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SURFACE CHARGE ASYMMETRY AND A SPECIFIC CALCIUM ION EFFECT IN CHLOROPLAST PHOTOSYSTEM II

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Summary

We have used the decay kinetics of Signal IIf in Tris-washed chloroplasts as a direct probe to reactions on the oxidizing side of Photosystem II. A study of the salt concentration dependence of the rate of reduction of Z^+ by the ascorbate monoanion has been interpreted by using the Gouy-Chapman diffuse double layer model and allows the calculation of an inner membrane surface charge density of -3.4 ± 0.3 $\mu C \cdot cm^{-2}$ at pH = 8.0 in the vicinity of Photosystem II. We have also measured the outer membrane surface charge density at this pH in Tris- and sucrose-washed chloroplasts by monitoring the rate of potassium ferricyanide oxidation of Q^-, and arrive at values of -2.2 ± 0.3 $\mu C \cdot cm^{-2}$ and -2.1 $\mu C \cdot cm^{-2}$, respectively. From these experiments we conclude that in dark-adapted chloroplasts at pH 8.0 there exists a transmembrane electric field in the vicinity of Photosystem II which arises from this surface charge asymmetry. In the presence of 10 mM monovalent salts, the transmembrane potential difference is of the order of 20 mV, corresponding to a field of $4 \cdot 10^4$ $V \cdot cm^{-1}$ (negative inside) for a 50 Å membrane. It is both smaller in magnitude and in the opposite direction compared to the photoinduced transmembrane field which gives rise to the 515 nm absorption change. We have also found non-double layer Ca^{2+} effects on the decay kinetics of Signal IIf with both charged (ascorbate monoanion) and neutral (diphenylcarbazide) donors. These results suggest a change in the environment of Z from lipophilic to hydrophilic upon specific binding of Ca^{2+}.

Abbreviations: Tricine, *N*-tris-(hydroxymethyl)-methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Introduction

We have recently completed an extensive analysis of the decay kinetics of EPR Signal IIf [1]. The photo-induced free radical giving rise to this signal is observed in chloroplasts whose oxygen evolving capability has been inhibited by one of a variety of methods, including incubation in alkaline Tris buffer (Tris-washing) [2]. The signal arises from the oxidation of an intermediate, Z, which is located near the inner surface of the thylakoid membrane and is involved in the electron transfer chain on the oxidizing side of Photosystem II. We have demonstrated that the decay kinetics of Z^{+} vary with the electron donor used and, in some cases, with both the pH and the ionic strength of the suspension medium. These last two factors are particularly important when ascorbic acid is used as the donor to Photosystem II. Both the neutral ascorbic acid (H_2Asc) and the ascorbate mono-anion ($HAsc^-$) serve as donors to Z^{+}. At high pH, however, reduction proceeds almost exclusively by the anion. At these pH values acidic surface groups are deprotonated [3] and the resulting negative surface charge influences the rate of Z^{+} reduction by $HAsc^-$ through a double layer effect: at low ionic strength Z^{+} reduction proceeds relatively slowly and the rate increases as the ionic strength is increased [1]. We have quantified this ionic strength dependence and, by using the basic Gouy-Chapman double layer equations, have calculated the surface charge density of the inner membrane surface near Z.

Both experimental results and theoretical treatments [4—6] have appeared in the literature recently which suggest that the charge on the thylakoid inner membrane surface may be different to that of the outer surface. Recently reported techniques for measuring the outer surface charge density in the vicinity of Photosystem II [7—9] enable us to compare directly the surface charge densities on either side of the membrane in the vicinity of Photosystem II. To avoid ambiguities caused by comparisons of quantitative results between different laboratories, we have carried out both EPR and fluorescence experiments in our laboratory. From the results of these measurements, we have found that there is indeed surface charge asymmetry in the vicinity of Photosystem II. We have also observed specific effects of Ca^{2+} on the oxidizing side of Photosystem II and summarize here our initial observations.

Materials and Methods

EPR experiments

All EPR experiments were performed with Tris-washed chloroplasts prepared from market spinach as described previously [1]. The chloroplasts were suspended (3 to 5 mg chlorophyll per ml) [10] in 0.4 M sucrose, 10 mM NaCl and 50 mM Tricine (pH = 8.0) containing 10^{-4} M EDTA [11], 20 $\mu g \cdot ml^{-1}$ spinach ferredoxin and $5 \cdot 10^{-4}$ M NADP (Sigma) as an electron acceptor system and 2 mM ascorbic acid as a donor system. The acceptor system was present in order to insure a constant rate of electron flow under signal averaging conditions. All reagents were purchased from standard commercial sources and used as received.

The light source was a xenon flash lamp (ILC, Sunnyvale, CA) fired by a dis-

charge circuit constructed in the laboratory. The pulse duration was 14 μs (full width at 1/3 maximum); the electrical discharge energy was typically 20 J. The flashes were of saturating intensity as determined by a saturation curve for EPR Signal IIf formation. In signal-averaged experiments a flash repetition rate of one every 2 s was used. All experiments were performed at room temperature with a Varian E-4 spectrometer fitted with a TM_{110} mode cavity (Varian E-238) and a Scanlon EPR flat cell (S-814). Data were collected and stored in a Nicolet model 1074 signal averager with a model SD 72-2A analog-to-digital converter and a model SW-71A time base in the 1074 main frame. The number of passes accumulated and the experimental protocol are noted in the figure legends.

Fluorescence experiments

Fluorescence experiments were performed with either sucrose- or Tris-washed chloroplasts as noted. The chloroplasts were suspended (25 to 40 μg chlorophyll per ml) in 0.4 M sucrose, 10 mM NaCl and 50 mM Tricine (pH = 8.0) containing 1 μM DCMU. $K_3Fe(CN)_6$ (0.5 mM) was added as noted. The light source was a 200 W tungsten lamp (General Electrical model EJL) operated at 5 V (d.c.) whose output was passed through two glass filters (Corning CS4-96) and a short pass filter (Baird-Atomic, 600 nm) in order to select blue exciting light. The excitation intensity was $5 \cdot 10^3$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1}. The fluorescence, measured at a right angle to the excitation beam, was detected by a photomultiplier (EMI 9558QB) protected by two red filters (Corning CS2-58). A camera shutter was used to begin and end the illumination of the sample. The signal from the photomultiplier was recorded on a strip chart recorder. All measurements were made at room temperature.

Aliquots of the dark adapted chloroplasts were added to the reaction solution, including DCMU and $Fe(CN)_6^{3-}$, and allowed to incubate for 1 min in the dark. The sample was then illuminated for 90 s to insure complete formation of Q^-. The sample was subsequently re-illuminated after variable (5 s—80 s) dark delay times and the state of Q calculated from the equation [12]:

$$\frac{[Q^-]}{[Q_{tot}]} = \frac{F_t - F_0}{F_\infty - F_0} \tag{1}$$

where F_0 is the initial 'dead-fluorescence' level when the dark adapted sample is first illuminated. F_t is the initial fluorescence level upon re-illumination. F_∞ is the fluorescence level, measured in the presence of DCMU but absence of $Fe(CN)_6^{3-}$, at the end of a 90 s illumination period.

Results

Surface charge asymmetry in the vicinity of Photosystem II

The relation between the membrane surface charge density (σ) and the rate constant of an electron transfer reaction between a membrane bound component (Z,Q) and a charged redox reagent in the suspension medium ($HAsc^-$, $Fe(CN)_6^{3-}$) has been described as follows [13]:

$$\ln(k/\gamma) = \ln k^0 - \frac{Z}{Z_i}\left(\frac{2\pi}{RT\epsilon}\right)^{1/2} C_i^{-1/2}\sigma \tag{2}$$

This is the linearized form of the basic Gouy-Chapman equation and is valid for monovalent ($Z_i = 1$) symmetrical salts. For this reason, we have used only the KCl concentration dependence in calculating the surface charge density values from the data presented below. The terms γ and Z refer to the activity coefficient and charge of the redox reagent, $HAsc^-$ for the EPR experiments and $Fe(CN)_6^{3-}$ for the fluorescence experiments. The extended Debye-Hückel equation [32] was used to calculate γ; the ion size parameters were taken from Kielland [32] and the value for $HAsc^-$ estimated to be $a = 6$. Because we are working at relatively high ionic strengths, we have also calculated γ using the Hückel modification to the extended equation which is valid at high (a few molar) ionic strengths [32]. The difference in the calculated values of γ by these two methods leads to a difference in σ, the net surface charge density, which falls within our range of experimental error ($\pm 0.3\ \mu C \cdot cm^{-2}$). The use of relatively high ionic strength is dictated by the form of Eqn. 2 which is only valid if the surface potential is less than about 50 mV [13]. The terms Z_i and C_i refer to the charge and concentration of the salt in the medium. All other terms assume their standard values.

The salt dependence of EPR Signal IIf is apparent in Fig. 1, where we show a series of experimental traces which demonstrate the salt-induced enhancement of the decay kinetics. Rate constants are calculated from these data and have been plotted in Fig. 2 (left) as $(Z_i/Z)\ln(k/\gamma)$ vs. $C_i^{-1/2}$. As indicated by Eqn. 2, the slope is proportional to the surface charge density. The EPR data indicate a value for σ of $-3.4 \pm 0.3\ \mu C \cdot cm^{-2}$ for the inner surface of the thylakoid

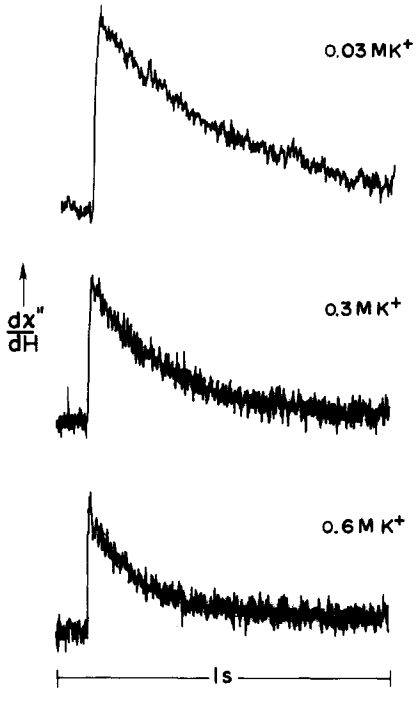

Fig. 1. Effect of [KCl] on reduction of Z^+ by $HAsc^-$ (2 mM) in Tris-washed chloroplasts, pH = 8.0, instrument time constant = 3 ms, 75 scans averaged.

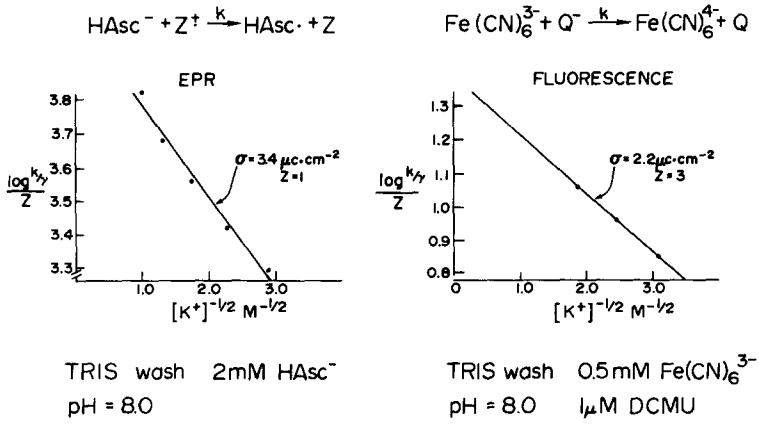

Fig. 2. Plot of equation 2 with $Z_i = 1$, (see text) for EPR experiments (left) on the rate of Z^+ reduction by ascorbate and fluorescence experiments (right) on the rate of Q^- oxidation by ferricyanide with Tris-washed chloroplasts, pH = 8.0. The salt concentration was adjusted by addition of the monovalent salt, KCl, in both series of experiments.

membrane in the vicinity of Z at pH 8.0. Fig. 2 (right) is a similar plot of the results of the fluorescence induction experiments on Tris-washed chloroplasts. The fluorescence data show an outer surface charge density near Q of $-2.2 \pm 0.3\ \mu C \cdot cm^{-2}$, also at pH = 8.0. We have carried out analogous fluorescence experiments with sucrose-washed, oxygen-evolving chloroplasts as a control. The surface charge density near Q calculated from these experiments is $-2.1 \pm 0.3\ \mu C \cdot cm^{-2}$. Therefore, under dark-adapted conditions, a surface charge asymmetry exists across the thylakoid membrane in the vicinity of Photosystem II.

Attempts to measure $\Delta\sigma$ at lower pH values have been complicated by the mechanism of electron donation to Z^+ by ascorbic acid. At pH values below 8.0, reduction by the neutral acid, H_2Asc ($pK_a = 4.1$), becomes increasingly important and the surface charge effect is lost [1].

Detergent effects on inner and outer surface charge densities

To provide assurance that the charge densities we have measured by EPR and fluorescence techniques do indeed correspond to those at the inner and outer membrane surfaces, respectively, we have explored the effects of cationic detergents on the rates of the two reactions. The detergents modify the surface potential not through a double layer effect but through specific adsorption. Itoh [8,9] has postulated that the hydrocarbon tails adsorb to the membrane and serve to anchor the positively charged head groups to the surface. He has demonstrated that the resulting decrease in surface charge leads to faster rates of oxidation of Q^- by $Fe(CN)_6^{3-}$. In contrast to this outer surface behavior, however, we have reported [1] that the addition of a cationic detergent, nonyltrimethylammonium bromide, up to its critical micellar concentration, ($1.4 \cdot 10^{-1}$ M at $T = 30°C$) shows no effect on the reduction kinetics of Z^+ by $HAsc^-$. We have concluded that this reaction is mediated by the inner membrane surface potential which remains unaffected, at least at short times, by the addition of cationic detergents.

We have now studied the effect of nonyltrimethylammonium bromide on the kinetics of Q^- oxidation by $Fe(CN)_6^{3-}$ in Tris-washed chloroplasts. In agreement with the results of Itoh on sucrose-washed chloroplasts in the presence of cetyltrimethylammonium bromide, we see an enhancement in the rate of reaction at detergent concentrations below those required to perturb the double layer through nonspecific cationic effects (results not shown). Thus, only one of the two surface charge densities that we have measured is susceptible to alteration by detergent adsorption, consistent with the hypothesis that one reflects outer, and the other inner, surface effects.

Ca^{2+} effects

In both EPR and fluorescence experiments we have used a variety of mono- (K^+, Na^+) and divalent (Mg^{2+}, Ca^{2+}) cations to probe the surface charge properties of the thylakoid membrane. In general, these species behaved in a manner analogous to that exhibited by K^+ in Fig. 1 and increased the observed reaction rates through a double layer effect as the ionic strength was increased. We have, however, observed anomalous effects with Ca^{2+} salts, as shown in Fig. 3. On the left are experimental EPR traces of Signal IIf decay in the presence of ascorbic acid (2 mM, pH = 8.0) and 40 mM Ca^{2+} (bottom) or 40 mM Mg^{2+} (top). Addition of Ca^{2+} leads to a significant enhancement in the decay rate. This effect saturates at low concentrations (<10 mM) (Fig. 4) and is specific to Ca^{2+}. Above this concentration Ca^{2+} exhibits a normal, divalent cation effect as seen by the parallel traces for Ca^{2+} and Mg^{2+} at higher concentrations. A trivial explanation for the Ca^{2+} specific rate enhancement (complex formation beween $HAsc^-$ and Ca^{2+}) can be ruled out based on the binding constant for this reaction [14]. This effect also seems to be specific to the oxidizing side of

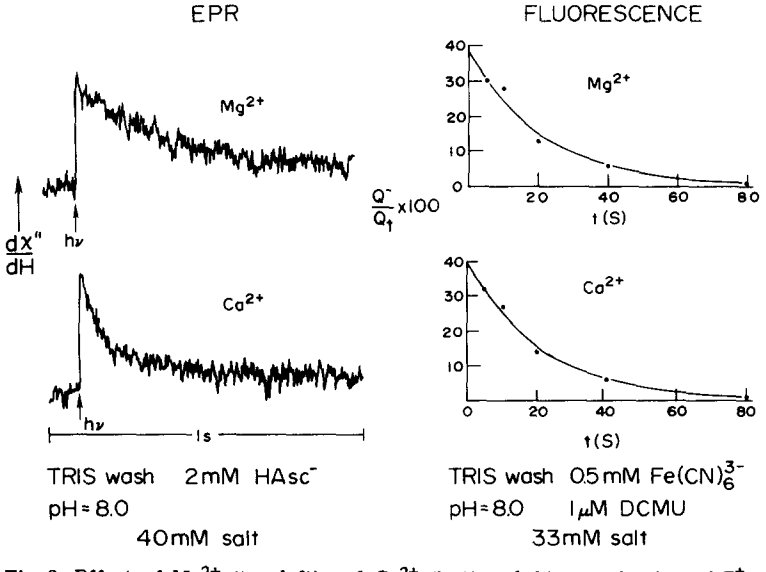

Fig. 3. Effect of Mg^{2+} (top left) and Ca^{2+} (bottom left) on reduction of Z^+ by $HAsc^-$ (2 mM) in Tris-washed chloroplasts, pH = 8.0; instrument time constant = 3 ms, 75 scans averaged. Effect of Mg^{2+} (top right) and Ca^{2+} (bottom right) on oxidation of Q^- by $Fe(CN)_6^{3-}$ (0.5 mM) in Tris-washed chloroplasts, pH = 8.0.

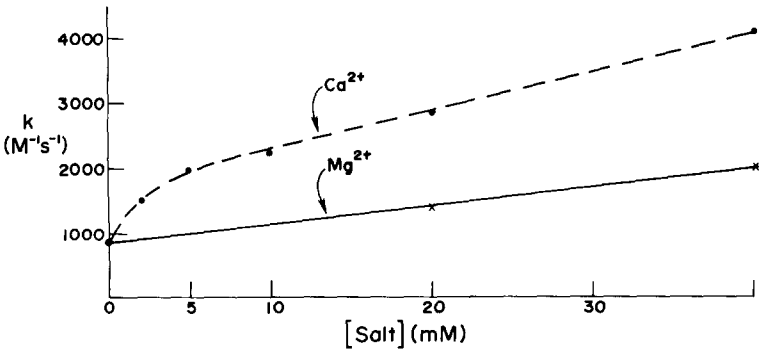

Fig. 4. Effect of Ca^{2+} and Mg^{2+} on the rate of reduction of Z^{+} by $HAsc^{-}$ (2 mM) in Tris-washed chloroplasts at pH = 8.0. A total of 75 scans were averaged for each trace, instrument time constant = 3 ms.

Photosystem II as demonstrated by the right hand portion of Fig. 3, a plot of the time course of fluorescence induction data for Tris-washed chloroplasts (pH = 8.0) with 0.5 mM $Fe(CN)_6^{3-}$ and 33 mM Ca^{2+} (bottom) or 33 mM Mg^{2+} (top). In these experiments, which probe the reducing side of Photosystem II, both Ca^{2+} and Mg^{2+} behave simply as divalent cations. Similar fluorescence results were noted by Itoh [9] in experiments with non-Tris-washed chloroplasts.

To support our hypothesis of a non-surface charge Ca^{2+} effect on the water side of Photosystem II, we have studied the effect of Ca^{2+} and Mg^{2+} on the decay kinetics of Signal IIf using diphenylcarbazide, a polar but neutral Photosystem II donor [1]. The results are shown in Fig. 5. Addition of Mg^{2+} in concentrations ranging from 10 to 40 mM in the presence of diphenylcarbazide (0.4 mM) has no effect on the rate of Signal IIf decay. The observed absence of a double layer effect is predicted for a neutral donor such as diphenylcarbazide. Similar concentrations of Ca^{2+}, however, lead to a 2.5-fold increase in the decay rate ($1.1 \cdot 10^4\ M^{-1} \cdot s^{-1}$ for no salt or Mg^{2+} to $2.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$ for Ca^{2+}), an effect which saturates at low Ca^{2+} concentrations (<10 mM). As we observed with Mg^{2+} no double layer effect is seen as the Ca^{2+} concentration is increased.

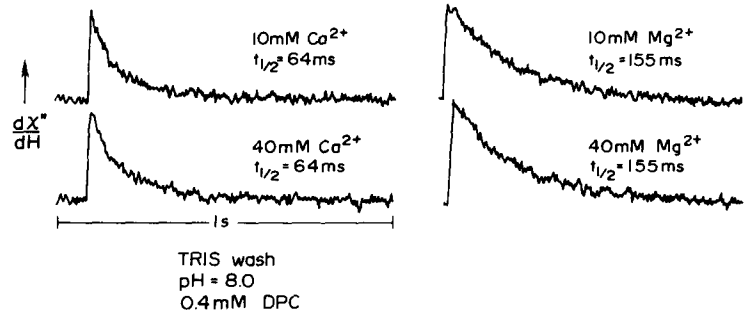

Fig. 5. Effect of Ca^{2+} (left) and Mg^{2+} (right) on the reduction of Z^{+} by diphenylcarbazide (0.4 mM) in Tris-washed chloroplasts, pH = 8.0, instrument time constant = 3 ms, 75 scans averaged.

Discussion

Ca^{2+} induced changes in Photosystem II

A number of research groups have identified Ca^{2+} specific effects in the electron transfer reactions of Photosystem II [15—20]. Our data further localize a Ca^{2+} effect on the oxidizing side of Photosystem II, between the Photosystem II reaction center and the water oxidizing enzyme. Our observations show that Ca^{2+} accelerates the reduction of Z^{+} by $HAsc^{-}$ in two ways. At low concentrations of the cation, Ca^{2+} is specific for the rate enhancement; this process saturates at a Ca^{2+} concentration of about 10 mM which is similar to the value reported in several of the studies on oxygen evolution cited above [16—18,20]. Above this threshold value, Ca^{2+}, like Mg^{2+}, exhibits a double layer effect on the reduction of Z^{+} by the anion, $HAsc^{-}$, as predicted by the Gouy-Chapman theory and quantified in Eqn. 2. For the neutral donor, diphenylcarbazide, neither Ca^{2+} nor Mg^{2+} influence the rate through a double layer effect; however the Ca^{2+} specific effect remains in operation (Fig. 5).

From Eqn. 2 we have calculated k^0 values for the $HAsc^{-}$ reaction rate in the presence of K^{+}, Mg^{2+} and Ca^{2+} salts. These values result from the extrapolation to infinite salt concentration of a plot of $[C_i]^{-1/2}$ vs. $\ln k/\gamma$ and should reflect the intrinsic rate of reaction between $HAsc^{-}$ and Z^{+} in the absence of any surface potential. We observe that the rates at infinite K^{+} and Mg^{2+} concentrations are the same ($\sim 8 \cdot 10^3\ M^{-1} \cdot s^{-1}$) within experimental error. This rate approaches that calculated for H_2Asc ($1.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$) a neutral but polar donor. We have previously [1] established a positive correlation between lipophilicity of neutral donors and their rate of reduction of Z^{+}. In the presence of Ca^{2+}, we find a k^0 for the $HAsc^{-}$ reaction of $2.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$, three times faster than that observed with K^{+} and Mg^{2+}. A possible explanation for this Ca^{2+} specific effect is that the ion binds to a membrane site in the vicinity of Z^{+} and as a result the free radical species becomes more accessible to polar donors. This interpretation is reinforced by the results of the Ca^{2+}/diphenylcarbazide experiments. The rate constant under these conditions is $2.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$ for all Ca^{2+} concentrations above 10 mM, as opposed to $1.1 \cdot 10^4\ M^{-1} \cdot s^{-1}$, the rate constant for the reaction in the absence of added cations or in the presence of Mg^{2+}. Again, the specific binding of low concentrations of Ca^{2+} leads to an enhanced reaction rate between a polar donor and Z^{+}, implicating a change in the Z^{+} environment. Experiments are now underway to explore in detail the nature and mechanism of this Photosystem II, Ca^{2+}-specific effect.

Surface charge densities

We have used two techniques, the decay of EPR Signal IIf and fluorescence induction in Tris-washed chloroplasts, to determine net surface charge densities for the inner and outer thylakoid membrane surfaces in the vicinity of Photosystem II. The values we have obtained, $-3.4 \pm 0.3\ \mu C \cdot cm^{-2}$ for the inner surface and $-2.2 \pm 0.3\ \mu C \cdot cm^{-2}$ for the outer surface, correspond to one electronic charge per 500 $Å^2$ on the inner surface and to one charge per 750 $Å^2$ on the outer surface near Photosystem II. The use of two different ionic species in these experiments does not appear to be a serious limitation judging by the similarity in values for surface charge near *P*-700 obtained by using either

ascorbate or potassium ferrocyanide as the charged species (see Table I). We have also repeated the measurements of Itoh [4—6] of the outer surface charge densities in non-Tris-washed chloroplasts and arrive at a value of -2.1 ± 0.3 $\mu C \cdot cm^{-2}$, essentially the same as that for the Tris-washed chloroplasts and in reasonable agreement with those most recently reported from other laboratories (see Table I for a summary). Under our experimental EPR conditions, one saturating flash every 2 s, we have shown that no pH gradient is built up during the course of an experiment [1]. These calculated surface charge densities are, therefore, valid at pH = 8.0, the pH of the suspension medium. We

TABLE I

SELECTED VALUES OF THYLAKOID SURFACE CHARGE DENSITIES

Conditions	Method	Membrane location, surface charge (σ)	Source
Tris-wash H_2Asc pH = 8.0	EPR	inside, PS II -3.4 ± 0.3 $\mu C \cdot cm^{-2}$	This work
Tris-wash $K_3Fe(CN)_6$ DCMU pH = 8.0	Fluorescence induction	outside, PS II -2.2 ± 0.3 $\mu C \cdot cm^{-2}$	This work
Sucrose wash $K_3Fe(CN)_6$ DCMU pH = 8.0	Fluorescence induction	outside, PS II -2.1 ± 0.3 $\mu C \cdot cm^{-2}$	This work
Sucrose wash DCMU pH = 7.4—8.0	Fluorescence changes	outside, PS II -2.5 $\mu C \cdot cm^{-2}$	[29]
Sucrose wash $K_3Fe(CN)_6$ DCMU pH = 7.6	Fluorescence induction	outside, PS II -1.3 $\mu C \cdot cm^{-2}$	[8]
Sucrose wash, phenazine-methosulfate DCMU pH = 7.6	Fluorescence induction	outside, PS II -1.47 $\mu C \cdot cm^{-2}$	[8]
Sucrose wash, sonicated H_2Asc pH = 7.8	Absorption	inside, PS I -0.86 $\mu C \cdot cm^{-2}$	[13]
Sucrose wash, sonicated $K_4Fe(CN)_6$ pH = 7.8	Absorption	inside, PS I -0.84 $\mu C \cdot cm^{-2}$	[13]
Chloroplasts pH = 7.0	Electrophoretic mobility	outside, total -1.1 $\mu C \cdot cm^{-2}$	[3]
Chloroplasts pH = 7.2	Electrophoretic mobility	outside, total dark -0.46 $\mu C \cdot cm^{-2}$ light -0.95 $\mu C \cdot cm^{-2}$	[30]
Chloroplasts 9-aminoacridine pH = 7.5.	Fluorescence quenching	outside, total -1.4 to -3.6 $\mu C \cdot cm^{-2}$	[31]

note, also, that the $\Delta\sigma$ measured in these experiments is specific to the membrane in the vicinity of Photosystem II. In other regions of the membrane, however, the net negative charge on the outer surface may be greater than that of the inner surface. This appears to be the case in the vicinity of chloroplast Photosystem I [4], although this conclusion was reached by somewhat indirect measurements.

We have also tested the effect of cationic detergents on the rates of both reactions, $Z^{\ddagger}$ reduction by $HAsc^-$ and Q^- oxidation by $Fe(CN)_6^{3-}$. As predicted by our model, the inner membrane mediated reaction between $Z^{\ddagger}$ and $HAsc^-$ was unaffected by the cationic detergent [1] which adsorbs to and selectively masks only the outer membrane surface charge. Addition of cationic detergents does enhance the rate of reaction between Q^- and $Fe(CN)_6^{3-}$ in both sucrose-washed [8,9] and, as we have now observed, Tris-washed chloroplasts.

All evidence, then, suggests a surface charge asymmetry in dark adapted (ΔpH = 0) chloroplasts. Surface potentials can be calculated from these charge densities at various salt concentrations using the equation:

$$\sigma = 0.00733C^{1/2}\sinh(^{Z}\psi_0/50.9) \qquad (3)$$

valid at 25°C [22]. If the chloroplast suspension is 10 mM in monovalent salts, this leads to a $\Delta\psi_0$ of 20 mV across the membrane or a field of $4 \cdot 10^4$ V $\cdot$ cm^{-1} for a membrane thickness of 50 Å. This dark, static field is an order of magnitude smaller [23] and in the opposite sense to the photo-induced field. The field arising from the surface charge asymmetry is not, therefore, the widely hypothesized permanent field that allows linearization of the electrochromic carotenoid absorption shift [24,25]. Rather, our results support the work of Reich and Sewe [26,27] which suggests a localized source for this linearizing static field.

The most obvious ramification of this static membrane field is its dependence on the 'history' of the chloroplast sample. We expect the field to be largest in dark adapted samples, when there is no pH gradient across the membrane. As the sample is illuminated and the inner pH decreases, we expect to see the field diminish as the groups giving rise to the inner surface charge protonate. The magnitude and even the orientation of the steady state field are difficult to predict as we have been unable to measure the effective pK_a of the inner surface with the system we are studying, i.e., using ascorbic acid as the donor. Average values for the inner (pK_a = 4.1) and outer (pK_a = 4.4) surfaces have been estimated [5] and indicate a charge asymmetry across the membrane, although these are average values for the two surfaces, not localized charge densities as we believe we have measured. We note that the transmembrane field near Photosystem II can also be modified by the ionic strength of the chloroplast suspension [4,22,25,29].

Two other aspects of the surface charge densities we have measured under dark-adapted conditions, in addition to the possible effects on proton uptake discussed in a recent paper by Duniec and Thorne [6], merit further consideration. The first is that the orientation of the static Photosystem II field, negative toward the inside, is such that charge separation in the reaction center is expected to be stabilized by the field. Assuming that the full 20 mV is experienced by the photogenerated electron and hole, we calculate that the field slows the rate of deleterious charge recombination by a factor of two. The

second point is that as a result of the stabilizing field, the surface pH in the vicinity of the oxygen-evolving enzyme is lower than the inner aqueous space bulk pH and consequently the water splitting protein requires a more positive midpoint potential in order to carry out this process efficiently. For example, in dark-adapted chloroplasts at pH 8 the inner surface pH, assuming a monovalent salt concentration of 10 mM and an inner surface charge density of -3.4 $\mu C \cdot cm^{-2}$, will be approximately 6.5. Thus a midpoint potential 90 mV more positive will be apparent for the oxygen-evolving center. We also note that under steady state conditions the prevailing inner bulk pH is near 5.0 [28] and, even in the absence of surface charge effects, the midpoint potential for the O_2/H_2O couple is approximately 930 mV. Thus in order to assure efficiency in the water-splitting process *P*-680 must have a midpoint potential more positive than 1.0 V.

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