Biochimica et Biophysica Acta, 504 (1978) 324—340
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BBA 47585

MEMBRANE SURFACE POTENTIAL AND THE REACTIVITY OF THE SYSTEM II PRIMARY ELECTRON ACCEPTOR TO CHARGED ELECTRON CARRIERS IN THE MEDIUM

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(Received March 14th, 1978)

Summary

A hypothesis is proposed to explain the change in the apparent rate constant for the reaction between the primary electron acceptor of System II situated in the thylakoid membrane and the artificial electron acceptors added in the medium. Dark oxidation rate of the primary acceptor by artificial electron acceptors was monitored by measuring the induction of chlorophyll fluorescence in the presence of an electron transport inhibitor, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, in spinach chloroplasts. The apparent rate constant for the oxidation changed widely when the medium pH or salt concentrations were varied, or ionic detergents were added. The change was quantitatively ascribed (1) to the change in the local concentration of electron acceptors at the thylakoid surface due to the electrical potential difference between the surface and the bulk aqueous phase (Gouy-Chapman diffuse double layer theory) and (2) to the situation whereby the apparent rate constant is determined with respect to concentration in the bulk phase.

Values for the surface potential in the vicinity of System II were estimated from the change in the apparent rate constant under various conditions. The results closely agreed with those obtained previously from the rate constant of the dark step of the System II-dependent Hill reaction with ferricyanide (Itoh, S. (1978) Plant Cell Physiol. 19, 149—166).

Application of the hypothesis to various reactions between the added ionic reagents and the endogenous components in the membrane or between the endogenous components situated in different parts of the membrane is discussed.

Abbreviations: CTAC, cetyltrimethylammonium chloride; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; MES, morpholinoethanesulfonate; SDS, sodium dodecyl sulfate.

Introduction

In green plants, the highly organized thylakoid membranes of the chloroplast provide special environments for photosynthetic energy conversion reactions. Electrostatic interaction between immobilized charge groups on the surface of these membranes and ionic species in the outer aqueous medium seems to play a significant role in determining the modes and efficiencies of various reactions performed on the membrane [1,2]. Such interaction may be treated quantitatively in terms of the Gouy-Chapman diffuse double layer theory [3,4]. A recent study by Barber et al. [5] pointed out the importance of such electrostatic interaction in various reactions in photosynthesis. However, as these authors noted [5,6] little is known about the mechanism how interaction between the membrane surface and the solution electrolytes affects membrane reactions or structure.

In a previous study by the author [7], changes in the apparent rate constant of the dark step of the Hill reaction with ferricyanide (reaction between ferricyanide and the System II secondary electron acceptors) were analyzed in sonicated or dibromothymoquinone-inhibited chloroplasts under various conditions and were almost quantitatively explained by a hypothesis in terms of the Gouy-Chapman theory. It was proposed that the apparent rate constant for the dark step changed with the change in local concentration of ferricyanide ion at the membrane surface, and that the local concentration was determined by the electrical potential difference at the membrane surface with respect to the bulk aqueous phase. The hypothesis can explain variations of the rate constant under conditions in which the electrostatic state of the membrane surface was systematically changed.

Itoh and Nishimura [8] recently showed that the accessibility of the System II primary electron acceptor, Q (quencher), to the added ferricyanide or dithionite in the presence of DCMU changed significantly with pH. They proposed that the decrease in electrostatic repulsion between these anionic reagents and negative charges on the membrane surface enhanced the accessibility at low pH where the negatively charged groups on the surface were protonated. Katoh [9] suggested a similar mechanism from his study of the reactivity of the primary acceptor (*C*-550) or of cytochrome *f* to added artificial electron carriers. These results suggest that an application of the hypothesis proposed in the previous study [7] to the reaction between the system II primary acceptor and charged artificial electron acceptors is also possible.

In the present study the hypothesis previously proposed was refined to cover the general case in reactions between membrane constituents and electrolytes in the aqueous medium. The theory was tested in the reaction between the System II primary acceptor and artificial acceptors added in the medium in various electrostatic states of the membrane. The dark oxidation rate of the primary acceptor was monitored by the dark-recovery time course of chlorophyll fluorescence induction [8]. The values for surface potential and net surface charge density calculated under various conditions are discussed.

Materials and Methods

Chloroplasts (Class II) were obtained from spinach leaves as described previously [7] except that medium containing 0.4 M sucrose and 0.03 M NaCl was used for the final washing and stock suspension of chloroplasts. In the measurement of fluorescence, an aliquot of the stock suspension of chloroplasts was diluted about 100 times with reaction medium to give 3—4 μg chlorophyll/ml. Chlorophylls were assayed according to the method of Arnon [10].

The redox state of the primary electron acceptor of System II, Q, was monitored by measuring induction of chlorophyll fluorescence during illumination [11,12]. The fluorescence was measured at 680 nm with an excitation light (480 nm) of low intensity (900 ergs/cm^2 · s) as described previously [8]. As in the previous study [8], the time course of dark oxidation of the primary acceptor was calculated from the dark recovery of fluorescence induction according to Malkin and Kok [12], i.e. from the initial intensity of the fluorescence, F_i, upon illumination after varied lengths of dark intervals according to the equation,

$$\frac{[\mathrm{Q}^-]}{[\mathrm{Q}_\mathrm{total}]} = \frac{F_\mathrm{i} - F_\mathrm{o}}{F_\infty - F_\mathrm{o}} \qquad (1)$$

where Q^- and $\mathrm{Q}_\mathrm{total}$ are the amount of the reduced form and the total amount of the primary electron acceptor, respectively. F_o is the initial fluorescence intensity upon excitation after a long dark adaptation of chloroplasts, and F_∞ the intensity after a sufficient illumination time in the presence of DCMU before addition of ferricyanide. F_∞ and F_o represent the intensities of fluorescence when all of Q is reduced and oxidized, respectively. Apparant rate constant for the Q^- oxidation was calculated by plotting $\log[\mathrm{Q}^-]/[\mathrm{Q}_\mathrm{total}]$ against dark time as shown in Fig. 1. The rate thus calculated was 0—20% larger than that calculated by using the Joliot equation [13], in which variation in the degree of energy transfer between photosynthetic units is taken into account for the relation between the redox state of Q and the fluorescence yield.

The pH value of the reaction medium was checked in each measurement. All measurements were done at room temperature (about 25°C).

Theoretical

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 phase. At equilibrium, electro-chemical potential difference between points near the surface and in the bulk solution distributes ions according to the potential and forms the diffuse double layer of ions adjacent to the membrane surface (Gouy-Chapman theory) [3,4]. Application of the theory to the interaction between the thylakoid membranes and electrolytes in the medium has been attempted by several investigators [5,6] and the limitation of the theory has also been discussed [3—6,14,15].

Concentration of ions at the membrane surface is given by the Boltzmann equation,

$$C_{i_\mathrm{s}} = C_{i\mathrm{b}} \exp(-z_i F\psi_0/RT) \tag{2}$$

where C_{i_s} and $C_{i\mathrm{b}}$ denote the concentrations of the ith ion at a point immediately adjacent to the surface and in the bulk phase. z_i and ψ_0 represent valence of the ion and the electrical potential difference at the surface with respect to the bulk phase. The other symbols have their usual meanings.

According to the Gouy-Chapman theory, the relation between ψ_0, $C_{i\mathrm{b}}$ and the net surface charge density, q, is expressed as follows:

$$q = \pm \left| \frac{RT\epsilon}{2\pi} \sum_i C_{i\mathrm{b}} \left[\exp\left(\frac{-z_i F\psi_0}{RT} \right) - 1 \right] \right|^{1/2} . \tag{3}$$

ϵ is the permittivity of water. When the membrane is suspended in the solution of a z-z symmetrical electrolyte Eqn. 2 reduces to

$$q = 2\left(\frac{RT\epsilon}{2\pi} \right)^{1/2} \cdot C_\mathrm{b}^{1/2} \sinh\left(\frac{zF\psi_0}{2RT} \right) . \tag{4}$$

At relatively low potentials ($\psi_0 < 2RT/zF$), a linear approximation equation is obtained,

$$q = \left(\frac{z^2F^2\epsilon}{2\pi RT} C_\mathrm{b} \right)^{1/2} \psi_0 . \tag{5}$$

Replacement and numerical substitutions at 25°C give,

$$\psi_0 = 4.42\; q/z \cdot C_\mathrm{b}^{-1/2} \tag{6}$$

where q is in μC/cm^2, C_b in mol/l and ψ_0 in mV.

Thus the potential is directly proportional to the net surface charge density and inversely proportional to the square root of salt concentration. This equation predicts that the change in the extent of protonation of the charged groups on the membrane surface, such as is induced by change in the medium pH, affects the magnitude and sign of the potential by changing the q value. In the thylakoid membranes it is expected that the sign of q, hence the sign of ψ_0, will change from negative to positive as the medium pH decreases across the isoelectric point of the membrane. Adsorption of lipophilic ions such as ionic detergents will also change q and make ψ_0 more negative or positive depending on the sign of their charges. Eqn. 2 also predicts that increase in the ionic strength of the medium will decrease the absolute value of ψ_0, but should not change its sign. At infinite ionic strength, ψ_0 will converge on zero at any pH.

Derivation of these equations and the detailed treatises on the multielectrolyte system have been published by several investigators [3—6,14,15].

The hypothesis relating surface potential to the apparent rate constant for the reaction between endogenous components in the membrane and ionic reagents in the bulk phase.

It seems reasonable to assume that a change in the concentration of added ionic reagent at the membrane surface, induced by the change in the ψ_0 value according to Eqn. 2, will affect the apparent rate constant for the reaction between the ionic reagent and endogenous electron transport components in the membrane, since the apparent rate constant is usually determined with respect to concentration in the bulk aqueous phase.

Let us take the case of the reaction between the reduced primary electron acceptor of System II, Q^-, and ferricyanide added in the medium. In the presence of DCMU, oxidation of Q^- by the endogenous secondary electron acceptors is blocked almost completely. The oxidation of Q^- by ferricyanide in the presence of DCMU is known to be essentially a pseudo-first order reaction with respect to the bulk ferricyanide concentration over a wide variety of medium pH values [8],

$$-\frac{d[Q^-]}{dt} = k[Q^-][\text{ferricyanide}]_b \tag{7}$$

where the term with subscript b represents the concentration in the bulk phase. The apparent rate constant, k, which is obtained with respect to the bulk concentration of ferricyanide, is a function of the medium pH.

On the other hand, concentration of ferricyanide ($z = -3$) at the surface with a potential, ψ_0, is given according to Eqn. 1 including activity coefficient terms, γ,

$$[\text{ferricyanide}]_s\gamma_s = [\text{ferricyanide}]_b\gamma_b \exp(3F\psi_0/RT) \tag{8}$$

where terms with subscripts s and b denote concentration or activity coefficient at the surface and in the bulk phase, respectively.

If the reaction rate is determined by the activity of ferricyanide at the membrane surface, the rate can also be expressed as follows,

$$-\frac{d[Q^-]}{dt} = k^0[Q^-][\text{ferricyanide}]_s\gamma_s \tag{9}$$

where k^0 is the rate constant with respect to the surface activity of ferricyanide. By comparing Eqns. 7—9 we obtain

$$k/\gamma_b = k^0 \exp(3F\psi_0/RT)\ . \tag{10}$$

Thus k^0 equals k/γ_b when ψ_0 is zero. After suitable rearrangements Eqn. 10 becomes

$$\psi_0 = 2.303\ RT/3F \cdot [\log(k/\gamma_b) - \log k^0]\ . \tag{11}$$

Numerical substitutions at 25°C give

$$\psi_0 = 20[\log(k/\gamma_b) - \log k^0] \text{ in mV}. \tag{12}$$

Thus the apparent rate constant, k, is a function of ψ_0 and k^0. If values of k^0 and γ_b are given, ψ_0 can be estimated from k. On the other hand, as already shown in Eqn. 5, ψ_0 is a function of q and of concentration of electrolytes in the medium including ferricyanide. In the presence of relatively high concentrations of indifferent salts such as KCl, ψ_0 can be assumed to be mainly a function of the indifferent salt concentration, since surface concentration of ferricyanide ion is negligibly low as far as ψ_0 is negative as seen from Eqn. 2. The value of k^0 can be obtained from the extrapolation of the k/γ_b values to infinite ionic strength, from the intercept value at the infinite concentration of a 1-1 salt such as KCl in the plot of log k/γ_b vs. the inverse square root of the ionic strength, as expected from Eqn. 6.

According to the Debye-Hückel theory with the modification by Robinson and by Guggenheim and Bates [16], activity coefficient, γ_b, at an ionic strength I, is given by the equation below at 25°C:

$$-\log \gamma_b = z^2\left(\frac{0.51\sqrt{I}}{1 + 0.33\,a\sqrt{I}} - 0.2\,I\right) \tag{13}$$

where a is the ionic radius of the ion (4 Å in case of ferricyanide). Thus the relation between the surface potential, ψ_0, and the apparent rate constant, k, can be directly obtained. However, at positive ψ_0 values, ferricyanide concentration at the surface will be very high and ferricyanide itself will work as a major potential-determining ion even in the presence of relatively high concentration of indifferent electrolyte carrying smaller number of charges. The value of k obtained under positive potential values probably gives only rough estimations of ψ_0 unless competition between all the ionic species in the medium is solved by using nonlinear equations, which can be derived from Eqn. 3 [5].

There also exist some uncertainties in the hypothesis which should be taken into account. (1) Estimation of γ_b according to Eqn. 13 has some deviation from the experimentally obtained values [17]. This gives an uncertainty of about ±20% in the calculated potential values in the present study. (2) If the actual reaction deviates from a pseudo-first order kinetics (Eqn. 7) with respect to ferricyanide activity in the bulk phase, the real ψ_0 value is expected to be somewhat different from that calculated according to Eqn. 12. (3) If the rate-limiting step for the reaction is the permeation of ferricyanide to the reaction site in the membrane, with its rate being almost proportional to the surface concentration, Eqn. 12 can be used as it is. Then k^0 is a function of the permeability of ferricyanide in the membrane in the vicinity of the reaction site. On the other hand, if the rate-limiting step is the electron transfer process itself between Q^- and ferricyanide, not only the change in surface activity of ferricyanide but also the change in its electrical potential in the diffuse layer with respect to the bulk phase should be considered, as known in the case of the Frumkin effect in electrode reactions [15]. In the latter case, the actual potential value will be smaller than that calculated according to Eqn. 12.

These treatments with ferricyanide should also be applicable to the reaction between charged reagents in the medium and the electron transport components in the membrane as far as the surface concentration of the added reagent does not saturate for the reaction.

Results

Effects of salts on the apparent rate constant for dark oxidation of the System II primary electron acceptor by ferricyanide

Dark oxidation of the photoreduced System II primary acceptor, Q^-, was followed by measuring the dark recovery time course of the fluorescence induction in the presence of DCMU as described in Materials and Methods. Fig. 1

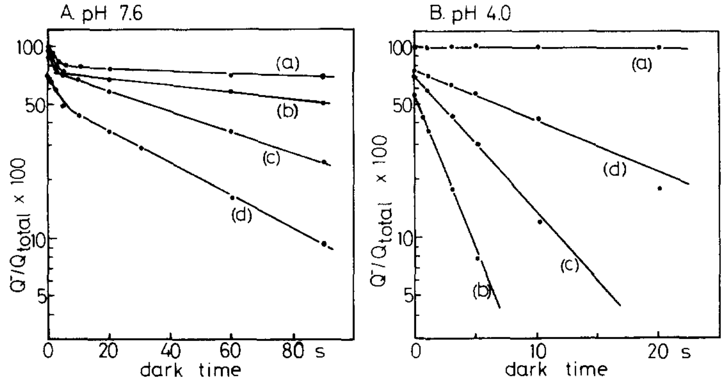


Fig. 1. Effect of KCl on the dark oxidation of Q^- by ferricyanide in the presence of DCMU. A, at pH 7.6: (a) no addition; (b) with 1 mM $K_3Fe(CN)_6$; (c) with 1 mM $K_3Fe(CN)_6$ and 50 mM KCl; (d) with 1 mM $K_3Fe(CN)_6$ and 300 mM KCl. B, at pH 4.0: (a) no addition; (b) with 20 μM $K_3Fe(CN)_6$; (c) with 20 μM $K_3Fe(CN)_6$ and 50 mM KCl; (d) with 20 μM $K_3Fe(CN)_6$ and 300 mM KCl. Reaction mixture contained 10 μM DCMU, 0.4 M sucrose and 5 mM Tricine/sodium buffer, pH 7.6 (A) or 5 mM MES/sodium buffer, pH 4.0 (B).

shows the effects of additions of ferricyanide and KCl on the dark oxidation. At pH 7.6 the oxidation was very slow except for a partial rapid phase just after the cessation of illumination in the low ionic strength medium with DCMU. The half-time of the slow oxidation phase was 550 s under the present experimental conditions. Addition of 1 mM ferricyanide accelerated this slow phase with little effect on the rapid phase (curve b). As previously reported [8] the rate of this slow phase in the presence of ferricyanide obeyed Eqn. 7, suggesting a direct reaction between Q^- and ferricyanide. Addition of KCl plus ferricyanide accelerated the slow phase significantly (curves c and d). A higher rate was obtained at the higher concentration of KCl. On the other hand, addition of KCl alone without ferricyanide little affected the rate (not shown).

In a similar set of experiments at pH 4.0, dark oxidation of Q^- in the presence of DCMU was also very slow without ferricyanide (Fig. 1B, curve a). At this pH, addition of ferricyanide increased the dark oxidation rate more markedly than at pH 7.6 without KCl in accordance with the previous observations [8]. With only 20 μM ferricyanide (curve b) the half time was shortened to 1.7 s (ferricyanide concentration was kept as low as possible in order to minimize deviation from Eqn. 7). This indicates that the apparent rate constant of the reaction between ferricyanide and Q^- increased more than 15 000-fold with a decrease of pH from 7.6 to 4.0. Further addition of KCl in addition to ferricyanide at this pH acted in a way opposite to that at pH 7.6 (curves c and d). With higher concentrations of KCl lower oxidation rates were obtained. Addition of 300 mM KCl decreased the rate to almost one-sixth that without KCl.

These results at pH 7.6 and 4.0 can be explained by assuming a change in the surface concentration of ferricyanide in terms of the Gouy-Chapman theory as proposed in the working hypothesis in the present study. At pH 7.6 the thylakoid membrane surface is assumed to have a negative ψ_0 value since the net surface charge density, q, is estimated to be negative at this pH [7,18,19]. This

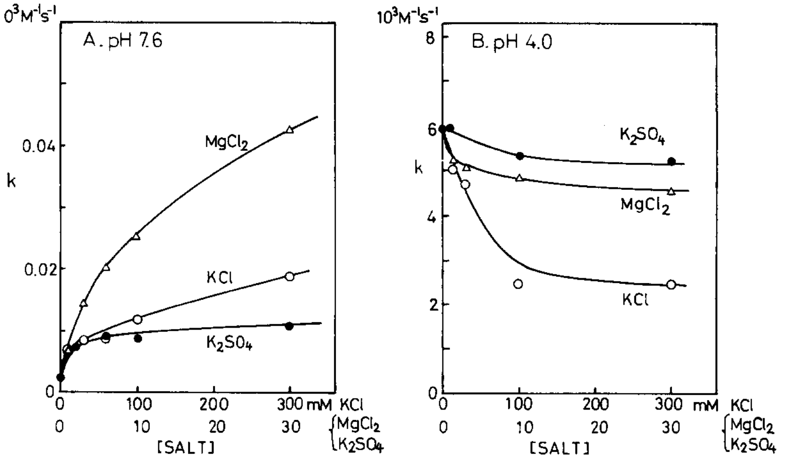


Fig. 2. Effects of various salts on the apparent rate constant, *k*, of the Q^- oxidation by ferricyanide. A, at pH 7.6. B, at pH 4.0. The *k* values were calculated from the oxidation rate of Q^- in the presence of 1 mM (A) or 20 μM (B) $K_3Fe(CN)_6$ according to Eqn. 7. Conditions for the measurements were similar to those in Fig. 1.

negative potential will make the surface concentration of ferricyanide lower than that in the bulk phase and will give a low *k* value in Eqn. 7 at low ionic strength. Addition of KCl will decrease the extent of the negative potential according to Eqn. 6. Thus the apparent rate constant, *k*, will increase by the addition of KCl at this pH due to the increase of surface concentration of ferricyanide resulting from the decrease of the negative surface potential. As the medium pH decreases the *q* value will become more positive due to the protonation of charged groups on the membrane. At pH 4.0, *q* is probably positive since the isoelectric point of the thylakoids is around pH 4.3—5.0 [18,19]. The positive ψ_0 at this pH will increase the surface concentration of ferricyanide and thus will give a large *k* value. Addition of KCl will decrease the magnitude of the potential. Then the apparent rate constant will be decreased with the decrease of surface concentration of ferricyanide. Decrease in the activity coefficient of ferricyanide in the bulk phase by addition of KCl should also be considered.

Thus the opposite effects of KCl on the rate constant at these high and low pH values seems to support the hypothesis proposed in the present study.

Effects of mono- and divalent ions on the apparent rate constant are compared in Fig. 2. Multivalent ions are more susceptible to the change in ψ_0 and will change their distributions greatly at the membrane surface as expected from Eqn. 2. Effects of divalent cation, Mg^{2+}, and anion, SO_4^{2-}, on the values of *k* were studied at pH 7.6 (A) and 4.0 (B) and compared with the effects of KCl. It is expected that $MgCl_2$ will more effectively reduce the extent of negative surface potential by being more strongly attracted to the negatively charged surface but will reduce the positive potential no more than KCl; while K_2SO_4 will be effective in reducing the extent of positive ψ_0 at low pH.

At pH 7.6, addition of $MgCl_2$ increased the *k* value more effectively than that of KCl or K_2SO_4 (Fig. 2A) supporting the hypothesis (note that the abscissa for $MgCl_2$ and K_2SO_4 is expanded 10 times). The effect of $MgCl_2$ was observed in a concentration range one order lower than that of KCl. On the other hand, effectiveness of K_2SO_4 was similar to that of KCl.

In Fig. 2B a similar set of experiments at pH 4.0 is also shown. At this pH, $MgCl_2$ did not significantly decrease the k value. K_2SO_4 was no more effective than KCl in decreasing k also at this pH. When changes in the activity coefficient terms are corrected, almost no decrease in the rate constant seemed to occur by additions of KCl, $MgCl_2$ and K_2SO_4 at this pH (not shown). Much higher concentrations of these salts were required to compete with $Fe(CN)_6^{3-}$ effectively when the surface is positively charged. Non-electrostatic specific adsorption of ferricyanide may also work when the surface is positive.

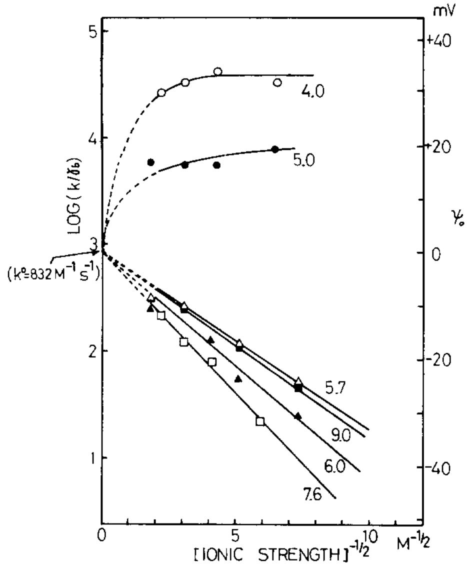
Estimation of surface potential and surface charge density from changes in the apparent rate constant

According to the hypothesis, the apparent rate constant should be related to the surface potential by Eqn. 12 and to the salt concentration by Eqn. 6. A plot of log k/γ_b vs. inverse square root of KCl concentration should give a straight line with a slope determined by the q value. This relation was studied.

The values of k were obtained in experiments similar to those in Fig. 1 at various pH values and at various ionic strength which was mainly varied by adding different concentrations of KCl. γ_b was calculated from the ionic strength, of which contributions from buffer and potassium ferricyanide were included, according to Eqn. 13. Log k/γ_b was plotted against the inverse square root of the ionic strength (Fig. 3). At pH values between 9.0 and 5.7 the plot gave straight lines with different negative slopes confirming the hypothesis. The largest negative slope was observed at pH 7.6. At lower pH the inclination became less with decrease of pH until 5.7. The plot showed little inclinations at pH 4 and 5. At pH values between 5.7 and 9.0, the plot gave the same value at the intercept at infinite ionic strength ($k/\gamma_b = 832\ M^{-1} \cdot s^{-1}$). Therefore, it seemed reasonable to assume the value at the intercept to be the k/γ_b value for ψ_0 = zero, i.e., the k^0 value in Eqn. 12. By using the k^0 value the surface potentials under various conditions were calculated from the k/γ_b values according to Eqn. 12. The ordinate on the right-hand side of Fig. 3 gives the scale for ψ_0 thus calculated. ψ_0 is zero at $k/\gamma_b = k^0$ and becomes about 20 mV more or less positive with the 10-fold increase or decrease of the k value, respectively. By the use of the scale, the ψ_0 value under given pH and salt conditions can be obtained from Fig. 3.

The flat lines obtained in the low pH range (at pH 5 and 4) suggest that Eqn. 12 does not hold at these pH values and that a more complex discussion on the competition of ions at the surface is required. The decrease in the k values seen in Fig. 2B by salt additions seems to be due to a change in γ_b. Changes in surface potential also accompany change in protonation of the surface groups resulting from surface pH change [3,4]. This effect should also be considered at pH values near the isoelectric point where q changes sharply with pH.

In Fig. 4A the value of ψ_0 obtained in Fig. 3 is replotted against pH in the absence and presence of 100 mM KCl. In the absence of added KCl, ψ_0 was most negative at pH 7.6. At pH 9.0 it became a little more positive. With decrease of pH below 7.6, ψ_0 became less negative and turned positive at about



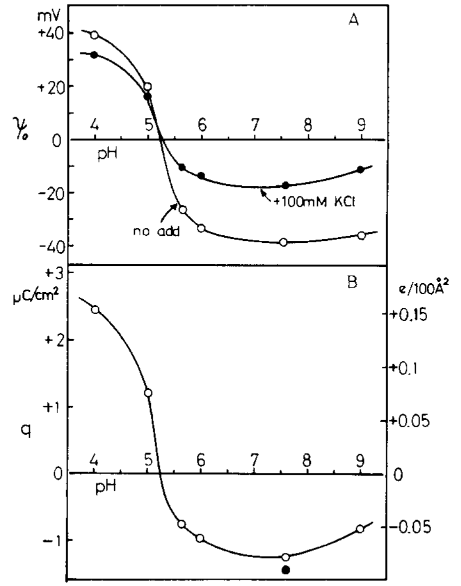


Fig. 3. The plot of log k/γ_b vs. the inverse square root of ionic strength of the medium which was mainly changed by varying KCl concentration at various pH values. The values of k for the oxidation of Q^- by ferricyanide were obtained in experiments similar to those in Fig. 1 and expressed in $M^{-1} \cdot s^{-1}$. The scale for the surface potential on the right-hand ordinate was determined by using a k^0 value of 832 $M^{-1} \cdot s^{-1}$. The reaction mixture contained 10 μM DCMU, 0.4 M sucrose and 5 mM Tricine/sodium buffer for pH values between 9 and 6, or 5 mM MES/sodium buffer for pH values between 5.7 and 4. Concentrations of ferricyanide used were 1 mM at pH values between 9.0 and 5.7, 100 μM at pH 5.0 and 20 μM at pH 4.0, respectively.

Fig. 4. A, pH-dependence of the surface potential in the presence and absence of KCl. Data similar to those in Fig. 3 were replotted against pH. B, pH-dependence of the net surface charge density. The value for net surface charge density was calculated from the surface potentials at 100 mM KCl in Fig. 4A according to Eqn. 5. Closed circle at pH 7.6 indicates the value calculated from the data with phenazine-methosulfate in Fig. 8.

pH 5.2. ψ_0 changed from −39 mV to +39 mV with change of pH from 7.6 to 4.0. Membrane surface in the vicinity of Q seemed to have the isoelectric point at pH 5.2. These values are similar to those obtained from the analyses of the System II-dependent Hill reaction in sonicated or dibromothymoquinone-treated chloroplasts [7] after some corrections of the latter values for the change in the activity coefficient term of ferricyanide. Addition of KCl decreased the extent of both the negative and positive ψ_0 values, with slight change in the isoelectric pH showing apparently opposite effects at pH above and below 5.2 as expected from the hypothesis.

The values of net surface charge density, q, at varied pH calculated according to Eqn. 5 are shown in Fig. 4B. For this calculation the values of ψ_0 at the corresponding pH at 100 mM KCl (Fig. 4A) were used. The q value of 1.3 μC/cm^2 (one negative charge per 1270 Å^2) was obtained at pH 7.6. This value is similar to those obtained in the previous study [7] after some corrections of the latter and is intermediate between that of whole thylakoids obtained from the electrophoretic mobility of chloroplasts [17] (0.82 μC/cm^2) and that obtained from a computer simulation of the dependence of the chlorophyll fluorescence intensity on the concentration of various salts [5] ($2.5 \mu C/cm^2$) which value also seems to indicate the characteristics of membrane surface in the vicinity of System II.

The value of q changed sharply between pH 6.0 and 4.0 suggesting titration of charged groups on the membrane surface. A midpoint pH of 5.1 was obtained from this titration. Protonation of the carboxyl groups of the membrane proteins may be responsible for this change. Number of the groups which protonated between pH 6 and 4 was estimated to be about 0.3—0.6 per chlorophyll molecule provided that chlorophyll molecules have an average density of 1 per 200—400 $Å^2$ on the membrane surface [20].

Effects of detergents on surface potential

Effects of detergents on the dark oxidation of Q^- were studied (Fig. 5). At pH 7.6, addition of a non-ionic detergent, Triton X-100 or of an anionic detergent, SDS, slightly increased the oxidation rate constant (Fig. 5A). On the other hand, a cationic detergent, CTAC, markedly increased the rate constant at very low concentrations. At 0.003% CTAC, k was increased almost 20-fold. On the right-hand ordinate of the figure, ψ_0 value, which was calculated from changes in k value according to Eqn. 12 by assuming γ_b and k^0 are constant, is presented. Increase by 0.003% CTAC corresponds to 30 mV reduction of the extent of negative ψ_0.

At pH 4.4 addition of Triton X-100 slightly increased the k value (Fig. 5B). SDS addition decreased k about 30%. On the other hand, addition of CTAC increased the k value 20-fold at 0.001%. These effects are probably not due to the destruction of membrane structure since they were used at low concentrations within a short incubation time.

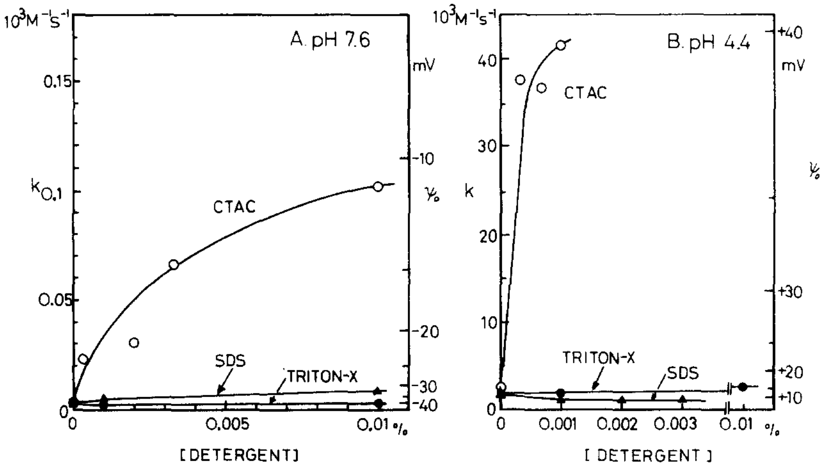


Fig. 5. Effects of detergents on the apparent rate constant, k, of Q^- oxidation by ferricyanide. A, at pH 7.6. B, at pH 4.4. The k values were calculated from the oxidation rate of Q^- in the presence of 1 mM (A) or 40 μM (B) $K_3Fe(CN)_6$ according to Eqn. 7. Other conditions were similar to those in Fig. 2 except that MES/sodium buffer, pH 4.4, was used in place of that at pH 4.0.

The detergents used are expected to be highly adsorbed on the membrane surface to give a changed surface charge density. Adsorption of CTAC on the surface will make ψ_0 more positive and increase k by increasing the surface concentration of ferricyanide, whereas in the case of SDS, the converse holds. In contrast to the salt effect, the detergent effect will not change its direction regardless of the sign of q, and hence, ψ_0, before adding detergent. The results with CTAC (increase of k at both high and low pH) confirm this hypothesis. On the other hand, SDS might be less strongly adsorbed on the negative membrane surface. It showed suppressive effect on the k value only at pH 4.0. Low effectiveness of Triton X-100 indicates that the change in k is mainly determined by the electrostatic characteristics of the surface. The transmembrane electrical potential gradient formed during illumination had little effect on the k value under the present experimental conditions.

Oxidation of Q^- by phenazinemethosulfate and p-benzoquinone

In order to test the hypothesis, Q^- oxidation in the presence of electron acceptors having positive (phenazinemethosulfate) and no charge (*p*-benzoquinone) were studied. In the analysis of effects of concentrations of phenazinemethosulfate and *p*-benzoquinone on the dark oxidation rate of Q^- at pH 7.6, the rate was proportional to the concentrations of the electron acceptors, giving pseudo-first order kinetics (Fig. 6). Judging from these concentration dependences, analyses similar to that with ferricyanide seemed to be possible with these reagents.

Q^- oxidation rate in the presence of these electron acceptors was measured at varied concentrations of KCl (Fig. 7A). With increase of KCl concentration the rate with phenazinemethosulfate decreased. Addition of 100 mM KCl

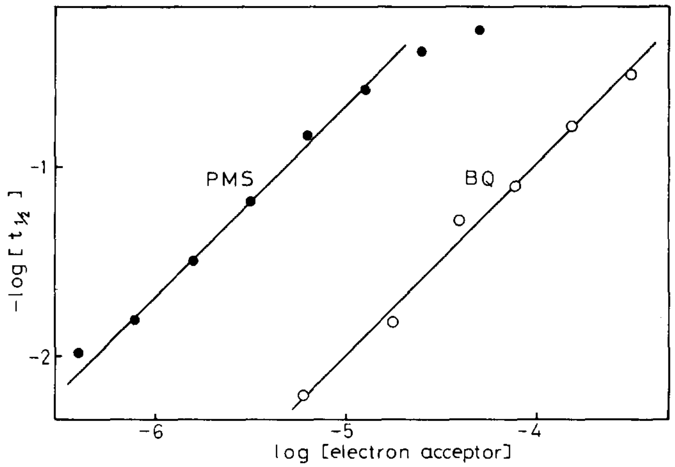


Fig. 6. Effects of phenazinemethosulfate (PMS) and *p*-benzoquinone (BQ) concentrations on the Q^- oxidation rate at pH 7.6. Q^- oxidation was measured with varied concentrations of phenazinemethosulfate (closed circles) or *p*-benzoquinone (open circles). Half-oxidation times, $t_{1/2}$, and concentrations were expressed in M and S, respectively. Solid straight lines represent the first-order kinetic relations between rates and concentrations of the electron acceptors. Reaction mixture contained 10 μM DCMU, 0.4 M sucrose and 5 mM Tricine/sodium buffer, pH 7.6.

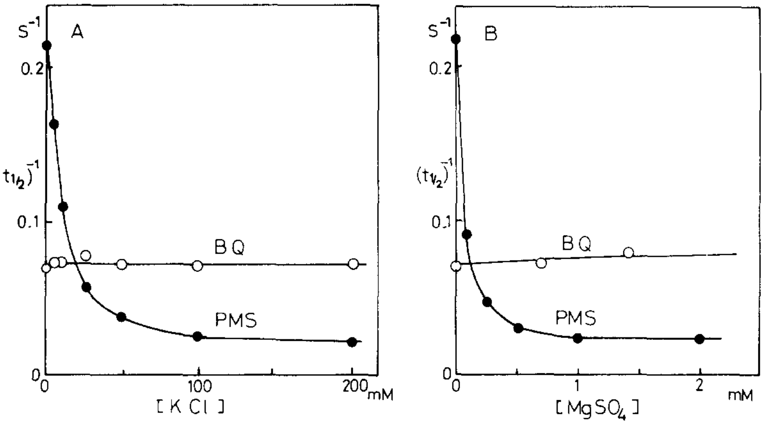


Fig. 7. Effects of KCl (A) and $MgSO_4$ (B) concentrations on the rate of Q^- oxidation by phenazinemethosulfate (PMS) or by *p*-benzoquinone (BQ) at pH 7.6. Concentrations of phenazinemethosulfate and *p*-benzoquinone were 80 μM and 0.4 mM, respectively. Reaction mixture contained 10 μM DCMU, 0.4 M sucrose, 5 mM Tricine/sodium buffer, pH 7.6 and varied concentrations of salts.

decreased the rate to $\frac{1}{10}$ of that without KCl. On the other hand, the rate with *p*-benzoquinone was not affected by the KCl addition. These results clearly suggest that KCl affects the Q^- oxidation rate with phenazinemethosulfate electrostatically. Decrease of the extent of negative surface potential by KCl addition probably decreased the surface concentration of phenazinemethosulfate as expected from the hypothesis. Non-dependence of the rate with *p*-benzoquinone on the KCl concentration indicates that conformational change of the thylakoid membrane by KCl, if any, has negligible effect on the reactivity of Q^- compared to that induced by the change in the electrostatic state of the surface.

As in the case with ferricyanide, $MgCl_2$ was more effective than KCl in changing the oxidation rate (Fig. 7B). Also in this case the rate with *p*-benzoquinone was little affected by the salt.

It was also noted that the effective concentrations of KCl or $MgCl_2$ were a little lower than those with ferricyanide seen in Fig. 2. This discrepancy was due to the difference in the ionic strength of the medium before addition of these salts, i.e. differences between the contributions from 80 μM phenazinemethosulfate and from 1 mM potassium ferricyanide.

Estimation of ψ_0 from the reactivity of Q^- with phenazinemethosulfate

As in the case with ferricyanide, relation between the changes in the rate constant of Q^- oxidation with phenazinemethosulfate and in the value of ψ_0 was studied. With a little modification of Eqn. 11 ($z = +1$ for phenazinemethosulfate), the following relationship is expected,

$$\psi_0 = 60(\log k/\gamma_b - \log k^0) \text{ in mV.} \qquad (14)$$

Fig. 8 shows the plot of $\log k/\gamma_b$ against the inverse square root of ionic strength which was changed by adding varied concentration of KCl. The plot gave a straight line giving a k^0 value of 120 $M^{-1} \cdot s^{-1}$. A scale on the right-hand ordinate gives a measure for ψ_0 calculated from Eqn. 14. Dependence of ψ_0 on the ionic strength was similar to that seen with ferricyanide (Fig. 3). From the

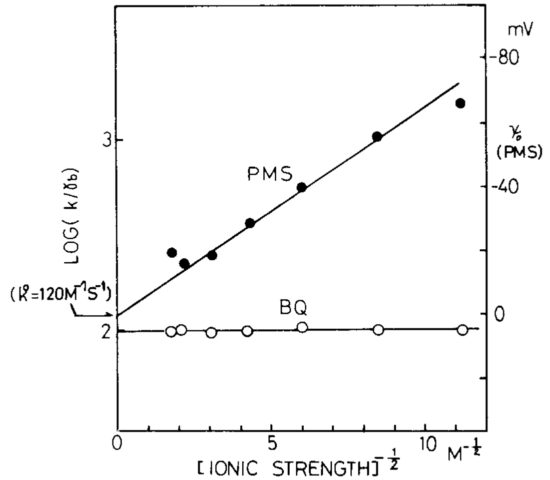


Fig. 8. The plot of log k/γ_b against the inverse square root of ionic strength at pH 7.6 for the Q^- oxidation by phenazinemethosulfate (PMS) and by p-benzoquinone (BQ). The values of k were calculated from the data in Fig. 7A and expressed in $M^{-1} \cdot s^{-1}$. The scale for the surface potential on the right-hand ordinate was calculated from the data with phenazinemethosulfate using a k value of 120 $M^{-1} \cdot s^{-1}$.

slope of the plot, a value of 1.47 $\mu C/cm^2$, which was almost the same as that obtained in Fig. 3, was obtained for the net surface charge density, q.

In the same figure the rate constants with p-benzoquinone are also shown. The rate constants were not affected by ionic strength, and therefore by ψ_0. It is noted that, although the apparent rate constant with phenazinemethosulfate was more than 10 times that with p-benzoquinone in the low ionic strength medium, k^0 values for both oxidants were almost the same. These k^0 values were lower than that for ferricyanide.

Effects of detergents on the reaction with phenazinemethosulfate and p-benzoquinone

According to the hypothesis proposed in the present study, addition of a cationic detergent but not an anionic or nonionic detergent is expected to make membrane surface more positive and decrease surface concentration of phenazinemethosulfate resulting in the lower apparent rate constant.

Effects of detergents on the Q^- oxidation rate with phenazinemethosulfate are shown in Fig. 9A. On addition of SDS or Triton X-100, the rate was little affected as in the case with ferricyanide at pH 7.6. Addition of CTAC, on the other hand, decreased the rate significantly as expected.

In Fig. 9B effects of these detergents on the Q^- oxidation rate with p-benzoquinone are also shown. In this case CTAC as well as the other detergents affected the rate constant slightly in the concentration range at which marked decrease in the rate constant with phenazinemethosulfate was observed. These results with p-benzoquinone and with phenazinemethosulfate suggest that the effect of CTAC on the rate constant with phenazinemethosulfate is mainly due to the change in electrostatic characteristics of the membrane surface and that the other effects of the detergents will produce little change in the reactivity of Q^- under the present experimental conditions.

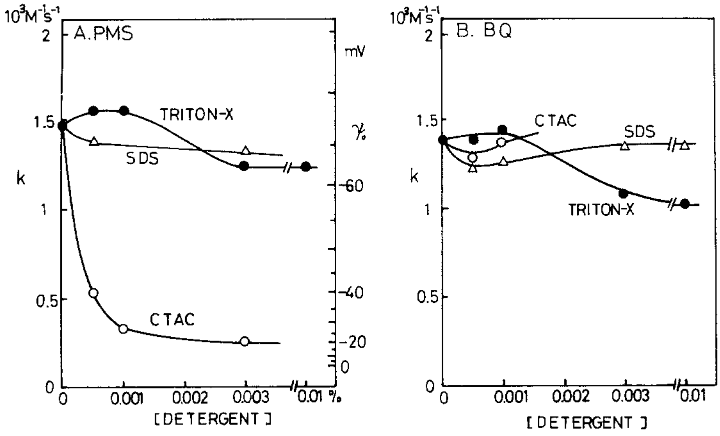


Fig. 9. Effects of detergents on the apparent rate constant for the Q^- oxidation by (A) phenazinemethosulfate (PMS) and (B) *p*-benzoquinone (BQ). The values of k were calculated from the oxidation rate of Q^- in the presence of 80 μM phenazinemethosulfate and 0.4 mM *p*-benzoquinone. Other conditions were similar to those in Fig. 6. The scale for ψ_0 on the right-hand ordinate of (A) was determined by using the same k^0 value as in Fig. 8.

On the right-hand ordinate of Fig. 9, a scale for ψ_0 which corresponds to the values of k with phenazinemethosulfate, calculated according to Eqn. 14 using the k^0 value of 120 $M^{-1} \cdot s^{-1}$, is also shown.

Discussion

The analysis in the present study suggests the validity of the hypothesis that the apparent change in the rate constant for the reaction between a charged electron acceptor and the primary acceptor of System II reflects the surface potential induced change in the local concentration of the acceptor in the electrical diffuse layer immediately adjacent to the membrane surface. The more positive the surface potential, the higher/lower the apparent rate constant with the negative(ferricyanide)/positive(phenazinemethosulfate) acceptor. Variation in the medium pH or the addition of detergents changes the surface potential by changing the net surface charge density directly, while addition of salt, by screening the surface charges. Change in the apparent rate constant under various conditions is quantitatively explained by a hypothesis based on the Gouy-Chapman theory.

Analysis with an uncharged electron acceptor, *p*-benzoquinone, suggests that changes in membrane structure probably occurring with the surface potential change, play a negligible role in determining the apparent rate constant for the Q^- oxidation by electron acceptors under the present experimental conditions.

The value for the surface potential and the net surface charge density estimated in the present study were similar to those estimated previously [7] from the rate constant for the dark step of the System II-dependent Hill reaction (possibly the reaction between ferricyanide and plastoquinone in the membrane) after some corrections of the latter for the change in the activity coefficient. Thus electrostatic characteristics of the thylakoid membrane surface in the vicinity of System II seems to have profound effects on photosynthetic electron transfer. The isoelectric pH of the surface was estimated to be about 5.2. This pH seems a little higher than the electrophoretically determined isoelectric pH of the whole thylakoid membrane (pH 4.3—5.0) [18,19]. The midpoint pH (about 5.1) in the span of titration of the surface charge in the present study was a little lower than the isoelectric pH. This value was also a little higher than those estimated by other methods [6,18,19] for the thylakoid membrane, but was similar to that estimated by titration for Ca^{2+}-binding to the thylakoids [21]. These values obtained in the present study may indicate the characteristics of local membrane surface in the vicinity of System II-reducing sites. However, it can be said that electrostatic characteristics of the membrane surface are not significantly different from those of the whole thylakoids.

The reactions between the endogenous components and charged electron carriers in the medium seem to give a reliable and convenient means of probing electrostatic characteristics of the lacal domain of the membrane surface in the vicinity of the reaction sites which cannot be measured by other methods.

It is also noted that the k^0 values for electron acceptors showed a different order of effectiveness from that of the apparent rate constants. The k^0 value for ferricyanide, which may be less permeable into membrane than the other acceptors studied in the present study, was the highest. The result suggests that the factor which determines the k^0 value is not only the permeability to the membrane and that Q^- molecule is situated close to the membrane surface.

Various observations on the electron transport reactions in the thylakoid membrane; changes in the reactivity of *C*-550 or of cytochrome *f* to external redox reagents by salt additions [9], increase of the reduction rate of a heteropolyanion, silicomolybdate, in the presence of DCMU at low pH [22] or by addition of Mg^{2+} [23], etc., are satisfactorily explained by the hypothesis in the present study.

Renger [24] reported that the trypsin digestion of chloroplasts increased the reactivity of the primary acceptor of System II to ferricyanide in the presence of DCMU. Itoh and Nishimura [8] confirmed this and showed that the increase of the reactivity by trypsin digestion was less marked than that observed by lowering medium pH. Analyses in the present study suggest that these results are explained by a mechanism invoking a partial elimination of the negative surface charges on proteins in the vicinity of the reaction sites, by digestion. The protein eliminated by trypsin digestion may not necessarily be a specific cover over the primary acceptor.

In contrast to the System II-reducing side, that of system I shows activity with ferricyanide too high for an analysis similar to that in the present study. The ferricyanide reduction was affected little by salt additions [7]. This suggests that the reactivity and its response to change in electrostatic environments vary depending on the localization of the component on the membrane.

Proton uptake during illumination may thus change the reactivity between endogenous electron transport components situated on the inner side of the membranes, such as the oxidizing sites of both Systems I and II, as well as their reactivities to artificial electron carriers, since a positive surface potential change of about 80 mV took place even in the presence of 10 mM KCl if the medium pH decreased from 7.6 to 4.0 (Fig. 3). Walz et al. [2] reported that electron flow from water to System I was suppressed in the presence of high concentration of salts. This may indicate that the reaction rate between endogenous components was affected by the change in surface characteristics of the thylakoid membrane. Study of the electrostatic state of the membrane surface in addition to the hydrophobicity of the membrane will give valuable information on the mechanisms of the energy conservation reactions in photosynthesis.

Acknowledgement

The author thanks Dr. M. Nishimura for his valuable suggestions and criticism during the work. This work was supported by financial aids from the Ministry of Education and from the Ito Science Foundation.

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