DOUBLE LAYER THEORY AND THE EFFECT OF pH ON CATION-INDUCED CHLOROPHYLL FLUORESCENCE

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1. Introduction

When freshly isolated, unwashed thylakoids treated with DCMU are suspended in a cation-free medium, the yield of chlorophyll fluorescence is high when irradiated with an intensity of light sufficient to close the photosystem II reaction centres [1,2]. As the level of monovalent cations is raised in the suspending medium this maximum yield of fluorescence initially drops to a low level and then rises again to the high level [2,3]. The position of the minimum occurs when the bulk level of monovalent cations is in the region of 10–20 mM while at 100 mM the fluorescence has increased to its high level again. This effect was first reported [4] and studied in some depth [2,3,5]. In attempting to explain the mechanism controlling this chlorophyll fluorescence phenomenon, it was argued [5] that the effect was due to changes in the electrical properties of the thylakoid membrane surface. In particular, the effect was pin-pointed to be a consequence of changes in the positive space charge density within a few nanometers of the membrane surface. It was argued [6] that changes in this parameter will alter the Coulombic interaction between the pigment–protein complexes within the membrane in such a way as to bring about changes in fluorescence yield. The calculations made were based on the assumption that the high fluorescence state observed in salt-free media could only occur when divalent cations act as the counterions for the fixed negative charges on the thylakoid surface. A number of observations support this concept [1,7,8] and indeed for unwashed thylakoids Mg^{2+} seems to fulfil this rôle [8,9].

However, in contrast to this argument, it is possible that changes in proton concentrations within the diffuse layer could also be responsible for the high fluorescence level observed with thylakoids suspended in a cation-free medium [10]. Under these conditions a substantial negative surface potential would exist [5] which could bring about a lowering of the pH at the membrane surface compared to the bulk (1 pH unit difference for every −58 mV) and effect the degree of dissociation of the surface negative groups. In so doing the surface charge density would be lowered and Coulombic interactions between adjacent chlorophyll–proteins altered. Thus, if this argument is correct, the high fluorescent state observed with salt-free media is due to 'protonation' while with high levels of monovalent cations the effect is due to 'electrostatic screening'. Both effects will result in a reduction of Coulombic repulsion.

Here we present data which suggests that changes in the dissociation of surface negatively-charged groups need not play a major role in cation regulation of chlorophyll fluorescence. The rationale of the experiments has been to monitor cation-induced chlorophyll fluorescence under conditions when protonation of surface charges does and does not occur.

2. Materials and methods

Chloroplasts were isolated from young pea leaves as in [11] and suspended in a small volume of 'cation-free' medium consisting of 0.33 M sorbitol adjusted to pH 7.5 with tris(hydroxymethyl)aminomethane

(Tris) base. For experiments, the chloroplasts were broken hypotonically in distilled water, followed by addition of an equal volume of the double strength cation-free media (see below). 3-(3′-4′-Dichlorophenyl)-1,1-dimethylurea (DCMU) (10 μM) and 9-aminoacridine (9-AA) (2 μm) were then added.

Chlorophyll and 9-AA fluorescence was measured in a laboratory-built fluorimeter: the sample, in a 10 × 10 cm cuvette was illuminated with broad band illumination (for chlorophyll fluorescence, 4 mm Schott BG18 + 4 mm BG38; for 9-AA, Schott 4 mm UG1). The fluorescence emission was detected at 90°C with EMI 9558B photomultipliers shielded with a Balzer B-40 693 nm + 2 mm Schott RG695 for chlorophyll and a Balzer B-40 498 + 2 mm Schott BG 18 for 9-AA.

The cation-free media used in the experiments reported below were:

(a) pH 8.25; sorbitol, 0.33 M; Tris base, 1 mM; KOH, 0.5 mM (total monovalent cation conc., 0.92 mM)

(b) pH 6.40; sorbitol, 0.33 M; 2(*N*-morpholino)-ethane sulphonic acid, 0.1 mM; Tris base, 0.12 mM (total monovalent cation conc., 0.12 mM)

3. Results and discussion

As shown in fig.1, when chloroplasts are isolated

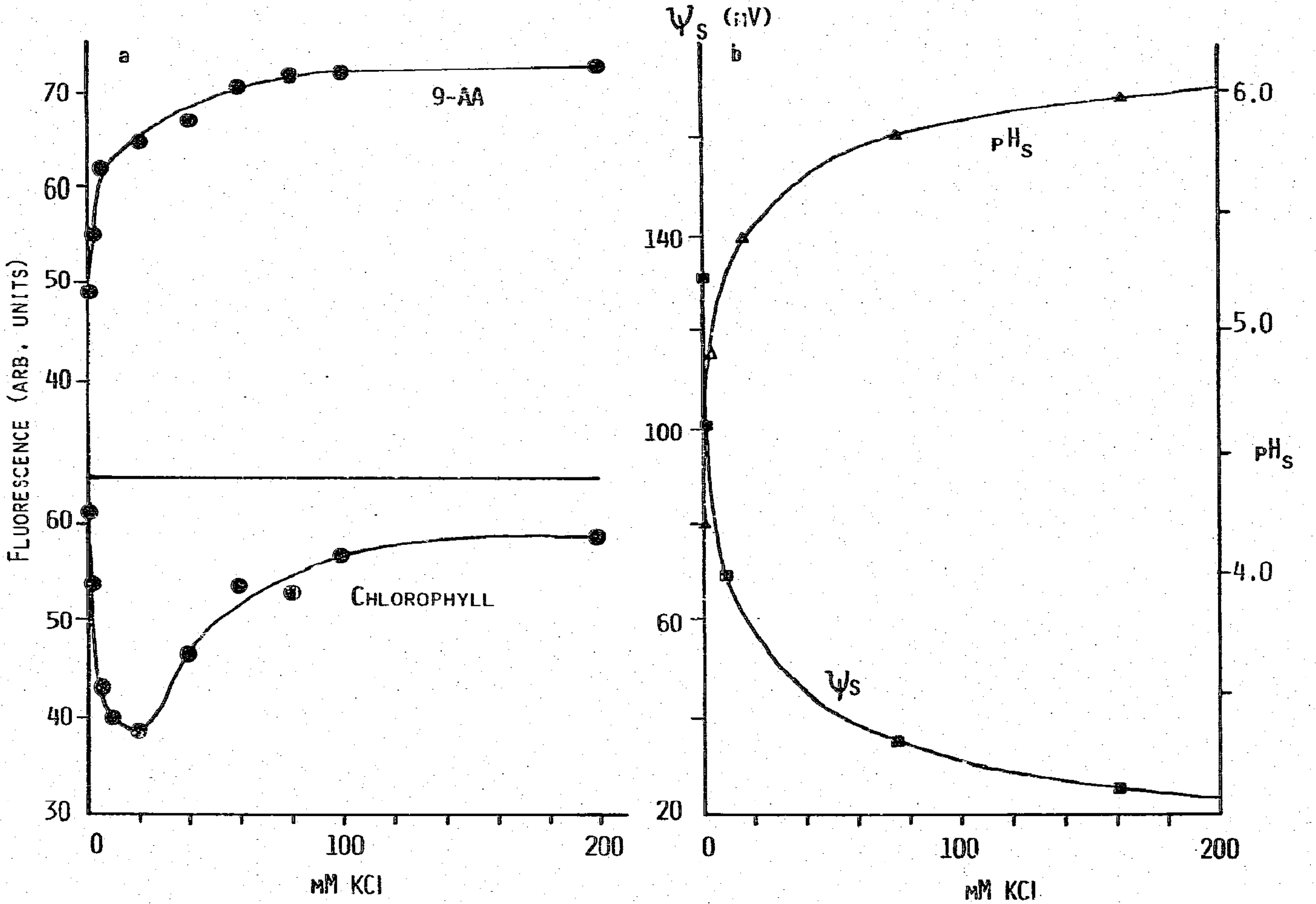

Fig.1. (a) Chlorophyll and 9-aminoacridine fluorescence changes induced by adding KCl to chloroplast thylakoids suspended in a 'cation-free' medium at pH 6.4 (see section 2). Chlorophyll was 5 μg/ml and the experiment was conducted at ~25°C. (b) Calculated values of surface potential (ψ_S) and surface pH (pH_S) for the various conditions used in the experiment shown in fig.1a (see text).

in the high chlorophyll fluorescing state and suspended in cation-free medium at pH 6.40, the addition of ⩽ 20 mM KCl causes a decrease in chlorophyll fluorescence intensity. The 9-AA fluorescence rises under the same conditions reflecting the release of quenching which accompanies the decrease in the magnitude of the negative surface potential (ψ_s) as explained [12,13]. However in our past considerations we had ignored the possibility that the surface potential changes may not only be controlled by changing the inorganic cation level in the diffuse layer but also by changes in protonation of the fixed surface charges.

The surface charge density σ has been related [10] with the dissociation reaction constant for fixed negative charges on the membrane surface in the following way:

$$\sigma = \frac{-\sigma_T K}{K + (H^+)_B \exp\left(\frac{-F\psi_s}{RT}\right)} \tag{1}$$

where K = dissociation constant of surface negative groups ($AH \rightleftharpoons A^- + H^+$), that is:

$$K = \frac{(A^-)(H^+)_s}{(AH)}$$

and since

$$(H^+)_s = (H^+)_B \exp\left(\frac{-F\psi_s}{RT}\right)$$

then

$$K = \frac{(A^-)(H^+)_B}{(AH)} \exp\left(\frac{-F\psi_s}{RT}\right) \tag{2}$$

where $(H^+)_B$ = bulk proton concentration (M); $(H^+)_s$ = surface proton concentration (M); ψ_s = surface potential; σ = actual surface charge density for a particular condition, i.e., $\sigma = -F(A^-)$; σ_T = total surface charge density when all the surface groups are fully dissociated, i.e., $\sigma_T = -F(|A^-| + |AH|)$.

Substituting definitions of σ and σ_T into eq. (2), yields eq. (1).

A general relationship between σ and ψ_0 for various salt conditions can be obtained from the Gouy-Chapman theory of diffuse layer [5,14]:

$$\sigma = \pm \left[2\epsilon_r\epsilon_0 RT \sum_i C_{iB} \exp\left(\frac{-Z_iF\psi_s}{RT} - 1\right)\right]^{1/2} \tag{3}$$

where ϵ_r is the dielectric constant of the solution, ϵ_0 the permittivity of a vacuum, C_{iB} concentration of ion i in the bulk.

For a mixture of monovalent salts eq. (3) can be reduced to:

$$4(C_1' + C_2') \sinh^2 \left(\frac{-F\psi_s}{2RT}\right) = \frac{\sigma^2}{A^2} \tag{4}$$

where C_1' = monovalent salt concentration in the 'cation-free' medium (M); C_2' = added monovalent salt concentration (M); RT/F = 25.85 at 25°C; A = 5.87 at 25°C; σ = surface charge density in $\mu C/cm^2$; ψ_s = surface potential in mV.

Combining eq. (1) and eq. (4) gives:

$$4(C_1' + C_2') \sinh^2 \left(\frac{-F\psi_s}{2RT}\right) = \frac{\sigma_T^2}{A^2} \left[\frac{K}{K + (H^+)_B \exp\left(\frac{-F\psi_s}{RT}\right)}\right]^2 \tag{5}$$

Using the Boltzmann equation and values of ψ_s obtained from eq. (5) then the surface concentrations of protons $(H^+)_s$ can be calculated, that is:

$$(H^+)_s = (H^+)_B \exp\left(\frac{-F\psi_s}{RT}\right) \tag{6}$$

As shown in fig.1b, eq. (5) and (6) have been used to calculate ψ_s and the local pH at the membrane surface (pH$_s$) for the salt conditions used in the experiment shown in fig.1a. In carrying out the calculation it has been assumed that the membrane dissociable groups have a pK_a = 4.5 (i.e., 3.162×10^{-5} mol/l) and that $\sigma_T = 2.5\ \mu C/cm^2$ (see [5]). Note that these calculations have ignored corrections for activity coefficient changes between the bulk and surface and assume that protons are concentrated at the negatively-charged membrane surface like any other cation, but unlike any other cations, protons are able to bind specifically to the negative groups (probably carboxyls of glutamic and aspartic acids, see [15]) in a way which is governed by the laws of mass action. Also ions are considered as point charges and it has been assumed that the surface charge is smeared out over a flat surface (see [5]). In addition, positive charges are also present on the thylakoid membrane surface [15,16] so that the absolute number of negative charges would be greater than assumed. Bearing in mind these points it can be seen in fig.1b that the surface pH is estimated to drop to ~4.25 when the thylakoids are suspended in a very low cation containing medium ($C_1' = 0.12$ mM). As a result the surface charge density changes from 2.5 μC/cm^2 to ~0.8 μC/cm^2 but even then there is still a significant surface potential equal to −132 mV. Thus in this case the high fluorescence yield observed could be partly due to neutralization of surface negative groups by protonation. The decrease in fluorescence observed when 20 mM K$^+$ is present can be attributed to a decrease in the positive space charge density as explained [2,3,5] but also clearly there will be an increase in the value of σ. In fact at 20 mM K$^+$ the surface pH is ~5.4 and σ is ~2.24 μC/cm^2. Both affects would tend to strengthen Coulombic repulsive forces between the surface negative charges. K$^+$ at >20 mM brings about effective electrostatic screening of the negative charges even though they would be fully dissociated and as a consequence the Coulombic repulsion would become minimal.

In the case of the experiment carried out with a bulk pH 8.25 (see fig.2a) the same characteristic chlorophyll fluorescence curve is obtained even

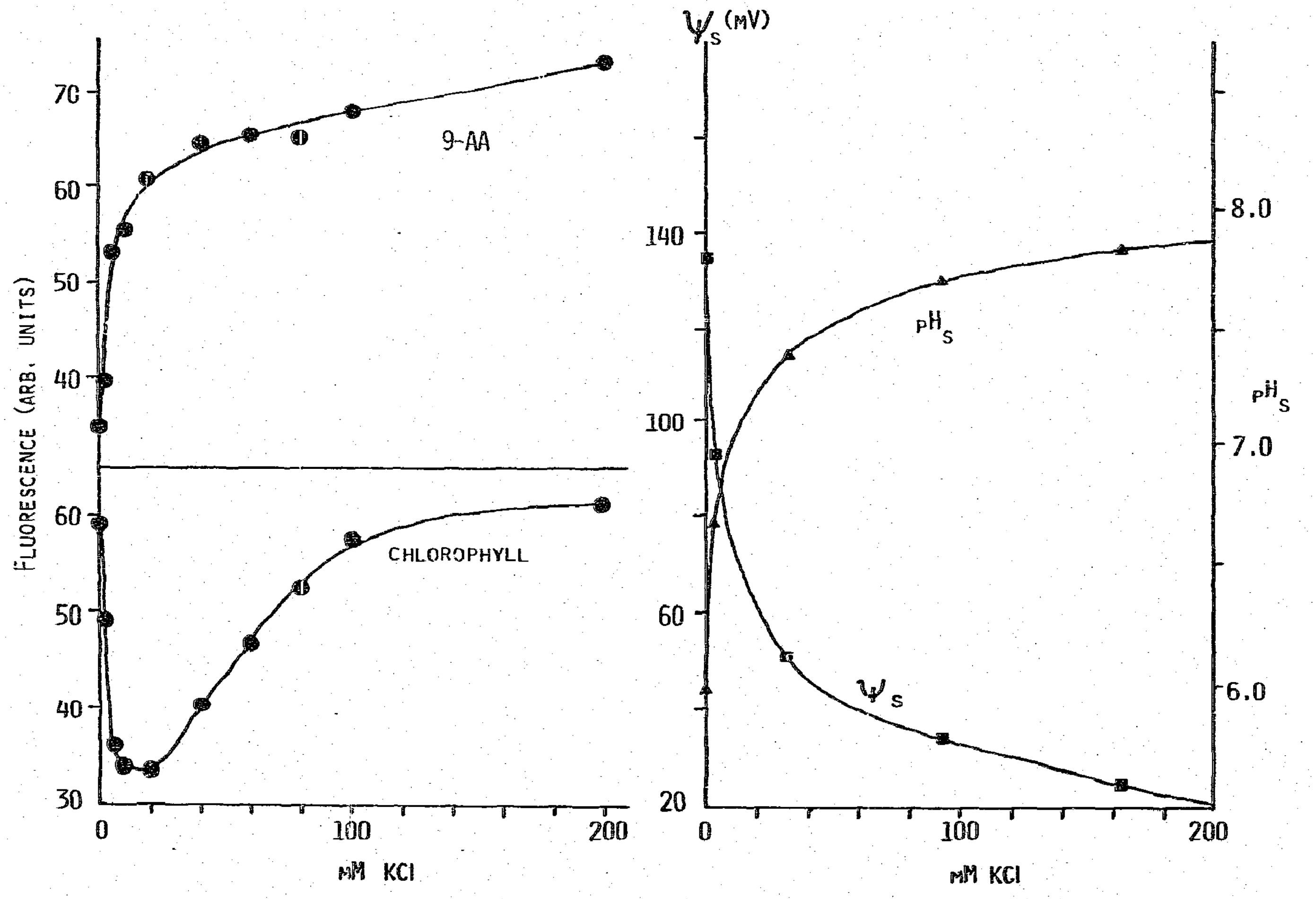

Fig.2. (a,b) as for fig.1 but at pH 8.25 and the initial monovalent cation level increased from 0.12 mM to 0.92 mM (see section 2).

though the surface charge density is at its maximum. In this case the surface potential is about −136 mV before addition of K$^+$, a value similar to that for the corresponding condition in fig.1b except the C_1' is now 0.92 mM. Although the surface pH is 6.0 under these conditions the σ value is essentially 2.5 μC/cm^2. Thus in this case the high fluorescence state must be totally attributed to the positive space charge density at the membrane surface resulting from residual divalent cations carried over during preparation of the unwashed isolated membranes. As before, the characteristic changes of chlorophyll fluorescence induced by adding increasing levels of K$^+$ can be attributed to the decrease and increase, respectively, in the space charge density at the surface. Both fig.1a and 2a show that the fluorescence changes of 9-aminoacridine do not follow those of the chlorophyll fluorescence on addition of K$^+$ and emphasise that it is controlled by changes in the surface potential (ψ_s).

Since the value of 4.5 taken for the pK_a of the surface negative charges on the thylakoid membrane is in line with a number of observations [1,12,15,16] then our results demonstrate that although protonation of surface charges could be important when the bulk pH is low this effect cannot explain the monovalent cation-induced chlorophyll fluorescence changes at high pH. It seems that at normal pH values the chlorophyll fluorescence changes (and probably thylakoid stacking) are controlled by the positive space charge density within a few nanometers of the membrane surface as emphasised [2,3,5,6]. It is not surprising that this parameter is important in controlling membrane conformational changes since it will govern the degree of interaction between similarly charged surfaces by affecting the balance between attractive Van der Waal's forces and repulsion due to Coulombic forces [6].

Acknowledgements

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