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ELECTRIC SURFACE CHARGE DYNAMICS OF CHLOROPLAST THYLAKOID MEMBRANES

TEMPERATURE DEPENDENCE OF ELECTROKINETIC POTENTIAL AND AMINOACRIDINE INTERACTION

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Electric surface charge dynamics of unstacked broken chloroplasts at low-ionic strength were studied by free-flow electrophoresis and aminoacridine fluorescence and binding changes over the temperature range 4–36°C. Both illumination and ATP hydrolysis in the dark cause a significant increase of net negative surface charge. The light and dark electrokinetic (ζ) potentials have a broad temperature optimum between 20 and 36°C. The decline at lower temperature shows a transition at about 18°C. The ATP-induced increase of the ζ potential requires preactivation of the ATPase and is dicyclohexylcarbodiimide sensitive. Aminoacridine binding shows a quite different temperature dependence. At lower temperatures there is an increased number of binding sites with a decreased affinity and the binding becomes positively cooperative. It is demonstrated that aminoacridines aggregate to dimers upon binding to the membranes. This phenomenon is stimulated by light and favoured at lower temperatures. The light-dependent extra binding increases sigmoidally with increasing temperature, similar to the increase of ζ potential, but with a less abrupt transition. The different effects of temperature on the electrokinetic and binding data are explained in terms of surface charge screening in the electric double-layer of the thylakoid membrane.

Introduction

A light-induced increase of net negative surface charge was observed by Nobel and Mel [1] and by Schapendonk et al. [2] by free-flow particle electrophoresis. Quintanilha and Packer [3] detected only small light-induced changes and Nakatani et al. [4] did not observe any light-induced increase of surface charge in both intact and cation-depleted chloroplast membranes, later attributed to different chloroplast preparation procedures [2,5]. On the other hand, 9-amino-substituted acridine probes interact electrostatically with the bound negative charges of the chloroplast thylakoid membrane, identified as protein carboxyl groups [2,5–9] and a fair correlation was observed between the

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; Chl, chlorophyll; DCCD, dicyclohexylcarbodiimide; PMS, phenazine methosulphate; Tricine, *N*[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine

electrokinetic properties and the behavior of such cationic probes, be it at a fixed temperature [2,10–12].

If appreciable agreement of the free-flow electrophoresis data and binding of these fluorescence probes indeed exists, the latter would provide reliable monitors for the dynamic behavior of the membrane-bound surface charges in kinetic studies. Rapid kinetics cannot be analyzed with the former technique; single measurements with a relatively fast version, described here, still require 5 s or more.

However, earlier work with the uncoupling aminoacridine atebrin [13,14] indicated that the binding of this compound increases upon lowering the temperature. It is therefore of interest to investigate the two surface charge-indicating methods as a function of temperature, and also to compare their possible temperature optima and transition temperatures with those of other energy-linked phenomena in chloroplast membranes.

In this paper comparative studies are reported on the electrokinetic (ζ) potential and the interaction with different fluorescent aminoacridine probes of unstacked broken chloroplasts at low-ionic strength.

The effect of ATP hydrolysis on the ζ potential in the absence of actinic light (thus excluding direct thermal effects) is investigated as well.

A brief account of part of this work was presented in a proceedings paper [15].

Materials and Methods

Chloroplast preparation

Broken chloroplasts (without outer envelope membranes) were prepared from spinach (*Spinacia oleracea* L.) leaves, obtained from local market sources, according to a modification of the procedure in Ref. 2. The isolation medium contained 345 mM sorbitol and 2 mM Tricine-NaOH buffer (pH 8.0). Broken chloroplasts were obtained by suspending the pellets in 1 ml of 2 mM Tricine-NaOH (pH 8.0), for 1 min, after which 1 ml of 690 mM sorbitol and 4 mM Tricine-NaOH was added. Chlorophyll concentrations were determined according to Kirk [16].

Electrophoresis experiments

Electrophoretic migration of chloroplast populations was followed in a thermostated laterally oriented quartz chamber (depth, 0.55 mm; height, 14.4 mm) connected to reversible $Cu/CuSO_4$ electrode compartments. The instrument used was a modified Rank Brothers (Cambridge, G.B.) particle micro-electrophoresis apparatus Mark II, provided with a slowly-rotating prism system in the observation path and a Philips TV monitor facilitating the rapid timing of electrophoretic migration of chloroplast populations. The particle micro-electrophoresis apparatus was equipped with phase contrast optics and an actinic illuminator providing red light (cut-off below 610 nm) with an intensity of 22 $mW \cdot cm^{-2}$ at the electrophoresis chamber. The observation light was filtered by a 545 nm interference filter. In both cases the light was also passed through a 1 cm cuvette containing 100 mM $CuSO_4$ to minimize heat convection currents. The objective was focussed on one of the two predetermined stationary levels. The electrical conductivities and viscosities of the electrophoretic media at different temperatures were measured with a conductivity meter (Philips PW 9501) in conjunction with a conductivity cell (Philips PR 9512/01) and with an Oswald-type viscosimeter, respectively. The electrophoresis medium (10 ml) contained 345 mM sorbitol, 2 mM Tricine-NaOH (pH 8.0), chloroplasts equivalent to 10 μg Chl $\cdot$ ml^{-1}, and in addition (in temperature dependence studies) 20 μM methylviologen or (in ATP hydrolysis studies) 0.25 mM $MgCl_2$/1 mM dithioerythritol/5 μM PMS and different ATP concentrations. Electrophoretic mobilities were measured at constant current (50 μA) resulting in electric field strengths between 15 and 25 $V \cdot cm^{-1}$.

Fluorescence and binding experiments

Aminoacridine fluorescence changes were measured front-face (0°) with a modified Aminco-Chance spectrophotometer equipped with a multipurpose cuvette described elsewhere [17] and a dichromatic mirror and filter combination (Zeiss Ploemopak TK 455); the detector was an E.M.I. 9558 QC photomultiplier and the preamplified signal was recorded by a Watanabe MC 641-4 Z multichannel recorder. Actinic light was provided from the bottom of the cuvette (1.8 ml) via a

fiber-optic light guide by a tungsten-halogen illuminator (250 W), filtered by a red filter, cutting-off below 610 nm and a heat-absorbing filter (Calflex-C) from Balzers, Liechtenstein. The light intensity in the cuvette was 15 $mW \cdot cm^{-1}$. The fluorescence and binding assay media contained 345 mM sorbitol, 2 mM Tricine-NaOH (pH 8.0), 20 μM methylviologen, different aminoacridine concentrations (legends) and 40 μg Chl $\cdot ml^{-1}$, unless stated otherwise. The binding of aminoacridines was determined in parallel experiments by rapid filtration of the suspension from a 2 ml glass syringe, equipped with a 1 cm filter holder containing a Whatman GF/B glass fiber filter. For dye adsorption to the filter assembly (negligible in most cases) correction was made. This system allows binding measurements both in darkness and during illumination. Indirect binding data derived from fluorescence quenching were in good harmony with this more direct method.

Absorbance measurements

Corrected and difference absorbance spectra were recorded on an Aminco DW-2a dual-wavelength spectrophotometer, also equippped with the cuvette mentioned above, and with an on-line microprocessor (PDP 11-03, DEC)/minicomputer (HP 1000) system.

Activation of ATP hydrolysis

The ATP hydrolysis activity of the membrane-bound ATPase was triggered by a 2 min illumination period at saturating light intensity in the presence of 0.25 mM $MgCl_2$, 1 mM dithioerythritol and 5 μM PMS (cyclic electron flow). After a 1 min dark period different concentrations of ATP were injected. In the controls (masked ATPase) the same procedure was followed, except that pre-illumination was omitted.

Electron microscopy

The chloroplast suspension used for the electrophoresis experiments were examined under the electron microscope in order to inspect the state of membrane appression. The procedure for fixation, staining and embedding was as described by Gross and Prasher [18]. The electron microscope was a Zeiss EM 10A.

Chemicals

All chemicals were of analytical grade. Dithioerythritol was obtained from Sigma and freshly prepared for each experiment. ACMA was synthesized according to the general recipes of Albert [19].

Results

Free-flow electrophoresis studies

The electrokinetic potential at the hydrodynamic plane of shear was determined by free-flow

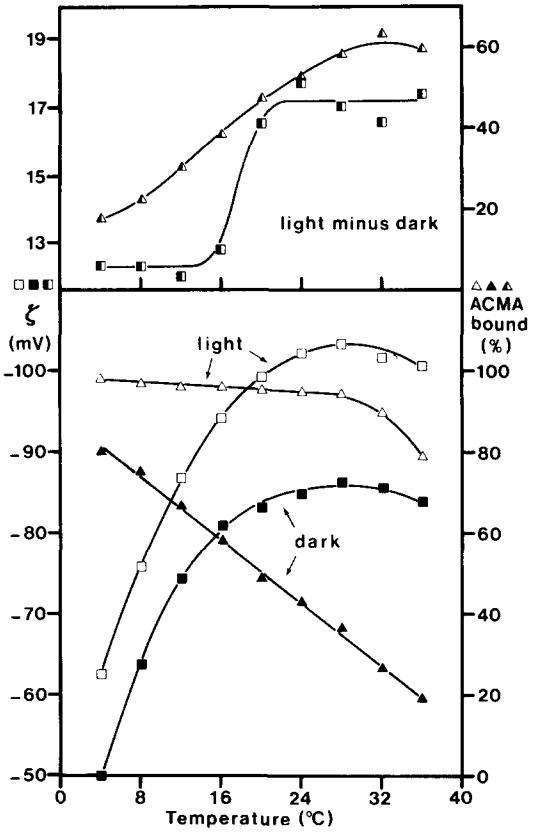

Fig. 1. Temperature dependence of the electrokinetic (ζ) potential and ACMA binding of unstacked thylakoid membranes and the effect of energization by light. In the bottom part the measured data are plotted; in the upper part the differences between the light and dark values for ζ are given on an expanded scale. Experimental conditions are given in Materials and Methods. The specific medium conductivity increased linearly from 106 μsiemens $\cdot cm^{-1}$ at 4°C to 224 μsiemens $\cdot cm^{-1}$ at 36°C; the kinematic medium viscosity decreased non-linearly from $1.89 \cdot 10^{-3}$ N $\cdot$ s $\cdot m^{-2}$ at 4°C to $0.84 \cdot 10^{-3}$ N $\cdot$ s $\cdot m^{-2}$ at 36°C.

particle electrophoresis of broken and unstacked spinach chloroplasts (at low ionic strength) in dark and light and as a function of temperature. The results are shown in Fig. 1. Under these experimental conditions (no Mg^{2+} added, low-ionic strenght) the values for the ζ potential vary between -50 and -85 mV in the dark and between -63 and -104 mV in the light, in the temperature range of 4–36°C. When these data are put into an Arrhenius plot (Fig. 2) major phase transitions seem to occur at 12°C (dark) or 17°C (light); on the whole it rather shows a gradual activity change. In Fig. 1 (top) the light minus dark values indicate a transition temperature at about 19°C for the light-induced increase of net negative surface charge. This would be in line with earlier observed transitions in a variety of energy-linked chloroplast activities [13,14,20]. The ζ potentials shown in this work are higher than those previously reported [2] due to the absence of added Mg^{2+} ions in the isolation and incubation media. For the same reason the light-induced increase of ζ potential is smaller since at the low endogenous Mg^{2+} concentration electron transfer and membrane energization are less efficient [21].

For the interpretation of these data it is important to know what is the state of thylakoid membrane appression (stacking) because mutual charge screening by appressed membranes undoubtedly influences the net charge detected in the hydrodynamic plane of shear around the particles. We have examined the chloroplast preparation used in this study by electron microscopy according to Gross and Prasher [18] and observed that the thylakoid membranes were essentially unstacked and loosely arranged in parallel or completely separated. The membranes were also unstacked in the presence of 0.25 mM $MgCl_2$, used in the experiments on the effect of ATP (see below). Stacking reappeared to some extent after addition of 1.5 mM $MgCl_2$.

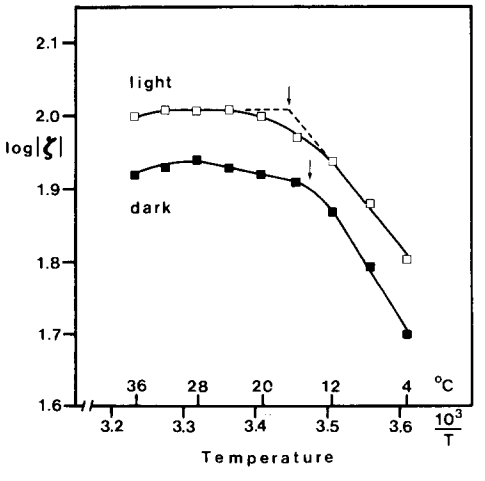

Fig. 2. Arrhenius plot of dark and light ζ potentials of unstacked thylakoid membranes. Data derived from Fig. 1; on the ordinate the ^{10}log of the absolute values of ζ are plotted.

Some other control experiments (not shown) were the following. In the absence of electron flow and/or membrane energization illumination does not cause a change of ζ potential by itself [2]. The electrophoresis cuvette is completely submerged in the circulating thermostate fluid and neither actinic nor observation light cause a detectable rise in temperature (measured with a micro temperature sensor). The dark ATP effects (below) further exclude direct thermal effects as the cause of potential changes. In one case we have tested cyclic photophosphorylation activity in the rather dilute chloroplast suspension before and after the electrophoresis procedure (5 min) and did not observe any impairment due to this treatment.

If the light-induced increase of ζ potential is indeed the result of a charge rearrangement associated with membrane energization, rather than the direct consequence of electron flow [1,4], ATP hydrolysis in the dark should also been able to produce this effect, although we may not expect that the extent is as large as that induced by electron flow since the 'energy pressure' will be smaller in the former case [6,8]. Fig. 3 indeed demonstrates that in the presence of ATP hydrolysis there is a small but significant increase of ζ potential, which is dependent on the preactivation of the ATPase enzyme [22]. The increase of ζ potential in the controls (masked ATPase) is probably due to the introduction of additional negative charges by ATP itself and/or by its association with residual membrane-bound Mg^{2+} ions (0.25 mM $MgCl_2$ was present here) in the diffuse double-layer. Note also that ATP also significantly alters the medium specific conductivity (legend Fig. 3). The energy transfer inhibitor DCCD prevents the ATP-induced increase of ζ potential after

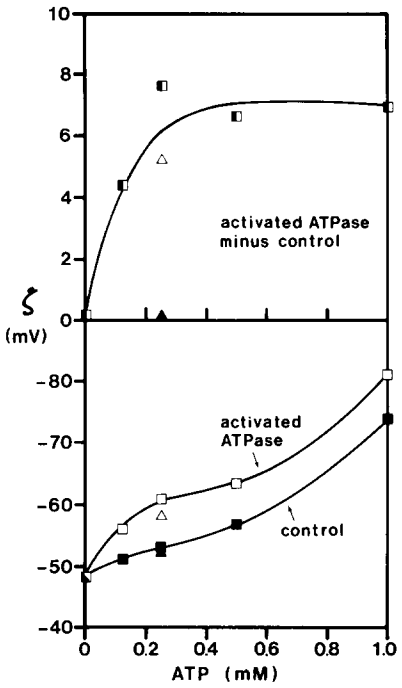

Fig. 3. ATP hydrolysis-dependent increase of ζ potential of unstacked thylakoid membranes in the dark. Bottom, measured data in the presence of activated and masked (control) ATPase enzyme; top, difference on expanded scale; △ and ▲, data of activated ATPase plus 5 and 20 μM DCCD, respectively (DCCD had no significant effect on control values). The temperature was 20°C; further experimental details in Materials and Methods. The specific medium conductivities at 0, 0.125, 0.25, 0.5 and 1.0 mM ATP were 165, 200, 244, 322 and 494 μsiemens·cm^{-1}, respectively.

triggering the ATPase, but did not significantly change the potentials of the controls. Additional control experiments in the presence of 1 mM ADP or 1 mM inorganic phosphate did not show significant changes either. The combination of these compounds and light (phosphorylating conditions) slightly lowered the light values, as expected from the partial membrane deenergization in this case.

The ζ potentials in Fig. 3 are lower than that in Fig. 1, at 20°C. This is due to the partial charge screening by the Mg^{2+} ions, required in the ATP hydrolysis experiments, but as said before, this is not accompanied by membrane stacking (at 0.25 mM $MgCl_2$).

Among different chloroplast membrane preparations, the standard deviations of the electrophoresis data never exceeded 3% at a given temperature.

Aminoacridine interaction studies

The fluorescence quenching occurring immediately after addition of aminoacridine probes to negatively charged membranes and its energy-dependent enhancement are plausibly explained by the electrostatic interaction of these cationic probes with the external surface-bound charges [2,4,10–12]. The binding of aminoacridines to chloroplasts, defined as the sum of electrostatic and hydrophobic interaction and internal uptake, goes hand in hand with the fractional quenching of the initial fluorescence signal. At low ionic strength and in the absence of multivalent cations this binding is largely determined by the electrostatic interaction [4,6] so that under these conditions amioacridine fluorescence and binding changes can be considered as measure of the surface charge density at the average distance of the acridine moiety to the membrane surface [2]. The 'surface potentials' thus measured with fluorescent aminoacridine probes are higher than the potentials experienced in the plane of shear (at a larger average distance) but the results of both approaches go hand in hand under comparable conditions (same medium and temperature) [2,11,12].

However, if the temperature is changed, aminoacridine binding does not follow the ζ potential change. This is shown in Fig. 1 for the probe ACMA. Binding of ACMA in the dark linearly decreases with increasing temperature while the dark ζ potential increases with a typical saturation profile. At the used low concentration of ACMA this compound is almost completely bound in the light; binding decreases from 99% at 4°C to 96% at 30°C, then rather sharply declines at higher temperatures. The net light-dependent ACMA binding (Fig. 1, top) increases sigmoidally with increasing temperature, not showing the sharp transition observed in the ζ potential change.

The kinetics of the light-induced fluorescence quenching and dark relaxation of an analogous compound, atebrin, show clear temperature transitions at about 19°C [13]; this is also observed for ACMA (not shown).

In order to find a possible reason for the apparent lack of correlation between the two 'surface-potential'-indicating methods when the temperature is changed, we have studied the light-

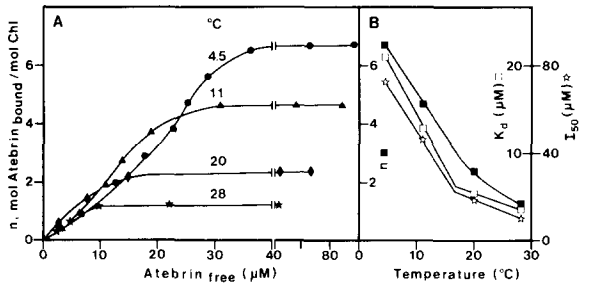

Fig. 4. Light-induced atebrin binding characteristics and its uncoupling effect in broken chloroplasts at different temperatures. The medium contained 50 mM NaCl/50 mM KCl/0.3 mM $MgCl_2$/1 mM ADP/3 mM KH_2PO_4/2 mM Tricine buffer (pH 8.0)/20 μM pyocyanine/30 μg Chl·ml^{-1}. (A) Binding data; (B) determined values for the number of binding sites (n), the K_d and I_{50} (see text).

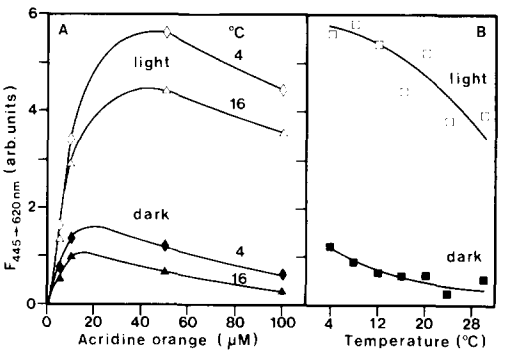

Fig. 5. Light and temperature dependence of acridine orange dimer formation in broken and unstacked chloroplasts. Reaction conditions were as in Fig. 1. (A) Concentratioan dependence; (B) temperature dependence at 50 μM acridine orange.

induced binding of several aminoacridines as function of temperature and probe concentration. An example is given for atebrin in Fig. 4, but other derivatives like ACMA behave qualitatively similar. Binding titrations clearly show that the number of atebrin binding sites (n) increases and that the binding becomes positively cooperative in nature (cf. sigmoidal curves in Fig. 4A) at lower temperatures. Fig. 4B shows that both n, the K_d for binding and also the atebrin concentration giving half-maximal inhibition of ATP synthesis (I_{50}) decrease in parallel with increasing temperature. Possible temperature transitions occur at about 18°C. In this experiment a different medium [15] was used to allow simultaneous photophosphorylation measurements. The increased and positively cooperative aminoacridine binding suggests that another process in addition to electrostatic interaction (and uptake) contributes to their binding to the thylakoid membranes. In line with earlier studies with acridine orange and neutral red [12,23] this process may be related to the concentration-dependent molecular aggregation, long known for this family of dyes [24,25]. Such aggregation will most likely be facilitated at lower temperatures. Fig. 5 shows that this assumption is probably correct for the case of acridine orange. This dye has the advantage that dimer formation can be followed by the appearance of its characteristic fluorescence at 620 nm. The fluorescence at 620 nm increases both upon energization and at lower temperatures. At very high acridine orange concentration dimer fluorescence decreases again, possibly due to the formation of higher-order aggregates. The total binding of this dye follows the

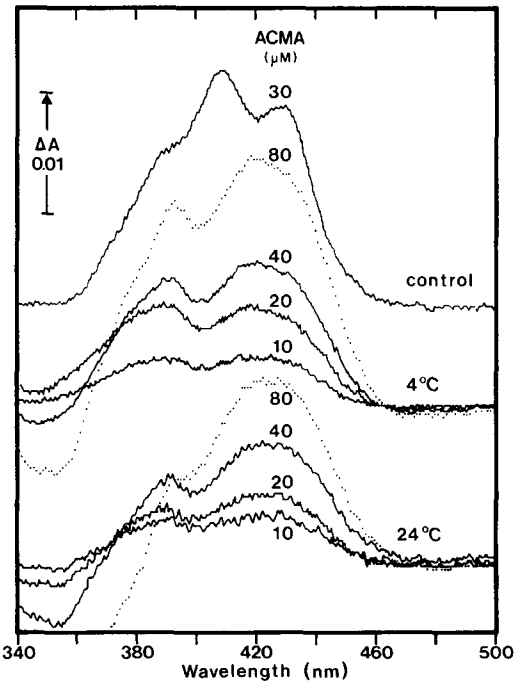

Fig. 6. ACMA absorbance spectra in the absence and presence of illuminated chloroplasts at different probe concentrations and temperatures. The reaction medium was as in Fig. 1. The control (uppermost) spectrum was recorded in the absence of chloroplasts (versus medium) at 24°C; in this case the indicated ΔA is 0.1. All other spectra ($\Delta A = 0.01$) represent the difference of (ACMA + chloroplasts) minus chloroplasts. ACMA concentrations and temperatures as indicated.

same pattern as that of atebrin (cf. Fig. 4). The acridine orange monomer fluorescence is quenched more rapidly than the dimer fluorescence appearance upon illumination [12] indicating that electrostatic attraction preceeds the slower aggregation.

Such a specific dimer or multimer fluorescence is not observed with ACMA (and atebrin) but we have demonstrated dimer formation of ACMA upon association with chloroplast membranes by the characteristic change of the absorbance spectrum in the visible region, as earlier shown for similar compounds [24,25]. This experiment is shown in Fig. 6 in which the corrected absorbance spectra of ACMA are recorded at 4 and 24°C and at different probe concentrations in the presence of illuminated chloroplasts. Compared to the control ACMA spectrum (24°C, no chloroplasts), top curve in Fig. 6, these spectra show a decrease at the major 409 nm band, giving way to the 392 nm and 427 nm bands. This behavior is associated with dimer formation [19,24,25], which is indeed more obvious at the lower tempeorature. The peak separation is less pronounced at 80 μM ACMA (dotted curves), comparative to acridine orange at higher concentrations (cf. Fig. 5).

Discussion

In this work we have studied some dynamic properties of the chloroplast thylakoid membrane charges by free-flow electrophoresis and by aminoacridine probe adsorption at different temperatures. The negative charges at the outer surface of these membranes are mainly derived from protein carboxyl groups [2,4,5] and these interact with medium counterions to form a diffuse double-layer. The formation of this double-layer is influenced by Coulombic forces and thermal motion. At very low temperatures (negligible thermal motion) an extremely compact double-layer would form and hardly any electrokinetic potential would be experienced in the hydrodynamic plane of shear, as argued by Mysels [26]. However, within the tested temperature range (4–36°C) thermal motion will not be a major factor. At the lower end of this temperature range we observe a decreased electrokinetic potential, similar to the charge-screening effect of added multivalent cations. This suggests that the electrostatic interaction between membrane surface and residual cations is favoured at lower temperatures, besides a minor contribution of a somewhat decreased thermal motion [26].

A significant complication of measurements of this type is presented by the fact that we have no direct information on the surface conductivity of the membrane particles. The electrophoretic mobility of a particle in suspension depends on the ratio of the conductivities of particle and medium [27]. The higher the particle conductivity, the lower the mobility in an electric field. For the case that the radius of the particle is much greater than the thickness of its surrounding double-layer of counterions, the Helmholtz-Von Smoluchowsky equation [2,26,29] turns into Henry's equation:

$$u = \frac{\epsilon \zeta}{\left(1 + \frac{c_s}{2c_m}\right)\eta}$$

where u represents the electrophoretic mobility, ϵ the permittivity, and c_s and c_m the conductivities of the particle surface and medium, respectively. Thus, the decrease of ζ potential at lower temperatures might be due in part to a decrease of the ratio $c_s/2c_m$.

We have not considered it appropriate at this stage to express the electrokinetic data in terms of surface charge density. Haydon [30] states that the transformation of ζ potential into surface charge density requires the assumption that the particle surface is impermeable to ions which is obviously not the case for chloroplasts and other biological membranes. Under conditions of low ionic strength as in our experiments this assumption is of minor importance. However, we remain with the uncertainty whether we have to consider the broken chloroplasts as single particles, in which case the correction term $(1 + c_s/2c_m)^{-1}$ must be applied [27], or as a bunch of small vesicular or ruffled structures with radii of the same order of magnitude as the double-layer thickness.

Membrane surface dynamics is functionally significant and not appropriately accounted for in the usual equations [2,26,29–31]. Although they yield appreciable information for simple model systems, where statistical numbers of mobile ions are of major importance, more detailed knowledge about the nature of the thylakoid mebrane surface

is required for the adequate corrections of such equations [28,32–35].

In addition, it is noteworthy that most theoretical considerations in membrane physics visualize the surface charges as smeared out over the lateral plane of the membrane, whereas the identification of the negative charges generating the ζ potential in chloroplasts with protein carboxyl groups [2,4] strongly suggests that these charges would rather exist in discrete domains (patches). The energy-dependent occlusion of positively charged amino groups on the ATPase protein [7] will also contribute to the observed net increase of negative charge at the external surface.

The energy-dependent binding changes of aminoacridines to thylakoid and other biological membranes, as monitored by fluorescence quenching, can be used as a measure for surface charge and changes thereof [2,4,10,13]. At fixed temperature such data provide qualitatively the same information as free-flow electrophoresis. The former technique has the advantage over the latter in that it can be used in kinetic experiments. Recent studies of the ζ potential in yeast cells showed that the values for this potential constitute a constant fraction of the surface potential experienced by the monovalent cation carrier [11]. This relation also holds at fixed temperature.

The results presented here demonstrate that at varying temperature the correlation between the electrophoresis and fluorescence (or binding) methods does not hold. The partly counteracting temperature dependence of the binding of aminoacridines can be explained by their more effective participation in charge screening at lower temperatures. The number of aminoacridine binding sites increases with decreasing temperature, but their affinity decreases as well (Fig. 4). The increased binding at lower temperatures and by illumination or ATP hydrolysis [6] is facilitated in addition by their aggregation when high local concentrations are formed at the membrane surface. In a recent paper Huang et al. [36] also demonstrated that ACMA interaction with submitochondrial membranes is associated with nonfluorescent complex formation. These authors also showed by fluorescence polarization of atebrin that dyes of this type are maximally immobilized below 15°C.

The results on aminoacridine aggregation plausibly explain their apparently anomalous behavior with respect to the surface charge in the membrane-medium interface.

In order to arrive at quantitative determination of the actual surface charge density of thylakoid and other biological membranes by fluorescence probes, also in kinetic studies, it is necessary to calibrate the signals at any temperature and ionic composition of the medium used. The average membrane-probe distance is also a parameter that should be taken into account. Work is now in progress in which we attempt to perform such calibrations with a family of hydrophobically bound dyes [cf. 37] with varying spacer groups between the membrane and the fluorophore.

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References

1 Nobel, P.S. and Mel, H.C. (1966) Arch. Biochem. Biophys. 113, 695–702
2 Schapendonk, A.H.C.M., Hemrika-Wagner, A.M., Theuvenet, A.P.R., Wong Fong Sang, H.W., Vredenberg, W.J. and Kraayenhof, R. (1980) Biochemistry 19, 1922–1927
3 Quintanilha, A.T. and Packer, L. (1977) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.), pp. 55–60, Elsevier/North-Holland, Amsterdam
4 Nakatani, N.Y., Barber, J. and Forrester, J.A. (1978) Biochim. Biophys. Acta 504, 215–225
5 Barber, J. (1982) Annu. Rev. Plant Physiol. 33, 261–295
6 Kraayenhof, R. (1973) in Fluoresence Techniques in Cell Biology (Thaer, A.A. and Sernetz, M., eds.), pp. 381–394, Springer-Verlag, Berlin
7 Kraayenhof, R. and Slater, E.C. (1975) in Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), pp. 985–996, Elsevier, Amsterdam
8 Kraayenhof, R., Bocklehurst, J.R. and Lee, C.P. (1976) in Biochemical Fluorescence – Concepts, Vol. 2, (Chen, R.F. and Edelhoch, H., eds.) pp. 761–809, Marcel Dekker, New York
9 Kraayenhof, R. and Arents, J.C. (1977) in Electrical Phe-

nomena at the Biological Membrane Level (Roux, E., ed.), pp. 493–504, Elsevier, Amsterdam
10 Mansfield, R.W., Nakatani, H.Y., Barber, J., Mauro, S. and Lannoye, R. (1982) FEBS Lett. 137, 133–136
11 Theuvenet, A.P.R. and Borst-Pauwels, G.W.F.H. (1983) Biochim. Biophys. Acta, 734, 62–69
12 Kraayenhof, R. (1977) in Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), pp. 223–236, Elsevier, Amsterdam.
13 Kraayenhof, R., Katan, M.B. and Grunwald, T. (1971) FEBS Lett. 19, 5–10
14 Schuurmans, J.J., Veerman, E.C.I., Francke, J.A., Torres-Pereira, J.M.G. and Kraayenhof, R. (1984) Plant Physiol. 74, 170–175
15 Kraayenhof, R., Torres-Pereira, J.M.G., Peters, F.A.L.J. and Wong Fong Sang, H.W. (1984) in Advances in Photosynthesis Research, Vol. II (Sybesma, C., ed.), pp. 289–292, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
16 Kirk, J.T.O. (1968) Planta 78, 201–207
17 Kraayenhof, R., Schuurmans, J.J., Valkier, L.J., Veen, J.P.C., Van Marum, D. and Jasper, C.G.G. (1982) Anal. Biochem. 127, 93–99
18 Gross, E.L. and Prasher, S.H. (1974) Arch. Biochem. Biophys. 164, 460–468
19 Albert, A. (1966) The Acridines – Their Preparation, Physical, Chemical and Biological Propeorties and Uses, Edward Arnold, London
20 Torres-Pereira, J., Mehlhorn, R., Keith, A.D. and Packer, L. (1974) Arch. Biochem. Biophys. 160, 90–99
21 Gross, E.L., Dilley, R.A. and San Pietro, A. (1969) Arch. Biochem. Biophys. 134, 450–462
22 Carmeli, C. and Avron, M. (1967) Eur. J. Biochem.. 2, 318–326
23 Dell'Antone, P., Colonna, R. and Azzone, G.F. (1972) Eur. J. Biochem. 24, 553–576
24 Levshin, L.V. (1955) Soviet Physics – JETP 1, 235–253
25 Haugen, G.R. and Melhuish, W.H. (1964) Trans. Faraday Soc. 60, 386–394
26 Mysels, K.J. (1959) Introduction to Colloid Chemistry, Interscience Publishers Inc., New York
27 Einolf, C.W. and Carstensen, E.L. (1967) Biochim. Biophys. Acta 148, 506–516
28 Brinton, C.C. and Lauffer, M.A. (1959) in Electrophoresis (Bier, M., ed.), pp. 427–492, Academic Press, New York
29 Aveyard, R. and Haydon, D.A. (1973) An Introduction to the Principles of Surface Chemistry, University Press, Cambridge, MA
30 Haydon, D.A. (1961) Biochim. Biophys. Acta 50, 450–457
31 McLaughlin, S., Szabo, G. and Eisenman, G. (1971) J. Gen. Physiol. 58, 667–687
32 McLaughlin, S. (1977) Curr. Top. Membr. Transp. 9, 71–144
33 Overbeek, J.T.G. and Lyklema, J. (1959) in Electrophoresis (Bier, M., ed.) pp. 1–33, Academic Press, New York
34 Friedenberg, R.M. (1967) The Electrostatics of Biological Cell Membranes, North-Holland, Amsterdam
35 Wunderlich, R.W. (1982) J. Colloid Interfac. Sci. 88, 385–397
36 Huang, C.-S., Kopacz, S.J. and Lee, C.P. (1983) Biochim. Biophys. Acta 722, 107–115
37 Van Walraven, H.S., Lubberding, H.J., Marvin, H.J.P. and Kraayenhof, R. (1983) Eur. J. Biochem. 137, 101–106