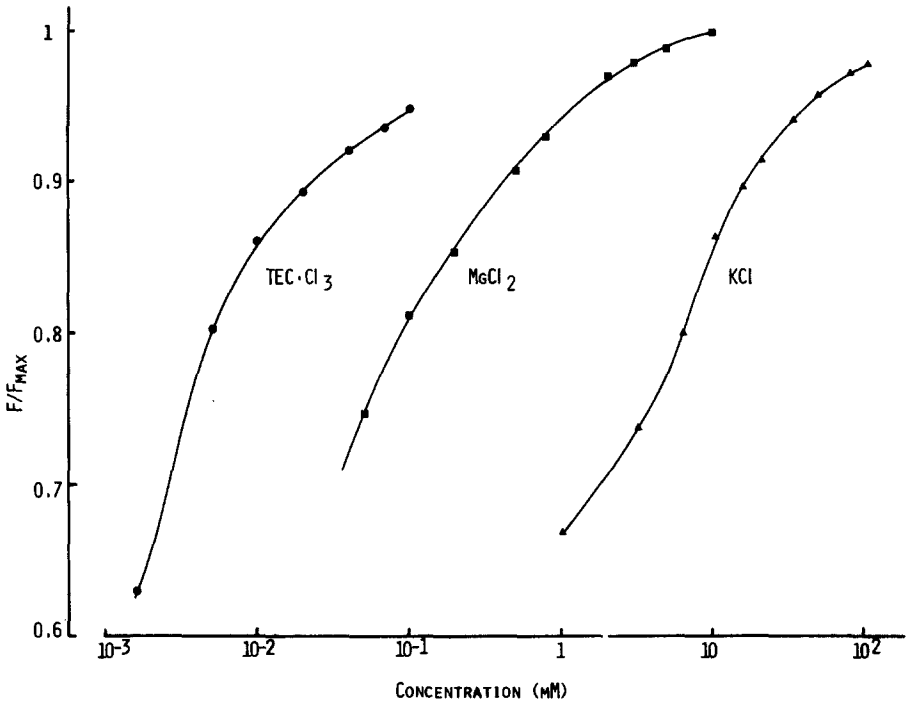

Fig. 1. Dependence of 9-aminoacridine fluorescence on the presence of mono-, di- and trivalent cations in chloroplast suspensions. The concentrations refer to the nominal concentrations of salts added. The concentration of 9-aminoacridine was 20 μM, and chlorophyll concentration 7 μg/ml. F_{max} is the maximum fluorescence intensity observed on adding 20 mM $MgCl_2$. TEC^{3+}, tris(ethylenediamine)-cobaltic cation.

to estimate the surface charge density. For an electrolyte consisting of a 1 : 1 salt, the surface potential ψ_0 is related to the surface charge density σ in the Gouy-Chapman theory by

$$\sigma = (8RT\epsilon_r\epsilon_0 C')^{1/2} \sinh\left(\frac{F\psi_0}{2RT}\right) \tag{1}$$

where R, T and F are the gas constant, temperature in K, and the Faraday, respectively. The dielectric constant is denoted by ϵ_r, the absolute permittivity of free space by ϵ_0, and the bulk concentration of 1 : 1 salt by C'.

When divalent cations were added to a chloroplast suspension the resulting medium contained a mixture of both monovalent and divalent cations. However, since the background monovalent cation concentration was low (about 0.8 mM measured in the bulk), the surface potential can be approximated by that for a 2 : 2 salt solution of concentration C'' in the bulk.

$$\sigma = (8RT\epsilon_r\epsilon_0 C'')^{1/2} \sinh\left(\frac{F\psi_0}{RT}\right) \tag{2}$$

Assuming that ψ_0 is the same when a monovalent cation concentration gives an equal release of quenching of 9-aminoacridine fluorescence as an equivalent

divalent cation concentration, these two equations (Eqns. 1 and 2) can be solved simultaneously to give

$$\psi_0 = -(RT/F)\cosh^{-1}\left(\frac{C'}{2C''} - 1\right) \tag{3}$$

and

$$\sigma = -[2RT\epsilon_r\epsilon_0(C'^2 - 4C'C'')/C'']^{1/2}$$
$$= -[3.444 \cdot 10^{-6}(C'^2 - 4C'C'')/C'']^{1/2} \tag{4}$$

where $T = 298$ K, $\epsilon_r = 78.5$, concentrations in mol $\cdot$ m^{-3} (equivalent to mM), and σ in C/m^2.

Using data obtained with the addition of methyl viologen as the divalent cation and K^+ as the monovalent cation and applying Eqn. 4 we have calculated values of σ for different levels of fluorescence quenching and the results for thylakoid membranes are shown in Table I. These values have been obtained by direct determination of the bulk concentrations of the added cations rather than relying on the concentrations calculated from the addition which do not take into account the cations adsorbed into the diffuse layer. The uncertainty of the bulk concentration becomes serious for the multicharged cations and for this reason no calculations were made with the tris(ethylenediamine)-cobaltic cation data. Methyl viologen was chosen for the divalent species since its bulk concentration could be conveniently measured spectroscopically and because this cation seemed not to bind readily with the membrane surface.

As it can be seen this analysis indicates that σ varies under different ionic conditions. This could be a genuine effect indicating salt-induced conformational changes in the thylakoid membrane brought about by changes in electrostatic screening. For example, the addition of salts could induce redistribution of electrically charged protein complexes in the fluid lipid matrix of the membrane [9,10]. To test this hypothesis we have carried out similar experiments to those conducted with fresh thylakoid membranes but using glutaraldehyde-fixed thylakoids and carboxymethyl-cellulose particles obtained from Whatman and converted into the potassium form by adding sufficient KOH to bring the pH to 7.5. Clearly these test systems would be far less likely to

TABLE I

ESTIMATED CHARGE DENSITIES FOR CHLOROPLASTS

The chlorophyll concentration was 7 μg/ml, and 9-aminoacridine concentration 20 μM. F is the fluorescence yield for a particular experimental condition and F_{max} is the level obtained on adding 20 mM divalent cation. MV, methyl viologen. The background bulk K^+ level was 0.8 mM and neglected in the τ estimations.

F/F_{max}	MV_{bulk} (mM)	K^+_{bulk} (mM)	σ (C/m^2)
0.70	0.095	2.5	—0.014
0.75	0.17	4.5	—0.019
0.80	0.28	6.8	—0.022
0.85	0.40	10.0	—0.027
0.9	0.60	16.1	—0.036

TABLE II

ESTIMATED CHARGE DENSITIES FOR GLUTARALDEHYDE-FIXED THYLAKOIDS AND CARBOXYMETHYL-CELLULOSE PARTICLES

The 9-aminoacridine concentration was 20 μM, chlorophyll concentration 20 μg/ml and carboxymethyl-cellulose particles 0.6 mg/ml. F is the fluorescence yield for a particular experimental condition and F_{max} is the level obtained on adding 20 mM divalent cation. MV, methyl viologen. For chloroplasts the background bulk K^+ level was 1.3 mM and for carboxymethyl-cellulose particles was about 0.7 mM.

F/F_{max}	MV_{bulk} (mM)	K^+_{bulk} (mM)	σ (C/m^2)
(a) Glutaraldehyde-fixed, unstacked chloroplasts			
0.50	0.0123	2.6	—0.043
0.55	0.0289	3.5	—0.038
0.60	0.0516	4.9	—0.039
0.65	0.0822	6.6	—0.042
0.70	0.121	8.8	—0.046
0.75	0.186	12.0	—0.050
0.80	0.345	17.6	—0.053
(b) Carboxymethyl-cellulose particles			
0.29	0.038	3.6	—0.034
0.33	0.070	4.5	—0.031
0.445	0.199	8.4	—0.033
0.55	0.317	12.3	—0.038
0.66	0.487	18.3	—0.046

undergo conformational changes of the type suggested above for untreated thylakoid membranes.

The results shown for these two more rigid systems indicate that the calculated surface charge densities remain reasonably constant over the concentration ranges used for the analyses (Table II).

Discussion

As previously argued [1,2] it seems that by measuring the cation-induced changes of fluorescence yield of 9-aminoacridine it is possible to monitor the nature of the electrical properties of charged surfaces. The differential effect of cations of different valencies and the independence of the fluorescence changes to the nature of the associated anion or to the chemical properties of cations carrying the same net charge is strong evidence that 9-aminoacridine responds to changes in the electrostatic properties at surface/solution interface [1,2]. Thus this fluorescence probe technique has considerable potential to supplement other techniques currently being used to investigate surface electrical phenomena like particle electrophoresis [3—5,11].

Ideally for a quantitative analysis of the fluorescence changes based on Eqns. 3 and 4 it is necessary that no binding of cations occurs at the surfaces under study and that the differential effect of cations with different valencies is due to their effectiveness to electrostatically screen the fixed negative charges on the surfaces. For this reason we have been careful in our choice of cations and have used methyl viologen as a divalent cation in preference to cations like

Ca^{2+} which are likely to bind to the surface to some extent. Methyl viologen has its two positive charges well spaced from each other and is sufficiently bulky to minimise binding with surface negative groups. With trivalent cations the problem is even more acute and in the past we have found it necessary to use the tris(ethylenediamine)-cobaltic cation as a non-binding trivalent species [12].

Even when non-binding cations are used the approach does not take into account the concentration profile of 9-aminoacridine extending out from the surface which will vary under different salt conditions even when ψ_0 is identical. Thus the approach is simple minded but may have advantages over other techniques such as particle electrophoresis, in that it would tend to monitor the charge density of localized regions on the surface. This is likely to be important for considering measurements on biological membranes where the distribution of surface charge is almost certainly heterogeneous. With particle electrophoresis an average, and probably an underestimate of the surface potential, is obtained since this technique estimates the electrical potential at the plane of shear (so-called ζ-potential) which may be some distance from the surface itself [13]. On the other hand the small molecular size of 9-aminoacridine allows it to probe the surface more intimately. Thus any reorganisation in the heterogeneity of the charges at the surface may be detected by the 9-aminoacridine method.

It is this latter possibility which could give rise to the significant changes in σ calculated for fresh thylakoids on increasing the salt concentration but not observed under similar conditions with glutaraldehyde-treated membranes or carboxymethyl-cellulose particles. In the case of the fresh thylakoids the changes in σ would be occurring under conditions when the degree of thylakoid stacking is increasing. Recently Barber and Chow [10] have put forward the idea that salt-induced stacking is associated with a change in distribution of surface charges brought about by the lateral diffusion of protein complexes in the relatively fluid lipid matrix of thylakoid membranes. As a consequence of this the surface charge density would increase in the unstacked regions while membrane appression would occur in those areas of low surface charge density. Treatment of the thylakoids with glutaraldehyde inhibits stacking and associated chlorophyll fluorescence changes [14] and according to our analyses, stabilizes the value of the surface density in a way comparable with the more rigid carboxymethyl-cellulose surface. Our data also shows that glutaraldehyde fixation increases the overall value of surface charge on the thylakoid membrane an effect in line with the inability of these treated membranes to stack because of increased coulombic repulsion [10,14,16]. The idea of lateral diffusion of protein complexes in the thylakoid brought about by changes in the salt condition of the suspending medium has support from freeze-fracture studies [17,18].

The σ values reported for the untreated thylakoid membranes can be compared with those from other studies. Electrophoretic mobility studies yielded σ values in the range -0.008 to -0.011 C/m^2 [3,5], somewhat lower than reported here. As already discussed, estimation of σ from ζ-potential measurements tends to yield lower values. Other studies (see Refs. 1, 15, 19 and 20), based on chlorophyll fluorescence changes and thylakoid stacking/unstacking

concluded that σ was -0.025 C/m^2 in line with the values given in Table I. Another study gave a charge density of -0.013 to -0.015 C/m^2 for the membrane surface in the vicinity of the primary acceptor of Photosystem II [6]. All these methods have their particular limitations, but the use of 9-aminoacridine fluorescence appears to be very useful since it seems more sensitive to the heterogeneity of the thylakoid membrane surface and to changes in surface charge distribution.

Acknowledgements

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