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FURTHER STUDIES OF THE THYLAKOID MEMBRANE SURFACE CHARGES BY PARTICLE ELECTROPHORESIS

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Summary

1. Above pH 4.3 the outer surface of thylakoid membranes isolated from pea chloroplasts is negatively charged but below this value it carries an excess of positive charge.

2. Previously the excess negative charge has been attributed to the carboxyl groups of glutamic and aspartic acid residues (Nakatani, H.Y., Barber, J. and Forrester, J.A. (1978), Biochim. Biophys. Acta 504, 215—225) and in this paper it is argued from experiments involving treatments with 1,2-cyclohexanedione that the positive charges are partly due to the guanidino group of arginine.

3. The electrophoretic mobility of granal (enriched in chlorophyll *b* and PS II activity) and stromal (enriched in PS I activity) lamellae isolated by the French Press technique were found to be the same.

4. Treatment of the pea thylakoids with trypsin or pronase, sufficient to inhibit the salt induced chlorophyll fluorescence changes, increased their electrophoretic mobility indicating that additional negative charges had been exposed at the surface.

5. Polylysine treatment also inhibited the salt induced chlorophyll fluorescence changes but unlike trypsin and pronase, decreased the net negative charge on the surface.

6. The isoelectric point defined as the pH which gave zero electrophoretic mobility (about 4.3) was independent of the nature of the cations in the suspending medium (monovalent vs. divalent).

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Abbreviations: PS II, Photosystem II; PS I, Photosystem I; Chl, chlorophyll; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EDTA, ethylenediaminetetra-acetate; DNFB, dinitrofluorobenzene; *P*-700, reaction centre chlorophyll of PSI; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

Introduction

In previous papers we have stressed the importance of the surface electrical properties of the thylakoid membrane in controlling a number of photosynthetic phenomena [1—6]. It is becoming increasingly clear that phenomena such as chlorophyll fluorescence [2], thylakoid stacking [1,7], electron transport [1,8,9] electrochromic absorption change at 515 nm [10,11], ionic distribution within the intact chloroplast [4,12], membrane conformational changes [6], action of fluorescent probes [13—15] and pH indicating dyes [16], are influenced by the surface electrical charges on the thylakoids. Several workers have demonstrated that at physiological pH the outer surface of this membrane system carries a net negative charge [3,17,18] and estimates of its average density are in the region of 2.5 $\mu C/cm^2$ or one electronic charge per 625 $Å^2$ [1,2].

Particle electrophoresis studies have shown that the isoelectric point occurs at pH 4.3 [3,17] and that above this pH value the thylakoids carry a net negative charge. Nakatani et al. [3] investigated the origin of the surface charges and found no evidence for the involvement of sugar groups of the galactolipids or for basic groups having p*K* values in the range of 6 to 10. They did, however, find that the surface negative charges could be neutralized by treating the membranes with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and glycine methyl ester and concluded that they were due to the carboxyl groups of aspartic and glutamic acid residues exposed at the membrane surface. Although these chloroplast membranes carry a net positive charge below 4.3 the origin of these charges was not determined.

In this paper we report the results of further particle electrophoresis studies designed to investigate in more detail the nature and distribution of electrical charge on the outer surface of the thylakoid membrane.

Materials and Methods

Isolated thylakoid membranes were obtained from pea seedlings (Feltham First) as previously described [19]. However, the broken chloroplasts were resuspended in the wash buffer with addition of 1 mM each of KCl and $MgCl_2$ to preserve activity. Also for making French press particles, the leaf material was increased to approx. 100 g of leaves per 100 ml of grinding buffer.

(i) French press particles

French press particles were prepared by a method similar to that described by Sane et al. [20]. Isolated chloroplasts were resuspended in 10 ml of 0.6 M sorbitol, 50 mM Hepes-KOH at pH 7.6, 2 mM Na^+-EDTA and 2.5 mM $MgCl_2$ at a chlorophyll concentration of approx. 1 mg chlorophyll per ml. After passage throught the chilled French press at a pressure of 4000 to 5000 lbs/in^2, the suspension was increased to approx. 40 ml with the resuspension buffer above. The granal fraction (P_g) was collected as the pellet obtained from an initial centrifugation at 10 000 $\times g$ for 10 min in an MSE 18. The supernatant was respun at 38 000 $\times g$ for 30 min in the MSE 18 and the supernatant of this spin was respun at 190 000 $\times g$ for 2 h in an MSE 65 to yield the stromal frac-

tion (P_s). The pellets were resuspended in small volumes of 0.33 M sorbitol-Tris at pH 7.6.

A modification of the above procedure was carried out to obtain unstacked granal particles (P_{us}). The granal fraction was washed, resuspended and incubated for 15 min in an unstacking medium consisting of 0.1 M sorbitol-5 mM Tris at pH 7.6 before being subjected once again to French press treatment. The resulting suspension was diluted with 0.1 M sorbitol-5 mM Tris (pH 7.6) medium and then centrifuged at 38 000 × *g* for 30 min as above and the supernatant from this spin subjected to 190 000 × *g* for 2 h in the MSE 65 to yield the P_{us} fraction.

(ii) Chemical treatment of the membrane surface

(a) Trypsin treatment. Broken chloroplasts were diluted to 50 μg Chl/ml in 20 mM Tricine-KOH at pH 7.0 and incubated with trypsin (Sigma, type 1, 25 μg/ml 10 000 BAEE * units/mg) of sufficient duration to inhibit the cation induced increase in room-temperature chlorophyll fluorescence (usually about 5 min). Trypsin inhibitor (Sigma, type IS) was added at the end of the incubation period both in the treated sample and in the control. For fluorescence measurements the chlorophyll concentration was reduced 10-fold with 0.33 M sorbitol, 10 mM Hepes-Tris, pH 7.6 containing 10 μM DCMU and were carried out as previously described [21], ±10 mM $MgCl_2$.

(b) Pronase treatment. Broken chloroplasts were diluted at room temperature to 50 μg Chl/ml in 0.1 M sorbitol, 20 mM Tris-HCl at pH 7.6 containing a protease concentration of 50 μg/ml (Sigma Type V, 0.7 to 1.0 units/mg). As before, the incubation time was based on the minimal time needed to inhibit the cation induced increase in chlorophyll fluorescence and measured as above.

(c) 1,2-Cyclohexanedione treatment. Broken chloroplasts were diluted to 100 μg Chl/ml in buffer, containing 50 mM 1,2-cyclohexanedione (Kock Light Labs. Ltd.) in the presence of 0.1 M sodium borate at pH 8 to 9.5, incubated for 24 h at 40°C and then placed in an ice bath.

(iii) Particle electrophoresis

Particle electrophoresis measurements were conducted as previously described [3] except that either one or a combination of the following buffers were used at a concentration of about 10 mM; citrate, maleate and Tris.

(iv) Chlorophyll fluorescence

Cation induced chlorophyll fluorescence changes were measured at room temperature as previously described [2] and low temperature emission spectra were recorded using a Perkin-Elmer MPF44A. Chlorophyll levels were determined by the method of Arnon [21].

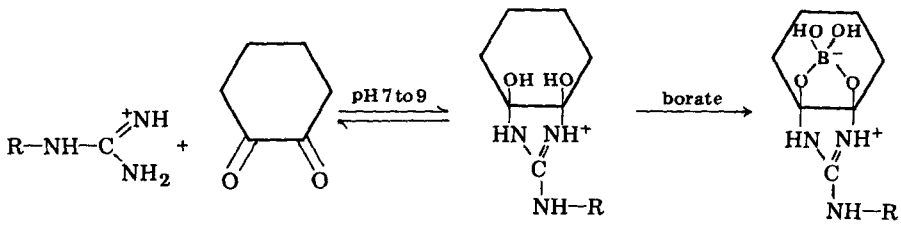
Results

(i) Nature of the surface positive charge

In our earlier electrophoretic mobility studies [3] we were unable to affect

* BAEE, *N*-benzoyl-L-arginine ethyl ester.

the pH titration of the positive charges existing on the thylakoid surface by treatment with the Sanger reagent, dinitrofluorobenzene (DNFB). Further attempts have therefore been made to determine the nature of the positive charge on the outer thylakoid surface using specific modifiers for lysine, maleic anhydride and arginine, cyclohexanedione (Refs. 22 and 23). Initial treatments with these two reagents up to two hours were unsuccessful and prolonged treatments were therefore undertaken. After 24 h, the maleic anhydride treatment was still unsuccessful (not shown), but the cyclohexanedione treatment had significantly modified the pH sensitivity of the electrophoretic mobility. The cyclohexanedione treatment had to be carried out in the presence of borate, so that the complex formed would be electroneutral:



As Fig. 1 shows, after this treatment the membranes carried a net negative charge which was neutralized at pH 3.0 (aggregation prevented meaningful determinations at pH values <3.0). The control membranes, treated only with borate, were positively charged below pH 4.3 as shown earlier [3] but the mobilities at higher pH values were greater than observed with normal thylakoid membranes, probably reflecting the formation of a borate complex with

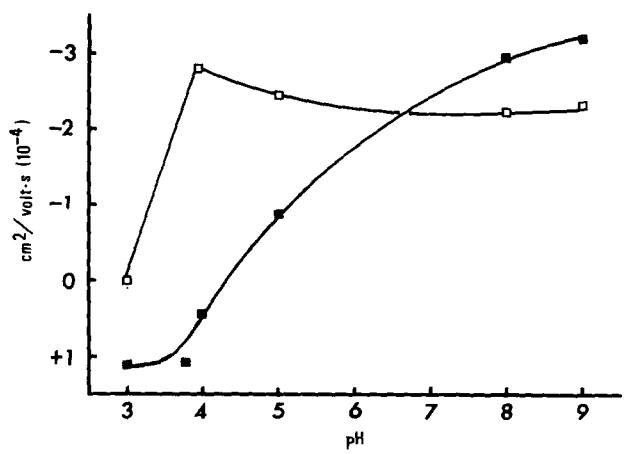


Fig. 1. pH electrophoretic mobility profile for pea chloroplast thylakoids (approx. 25 μg Chl/ml) plus (□) and minus (■) cyclohexanedione treatment, in presence of borate see Materials and Methods. The thylakoids were suspended in 0.33 M sorbitol and 20 mM KCl and pH was adjusted with the following buffers: Tris base, maleate, citrate and carbonate, total approx. 10 mM. All measurements were conducted in a Zeiss cytopherometer at 20°C with voltages adjusted to maintain measurement times between 7 and 10 s per particle. The mean of at least 20 particles is represented by each point.

the carbohydrate moieties of the membrane. Overall the results indicate that the positive charges on the thylakoid outer surface are mainly due to the guanidino group of arginine. The apparent shift in the isoelectric point (from pH 4.3 to 3.0) could be entirely due to the removal of the positive charges or could reflect the exposure of some charged groups of the membrane phospholipids. The latter possibility seems unlikely since further treatment with phospholipase C did not change the characteristic pH profile of the electrophoretic mobility of the cyclohexanedione treated membranes.

(ii) Electrophoretic mobility of stromal and granal lamellae

The electrophoretic measurements reported above and in our earlier paper [3] were conducted on whole isolated thylakoid membranes so that the information obtained reflects the surface properties of this complete membrane system as detected at the plane of shear. However the thylakoids can exist both as granal and stromal lamellae and we have therefore attempted to carry out measurements on these two types of membranes. In order to minimize any chemical or electrical modification to the membrane surfaces which might occur with detergent treatment, stromal and granal particles were isolated using a French press after the method of Sane et al. [20]. Table I shows the properties of the particles obtained by this procedure and it is clear that a satisfactory separation was obtained between stromal (P_s) and granal (P_g) lamellae as indicated by their chlorophyll *a*/chlorophyll *b* ratios, *P*-700/chlorophyll ratio and low temperature fluorescence emission properties. Moreover, the P_s fraction could not support light induced electron flow from water to ferricyanide or to methyl viologen. Only in the presence of reduced 2,6-dichlorophenol indophenol ($DPIPH_2$) was electron flow to methyl viologen possible and the rate was 1050 μequiv./mg Chl per h. In contrast, P_g was found to support a light induced rate of 270 μequiv./mg Chl per h when either ferricyanide or methyl viologen was present. Although the P_g and P_s preparations had different optical and photochemical properties their electrophoretic mobilities were essentially the same and comparable with the mobilities of the P_{us} fraction, grana which were unstacked (see Table I).

(iii) Effect of trypsin, pronase and poly(lysine)

Trypsin has been used by a number of workers [24—32] to study how

TABLE I

F_{685} and F_{730} are the intensities of chlorophyll fluorescence measured at 685 nm and 730 nm at liquid N_2 temperature. *P*-700 was measured chemically by ferricyanide oxidised dithionite reduced absorption spectra using a Perkin-Elmer 554.

Preparation *	Electrophoretic mobility ** $(cm^2/V/s) \times 10^4$	Chl *a*/Chl *b*	*P*-700/Chl	F_{730}/F_{685}
P_g	−1.97 ± 0.12	2.72	1:437	0.51
P_s	−1.96 ± 0.09	7.54	1:203	1.34
P_{us}	−1.94 ± 0.06	2.78	1:426	1.22

* See Materials and Methods.

** ± S.E. (mean of 40 or more measurements).

modifications or removal of surface peptides can affect photosynthetic processes. Amongst other explanations, it has been argued that this enzyme attacks the chlorophyll *a*/chlorophyll *b* pigment-protein complex so as to impede grana formation and salt induced chlorophyll fluorescence changes [31,32]. To understand the action of trypsin further we have measured the electrophoretic mobility of thylakoid membranes treated with this enzyme. For comparison we have also treated the membranes with pronase and poly-(lysine).

Hydrolytic action of trypsin depends upon the length of treatment and the pH as well as on the substrate enzyme ratio. We conducted treatments according to the details given in Materials and Methods until the salt induced component of chlorophyll fluorescence was inhibited (see Table II). As can be seen in Table II this mild tryptic digestion significantly inhibited electron flow from H_2O to methyl viologen (from PS II to PS I) without dramatically changing the electron flow in PS I or PS II monitored separately. (In fact there appears to be enhanced ability for $DPIPH_2$ to donate electrons to PS I.) Gerola et al. [29] also used trypsin treatment to inhibit the salt induced chlorophyll fluorescence change and concluded that the membrane protein which was affected was likely to be involved in divalent cation 'binding'. However as shown in Table II, this trypsin treatment does not remove surface negative charges, on the contrary, the surface becomes more negative as observed by an increase in electrophoretic mobility. Trypsin specifically attacks lysine and arginine so as to bring about an increase in both the number of N-terminal and C-terminal groups which in itself would not lead to an increase in the negative surface charge density. It would therefore seem that the trypsin treatment brings about the exposure of more negative charges at the membrane sur-

TABLE II

ELECTRON TRANSPORT AND ELECTROPHORETIC PROPERTIES OF THYLAKOID MEMBRANES SUBJECTED TO VARIOUS TREATMENTS

The uncoupler used was 10^{-6} M nigericin. Electrophoretic mobilities are given with S.E. determined by 20 or more measurements. The percentage increase in chlorophyll fluorescence (ΔF_s) was induced by adding 10 mM $MgCl_2$ in the presence of 10^{-6} M DCMU. Poly(lysine) (mol.wt. approx. 2000 with 9 degrees of polymerization) was used at 50 μg/ml. MV, methyl viologen; $DPIPH_2$, reduced 2,6-dichlorophenol indophenol.

Condition	Uncoupled electron transport (μequiv./mg Chl/h)		ΔF_s	Electrophoretic mobility ($cm^2/V/s$) × 10^4
1. Control	$H_2O \rightarrow MV$ (PS II + PS I)	565		
	$H_2O \rightarrow K_3Fe(CN)_6$ (PS II)	732	90	−1.30 ± 0.06
	$DPIPH_2 \rightarrow MV$ (PS I)	1368		
2. Trypsin	$H_2O \rightarrow MV$	52		
	$H_2O \rightarrow K_3Fe(CN)_6$	312	11	−1.60 ± 0.08
	$DPIPH_2 \rightarrow MV$	2587		
3. Pronase	$H_2O \rightarrow MV$	24		
	$H_2O \rightarrow K_3Fe(CN)_6$	223	15	−1.54 ± 0.1
	$DPIPH_2 \rightarrow MV$	1085		
4. Poly(lysine)	$H_2O \rightarrow MV$	30		
	$H_2O \rightarrow K_3Fe(CN)_6$	387	2	−0.98 ± 0.08
	$DPIPH_2 \rightarrow MV$	966		

face. This result contradicts the work of Mercer et al. [17], where Nitella chloroplasts were incubated with 1% trypsin for 1 h and no increase in electrophoretic mobility was observed.

If trypsin treatment removes peptides so as to expose more negative charges on the membrane surface then the action of pronase may be similar. Short incubation treatments with this non-specific endopeptidase sufficient to inhibit the salt induced chlorophyll fluorescence, did indeed increase the negativity of the thylakoid membrane surface (see Table II). Like trypsin, pronase inhibited electron flow between PS II and PS I but had less effect on PS II and PS I monitored separately (see Table II). With both proteolytic enzymes, PS II was more sensitive to the treatments than PS I.

To compare with the enzymic treatments above, thylakoids were subjected to poly(lysine) treatment [33]. As Table II shows this large multivalent cation decreased the electrophoretic mobility but like the two enzymes tested, significantly inhibited electron flow between PS II and PS I and partially inhibited PS II and PS I separately. Also, like the enzymic treatments, poly-(lysine) inhibited the salt induced increase in chlorophyll fluorescence.

(iv) Effect of salts on the pH sensitivity of electrophoretic mobility

In our previous paper [3] we had confirmed the earlier observations of Mercer et al. [17] that thylakoids have zero electrophoretic mobility in the region of pH 4.3 to 4.5. Also, it was shown that the addition of salts decreased the electrophoretic mobility at neutral pH with divalent cations being far more effective than monovalent cations as expected for the reduction of the surface potential (electrostatic screening) according to the Gouy-Chapman theory [3]. This differential effect of mono- and di-valent cations is also observed for the salt induced chlorophyll fluorescence increase [1] and it has been suggested that cations of different valencies affect specific sites differently [34,35]. If this were the case then it could result in a shift of the isoelectric point when

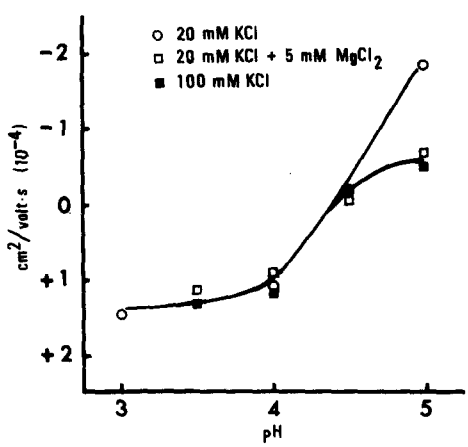


Fig. 2. pH-electrophoretic mobility profile for pea chloroplast thylakoids (approx. 25 μg Chl/ml) near the isoelectric point in the presence of additional salt as $MgCl_2$ or KCl as shown. The thylakoids were suspended in 0.33 M sorbitol and 20 mM KCl ± additional salt and pH adjusted with citrate-Tris, approx. 10 mM total. Other conditions as for Fig. 1.

measuring electrophoretic mobility in media containing either mono- or divalent cations. In fact as Fig. 2 shows, no such shift in the isoelectric point was observed when measurements were made either in 5 mM Mg^{2+} or 100 mM K^{+} both of which give about the same degree of electrostatic screening.

Discussion

At pH values above 4.3 the outer surface of the thylakoid membrane carries a net negative charge. This negative charge has been attributed mainly to the presence of surface proteins [3,17,33,36] rather than to the lipid or carbohydrate components of the membrane. Evidence for this comes from a number of observations [35,37] and in particular from the sensitivity of electrophoretic mobility to pH [3,17] and the action of various chemical agents including water soluble carbodiimides [3,33,37]. It has been concluded that the majority of the negative charge is conferred by the acidic amino acids, aspartic and glutamic [3]. Below pH 4.3 the thylakoids carry a net positive charge, the origin of which according to our studies, seems to be the guanidino group of arginine. Thus a simple picture emerges of a membrane for which the majority of the net surface charge can be attributed to three amino acid residues exposed on protein surfaces. Apparently the other amino acids and charged lipids to be found in this membrane system [38,39] do not significantly contribute to the surface charge density or if present do not readily interact with the chemical treatments tried.

There are many observations which indicate that the negative charges on the thylakoid membrane surface in some way control the cation induced chlorophyll fluorescence increase and thylakoid stacking [1]. We have observed that the inhibition of fluorescence, and presumably thylakoid stacking [31,32], by trypsin results in an increase in the surface charge density. There is evidence that the site of action of trypsin giving rise to these inhibitions is the chlorophyll *a*/chlorophyll *b* pigment-protein complex [31,32]. Thus our measurements indicate that trypsin and pronase treatments may increase the density of negative charges on the exposed portion of this complex. Thus any explanation for the inhibition of the salt induced stacking and associated chlorophyll fluorescence changes due to trypsin treatment must consider this change in the surface electrical properties. The simplest explanation is that the fluorescence and stacking phenomena are prevented by the additional increase in coulombic repulsion between surfaces which can no longer be overcome by the addition of screening cations [7,40]. Clearly the action of poly(lysine) is different, it reduces the surface charge density and reduces coulombic repulsion between surfaces. Thus it is not surprising that low molecular weight poly(lysine) is known to induce thylakoid stacking [33]. However, this type of stacking is different to that induced by the addition of cations like K^{+}, Mg^{2+}, etc., since there is no concomitant increase in chlorophyll fluorescence. Thus it seems likely that the mechanism of poly(lysine) stacking is different to that induced by adding salts. The explanation for the difference almost certainly lies in the fact that poly-lysine 'neutralizes' some of the exposed charges and reduces the magnitude of the surface charge density, while cations which bring about electrostatic sreening do not [1,3].

The question whether the various types of chloroplast lamellae, granal stromal and unstacked, carry different densities of charge may still not be resolved. It could be that the similarity in surface charge density observed in the above work is due to modification of the membranes from their normal in vivo state during preparation of the vesicles by the fractionation process adopted. Indeed, from their measurements of isoelectric points using a phase separation technique, Akerlund et al. [41] found evidence for reorganisation of surface charges after mechanical disintegration of spinach thylakoids.

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