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SURFACE POTENTIAL AND REACTION OF MEMBRANE-BOUND ELECTRON TRANSFER COMPONENTS

I. REACTION OF *P*-700 IN SONICATED CHLOROPLASTS WITH REDOX REAGENTS

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Summary

Salt- or pH-induced change of the rate of reduction of the phtooxidized membrane bound electron transfer components, *P*-700, by ionic and nonionic reductants added in the outer medium was studied in sonicated chloroplasts.

The rate with the negatively charged reductants increased with the increase of salt concentration at a neutral pH or with the decrease of medium pH. Salts of divalent cations were much more effective than those of monovalent cations. A trivalent cation was even more effective. The rate with a nonionic reductant was little affected by salts.

The change of the reduction rate was analyzed using the Gouy-Chapman theory, which explains the change of reduction rate by the changes of activities of ionic reductants at the charged membrane surface where the reaction takes place. This analysis gave more useful parameters and explained more satisfactorily the case with high-valence cation salts than the Brönsted type analysis. The values for the surface charge density and the surface potential of the membrane surface in the vicinity of *P*-700 estimated from the analysis were lower than those estimated for the surface in the vicinity of Photosystem II primary acceptor, suggesting the heterogeneity of the thylakoid surface.

The salt-induced surface potential change was shown to affect the activation energy of the reaction between *P*-700 and the ionic reagent.

Abbreviation: DCMU, 3(3′,4′-dichlorophenyl)-1,1-dimethylurea.

Introduction

The rate of reaction between ionic molecular species is known to vary depending on the ionic conditions of the reaction medium. Brönsted's treatment based on the Debye-Hückel theory has been applied to explain the phenomenon under the simplified conditions [1,2]. In biological reactions, we often encounter more complex situations. Reactant molecules usually have limited mobility on membranes and constitute a supramolecular membrane system, the electrostatic features of which are different from those of the individual molecules isolated from the membrane. Reactivities of membrane components are expected to be affected by the electrostatic characteristics of the membrane surface in their vicinity.

Chloroplast thylakoid membrane, as well as other biological membrane, is known to be negatively charged at neutral pH; the membrane surface is at a negative electrical potential with respect to the bulk aqueous phase [3—5]. Due to this negative potential, concentrations of cations at the surface are higher than those in the bulk aqueous phase, while those of anions become lower under usual experimental conditions as shown schematically in Fig. 1 (Gouy-Chapman theory [6], see Appendix).

The distribution of ions affects the reaction rate between the membrane components and the ionic reagents, since the thermodynamic activity of the reagent at the surface, but not that in the bulk phase, determines the rate.

In the previous studies [7,8], changes in the apparent rate constants of the reactions between small oxidants having various charges (ferricyanide^{3-}, phenazinemethosulfate^{+} and *p*-benzoquinone) and the primary electron acceptor of Photosystem II (Q) in the thylakoid membrane were extensively studied under various conditions. A hypothesis based on the Gouy-Chapman theory, which relates change in the apparent rate to the change in the surface activity of an ionic oxidant, satisfactorily explained the results [8]. The outer surface of the thylakoid membranes in the vicinity of Q was estimated to have a net surface charge density of $-1.3\ \mu C/cm^2$ at pH 6—9, which corresponds to the surface potential of about -60 mV at 0.01 M monovalent salt concentration. The change in the reaction rate of Q was interpreted in terms of the changes in the surface potential and in the local concentration of oxidant. The hypothesis was

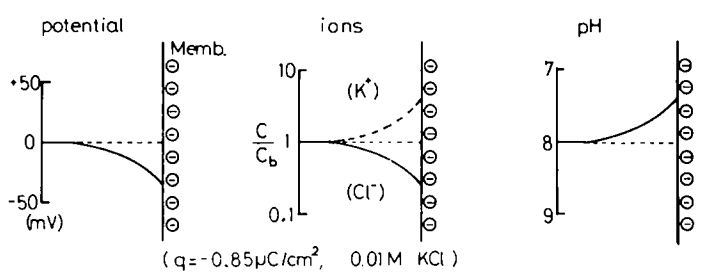


Fig. 1. Schematic profiles of electrical potential, ion concentrations, and pH on the thylakoid surface in the vicinity of *P*-700. The values were calculated for the case with $q = -0.85\ \mu C/cm^2$ in the presence of 0.01 M KCl at pH 7.8.

also shown to be applicable to the pH- or salt-dependent change in the rate of the Hill reaction with various electron acceptors [9].

In the present study the hypothesis is also applied to the reaction between the primary electron donor of the Photosystem I, *P*-700, and charged redox reagents to probe the surface characteristics of the different part of the thylakoid membrane. According to the currently accepted topology of thylakoid membrane [10], *P*-700 reacts on the inner surface of the thylakoid, a localization different from that of the Photosystem II primary acceptor situated on the outer surface. The analysis were done in sonically disrupted chloroplasts in order to expose *P*-700 reaction site [11] under various situations.

Materials and Methods

Chloroplasts were prepared from spinach leaves according to the methods described elsewhere [7] with a medium comprising 0.05 M Tris-HCl buffer (pH 7.8), 0.4 M sucrose and 0.01 M NaCl. The chloroplasts were disrupted in a sonic oscillator (Tomy Seiko, UR-200P) operated at medium power for 3 min in a medium containing 5 mM tricine-Na buffer, pH 7.8, and 0.4 M sucrose. The sonication was repeated four times with 4 min intervals. The temperature was kept at about 0° C throughout the treatment. Large unbroken fragments were removed by centrifugation of the sonicated suspension for 10 min at 3000 × *g*.

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

Chlorophylls were assayed according to Arnon [12].

Measurement of *P*-700 photooxidation and following dark reduction was done spectrophotometrically by monitoring absorption change at 705 nm (reference at 730 nm) with a Hitachi 356 dual wavelength spectrophotometer. The photomultiplier, which was kept apart from the sample in order to eliminate fluorescence, was protected with a glass filter (Toshiba V-R 69) from the blue excitation light from an illumination device through a glass filter (Corning 9782).

Theory

In the reaction between a component on the membrane (A_1) and a charged reagent in the medium (A_2), the relation between the apparent rate constant, k, which is calculated with respect to the bulk concentrations of A_2 and A_1, and the 'true' rate constant, k^0, which is referred to the surface activity of A_2, can be expressed as follows according to the hypothesis using the Gouy-Chapman theory (Appendix and Refs. 8 and 9).

$$\ln k/\gamma_b = \ln k^0 - \frac{z_2 F}{RT}\psi_0 \qquad (1)$$

γ_b and z_2 are the activity coefficient of A_2 in the bulk phase and the number of elementary charges carried by an A_2 molecule, respectively. ψ_0 is the electrical potential difference at the membrane surface with respect to the bulk phase. The second term on the right-hand side of Eqn. 1 comes from the difference

between the activity of A_2 at a point close to the surface and that in the bulk phase.

At relatively low surface potentials ($\psi_0 < 50$ mV) in the presence of a monovalent symmetrical salt at a sufficient level, a linear approximation equation is obtained (see Appendix).

$$\log k/\gamma_b = \log k^\circ - 0.078\, z_2 q C_b^{-1/2} \quad (2)$$

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

Eqn. 2 indicates that the plot of $\log k/\gamma_b$ vs. $C_b^{-1/2}$ should give a straight line, of which the intercept and slope are determined by the values of k° and q, respectively. The value of q and hence ψ_0, calculated from the slope, will give the electrostatic feature of the membrane surface in the vicinity of the reaction site. On the other hand, k° will depend on the nature of both the reagent and the membrane component.

Another approach is that of the Brönsted treatment. When we assume the thylakoid membrane as a large spherical molecule we can obtain the dependence of the apparent rate constant on the ionic strength by calculating the ionic-strength dependent changes of activity coefficients of the reactant and the transition-state-intermediate molecules (see Appendix). This approach was found to give results essentially similar to those of the former approach, but with less significant information.

Results

Effects of salts on the dark reduction rate of P-700 by ferrocyanide

Fig. 2 shows time courses of *P*-700 photooxidation and the following dark reduction in the sonicated chloroplasts. The dark reduction rate in the absence of artificial electron donors was very slow in the sonicated chloroplasts (curve *a*) probably due to the loss of plastocyanin [13], and was not changed by the addition of DCMU and methyl viologen. On addition of ferrocyanide the reduction rate became higher with little change in the rate and the extent of the preceeding photooxidation (curve *b*). The acceleration was dependent on the ferrocyanide concentration added (see Fig. 6A). *P*-700 reduction by ferrocyanide was further accelerated by addition of non-reacting salt, KCl (curves *c* and *d*). The endogenous rate without ferrocyanide was little affected by KCl (not shown).

These results suggest that the reduction rate of *P*-700 in the negatively charged membrane by ferrocyanide is controlled by the electrostatic interaction between the latter and the membrane surface (the isoelectric point of the thylakoid membrane lies around pH 4.2 [3]). Effect of KCl is explained by the increase of the surface activity of ferrocyanide due to the screening of negative charges on the surface by KCl.

Effects of various salts on the apparent rate constant of the reaction between ferrocyanide and photooxidized *P*-700 were studied (Fig. 3). 50- to 100-fold increase in the apparent rate constant (corrected for the endogenous rate) was usually observed in the presence of salts. A slight suppression in the high

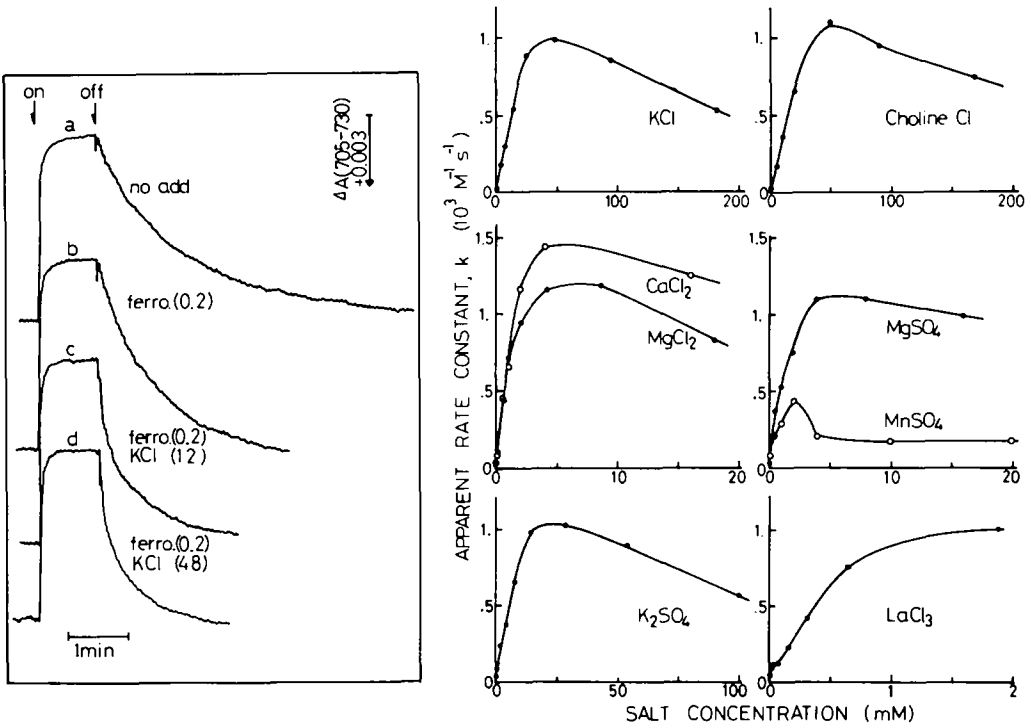


Fig. 2. Effects of KCl on the reduction rate of *P*-700 by ferrocyanide in the sonicated chloroplasts. Reaction mixture contained 5 mM Na tricine buffer, pH 7.8, 19 μM DCMU, 8 μM methyl viologen and the sonicated chloroplasts equivalent to 68 μg chlorophyll/ml. Concentrations of $K_4Fe(CN)_6$ (ferro) and KCl are shown in the figure in mM.

Fig. 3. Effects of various salts on the reduction rate of *P*-700 by ferrocyanide. Apparent rate constant of the reaction was calculated from the reduction rate of *P*-700 in the presence of 0.2 mM $K_4Fe(CN)_6$. The rate was corrected for the endogenous rate measured without ferrocyanide. Conditions for the measurement were the same as in Fig. 2.

concentration range may reflect the significant decrease of activity coefficient of ferrocyanide at high ionic strengths. Divalent cation salts were more effective and increased k at lower concentration ranges than monovalent ones. Ca^{2+} was a little more effective than Mg^{2+}. $MnCl_2$ was not effective at the higher concentration range. Trivalent cation salt, $LaCl_3$, increased the rate constant in a concentration range much lower than the other salts tested.

The enhancements of the apparent rate constant, k, by salts were dependent on the valence and concentration of cations (K_2SO_4 was not more effective than KCl). The higher the valence of cation, the higher the effectiveness of the salt in enhancing k value. These characteristics are explained by the hypothesis using the Gouy-Chapman theory more adequately than the Brönsted type treatment. The results with $MnCl_2$ may suggest that specific interactions between ions and the membrane surface also affect the reaction rate in some cases. This remains to be studied further.

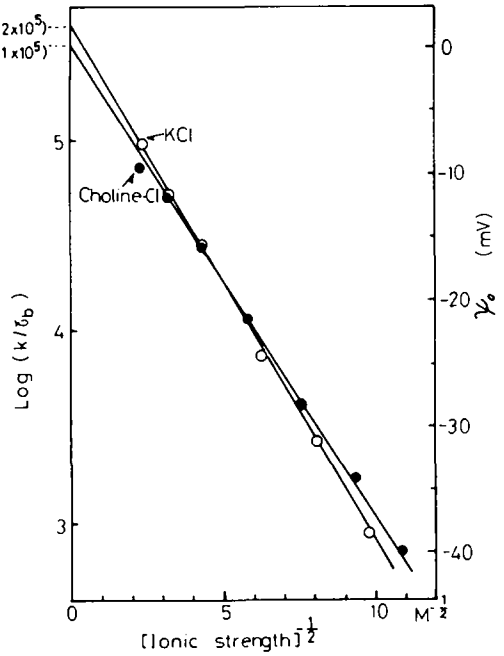


Fig. 4. Plot of log k/γ_b vs. (ionic strength)$^{-1/2}$; (ionic strength was changed by adding KCl or choline chloride) in the reaction between *P*-700 and ferrocyanide. The apparent rate constants in the presence of KCl or choline chloride were obtained from data in Fig. 3. In the calculation of the ionic strength, *I*, and hence of γ_b values, contributions from the buffer and other additives were also included.

Calculation of the surface potential from the apparent rate constant change in the presence of various salts

Log k/γ_b values were plotted against inverse square root of ionic strength from data with KCl and choline chloride (Fig. 4). The plot gave straight lines within the range tested as predicted by Eqn. 2. From the slope of the line, a surface charge density of $-0.84\ \mu C/cm^2$ was calculated. The k° value of $3-4 \cdot 10^5\ M^{-1} \cdot s^{-1}$ was obtained from the intercept at infinite ionic strength. On the right hand ordinate of the figure, values for the surface potential corresponding to the values of log k/γ_b (Eqn. 1) are also shown. The thylakoid surface in the vicinity of *P*-700 probably had the surface potential of about -35 or -40 mV at 0.01 M monovalent cation salt in the sonicated chloroplasts. These values for the net surface charge density and ψ_0 were a little smaller than those obtained by a similar analysis of the reaction between the Photosystem II primary acceptor, *Q*, and ferricyanide ($-1.3\ \mu C/cm^2$ and -60 mV at 0.01 M monovalent salt for q and ψ_0, respectively).

When the Brönsted type equation (Eqn. 17A) was used, relatively good agreements with the experimental results were obtained only at large radii for the membrane sphere, for instance, the theoretical curve using Eqn. 17A with a set of $r_1 = 300$ Å, $z_1 = -655$, and log $k_B^\circ = -21.6$ gave a straight line similar to that of the case with KCl in Fig. 4A. However, little information on the feature of the membrane surface and the reaction mechanism was deduced

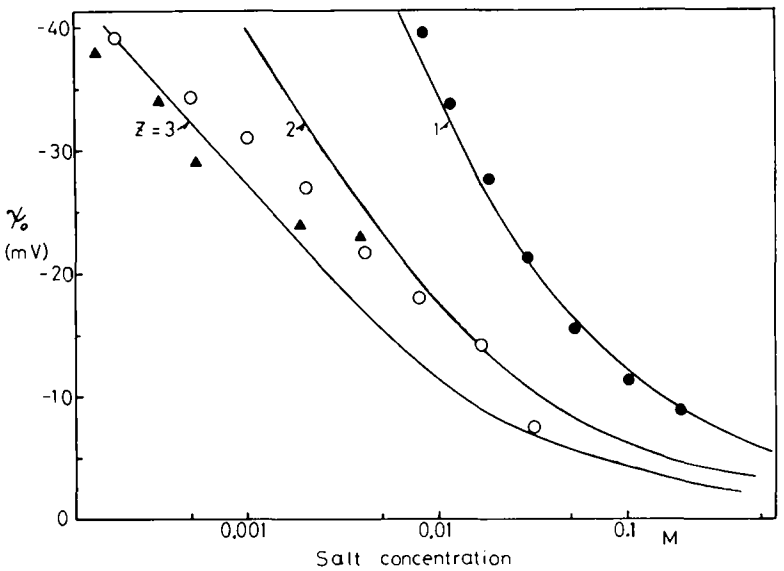


Fig. 5. Dependence of the estimated surface potential on the concentrations of various salts. Values of surface potential were calculated from the k value taken from the corresponding data in Fig. 2 using Eqn. 1 with k^o value of $3.1 \cdot 10^5\ M^{-1} \cdot s^{-1}$ obtained in Fig. 4 with KCl. ●, KCl; ○, $MgSO_4$; ▲, $LaCl_3$. Solid lines indicate theoretical lines calculated according to Eqn. 1A by assuming $q = -0.84\ \mu C/cm^2$ with 1 : 1, 2 : 2 amd 3 : 3 (z = 1, 2 and 3) symmetrical salts, respectively.

from these parameters except for an indication that the reaction site is on large membrane surface. Thus it seems better to carry out the analysis using the Gouy-Chapman theory to get meaningful results concerning the electrostatic feature of the thylakoid membranes.

Fig. 5 shows the dependence of the surface potential, which was calculated from the k values in Fig. 2 using Eqn. 1, on the concentrations of KCl, $MgSO_4$ and $LaCl_3$. In the calculation, the k^o value obtained with KCl in Fig. 4 was used. Solid lines indicate the theoretical dependence of ψ_0 on the concentrations of $z : z$ symmetrical salts calculated using the nonlinear equation (Eqn. 1A). Net surface charge density of $-0.84\ \mu C/cm^2$ obtained with KCl in Fig. 4 was used in these calculations. No correction for the contributions from coexisting buffer, ferrocyanide and other additives were made in the calculations of the curves with $z = 2$ and 3. The data with KCl fitted well the theoretical line of $z = 1$. The data with $MgSO_4$ or $LaCl_3$ were also relatively well correlated with the theoretical lines. Nonidealistic behavior of the higher-valence ions, which seems significant in calculations both of the surface potential and of the activity coefficient of ferrocyanide, may be responsible for the quantitative discrepancies in cases of $z = 2$ and 3. Thus the larger surface charge density or surface potential values are obtained from the analysis of the dependences of the reduction rate on the concentrations of the higher-valence cation salts. A q value of $-2.3 \pm 0.4\ \mu C/cm^2$ was obtained when the value was calculated from the sets of concentrations of KCl and $MgSO_4$ which produced the same change in k/γ_b value. This larger q value was almost the same as that for the outer surface of thylakoids estimated by Barber et al. [5] from the difference of the

effective concentrations of mono- and divalent cation salts in inducing the change of chlorophyll fluorescence in pea chloroplasts.

If the reduction rate of *P*-700 by ferrocyanide was measured in the presence of various concentrations of sucrose, suppression of the rate became marked at the sucrose concentrations higher than 1 M. It seemed that the reduction rate of *P*-700 by ferrocyanide was limited by a diffusive process at high concentrations of sucrose under this unstirred condition. In a medium with a relatively low viscosity as used in the present study, electron transfer rate at the membrane surface seemed to be the rate-limiting step in the overall reaction.

Effects of salts on the P-700 reduction rate by ascorbate and by phenazine methosulfate

Fig. 6 shows the dependence of the *P*-700 reduction rate on the concentrations of ascorbate, ferrocyanide and phenazine methosulfate in the low ionic medium. The apparent first-order rate of the reduction (corrected for the

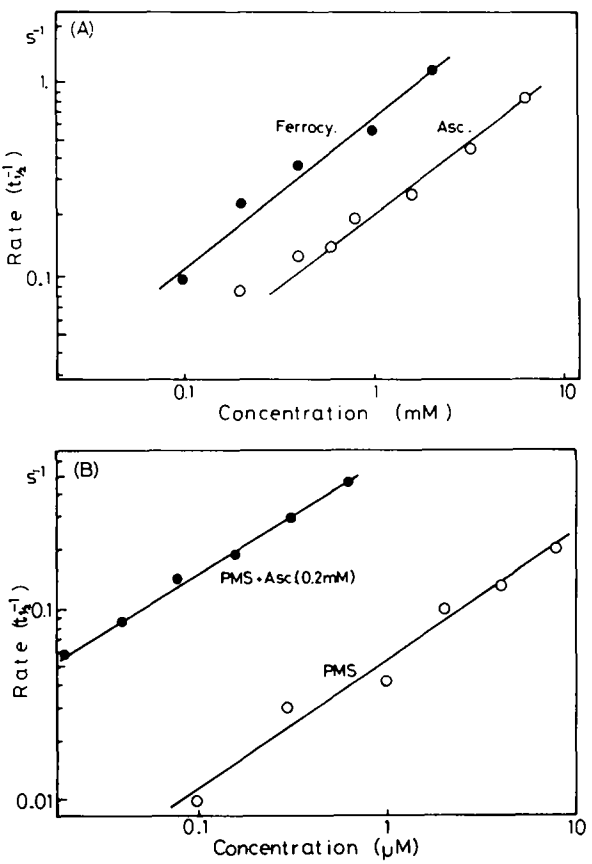


Fig. 6. Dependence of the reduction rate of *P*-700 on the concentrations of ferrocyanide, ascorbate (A) and on the concentration of phenazine methosulfate in the presence and absence of 0.2 mM ascorbate (B). The rate of *P*-700 reduction was corrected for the endogenous rate (0.025 s^{-1}) measured in the absence of added reductants. (A) ○, ascorbate; ●, ferrocyanide. (B) ○, phenazine methosulfate; ●, phenazine methosulfate plus 0.2 mM ascorbate. Experimental conditions were similar to those in Fig. 2.

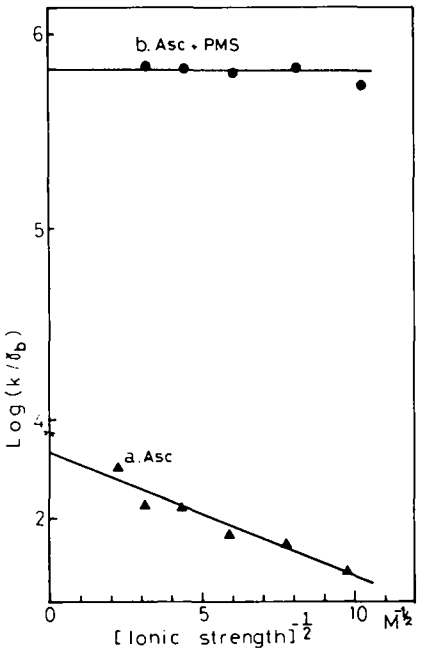


Fig. 7. Plots of log k/γ_b vs. (ionic strength)$^{-1/2}$ (ionic strength was changed by adding KCl in the reaction between *P*-700 and ascorbate or phenazine methosulfate). a, with 0.2 mM ascorbate; b, with 0.2 mM ascorbate and 0.64 μM phenazine methosulfate. Conditions for the measurement and calculations were similar to those in Figs. 2 and 3.

endogenous rate without reductants) depended on the 0.6—0.7th power of the concentration of these reagents in every case. The rate-limiting step seems to be the electron transfer reaction at the surface. This satisfies the prerequisite for the analysis according to the hypothesis with these reagents.

Effects of salts on the reduction rate by these reagents were also studied. The rate with ascorbate ($z = -1$) increased with increasing concentrations of KCl (Fig. 7). The extent of the enhancement with ascorbate was smaller than that with ferrocyanide as expected from the smaller negative charge on a molecule of ascorbate. The plot of log k/γ_b versus $I^{-1/2}$ gave a straight line with an intercept corresponding to k° of $2.2 \cdot 10^2\ M^{-1} \cdot s^{-1}$. This value of k° was about one-thousandth that with ferrocyanide. From the slope the net surface charge density of $-0.86\ \mu C/cm^2$ ($\psi_0 = -38$ mV at 0.01 M ionic strength with KCl) was obtained. This value of q was similar to that obtained with ferrocyanide and probably indicates electrostatic characteristics of the membrane surface in the vicinity of *P*-700.

The reduction rate by ascorbate-reduced phenazine methosulfate was not affected by the salt addition, as was expected — since the reagent will be uncharged when reduced (Fig. 7). A k° value of $7 \cdot 10^5\ M^{-1} \cdot s^{-1}$, which was a little higher than that with ferrocyanide, was obtained. That the rate with phenazine methosulfate was not affected by ionic strength suggests that structural changes of the membrane induced by the addition of salt, if they do exist, have little effect on *P*-700 reduction. Addition of other salts of mono- or

divalent cations did not affect the rate with reduced phenazine methosulfate (not shown). That the rate with oxidized form of the reagent was much lower than that with reduced one (Fig. 6) indicates that the rate of cyclic reaction induced by phenazine methosulfate was negligible compared to the reduction rate of *P*-700 in the presence of phenazine methosulfate and ascorbate.

It should be noted that the apparent rate constant of the membrane components is a function of the ionic conditions of the medium, of the valence of the reacting reagent and of the k° value which is specific for each case. Without the consideration of these points discussion of the reaction of membrane components with different redox reagents may be incomplete and sometimes misleading.

pH dependence of the reduction rate of P-700 by ferrocyanide

As seen from Eqn. 2, the reduction rate should also change as the q value of the membrane surface changes. Isoelectric point of the membrane is known to be around pH 4.2 [3,4]. The lower the medium pH, the more positive is the q value expected. The effect of salt on the reaction with ferrocyanide was studied at various pH values between 3.9 and 7.5, and q and k° values were calculated (Fig. 8). At any pH, the plot of log k/γ_b versus $I^{-1/2}$ changed with KCl gave an almost straight line within the range. At pH 3.9, q was close to

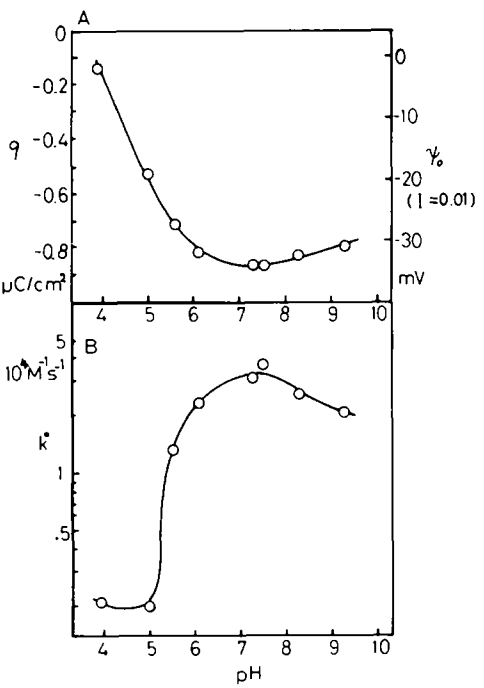


Fig. 8. Effects of medium pH on the values of q (A) and k° (B). The scale on the right-hand ordinate (A) shows the values of ψ_0 corresponding to q at 0.01 M monovalent cation concentration. The value of q and k° were calculated from the reaction of *P*-700 with ferrocyanide in experiments similar to those in Figs. 2 and 3 at various pH values, 5 mM morpholinoethane sulfonate buffer was used at pH values lower than 6.

zero. However, at this pH, a more positive value of q is likely, since some extent of specific adsorption of ferrocyanide on the positively charged surface, which cannot be removed by adding KCl, may occur [8]. A sharp decrease of the value of q was observed when pH was raised from 3.9 to 6. The q value attained the negative maximum at about pH 7—8 and increased slightly at higher pH values. On the right hand ordinate of the figure, a scale for the value of surface potential at 0.01 M monovalent cation salt is also shown. This pH dependence was a little different from that obtained in a similar analysis of the reaction between ferricyanide and the Photosystem II primary acceptor (q became zero at about pH 5). However, it resembles the pH dependence of zeta potential measured by electrophoresis of chloroplasts [3,4].

The k° value was also pH dependent. This might be due mainly to the protonation of ferrocyanide in the low pH region since pH of the first protonation of ferrocyanide is 4.2 and such pH dependent decrease of k° is not marked in the reaction between ferricyanide and the Photosystem II primary acceptor. A possibility of change in the electrostatic or structural state of the reaction site cannot be excluded.

The pH dependences of ψ_0 and k° values shown in Fig. 8 are merely the apparent quantities, since the q value also changes as the change of surface pH, even at the same bulk pH. Thus to get the dependence of q value on the surface pH, more knowledge of the surface charged groups is required (Masamoto, K., Itoh, S. and Nishimura, M., unpublished results).

Activation energy for the reduction of P-700⁺ under different salt conditions

Arrhenius plots of the reduction rate of *P*-700 by ferrocyanide or by ascorbate under different ionic conditions are shown in Fig. 9. The reduction rate in the presence of ferrocyanide or ascorbate gave a value of activation energy similar to that of the endogenous rate (about 8 kcal/mol). From the plots in

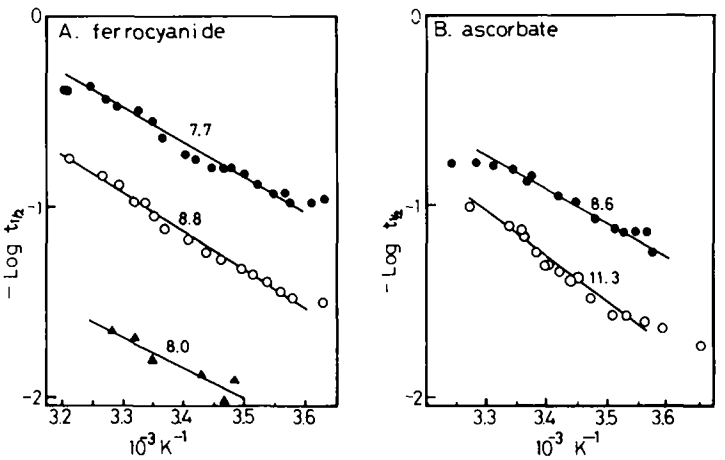


Fig. 9. Arrhenius plot of the reduction rate of *P*-700. A. ▲, no addition; ○, with 4 mM $K_4Fe(CN)_6$; ●, with 0.2 mM $K_4Fe(CN)_6$ and 3.6 mM $MgCl_2$. B. ○, with 4 mM sodium ascorbate; ●, with 2 mM sodium ascorbate and 3.6 mM $MgCl_2$. Other experimental conditions were similar to those in Fig. 2.

TABLE I

ACTIVATION ENERGY FOR THE REDUCTION OF $P700^+$

$\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta G^{\neq}$ values were calculated from data in Fig. 10.

Reactant	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal/deg · mol)	$\Delta G^{\neq}$ (kcal/mol)	$\Delta G^{\neq}(-MgCl_2) - \Delta G^{\neq}(+MgCl_2)$ kcal/mol (eV)
Ferrocyanide				
$-MgCl_2$	8.4	−19.8	14.3	1.5 (0.065)
$+MgCl_2$	7.7	−17.1	12.8	
Ascorbate				
$-MgCl_2$	10.0	−17.8	15.3	0.8 (0.035)
$+MgCl_2$	7.2	−24.3	14.5	

Fig. 9, apparent $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta G^{\neq}$ values were calculated (Table I). In either case with ferrocyanide or with ascorbate a decrease in $\Delta H^{\neq}$ was observed in the presence of $MgCl_2$. In the case of ferrocyanide difference between the $\Delta G^{\neq}$ values in the presence and absence of $MgCl_2$ was 1.5 kcal/mol (0.065 eV). About half of this decrease of $\Delta G^{\neq}$ comes from the change in $\Delta S^{\neq}$. In a similar set of experiments with ascorbate, a $\Delta G^{\neq}$ decrease of 0.8 kcal/mol (0.035 eV) was observed in the presence of $MgCl_2$. In this case contribution of the $\Delta H^{\neq}$ decrease to the $\Delta G^{\neq}$ decrease was partially compensated by the $\Delta S^{\neq}$ change. This result may indicate some difference between the mechanisms of reactions of *P*-700 with ascorbate and ferrocyanide.

These results indicate that the decrease of the electrostatic barrier due to the surface-potential decrease induces the decrease of activation energy. However, the process which is mainly responsible for the most of observed activation energy seems to be little affected by the surface potential change induced by salts. Probably the conformation change of the *P*-700 molecule upon reduction in the membrane or some other process which is sensitive to the membrane fluidity may be responsible for the salt-independent portion of the activation energy. The fact that similar activation energy values were observed for the reactions with ferrocyanide, ascorbate and endogenous electron donating components under different ionic conditions seems to support this view.

Discussion

Study of the reaction of *P*-700 molecule with ionic redox reagents in the present work indicated that the reaction rates are determined by the electrostatic interactions between the membrane surface and the reagents as predicted by the hypothesis using the Gouy-Chapman theory proposed in the previous study [8]. In essence, the reaction rate is determined by the surface activity of the reagent which depends on the surface potential of the membrane, valence of the reagent, and the valence and concentration of the neutral salt added.

At pH 7.6, analysis with ferrocyanide and ascorbate gave net surface charge density values of −0.84 and −0.86 $\mu C/cm^2$. These values probably represent the electrostatic characteristics of the membrane surface in the vicinity of

P-700. They were lower than that calculated for the membrane surface in the vicinity of Photosystem II primary acceptor (−1.3 to −1.5 $\mu C/cm^2$) [8]. According to the currently accepted picture of the chloroplast membranes, the reaction site of the *P*-700 molecule is situated on the inner surface of the thylakoid membrane in normal chloroplasts. In the extensively sonically disrupted chloroplasts, *P*-700 may be accessible directly to the ionic reagents added in the outer medium in a sufficiently high rate [13]. The high k° value with ferrocyanide obtained in the present study suggests such a situation. Then the q value obtained for *P*-700 in the present study may represent that of the inner surface of the thylakoid membrane in the vicinity of the molecule. The q value for the Photosystem II acceptor, which seems to be situated on the outer surface, on the other hand, will represent the characteristics of the outer surface. The outer surface of the thylakoid vesicles may have more negative charges than the inner surface.

From the measurement of zeta potential by the electrophoresis of chloroplasts [3,4], surface charge density values of −0.84 to −1.1 $\mu C/cm^2$ were reported. These values seem to be underestimated since the zeta potential is considered to reflect potential at a point just outside the Stern layer [4]. Nakatani et al. [4] report that the q value of −2.5 $\mu C/cm^2$ for the outer surface of thylakoids, estimated from the dependence of chlorophyll fluorescence on mono- and divalent cation concentrations [5], explains satisfactorily the various phenomena they studied. This value, however, might be a little overestimated because some nonidealistic behaviors of divalent cation as seen in this work may exist. The q value of −1.35 $\mu C/cm^2$ for the outer surface kinetically estimated in the previous study was between these two values and seems to reasonably well indicate the characteristics of outer thylakoid surface, at least in the vicinity of Q. This value also may be a slight underestimation since the dependence of the k value on the concentration of ferricyanide was not exactly first-order. A part of discrepancy between −2.5 and −1.35 $\mu C/cm^2$ at neutral pH may reflect three dimensional heterogeneity of the membrane surface. Further work is required to clarify these points.

pH dependent change in the q value in the present study suggests that proton uptake of chloroplasts during illumination of chloroplasts will change the q value and hence, the surface potential of the inner surface and will affect the reaction of *P*-700 with other electron transfer components, e.g., plastocyanin, which reaction is known to be sensitive to pH [14] and salt [15] conditions.

Surface potential due to the immobilized surface charges will have significant roles in the membrane reactions. As can be judged from the changes in the reduction rate of *P*-700, the surface potential as well as the intramembrane potential gradient [16,17] will affect the redox levels of the membrane components. Effects of salt on the redox state of membrane-bound electron transfer components in chromatophore membranes of *Chromatium vinosum* [18] indicate this possibility. Electrical potential difference between the aqueous phases of either side of the membrane can be divided into the intramembrane potential difference between the surfaces and the surface potentials between the surface and the bulk solution on both sides of the membrane. Change in the surface potential on one side of the membrane then results in the change in the intramembrane potential gradient under certain conditions

[19] as observed in *Rhodopseudomonas sphaeroides* chromatophores [20,21] and will affect the redox levels of the membrane components. Studies on these aspects, in addition to the kinetic approach used in the present study, will provide fuller information on the mechanism of energy conservation.

Appendix

The Gouy-Chapman theory

Immobilized charges on the membrane surface give rise to a difference in electrical potential at the surface with respect to the bulk aqueous phase. The relation between the net surface charge density, q, and the electrical potential of the surface, ψ_0, is given as follows according to the Gouy-Chapman diffuse double layer theory [5,6,8].

$$q = \pm\left|\frac{RT\epsilon}{2\pi}\sum_i C_{ib}(\exp(-z_i F\psi_0/RT) - 1)\right|^{1/2} \tag{1A}$$

where z_i and C_{ib} represent valence and the bulk concentration of ions. ϵ is the dielectric constant of water. R and F represent the gas constant and the Faraday constant, respectively.

For low values of ψ_0 ($\psi_0 < 50/z$ mV) in the case of a $z:z$ symmetrical salt, Eqn. 1A reduces to

$$\psi_0 = \left(\frac{2\pi RT}{F^2\epsilon C_b}\right)^{1/2} q/z \tag{2A}$$

where C_b is the bulk concentration of the salt.

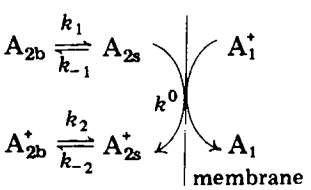
Activity of i-th ion at the membrane surface, a_{is}, with respect to that in the bulk phase, a_{ib}, can be obtained assuming the Boltzmann distribution of the ions.

$$a_{is} = a_{ib}\exp\left(-\frac{z_i F\psi_0}{RT}\right) \tag{3A}$$

Thus the higher-valence cations (anions) are expected to be more concentrated at the negatively (positively) charged membrane surface and to affect the ψ_0 value at low bulk concentration. On the other hand, the concentration of the higher-valence anions is expected to be very low at the negative surface.

The relation between the apparent rate constant and the surface potential

Electron transfer reaction between a component, A_1, on the membrane and a redox reagent, A_2, in the outer medium can be represented as follows:



Subscripts b and s denote molecules in the bulk phase and at the surface. As far as the initial rate is concerned, the reverse reaction at the surface can be

ignored. The rate of reduction of A_1^+ can be expressed as

$$-\frac{d[A_1^+]}{dt} = k^0 a_{2s}[A_1^+] \tag{4A}$$

where a_{2s} represents activity of A_2 molecule at a point close to the surface.

Assuming a steady state condition wtih respect to A_{2s},

$$a_{2s} = \frac{k_1}{k_{-1} + k^0[A_1^+]} a_{2b} \tag{5A}$$

If the rate limiting step of the overall reaction is the reaction at the membrane surface ($A_{2s} + A_1^+ \rightarrow A_{2s}^+ + A_1$), then $k_{-1} + k^0[A_1^+] \approx k_{-1}$. The ratio k_1/k_{-1} can be obtained from the Boltzmann distribution of A_2 molecules between the surface and the bulk phase as given by Eqn. 3A.

$$a_{2s} = a_{2b}\frac{k_1}{k_{-1}} = a_{2b}\exp\left(-\frac{z_2 F\psi_0}{RT}\right) \tag{6A}$$

where z_2 is the valence of A_2.

On the other hand, we calculate the apparent rate constant, k, from the bulk concentration of A_2.

$$-\frac{d[A_1^+]}{dt} = k[A_2][A_1^+] \tag{7A}$$

Then the relation between k and k^0 is given by comparing Eqns. 4A, 6A and 7A.

$$k = k^0\gamma_b \exp\left(-\frac{z_2 F\psi_0}{RT}\right) \tag{8A}$$

or

$$\ln k/\gamma_b = \ln k^0 - \frac{z_2 F}{RT}\psi_0 \tag{9A}$$

and

$$\psi_0 = \frac{RT}{z_2 F}(\ln k_0 - \ln k/\gamma_b) \tag{10A}$$

where γ_b is the activity coefficient of A_2 in the bulk phase which can be calculated by the extended Debye-Hückel expression.

$$\ln \gamma_b = -\frac{z_2^2 \alpha \cdot \mathcal{X}}{1 + r_2\mathcal{X}} \tag{11A}$$

$\mathcal{X}$ is the Debye-Hückel parameter,

$$\mathcal{X} = \left(\frac{8\pi F^2 I}{RT\epsilon}\right)^{1/2} \tag{12A}$$

r_2 is the ionic radius of the A_2 molecule, I is the ionic strength (M). α is a constant expressed as

$$\alpha = \frac{e^2}{2\epsilon kT} \tag{13A}$$

where e is the charge on an electron, and k is the Boltzmann constant.

Substitution of Eqn. 9A by Eqn. 2A gives a linear approximation equation.

$$\ln k/\gamma_b = \ln k^0 - \frac{z_2}{z}\left(\frac{2\pi}{RT\epsilon}\right)^{1/2} C_b^{-1/2} q \tag{14A}$$

Numerical substitution gives (q expressed in $\mu C/cm^2$),

$$\log k/\gamma_b = \log k^0 - 0.078q\,\frac{z_2}{z} C_b^{-1/2} \tag{15A}$$

In practical cases in the thylakoid membranes Eqn. 15A can only be used with monovalent symmetrical salts ($z = 1$).

When the membrane surface is negatively charged ($\psi_0 < 0$), the activities of high-valence cations at the surface are much higher than those of monovalent ones as expected from Eqn. 3A. Thus in the case of symmetrical salt, effectiveness of the salt in decreasing the extent of negative surface potential (and hence in changing the k value) mainly depends on the valence of cation but not on anion. In the case of positively charged membrane surface, the converse holds.

Treatment by Brönsted theory

Assuming the membrane system as a large spherical molecule, of which activity coefficient can be calculated according to the extended Debye-Hückel expression as in the case of protein molecules (Eqn. 11A) the reaction may be considered to be an usual bimolecular reaction between a membrane system (A_1) and a redox reagent (A_2). For the simplicity of the discussion each membrane system is assumed to have only one *P*-700 molecule. Ionic response of the apparent rate constant, k, of the reaction is known to be expressed as follows [1]:

$$\ln k = \ln k_B^0 - \frac{z_1^2\alpha\mathfrak{K}}{1+\mathfrak{K}r_1} - \frac{z_2^2\alpha\mathfrak{K}}{1+\mathfrak{K}r_2} + \frac{(z_1+z_2)^2\alpha\mathfrak{K}}{1+\mathfrak{K}r^*} \tag{16A}$$

where k_B^0 is the rate constant at infinite dilution. The r_i terms and r^* are the radii of the reactants and activated complex expressed in Å.

When the radius of the membrane sphere, r_1, is very large compared to r_2, it can be assumed that $r^* = r_1$. Then, Eqn. 16A reduces to

$$\ln k/\gamma_b = \ln k_B^0 + \frac{(2z_1z_2 + z_2^2)\alpha\mathfrak{K}}{1+\mathfrak{K}r_1} \tag{17A}$$

In a relatively low ionic strength range between 0.01—0.1 M where $\kappa r_1 >> 1$ and when $z_1 >> z_2$, Eqn. 17A reduces to an equation as follows after replacing z_1 by a net surface charge density of the sphere; $q = z_1e/4\pi r_1^2$, and κ by the expression using bulk salt concentration (Eqn. 12A).

$$\ln k/\gamma_b = \ln k_B^0 + \frac{4\pi r_1 q z_2 e}{\epsilon kT} - \frac{z_2}{z}\left(\frac{2\pi}{RT\epsilon}\right)^{1/2} C_b^{-1/2} q \tag{18A}$$

It is obvious that Eqns. 14A and 18A are quite similar to each other. The salt-independent first two terms in Eqn. 18A apparently correspond to $\ln k^0$ in Eqn. 14A. These terms also take the effect of assumption of placing only one

P-700 on each membrane sphere into account. Thus the same salt-induced change in the apparent rate constants are predicted by these two types of treatment at relatively low surface potential values as far as symmetrical salts are used. However, in the case of asymmetrical salts, both the higher-valence cations and anions are assumed to contribute to change k in the same effectiveness according to Eqn. 18A but not according to the precise expression of Eqn. 14A (i.e., Eqns. 9A and 1A) derived using the Gouy-Chapman theory. This discrepancy partially comes from the fact that in the former treatment using the Debye-Hückel expression, ionic strength, but not the contributions of each ions as in Eqn. 1A, is used to evaluate the contributions of salts.

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