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SURFACE POTENTIAL AND REACTION OF THE MEMBRANE-BOUND ELECTRON TRANSFER COMPONENTS

II. INTEGRITY OF THE CHLOROPLAST MEMBRANE AND REACTION OF *P*-700

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Summary

Electrostatic characteristics of the membrane surface in the vicinity of *P*-700 were estimated by analyzing the salt and detergent effects on its reaction rate with ionic reagents using the Gouy-Chapman diffuse double layer theory in various preparations of chloroplasts.

Upon disruption of thylakoid membranes by sonic treatment or by treatment with digitonin, the reaction rate markedly increased, while the estimated surface charge density became smaller.

It was concluded that the membrane surface which determines the reaction rate between *P*-700 and the ionic reagents changed as the disruption of thylakoid structure. The outer thylakoid surface had more negative charges than the inner one.

Changes in the electrical potential profile across the thylakoid membrane during the illumination were also discussed from these results.

Introduction

In the preceding paper [1], changes in the reaction rate of *P*-700 on the membrane of sonicated chloroplasts with the ionic reagents were explained by assuming electrostatic interactions between the membrane surface and the reagents according to the hypothesis using the Gouy-Chapman diffuse double layer theory [2,3].

According to the currently accepted topology of chloroplast membrane [4], the reaction site of *P*-700 molecule is assumed to be situated on the inner surface of the chloroplast thylakoids. In unbroken thylakoids, *P*-700 may react

with reagents added in the outer solution either mediated by other membrane components situated on the outer surface or directly after permeation of the reagent through the membrane. Upon the disruption of vesicular structure, *P*-700 is expected to react directly with the reagents in the outer solution [5]. Upon such disruption of chloroplasts, the reaction mode itself is expected to change. Electrostatic interaction between the surface and the reagent is also expected to change after such disruption, since the surface on which the reaction with the external reagent takes place may change from the outer to the inner one.

In this study the reaction rate of *P*-700 with the redox reagents in the outer medium was analyzed in various preparations of chloroplast membrane and the relation between the localization of the membrane component and the reaction rate was studied.

Materials and Methods

Preparation of chloroplasts from spinach leaves and sonic treatment of the chloroplasts were carried out as previously described [1]. The chloroplasts or sonically disrupted chloroplasts were stored in a medium containing 5 mM tricnine-Na buffer, pH 7.8, and 0.4 M sucrose. The sonicated chloroplasts were frozen and stored at −20°C for several days and used after thawing (freeze-thawed chloroplasts) in some cases.

System I particles were prepared according to the methods of Ohki and Takamiya [6].

In the measurement of absorption change suspension of chloroplasts was diluted 25—50 times with the reaction medium.

Chlorophylls were assayed according to Arnon [7].

Measurement of *P*-700 oxidation and reduction was done spectrophotometrically by monitoring difference absorption change at 705 nm with a reference at 730 nm as described previously [1]. In some cases a xenon flash (duration 40 μs) passed through a Corning 9782 glass filter was used as the excitation light. Suspension of chloroplasts was vigorously stirred by a magnetic stirrer in most of the measurements. The time required to mix fully reagents added by an air pipette (usually 5—10 μl) with the reaction medium was less than 0.3 s.

Results and Discussion

Surface potential and the reaction rate between the membrane components and the reagents in the aqueous phase

The apparent rate constant, k, of the reaction between the component on the membrane and an ionic reagent with a valence z is expected to follow the equation below at a membrane surface potential, ψ_0 [1,3],

$$\ln k/\gamma_b = \ln k^\circ - \frac{zF}{RT}\psi_0 \qquad (1)$$

where γ_b represents the activity coefficient of the reagent in the bulk phase, k° is the rate constant at the infinite salt concentration and equals the rate con-

stant taken with respect to the surface activity of the reagent.

At relatively low surface potentials ($\psi_0 < 50$ mV) in the presence of 1 : 1 symmetrical salt, Eqn. 1 reduces to a simple approximation equation [1],

$$\log k/\gamma_b \approx \log k^\circ - 0.078\, z\, C_b^{-1/2}\, q \qquad (2)$$

where C_b represents the bulk molar concentration of the salt. q is the net surface charge density expressed in $\mu C/cm^2$.

Thus the slope of the plot of $\log k/\gamma_b$ versus $C_b^{-1/2}$ gives q value, and hence ψ_0 of the membrane surface in the vicinity of the reaction site. The intercept at the infinite salt concentration gives k° which is determined by the particular combination of the reagent and the membrane component.

Effects of salt on the reaction of P-700 with ferricyanide and ascorbate in class II chloroplasts

Reaction of *P*-700 with ferricyanide or ascorbate was measured by following the time course of *P*-700 oxidation or reduction after adding these reagents. Artifacts due to light scattering change which is significant upon illumination of chloroplasts can be minimized by this mode of measurement. Fig. 1A shows the time courses of oxidation of *P*-700 in the class II chloroplasts by addition of 1 mM ferricyanide in the presence of varied concentrations of KCl. In the low ionic medium without KCl very slow oxidation of *P*-700 was observed. Lag of the oxidation after adding ferricyanide was significant. The half time for the oxidation was more than 40 min. At the same concentration of ferricyanide, the rate became higher as the increase of KCl concentration in the medium. The lag became less marked as the increase of KCl concentration. Such enhancement of the oxidation rate by KCl can be explained by the salt-induced decrease of electrostatic repulsion between ferricyanide^{3-} and the membrane surface which is negatively charged at this pH. Similar enhancing effects were also observed when other salts were added (not shown). Addition of a neutral salt is expected to screen the negative charges on the membrane surface and to increase activity of ferricyanide at the surface.

Effects of salts on the reduction rate of ferricyanide-oxidized *P*-700 by ascorbate was also studied (Fig. 1B). The rate of *P*-700 reduction by ascorbate^{-1} was also increased by addition of KCl. No lag of reduction was observed after addition of ascorbate. This can also be explained by the KCl-induced decrease in the electrostatic repulsion between the negatively charged surface and ascorbate.

The lag phase observed upon oxidation of *P*-700 but not on reduction seems to indicate that the *P*-700 molecule is interconnected with other membrane components of lower midpoint potentials. *P*-700 will not be chemically oxidized until these components are oxidized but will be reduced prior to them.

Effects of sonication of chloroplasts on the reaction of P-700 with ferricyanide and ascorbate

Fig. 2A compares the time courses of the oxidation of *P*-700 by ferricyanide in the sonicated and class II chloroplasts in the presence of KCl. In the sonicated chloroplasts the oxidation rate was significantly accelerated and the

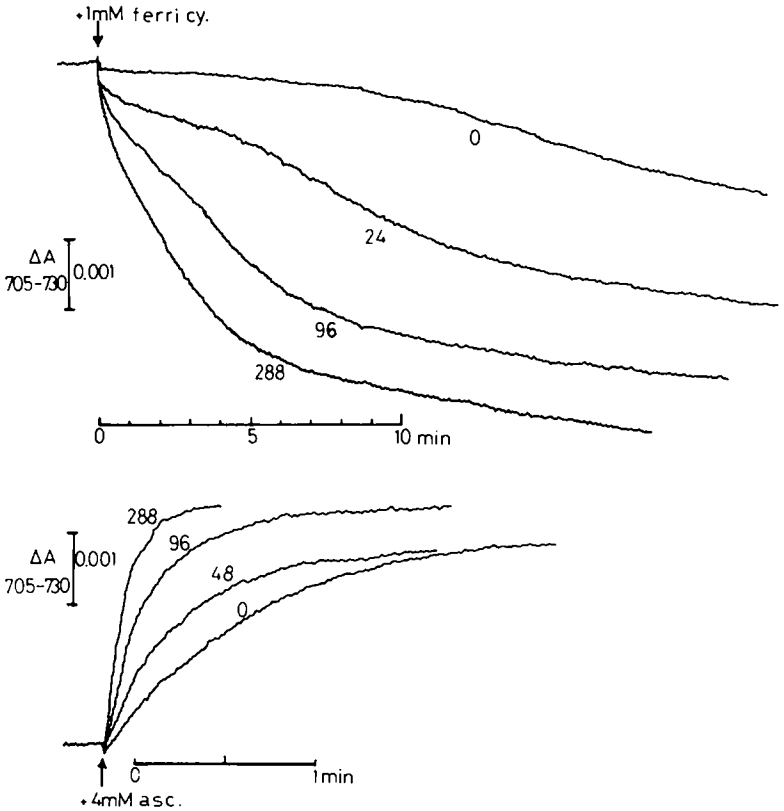


Fig. 1. Oxidation of *P*-700 by ferricyanide (A) and reduction by ascorbate (B) in the class II chloroplasts at varied concentrations of KCl. Reaction mixture contained 5 mM tricine-Na buffer, pH 7.6, 19 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, 8 μM methyl viologen, chloroplasts equivalent to 71 μg chlorophyll/ml and varied concentration of KCl as indicated in mM in the figure. In A, 10 μl solution of $K_3Fe(CN)_6$ (final concentration of 1 mM) was added in the dark at times indicated by an arrow to the suspension of chloroplasts which was rigorously stirred by a magnetic stirrer. In B, 10 μl solution of sodium ascorbate (final concentration of 4 mM) was added in the dark to the suspension of chloroplasts in which P-700 was preoxidized by 1 mM $K_4Fe(CN)_6$.

initial lag of the oxidation after addition of ferricyanide was not observed. This is consistent with the generally accepted idea that sonic treatment of chloroplasts breaks the membrane vesicles and disrupts the link of *P*-700 to other electron transfer components situated between system II and system I by dissociating plastocyanin [5]. The reduction of *P*-700 by ascorbate in the sonicated and class-II chloroplasts are also shown in Fig. 2. In this case, too, a significant increase of the reduction rate was observed on sonication.

Effect of sonication time on the reaction rate of *P*-700 is shown in Fig. 3. An only 15 s sonication was sufficient to increase the oxidation rate with ferricyanide 15-fold. The reduction rate with ascorbate also increased on sonication and required a little longer sonication time for the full gain in the rate. It is probable that the reaction rate of *P*-700 with ferricyanide, which has higher negative valence and is expected to be less permeable, was more accelerated by the sonication than that with ascorbate. Such an increase of the reac-

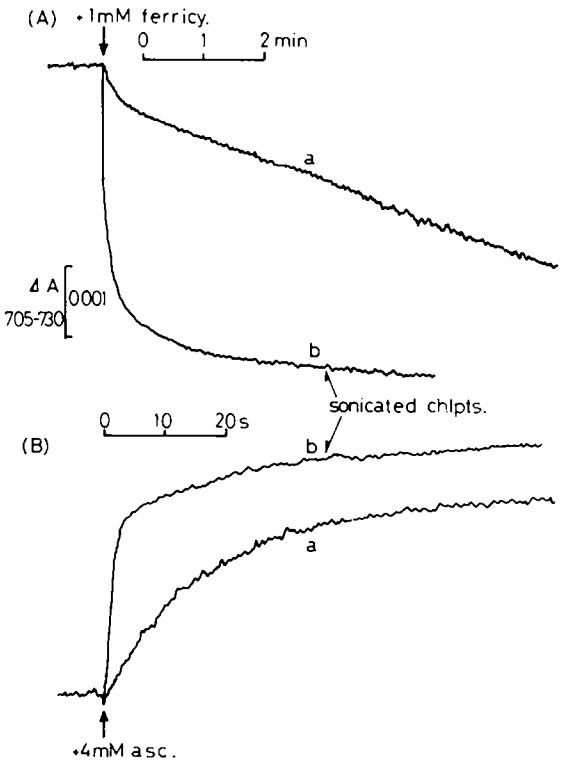


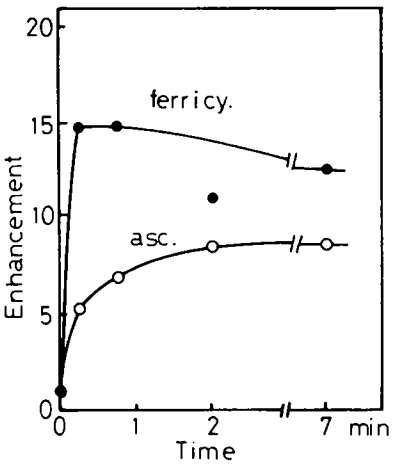
Fig. 2. Effects of sonication of chloroplasts (chlpts.) on the oxidation rate of *P*-700 by ferricyanide (A) and on the reduction rate of *P*-700 by ascorbate (B). a, class II chloroplasts; b, chloroplasts sonicated for 45 s. Experimental conditions were similar to those in Fig. 1 except that KCl concentration was 96 mM.

tion rate with the ionic reagents by the sonication agrees with the currently accepted topology of the electron transfer components on the thylakoid membrane which locates the reaction site of *P*-700 molecule on the inner surface of the vesicles [4].

Estimation of the surface potential and the net surface charge density from the salt effect on the reaction of P-700

In order to examine further the effects of salts, logarithms of the apparent rate constant, k, divided by the bulk phase activity coefficient of the reagent, γ_b, were plotted versus inverse square root of ionic strength, I, which was changed by adding KCl (Fig. 4).

In the case with ferricyanide in the class II chloroplasts, the values of q and k° were calculated to be $-1.5\ \mu C/cm^2$ and $458\ M^{-1} \cdot s^{-1}$ from the slope and the intercept, respectively, according to Eqn. 2. This q value corresponds to a surface potential of -53 mV in a solution containing 0.01 M salt of monovalent cation. These q and ψ_0 values were almost the same to the corresponding values obtained from the reaction rate of the system II primary acceptor, Q [3] ($q = -1.34\ \mu C/cm^2$). It may be said that this value of q indicates an electrostatic feature of the outer thylakoid surface. In the sonicated chloroplasts, the



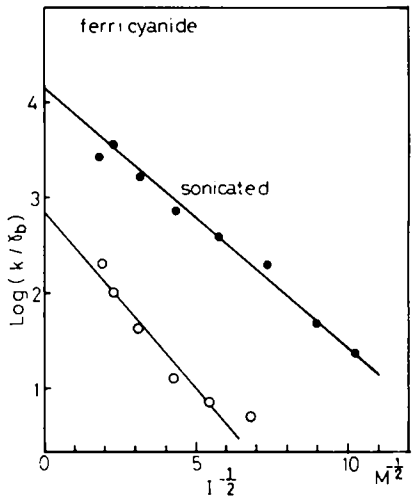


Fig. 3. Effects of sonication time on the reaction rates of *P*-700 with ferricyanide and ascorbate. Experimental conditions were similar to those in Fig. 2. •, oxidation rate with ferricyanide; ○, reduction rate with ascorbate.

Fig. 4. Plot of log k/γ_b vs. inverse square root of the ionic strength changed by adding KCl. Apparent rate constants were calculated from the oxidation rate of P-700 by ferricyanide in the class II and 10 min-sonicated chloroplasts in a set of experiments similar to those in Fig. 2. •, sonicated chloroplasts; ○, class II chloroplasts.

k° and the q values of $1.7 \times 10^4\,M^{-1} \cdot s^{-1}$ and $-1.1\,\mu C/cm^2$ (which corresponds to a surface potential of -42 mV at 0.01 M monovalent cation salt), were obtained in a similar plot. The k° value was hundred times larger while the q value was a little lower than the corresponding values in the class II chloroplasts. This q value was a little higher than that obtained by the analysis of the reduction rate of photooxidized *P*-700 preincubated with reductants (Table I and see Ref. 1). The reason for this discrepancy is not clear.

A similar plot of the results with monovalent anion, ascorbate, also gave straight lines with smaller slopes than those with trivalent anion, ferricyanide, as expected (not shown). In the class II chloroplasts the k° and q values were calculated to be $1.2 \times 10^2\,M^{-1} \cdot s^{-1}$ and $-2.6\,\mu C/cm^2$, respectively. The large negative q value in this case may be partially due to the contribution of coexisting ferrocyanide to the reduction rate in the higher ionic strength region. In the sonicated chloroplasts, values of $4 \times 10^2\,M^{-1} \cdot s^{-1}$ and $-1.4\,\mu C/cm^2$ ($\psi_0 = -51$ mV at 0.01 M monovalent cation salt) were obtained for k° and q. Also in this case a larger k° value and a smaller q value were observed in the sonicated chloroplasts than those in the class II chloroplasts, suggesting the change in the characteristics of the rate-determining membrane surface, probably corresponding to the exposure of the inner surface to the externally added reactant molecules.

Measurement of the reaction of P-700 in different methods in the sonicated chloroplasts

As already mentioned, the precise measurement of the reduction rate of

P-700 by ascorbate is difficult due to the coexisting ferrocyanide which was produced by the reduction of ferricyanide. In order to measure the reduction rate of *P*-700 more accurately, *P*-700 was preoxidized by illumination and then the reduction rate was measured by adding ascorbate immediately after the cessation of illumination (Fig. 5 trace b). The chloroplast preparation used was frozen and thawed after a sonication for 10 min. If the chloroplasts were preincubated with the same amount of ascorbate, a littke higher reduction rate was observed after the cessation of illumination (curve c). The reduction rate measured in both methods depended on the cation composition of the medium.

Salt-induced change in the rate of reduction by ascorbate was also analyzed (Fig. 6). The rate determined by mixing ascorbate immediately after the cessation of illumination was lower than that measured in the preincubated system. By plotting log k/γ_b vs. $I^{-1/2}$ which was changed by adding KCl, values of k^o and q were calculated to be $1.5 \times 10^3\,M^{-1} \cdot s^{-1}$ and $-1.1\ \mu C/cm^2$, respectively, in the former method. In the latter method, k^o and q were $3.5 \times 10^3\,M^{-1} \cdot s^{-1}$ and $-1.3\ \mu C/cm^2$, respectively. Although the higher k^o value was obtained in the latter method, similar q values were observed in both methods, suggesting the same membrane surface as the reaction site. In the same figure the rate of reduction of *P*-700 measured after the cessation of illumination in the

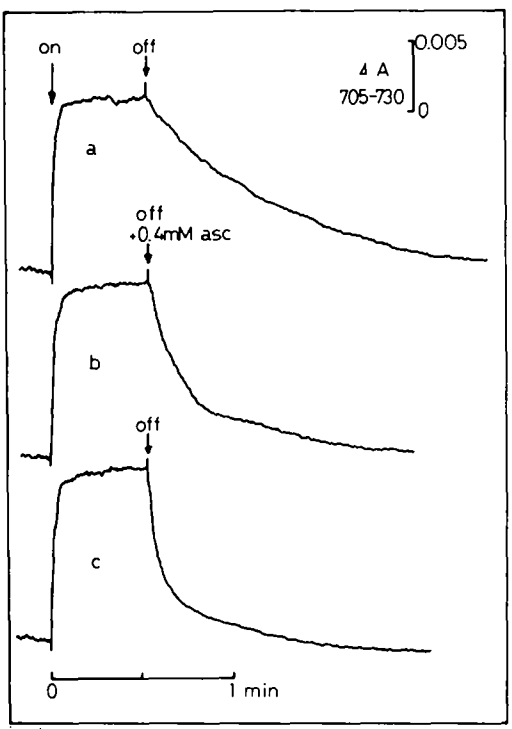


Fig. 5. Time courses of photooxidation and dark reduction of *P*-700 under various conditions in the 10-min sonicated chloroplasts. a, no addition. b, 0.4 mM ascorbate was added immediately after the cessation of illumination. c, 0.4 mM ascorbate was added before the illumination. The chloroplast preparation was frozen and thawed after the sonication. Experimental conditions were similar to those in Fig. 1. KCl concentration was 12 mM.

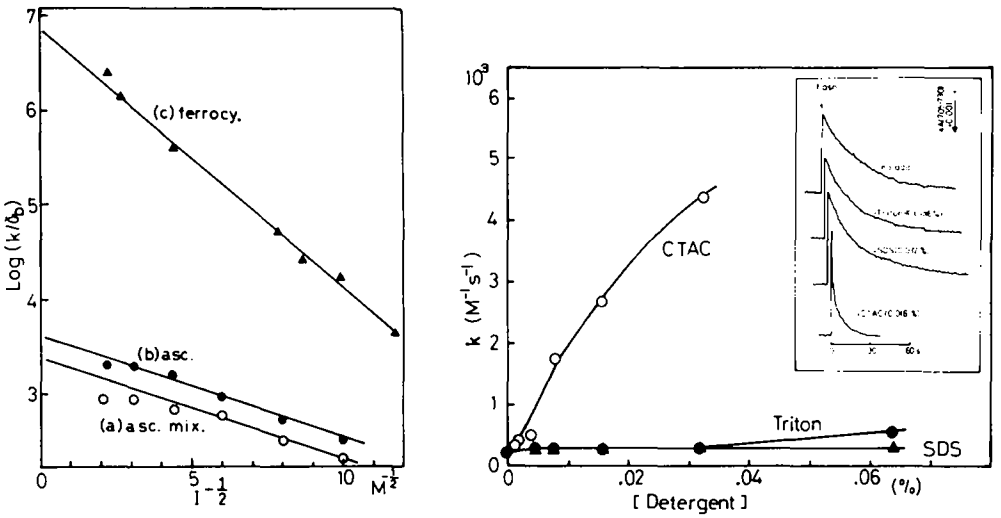


Fig. 6. Plot of log k/γ_b vs. inverse square root of the ionic strength changed by adding KCl in sonicated chloroplasts. (a) the rate of *P*-700 reduction was measured by adding 0.4 mM sodium ascorbate immediately after the cessation of illumination as shown in Fig. 5b. (b) The rate of *P*-700 reduction was measured after the illumination in the presence of 0.4 mM sodium ascorbate which had been added before illumination as in Fig. 5c. (c) The reduction rate was measured after the illumination in the presence of 1 mM ferrocyanide which had been added before the illumination. Experimental conditions were similar to those in Fig. 5.

Fig. 7. Effects of concentrations of various detergents on the *P*-700 reduction rate by ferrocyanide in the sonicated chloroplasts. Apparent first-order rate constants for the *P*-700 reduction are compared. *P*-700 was oxidized by a single-turn-over flash light and the dark reduction rate was followed. Reaction mixture contained, 5 mM Na tricine buffer, pH 7.6, 0.4 M sucrose, 0.2 mM ferrocyanide and 10 min-sonicated chloroplasts equivalent to 67 μg chlorophyll/ml. Insert indicates typical time courses. CTAC, cetyltrimethylammonium chloride. SDS, sodium dodecyl sulfate.

sonicated chloroplasts preincubated with ferrocyanide were also plotted. The plot gave also a straight line and gave the k° and q of 7.0×10^6 $M^{-1} \cdot s^{-1}$ and -0.88 $\mu C/cm^2$. This q value was quite close to those obtained with ferricyanide or with ascorbate in the sonicated chloroplast (cf. Fig. 4).

Effects of detergent on the rate of P-700 reduction by ferrocyanide in sonicated chloroplasts

Effects of various detergents on the reduction rate of *P*-700 by ferrocyanide were studied in sonicated chloroplasts by measuring dark reduction of flash-oxidized *P*-700 in the presence of ferrocyanide and various detergents. Dependence of the reduction rate on the concentration of detergents is shown in Fig. 7. Addition of a nonionic detergent, Triton X-100, or that of an anionic detergent, sodium dodecyl sulfate, little affected the rate. Addition of a cationic detergent, cetyltrimethylammonium chloride, on the other hand, enhanced the rate about 10-fold at a concentration of 0.03% where no effects by the other detergents were observed. A little enhancement of the rate at a high concentration of Triton X-100 (0.06%) accompanied a biphasic dark reduction time course. The rate again became lower due to the increase of the irreversible portion in the higher concentration range (not indicated in the figure).

These results suggest that the increase in the ion permeability of the membrane which should have occurred on detergent additions did not enhance the reduction rate in the sonicated chloroplasts and that the permeation of ferrocyanide through the membrane was not the rate-limiting step. The marked increase of the rate induced by the cationic cetyltrimethylammonium chloride can be explained by its high adsorption onto the negative membrane surface, which decreases the negative surface charge density, and hence the negative surface potential.

Conclusion

In Table I, values of k° and q obtained from the salt-induced changes of the reaction of *P*-700 with ferricyanide, ferrocyanide and ascorbate in various preparations of chloroplasts are summarized. The values obtained in the diginonin-prepared system I particles are also included in the table.

The q value obtained in the class II chloroplasts were generally higher than those in the sonicated chloroplasts or in the photosystem I particles. On the other hand, the k° values with both ascorbate and ferricyanide increased significantly by sonicating chloroplasts. In the sonicated chloroplasts once frozen and thawed, the highest k° value was obtained with ferrocyanide or with ascorbate, suggesting that the reaction site became most accessible to these reagents in this type of preparation. It seems that the treatment which increases the membrane permeability or destroys the membrane integrity increased k°. However, the k° value in the system I particles was lower than that in the sonicated chloroplasts. Some changes in the *P*-700 molecule itself or in the membrane surface in the vicinity of *P*-700 might have occurred by the action of digitonin. Adsorption of digitonin onto the membrane surface might have hindered the access of ascorbate. The low q value observed in the photosystem I particles suggests that digitonin treatment decreased the negative charges on the surface in the vicinity of *P*-700 by releasing some of the protein molecules from the membrane.

In the reaction of *P*-700 with ferricyanide or ascorbate in the class-II chloroplasts, *P*-700 molecules probably do not react with these reagents directly since *P*-700 is expected to be reacting on the inner surface of the thylakoid membrane. Some electron transfer components situated on the outer surface are expected to mediate the redox reaction in this case. The observed low k° values in the class II chloroplasts may thus represent those in the reactions between redox reagents and such components. However, even in such a situation, the present analysis will give the information about the membrane surface as far as the rate-limiting step is the reaction between the redox reagents and the electron transfer components on the membrane surface. In the sonicated chloroplasts, the k° values obtained probably correspond to the rate constants of the direct reaction between *P*-700 and these reagents since the increase of the ion permeability of the membrane by Triton X-100 did not significantly enhance the rate.

If the q values obtained in the class II chloroplasts and in the sonicated chloroplasts can be assumed to indicate the electrostatic features of the outer and inner surfaces of the thylakoid membrane, the outer surface is expected

TABLE I

VALUES OF k^o, SURFACE POTENTIAL AND SURFACE CHARGE DENSITY OBTAINED WITH VARIOUS REAGENTS IN DIFFERENT PREPARATIONS OF CHLOROPLASTS

Values were obtained from curves in Figs. 4 and 6, except those of system I particles. They are expressed in $M^{-1} \cdot s^{-1}$, mV (at 0.01 M monovalent cation salt) and $\mu C/cm^2$, respectively.

Preparation (Method)	Reagent								
	Ferricyanide			Ferrocyanide			Ascorbate		
	k^o	ψ_0	q	k^o	ψ_0	q	k^o	ψ_0	q
Class II (mixing)	$4.6 \cdot 10^2$	−53	−1.5				$(1.2 \cdot 10^2$	−77	−2.62) *
Sonicated (mixing)	$1.7 \cdot 10^4$	−42	−1.1				$(4.0 \cdot 10^2$	−51	−1.4) *
Sonicated (preincubated)				$3.1 \cdot 10^5$	−31	−0.77			
Sonicated and freeze-thawed (mixing)							$1.5 \cdot 10^3$	−42	−1.1
Sonicated and freeze-thawed (preincubated)				$7.0 \cdot 10^6$	−35	−0.88	$3.5 \cdot 10^3$	−48	−1.3
System I particles (preincubated)							$6.2 \cdot 10^2$	−24	−0.59

* The contribution of coexisting 1 mM ferrocyanide was not corrected.

to have more negative charges than the inner surface at neutral pH, at least in the vicinity of *P*-700 This means that even when the electrical potential in the inner and outer bulk phases are kept equal, some intramembrane electrical potential gradient (inside positive) should exist due to the difference of the potentials at the both surfaces as shown in Fig. 8. Surface pH values on both sides are also expected to be different (higher pH at the inner surface) due to the equilibration of the electrochemical potential. Existence of more negative charges on the outer surface may give an explanation for the stability of the sidedness of the thylakoid membrane.

The value of the inner surface potential estimated is in a range similar to that estimated by Rumberg and Muhle [10]. As they pointed out light induced proton uptake into the thylakoids will change the charge density of the inner surface more positive and will cause a large intramembrane electrical potential gradient (inside positive) even when no potential difference exists between the bulk aqueous phases (Fig. 8b). According to the results of pH dependence of the surface potential in the vicinity of *P*-700 in the sonicated chloroplasts in the accompanying paper [1], a surface potential change of at least +40 mV (the larger charge density may exist on the inner surface since another method gave a higher charge density on the outer surface than that estimated in the present analysis [11,12]) is expected to occur as the internal pH changes from 7.6 to 4.0 at 0.01 M monovalent cation sslt in the vicinity of *P*-700. This calculation indicates that the contribution of the surface potential difference to

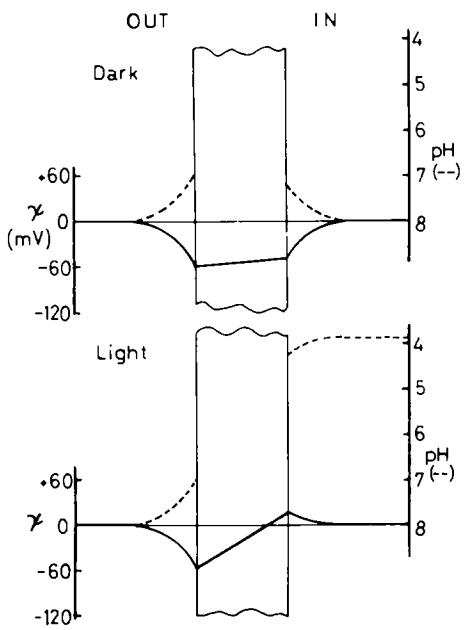


Fig. 8. Schematic representation of the potential profiles across the thylakoid membrane in the vicinity of *P*-700 in the dark (upper) and in the light (lower). Broken lines indicate pH profiles. The values of q were chosen to be −1.5 and −0.84 $\mu C/cm^2$ for the outer and inner surfaces. pH-dependent change of q of the inner surface was estimated from the results of the pH dependence of the *P*-700 reaction rate with ferrocyanide in the sonicated chloroplasts [1]. The difference of electrical potential between the bulk solutions was assumed to be negligible in the calculation. I = 0.01 M with monovalent cation salt.

the intramembrane potential gradient may be significant under some experimental conditions. These values of surface potential of the inner and outer surfaces of chloroplasts were lower than that of outer surface of chromatophore membrane of the photosynthetic bacterium, *Rhodopseudomonas sphaeroides*, in which intramembrane electrical field was shown to change by the surface potential change of the outer membrane surface [13,14] by the measurement of carotenoid band shift.

When the electrochemical potential difference between the surfaces of the membrane is identical with that between the bulk solutions on each side, the electrical and the chemical terms of the electrochemical potential difference are not necessarily the same. In the presence of a certain level of the surface potential, one should measure the pH and the electrical-potential differences between the membrane surfaces or between the inner and outer bulk phases at the same time, except when the surface potential value is known, in precise calculation of the protonmotive force or of the electrochemical potential difference of ions across the membrane. If the calculation of the potential difference is done by measuring the responses of intrinsic potential probes such as the carotenoid band shift in bacterial chromatophore or the 515 nm absorption change in chloroplasts, one had better measure the pH difference between the surfaces. When the protonmotive force is calculated from a set of the values of pH difference between the bulk solutions measured by the isotope distribution or by the fluorescence probe, and intramembrane potential difference measured by the intrinsic probe, the calculation contains unceartainty of several tens of mV in the chloroplast (as shown in the present study), depending on the experimental conditions.

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