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PHOTOSYSTEM II OXIDATION OF CHARGED ELECTRON DONORS

SURFACE CHARGE EFFECTS

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Summary

Reactions occurring on the oxidizing side of Photosystem II have been studied in Tris-washed chloroplasts by monitoring the decay kinetics of EPR signal IIf, arising from the photoinduced oxidation of Z, an intermediate in the electron transport chain between *P*-680 and the water-splitting enzyme. Upon addition of electron donors, signal IIf follows pseudo-first order decay kinetics with rates dependent on the chemical nature of the donor. Negatively charged donors (I^-, $Fe(CN)_6^{4-}$, $W(CN)_8^{4-}$) are poor reducing agents for Z^+ whereas neutral donors (benzidine, hydroquinone, diphenylcarbazide) are more efficient, their effectiveness paralleling their lipophilicity. The slow signal IIf reduction observed with the charged donors is consistent with the non-polar nature of the thylakoid membrane and a location for Z toward the inner membrane surface. It most probably exists in a hydrophobic site as indicated by the positive correlation between rate constant and lipophilicity for the neutral donors.

A detailed study of the mechanism of Photosystem II reduction by ascorbic acid has been carried out. The pH dependence of the decay kinetics of signal IIf in the presence of this donor is consistent with a model in which both the neutral acid and the ascorbate mono-anion serve as reducing agents to Z^+. The second-order rate constant for reduction by the mono-anion is less than that of the neutral acid and is found to vary with the suspension pH. This observation is interpreted to indicate the occurrence of negative charge on the inner membrane surface in the vicinity of Z. Additional experiments, which assessed the effect of mono- and divalent cations and of cationic detergents on the signal

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Abbreviations: H_2Asc, neutral ascorbic acid; $HAsc^-$, ascorbate mono-anion; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

IIf reaction rate constants, support both the presence of negative surface charge and its location on the membrane inner surface.

Introduction

The number and location of electron transfer components on the oxidizing side of Photosystem II is, at present, ambiguous. Current models locate at least three species in this region: the reaction center complex, *P*-680; an intermediate electron carrier, Z, most likely derived from a quinone species, which gives rise to EPR signal IIvf in its oxidized, Z^+ state; and a manganese-containing protein which carries out the water oxidation reaction [1,2]. Several treatments, including mild heating, Tris-washing and treatment with chaotropic agents, serve to inhibit oxygen evolution in this system by interrupting electron transfer between the water-oxidizing protein and the Z species [3]. Under these conditions, the reduction half-time of the photoinduced Z^+ radical is slowed considerably and is typically in the 500 ms range in the absence of exogenous electron donors. The EPR signal observed from Z^+ in inhibited chloroplast suspensions has been disignated signal IIf [3]; its decay kinetics are taken to reflect the reduction of Z^+. In previous work [4] it was shown that the decay of signal IIf follows pseudo-first order kinetics in the presence of several commonly used electron donors including hydroquinone, phenylenediamine and diphenylcarbazide. The kinetic simplicity of this system makes it especially useful because quantitative models of the system can be developed and tested. Of greater significance, however, is the intrinsic importance of the reaction itself. Aside from the recent observation of absorbance changes associated with the photooxidation of *P*-680 [5], the rise and decay of signal IIf and of its physiological counterpart, signal IIvf, are the only directly observable reactions occurring on the oxidizing side of Photosystem II. Thus, a detailed study of the kinetic and structural factors which influence electron transfer through the Z species will provide insight into membrane phenomena associated with the water-splitting process.

In the present study, ascorbic acid has been used not only as a reducing agent for Z^+, but as a probe to reactions occurring on the water side of Photosystem II. Ascorbic acid has two characteristics which make this possible. First, like the donors listed above, it effectively alters the kinetic rate of Z^+ reduction at relatively low concentrations. However, unlike these donors which are all neutral at physiological pH values and, therefore, of little use in studying ionic or surface charge effects, ascorbic acid exists largely as the ascorbate mono-anion in the pH range from 4.5 to 9.5, providing the system with a negatively charged donor. We show below that other negatively charged donors, among them I^-, $Fe(CN)_6^{4-}$ and $W(CN)_8^{4-}$, are effective only at prohibitively high concentrations.

From our results, we have developed a kinetic model which postulates that both neutral ascorbic acid and its mono-anion serve as electron donors to Z^+. Through this model, it has been possible to situate Z on the inside of the thylakoid membrane. Furthermore, our results allow us to postulate the existence of a net negative charge on the inner surface of the membrane in the vicinity of Z.

Materials and Methods

Sucrose-washed and Tris-washed broken spinach chloroplasts were prepared from market spinach as previously described [3]. The final reaction solution was 0.4 M sucrose, 10 mM NaCl and 50 mM buffer (Mes for pH 5.5—6.5, Hepes for pH 7.0—7.5 and Tricine for pH 8.0—8.5). In oxygen evolution experiments, these buffers were overlapped at the limits of their pH ranges and no deleterious buffer effects were observed. Chlorophyll concentrations in all experiments were between 3 and 5 mg/ml as assayed by the method of Sun and Sauer [6]. 10^{-4} M EDTA was added to all samples to suppress the hexaquo Mn^{2+} EPR signal present in Tris-washed chloroplasts [7]. 20 μg/ml spinach ferredoxin and $5 \cdot 10^{-4}$ M NADP, obtained from Sigma, were added to the final chloroplast suspensions as an electron acceptor system. The $K_4W(CN)_8$ was prepared as described previously [8]; all other reagents were purchased from standard commercial sources and used as received.

The light source was a model DL-1100 pulsed dye laser (Phase-R Corporation, New Durham, NH) with a rated output energy of 250 mJ (Rhodamine 6G, λ_{max} = 590 nm) and pulse duration of 450 ns. The dye was a solution of cresylviolet perchlorate ($4 \cdot 10^{-5}$ M) and Rhodamine 6G ($2.7 \cdot 10^{-5}$ M) in CH_3OH (λ_{max} = 660 nm) or Rhodamine 640 ($1.1 \cdot 10^{-4}$ M) in water/CH_3OH (6 : 4, v/v) (λ_{max} = 640 nm). The flash intensity was monitored as described by Van Best [9] and was of saturating intensity as determined by a saturation curve for EPR signal IIf formation. In signal-averaged experiments, a flash repetition rate of one every 3 s was used.

All experiments were performed at room temperature with a Varian E-4 spectrometer fitted with a TM_{110} mode cavity (Varian E-238) and a Scanlon EPR flat cell (S-814). Microwave power of 20 mW and modulation amplitude of 5.0 G were used. Data were collected and stored in a Nicolet model 1074 signal averager with a model SD 72/2A analog to digital converter and model SW-71A time base in the 1074 main frame. The number of passes accumulated and the experimental protocol are noted in the figure legends.

Because of the high ionic strength of the solutions used, concentration values have been corrected, as noted in figure legends, to activities using the Debye-Hückel extended equation [10].

Results

Fig. 1 is a collection of experimental traces of signal IIf decay from Tris-washed chloroplasts under different experimental conditions. Fig. 1a is the decay transient observed from chloroplasts (pH 7.5) in the absence of exogenous electron donors. Under these conditions, the decay kinetics are biphasic. Preliminary results suggest that the decay half-times for the two phases exhibit different pH dependencies and that the relative magnitudes of the two signals also vary as a function of pH [11].

Upon addition of exogenous electron donors, ascorbic acid in Fig. 1b and c and hydroquinone in Fig. 1d, signal IIf exhibits exponential decay kinetics. Fig. 2 illustrates the variety of donors that have been studied. Hydroquinone and other neutral, lipophilic donors increase the decay rates of signal IIf at very low (μM) concentrations [4]. However, their electroneutrality diminishes

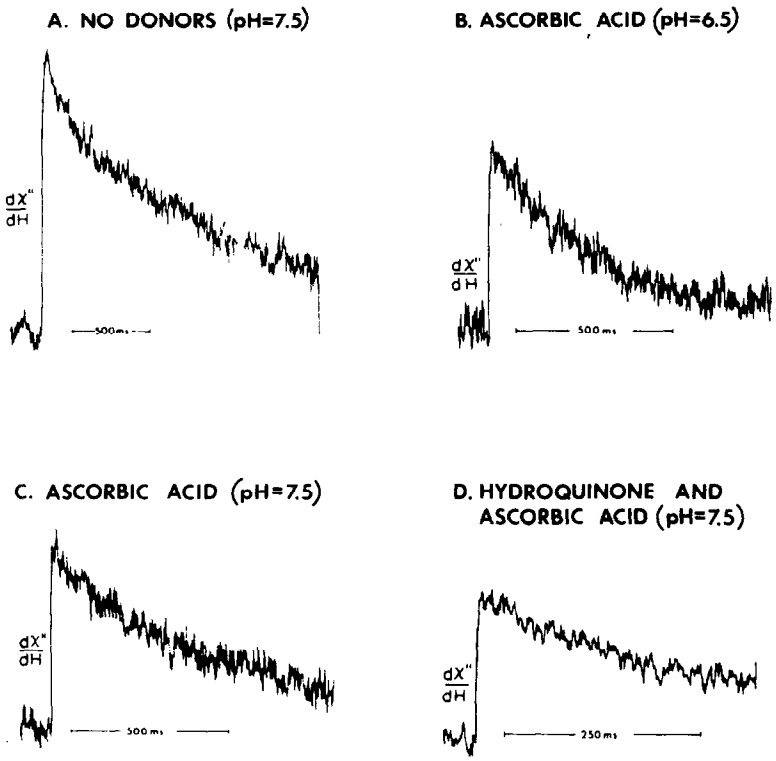

Fig. 1. Effect of exogenous electron donors on EPR signal IIf decay kinetics in Tris-washed chloroplasts. (A) No exogenous donor, pH 7.5, instrument time constant = 10 ms, 64 scans averaged; (B) $2 \cdot 10^{-3}$ M ascorbic acid, pH 6.5, instrument time constant = 3 ms, 120 scans averaged; (C) $2 \cdot 10^{-3}$ M ascorbic acid, pH 7.5, instrument time constant = 3 ms, 120 scans averaged; (D) 10^{-5} M hydroquinone and $2 \cdot 10^{-3}$ M ascorbic acid, pH 7.5, instrument time constant = 3 ms, 200 scans averaged. Note the expansion of the time axis in (D).

their usefulness in studying ionic effects. We have investigated the effectiveness of several anionic donors, including iodide (Fig. 2), ferrocyanide (Table I) and cyanotungstate (Table I) and have found that these species are effective only at donor concentrations in the 10—100 mM range.

TABLE I

RATE CONSTANTS FOR THE REDUCTION OF Z^+ BY EXOGENOUS ELECTRON DONORS

Samples prepared and experimental protocol as described in Materials and Methods.

Donor	$k(M^{-1} \cdot s^{-1})$	Reference
Benzidine	$1.3 \cdot 10^6$	4
Phenylenediamine	$4.6 \cdot 10^5$	4
Hydroquinone	$2.5 \cdot 10^5$	4
H_2Asc	$1.4 \cdot 10^4$	This work
Diphenylcarbazide	$8.0 \cdot 10^3$	4
$HAsc^-$ (infinite salt)	$3.6 \cdot 10^3$	This work
I^-	$2.4 \cdot 10^1$	This work
$Fe(CN)_6^{4-}$	$2.4 \cdot 10^1$	This work
$W(CN)_8^{4-}$	$1.8 \cdot 10^1$	This work

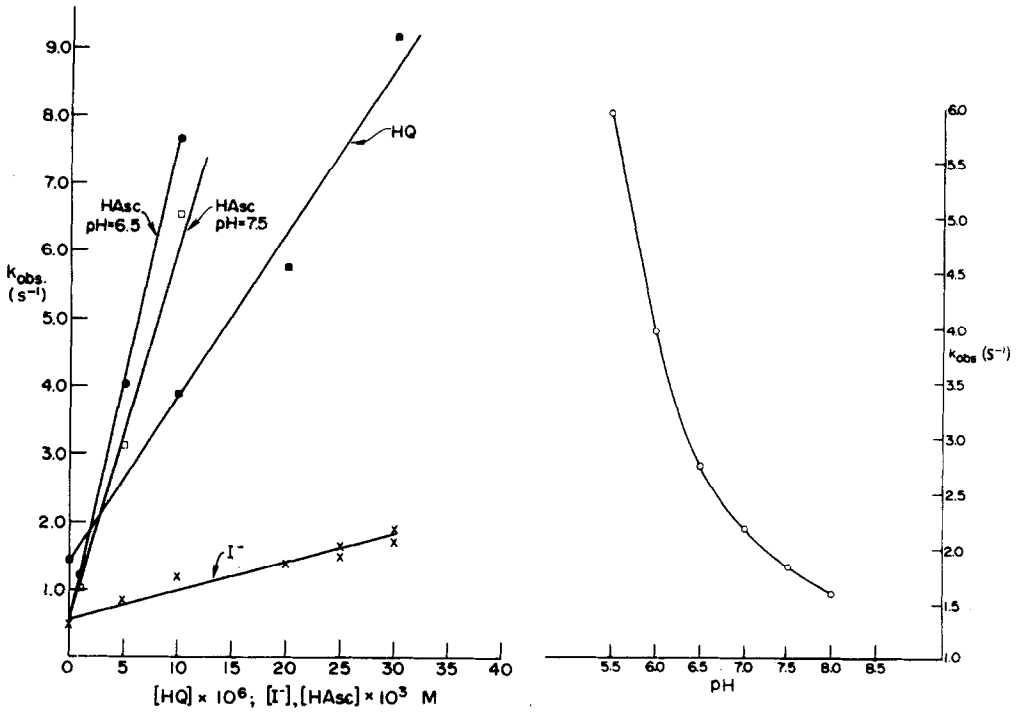

Fig. 2. Donor concentration dependence of the signal IIf pseudo-first order decay constant in Tris-washed chloroplasts at pH 7.5, except where noted, with a 3 ms time constant and 120 scans averaged.

Fig. 3. pH dependence of the signal IIf pseudo-first order decay constant in Tris-washed chloroplasts with 2 mM ascorbic acid as the exogenous electron donor. The experimental conditions are described in Fig. 2.

Ascorbic acid, with $pK_a = 4.10$, exists largely as the ascorbate mono-anion in the pH range studied (5.5—8.5) and thus may provide a charged donor to the system. Moreover, the ascorbic acid system is an effective electron donor to Z^+ at low concentrations (Fig. 2). These two characteristics combine to make ascorbic acid an ideal probe. Although it has been used as an electron donor to Photosystem II [12,13], the kinetics of these reactions have not been examined in detail. In particular, it has not been established whether both the mono-anion and the fully protonated neutral acid are effective as donors.

As the ascorbic acid data in Fig. 2 demonstrate, there is a pseudo-first order reaction between ascorbic acid and Z^+ and this reaction is pH dependent. Fig. 3, a plot of the pseudo-first order rate constants versus pH, verifies this pH dependence over the entire experimental pH range. We have examined a number of models to explain this pH dependence and have calculated theoretical rate constants corresponding to each of the models. These calculated rate constants, k_{calc}, can be compared to observed rates, k_{obs}, obtained from data such as those shown in Fig. 3. The correlation of these values is a good indication of the accuracy of the model. The most simple scheme to explain the pseudo-first order kinetics as well as the pH dependence of the reaction postulates electron donation by the neutral species only and is summarized

by the following reactions:

$$Z^{\cdot+} + H_2Asc \xrightarrow{k} Z + H_2Asc^{\cdot+} \quad (1)$$

where the decay of Signal IIf is given by:

$$\frac{-d[Z^{\cdot+}]}{dt} = k[Z^{\cdot+}][H_2Asc] \quad (2)$$

and the pH dependence is explained by the equilibrium reaction:

$$H^+ + HAsc^- \overset{K_a}{\rightleftharpoons} H_2Asc \quad (3)$$

The correlation between the second-order rate constant calculated for this reaction mechanism and the observed rate is shown in curve (a) of Fig. 4. The fit to the experimental data based on this model is clearly unsatisfactory. We have also found that a model which postulates only the ascorbate mono-anion as a reductant is unable to explain the observed pH dependence.

In a third model, both the neutral acid and its mono-anion are postulated as reducing agents as follows:

$$Z^{\cdot+} + H_2Asc \xrightarrow{k_1} Z + H_2Asc^{\cdot+} \quad (4)$$

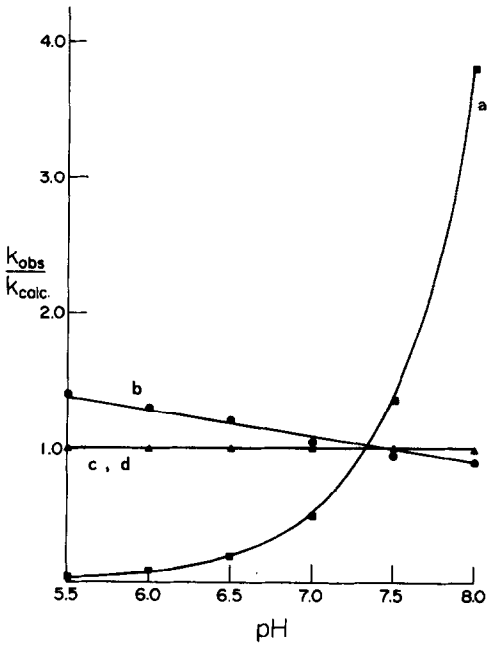

Fig. 4. Correlation between observed (k_{obs}) and calculated (k_{calc}) second-order rate constants for models of $Z^{\cdot+}$ reduction by ascorbic acid. (a) neutral ascorbic acid, H_2Asc, as role reducing agent for $Z^{\cdot+}$; (b) neutral acid, H_2Asc, and ascorbate mono-anion, $HAsc^-$, as reducing agents for $Z^{\cdot+}$; (c) neutral acid and mono-anion as reducing agents, as well as a pH dependent ΔpH across the thylakoid membrane, ΔpH = 0.7 at pH 5.5, ΔpH = 2.0 at pH 8.5; (d) neutral acid and mono-anion as reducing agents with k_2, associated with reduction of $Z^{\cdot+}$ by $HAsc^-$, as a function of pH, ranging from $2.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at pH 5.5 to $8.0 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ at pH 8.5.

$$Z^{+} + HAsc^{-} \xrightarrow{k_2} Z + HAsc^{\cdot} \tag{5}$$

$$H^{+} + HAsc^{-} \overset{K_a}{\rightleftharpoons} H_2Asc \tag{6}$$

The decay of Signal IIf is now given by

$$\frac{-d[Z^{+}]}{dt} = [Z^{+}][HAsc^{-}]\left(\frac{k_1[H^{+}]}{K_a} + k_2\right) \tag{7}$$

In this model, the observed second-order rate constant is associated with the quantity $((k_1[H^+]/K_a) + k_2)$. Using the data of Fig. 2, we calculate k_1 to be $1.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and k_2 to be $6.6 \cdot 10^2\ M^{-1} \cdot s^{-1}$ and with these values we find that the ratio k_{obs}/k_{calc} is close to unity for all pH values between 5.5 and 8.5 (Fig. 4b). Thus the model summarized in Eqns. 4—6 provides a reasonable, first order explanation for the experimental data. The non-zero slope in Fig. 4b indicates, however, a residual pH dependence in the experimental rate constant not explained by this model.

Two additional postulates to the basic model of Eqns. 4—6 have been tested to resolve this pH dependence (Fig. 4c and d). In the first, a variable pH gradient across the thylakoid membrane is postulated to account for the non-zero slope. In the second, k_2, the rate constant associated with reduction of Z^{+} by the ascorbate mono-anion, is assumed to be pH dependent and decreases as the pH is increased from 5.5 to 8.5.

The first model assumes that the species Z is located near the inner surface of the thylakoid membrane. This situation of Z has been postulated [4] and implies that the relevant $[H^+]$ in Eqns. 6 and 7 is that inside the thylakoid ($[H^+]_{in}$), not that of the suspending medium ($[H^+]_{out}$). By assuming a ΔpH across the membrane ($[H^+]_{in} \neq [H^+]_{out}$) and by allowing it to vary from about 0.6 pH unit at pH_{out} 5.5 to about 1.7 pH units at pH_{out} 8.5, the observed and calculated rates are brought into excellent agreement (Fig. 4c). The existence of a pH gradient across the membrane under these experimental conditions is not unreasonable. Yamashita and Butler [14] have shown that upon addition of electron donors, Tris-washed chloroplasts continue to phosphorylate, an indication that a pH gradient develops even after inhibition of oxygen evolution. To test this model, experiments have been carried out using the uncoupler gramicidin, which should collapse any pH gradient, independent of the suspension pH [15]. The model predicts that the rate at high pH values should be most susceptible to uncoupler action as the ΔpH is largest at those pH values and that the rate should decrease as the ΔpH approaches zero. We observe, however, that gramicidin addition produces only a slight increase in rate at pH 8.0. We conclude, therefore, that in Tris-washed chloroplasts under the intermittent flash conditions of our experiments (one flash every 3 s) there is no appreciable buildup of a membrane pH gradient.

The physical basis for the alternative postulate (Fig. 4d) is suggested by the 20-fold difference in magnitude between k_1, the rate constant associated with neutral ascorbic acid, and k_2, associated with the mono-anion in Eqn. 7. This difference indicates that Z^{+} may be more accessible to the neutral form of the reducing agent than to its anion. An explanation for this behavior may be the existence of a negative charge on the surface of the thylakoid membrane.

Natakani and coworkers [16] have conducted electrophoretic experiments which demonstrate the presence of a net negative charge on the exterior of the thylakoid membrane at physiological pH values. They attribute this charge to protein carboxyl groups which are found to have an effective pK_a of about 4.3. If the inner membrane surface were to carry a similar charge in the vicinity of Z, this would explain the efficiency of neutral ascorbic acid relative to the mono-anion in reducing Z^+. According to the Gouy-Chapman diffuse layer model, the approach of a neutral species to such a charged surface is unimpeded, leading to faster kinetic rates. However, the distribution of the ascorbate mono-anion near the charged membrane surface is governed by the surface charge density and the associated surface potential. At high pH, the membrane-bound groups would be largely deprotonated resulting in a large, negative surface potential, a lower concentration of the ascorbate mono-anion near the membrane surface and, thus, to a lower value for k_2. As the pH is decreased these groups would protonate, decreasing the surface charge density, and the value of k_2 would show a corresponding rise. This trend in k_2 is in agreement with that suggested above, i.e., that k_2 is pH dependent and increases as the pH decreases.

To test this model, we have made use of the inverse relationship between the surface potential and the square root of the salt concentration [17]. As the salt concentration of the solution is raised, an increase in the signal decay rate should be observed if the residual pH dependence is a surface charge phenomenon. The experiments were performed at pH 8.0 because under these conditions, over 99% of Z^+ is reduced by the mono-anion (Eqn. 7). We have used KCl, primarily, in these experiments since previous work localized signal IIf near the inner surface of the thylakoid membrane [4] and it has been shown that both K^+ and Cl^- diffuse readily through thylakoid membranes [18]. Fig. 5 shows the results of a series of salt concentration experiments. As the concentration of K^+ is increased, the reaction rate increases as predicted. To insure that the diffusion time of K^+ is not rate limiting, chloroplasts were incubated in a 400 mM salt solution and the signal IIf decay rate in the presence of 2 mM ascorbic acid was determined at various times. During the course of the incubation, no time dependence is observed in the decay rate of signal IIf as seen in the inset to Fig. 5. A divalent cation, Mg^{2+}, was also examined and its effect on the decay kinetics of signal IIf is summarized in Fig. 5. It is effective at lower concentrations than K^+, as predicted by the Gouy-Chapman model, which further substantiates the mediation of a surface charge effect in Z^+ reduction kinetics.

Additional evidence that the mediating surface charge exists on the inner thylakoid surface has been gathered from experiments with cationic detergents. Itoh and coworkers [17,19] have shown that the addition of low concentrations of cetyltrimethylammonium chloride to chloroplast suspensions effectively lowers the outer surface potential. The hydrocarbon tail is adsorbed to the outer membrane surface, preventing the diffusion of the molecule through the membrane, while the positively charged head group lowers the negative surface charge density. In this way, one may selectively mask the surface charge on the outer membrane. In our experiments, however, the choice of detergent is severely limited. EPR spectroscopy requires high chloroplast concentrations

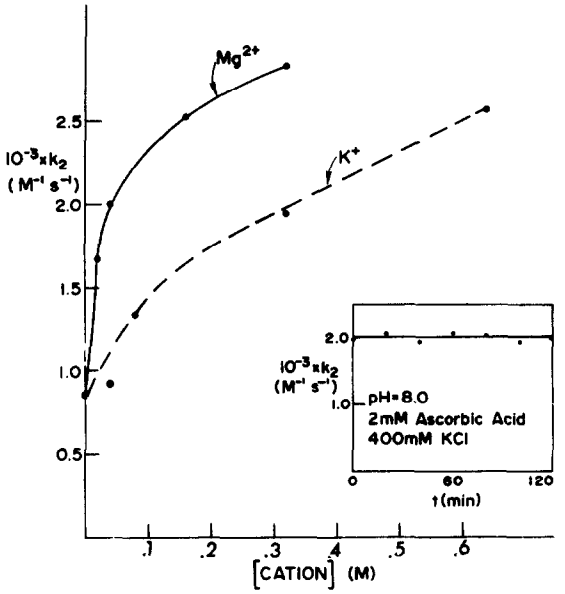

Fig. 5. Salt concentration dependence of the signal IIf second-order decay constant, k_2, in Tris-washed chloroplasts at pH 8.0 and 2 mM ascorbic acid. Chloroplasts were incubated in the salt solution for 5 min prior to initiation of the experiment. Inset: salt incubation time dependence of signal IIf second-order decay constant k_2. The experimental conditions are described in Fig. 2.

(3—5 mg chlorophyll/ml suspension) and correspondingly high detergent concentrations. One is constrained, however, to remain below the critical micellar concentration of the detergent. We have found with cetyltrimethylammonium bromide that above the critical micellar concentration ($9 \cdot 10^{-4}$ M)

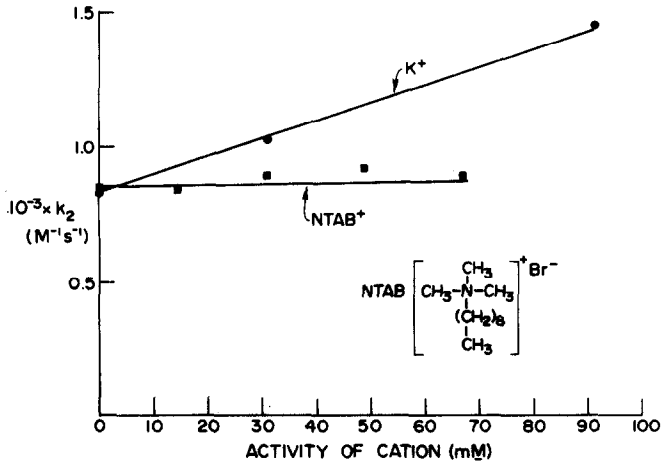

Fig. 6. Cationic detergent activity dependence versus salt activity dependence of the signal IIf second-order decay constant, k_2, in Tris-washed chloroplasts at pH 8.0 with 2 mM ascorbic acid. The experimental conditions are described in Fig. 2.

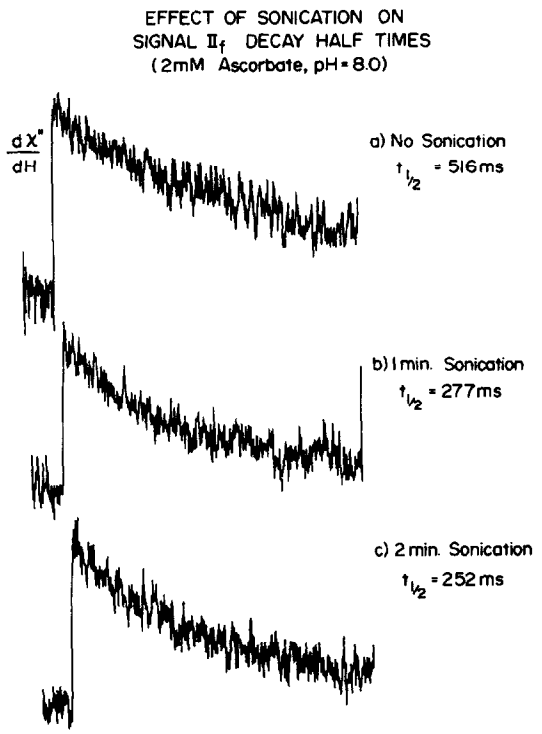

Fig. 7. Effect of sonication on the signal IIf decay half-time in Tris-washed chloroplasts at pH 8.0 with 2 mM ascorbic acid. Other conditions are described in Fig. 2.

[20], there is a complete loss of signal IIf, most likely due to the solubilization of the membrane. One detergent meeting our requirements is nonyltrimethylammonium bromide with a critical micellar concentration of $1.4 \cdot 10^{-1}$ M [20]. Its structure and the results of these experiments are shown in Fig. 6. Addition of the cationic detergent up to its critical micellar concentration has no appreciable effect on the rate of Z^+ reduction by ascorbic acid while in the same concentration range, K^+ concentration effects are readily discerned. Above the critical micellar concentration, there is again a complete loss of signal IIf. These results substantiate the proposal of a surface charge effect arising from the inside surface of the thylakoid membrane.

During the course of this study, we have observed a variation in the absolute kinetic rates among the chloroplast suspensions. We have used market spinach in these experiments and have little control over its age. We have investigated, therefore, the effect that membrane integrity plays in the decay kinetics of signal IIf. A chloroplast suspension was sonicated and these treated samples examined for changes in decay rates. The results are shown in Fig. 7. Upon mild sonication, there is a gradual increase in the reaction rates. The condition of the thylakoid membrane does appear to contribute to the observed rate of reduction of Z^+ by ascorbic acid and is the most likely explanation for the variation in quantitative kinetic rate constants among chloroplast suspensions.

Discussion

The origin of signal IIf, a photoinduced, EPR-detectable radical observed in non-oxygen evolving chloroplast suspensions, has been attributed to an as yet unidentified quinone derivative, $Z^{\cdot}$, which lies along the electron transport chain between the Photosystem II reaction center, *P*-680, and the site of water oxidation. In inhibited chloroplasts, $Z^{\cdot}$ is the principal site of photosystem II-mediated exogenous donor oxidation [4]. In the present study, we have investigated the signal IIf decay rate enhancement induced by several charged donors and have focused, in particular, on the mode of ascorbic acid donation. From the observed pH dependence of the reduction of $Z^{\cdot}$ by ascorbic acid, the kinetic scheme summarized in Eqns. 4—6 has been hypothesized. The overall kinetic rate of signal IIf decay is given by Eqn. 7. Values of $k_1 = 1.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and $k_2 = 6.6 \cdot 10^2\ M^{-1} \cdot s^{-1}$ have been calculated from a set of experiments (Fig. 2) in which the decay rate was examined as a function of both pH and the ascorbic acid concentration. As noted above, we have found the quantitative values of k_1 and k_2 for different chloroplast preparations to be somewhat variable. For example, in a second set of experiments, identical to those described in Fig. 2, values of k_1 and k_2 were found to be $4.0 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and $8.0 \cdot 10^2\ M^{-1} \cdot s^{-1}$, respectively. One cause for this variation appears to arise from alteration of the integrity of the chloroplast membrane as detailed in the sonication experiments of Fig. 7. In addition, the greater variability in the value of k_1, the constant associated with the neutral acid, can probably be explained by the extreme sensivity of the concentration of this species to variation in pH, arising from the low, first pK_a value of ascorbic acid (4.1). In going from pH 6.0 to 6.5 for example, the concentration of neutral ascorbic acid decreases by 70%, whereas, the concentration of the mono-anion, associated with k_2, varies less than 5% over the entire pH range (5.5—8.5). Thus, small pH errors can be magnified in the calculated values of k_1.

These calculated rate constants provide a first-order explanation for the pH dependence of the reduction kinetics of $Z^{\cdot}$ by ascorbic acid but do not explain the residual pH dependence shown in Fig. 4b. The primary cause of this residual dependence appears to arise from surface charge effects which have been incorporated into the reaction mechanism, described by Eqns. 4—6, by allowing k_2, the rate constant associated with the ascorbate mono-anion, to become a function of pH. This relationship between membrane surface charge effects, pH and the value of k_2 has been explained in terms of the Gouy-Chapman diffuse double layer theory (see Results). In this way, calculated and observed rates are brought into excellent agreement (Fig. 4d). The double layer model predicts that the rate of reaction between $Z^{\cdot}$ and $HAsc^-$ should vary not only as a function of pH but with the salt concentration as well. Fig. 5 illustrates that this is indeed true when either KCl or $MgCl_2$ is added to the chloroplast suspension. We have used these data to construct a plot of the inverse square root of the salt activity versus the natural log of k_2 as shown in Fig. 8. A linear relationship is observed, for both salts, as predicted by the double layer model. Moreover, the difference in the slopes of the K^+ and Mg^{2+} data in Fig. 8 indicates that the divalent cation effectively masks the surface charge at lower activities than the monovalent cation, again as

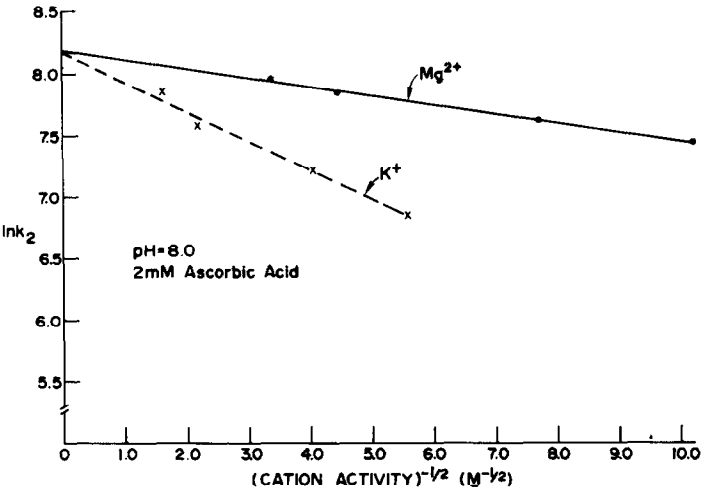

Fig. 8. A plot of the inverse square root of cation activity versus the natural log of k_2, taken from the data in Fig. 5.

predicted by the double layer model. For either salt, the extrapolated rate at infinite salt concentration should reflect the intrinsic ability of $HAsc^-$ to reduce Z^+ when the membrane surface charge has been completely masked. The data of Fig. 8 show that the rate is, as it should be, independent of both the charge and chemical identity of the masking cation. From the data in Fig. 8, we assign k_2^∞ the value $3.6 \cdot 10^3\ M^{-1} \cdot s^{-1}$. A comparison of the value of k_2 in the absence of added salt with k_2^∞ shows that a 4-fold enhancement in k_2 can be achieved through salt addition. Although values of k_2 vary somewhat from chloroplast preparation to preparation, as discussed above, this same degree of enhancement is observed, independent of the chloroplast sample.

As Saha et al. have done for exogenous acceptors to Photosystem II [21] one can characterize the donors we have studied here and in an earlier report [4] according to their lipophilicity as shown in Table I. The progression from benzidine, which exhibits the most rapid reduction of $Z^{+\cdot}$, through biphenylcarbazide roughly parallels the increasing polarity of the donors. These data suggest that Z may be buried in a lipophilic part of the membrane as the lipophilicity of the donor appears to define the efficiency with which it reduces Z^+. At the other end of Table I, I^- through $W(CN)_8^{4-}$, the reaction rates are slow. The negative charge on the outer surface of the thylakoid membranes [16] as well as the low dielectric constant of the membrane should severely hinder the passage of these anions through the membrane and render good reductants ineffective.

The ascorbate mono-anion occupies an anomolous position in Table I; it exhibits a very high reaction rate yet carries a negative charge. This is resolved by postulating that it is the neutral form of the acid which diffuses across the membrane and subsequently dissociates to the mono-anion in the inner thylakoid volume. In this model, $HAsc^-$ is insensitive to the outer surface charge and the observed surface charge mediation of the reaction between

$HAsc^-$ and Z^+ must therefore arise from a charge on the inner thylakoid membrane. This conclusion is strengthened by the results of the detergent experiments (Fig. 6). In these, we added a cationic detergent, nonyltrimethylammonium bromide, to the chloroplast suspension at a concentration below its critical micellar concentration, and recorded its effect on the reduction kinetics of Z^+ by $HAsc^-$. Previous experiments, reported by Itoh and coworkers [17,19], have suggested that cationic detergent addition should selectively modify only the membrane outer surface charge. As our data in Fig. 6 demonstrate, the addition of nonyltrimethylammonium bromide up to its critical micellar concentration has no effect on the reduction kinetics of Z^+ by $HAsc^-$ while addition of similar concentrations of K^+, which freely diffuses across the membrane, significantly alters the rate of reaction. We conclude that the species Z, which gives rise to signal IIf, lies near the inner surface of the thylakoid membrane, most probably in a lipophilic site. We have shown as well that the reduction of Z^+ by a charged donor, the ascorbate mono-anion, is mediated by surface charge effects and that the surface charge exists on the inner surface of the thylakoid membrane in the vicinity of Z. The magnitude of this inner surface charge should now be accessible using data such as those in Fig. 8; these experiments are now in progress.

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