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pH DEPENDENT CHANGES IN THE REACTIVITY OF THE PRIMARY ELECTRON ACCEPTOR OF SYSTEM II IN SPINACH CHLOROPLASTS TO EXTERNAL OXIDANT AND REDUCTANT

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SUMMARY

Changes in the rates of dark oxidation and reduction of the primary electron acceptor of System II by added oxidant and reductant were investigated by measuring the induction of chlorophyll fluorescence under moderate actinic light in 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea-inhibited chloroplasts at pH values between 3.6 and 9.5. It was found that:

(1) The rate of dark oxidation of photoreduced primary acceptor was very slow at all the pH values tested without added electron acceptor.

(2) The rate was accelerated by the addition of ferricyanide in the whole pH range. It was dependent approximately on the 0.8th power of the ferricyanide concentration.

(3) The rate constant for the oxidation of the primary acceptor by ferricyanide was pH-dependent and became high at low pH. The value at pH 3.6 was more than 100 times that at pH 7.8.

(4) The pH-dependent change in the rate constant was almost reversible when the chloroplasts were suspended at the original pH after a large pH change (acid treatment).

(5) An addition of carbonylcyanide *m*-chlorophenylhydrazone or heavy metal chelators had little effect on the rate of dark oxidation of the primary acceptor by ferricyanide.

(6) The dark reduction of the primary acceptor by sodium dithionite also became faster at low pH.

From these results it is concluded that at low pH the primary acceptor of System II becomes accessible to the added hydrophilic reagents even in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

Studies on the fluorescence induction of chlorophyll in green plants and in

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

various algae by Duysens and Sweers [1] showed the existence of a fluorescence-quenching component named Q which functions as the primary electron acceptor of Photosystem II. They also showed that oxidation of the reduced primary acceptor, QH(Q^-), by the secondary acceptor is inhibited by the addition of an electron transport inhibitor, DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea). DCMU also inhibits almost completely the reduction of added Hill oxidants by System II. These results suggest that the primary electron acceptor cannot react directly with the added Hill reagents in aqueous phase. Ikegami and Katoh [2], from a study on the effect of dithionite on the fluorescence induction, concluded that the primary acceptor of System II is situated in a special hydrophobic site where added dithionite cannot react directly. A similar conclusion was drawn by Barr et al. [3], who showed that a rather lipophilic heteropolyion, silicomolybdate, was reduced by System II in the presence of DCMU. Recent studies by Renger et al. [4] and by Renger [5] suggested that the primary electron acceptor is in a hydrophobic region covered by a proteinaceous shield which can be removed by mild trypsin digestion of chloroplasts. All these studies suggest that the primary acceptor of System II is situated in a hydrophobic region to where hydrophilic redox reagents cannot penetrate.

On the other hand, recent studies on electron flow [6] and on luminescence [7] in chloroplasts suggest that the primary acceptor is situated on the outside of the thylakoid membrane.

In this study, the reactivity of the primary electron acceptor to added electron donors and acceptors was analyzed in a wide pH range. The DCMU-insensitive electron flow from the primary acceptor at low pH, which seems to be similar to that noted by Gorkom et al. [8], was extensively studied. The induction of chlorophyll fluorescence was used to obtain information on the redox state of the primary electron acceptor of System II [1].

MATERIALS AND METHODS

Chloroplasts (class II) were prepared from spinach leaves as described previously [9] by using a medium containing 0.05 M phosphate buffer, pH 7.8, 0.4 M sucrose and 0.01 M NaCl. For measurement of fluorescence induction, the chloroplasts were suspended in a medium containing 0.4 M sucrose, 0.05 M KCl and 0.05 M phosphate buffer for pH values between 9.5 and 5.3 or 0.05 M phosphate-succinate buffer for pH values between 5 and 3.6. The final pH of the chloroplast suspension was checked in each measurement.

Acid-treatment of chloroplasts was done by washing the chloroplasts once in succinate buffer, 0.05 M, pH 3.5.

Trypsin digestion of the chloroplasts was carried out at room temperature by injecting a solution of trypsin (from bovine pancreas, Sigma) to the cuvette used for the fluorescence measurement. The digestion was stopped after the desired time by adding a saturating amount of trypsin inhibitor (from soybean, Sigma) to the cuvette. Chlorophyll concentrations were measured according to Arnon [10].

In the measurements of fluorescence induction, a suspension of chloroplasts (about 7 μg chlorophyll/ml), in a 1 × 1 × 4 cm cuvette was illuminated by a 50 W tungsten lamp operated at 9 V(d.c.), through a glass filter (Corning 9782) and an interference filter with a transmission peak at 480 nm (15 nm half band width, Japan

Vacuum Optics). The excitation intensity at the surface of the cuvette was 910 ergs/cm^2 unless stated otherwise. The fluorescence emitted at a right angle to the excitation beam was detected by a photomultiplier (6199, Hamamatsu TV) combined with a red cut-off filter (VR-66, Toshiba) and an interference filter with a transmission peak at 694 nm (15 nm half band width, Japan Vacuum Optics). The signal from the photomultiplier was amplified and recorded on a strip chart recorder (SP J-3, Riken Denshi). All measurements were performed at room temperature (about 20 °C).

Electron transport was measured polarographically with a Clark-type oxygen electrode at 25 °C with an actinic light ($1.2 \cdot 10^5$ ergs/cm^2) from a 300 W tungsten iodine lamp through glass filters, VR-65 (Toshiba) and HA 50 (Hoya).

RESULTS

Effect of ferricyanide on the steady level of fluorescence yield in the presence of DCMU

Fig. 1 shows the effects of additions of DCMU, ferricyanide, ferrocyanide and ascorbate on fluorescence at pH 7.8 and 4.5. In these measurements, a rather low intensity of excitation light was used because at high intensity a slow oxidation of Q^- by ferricyanide would not result in a significant fluorescence decrease. At pH 7.8, a slow increase of fluorescence yield was observed during illumination from the initial low level, the F_0 level, in dark-adapted chloroplasts. According to studies on fluorescence induction by Duysens and Sweers [1] and other investigators [11, 12], this slow increase corresponds to an accumulation of reduced primary electron acceptor, Q^-, by the excitation (a partial shift to the more reduced state). On addition of DCMU, the fluorescence yield showed a marked increase, suggesting an increase of Q^- due to the inhibition of reoxidation of Q^- by DCMU. Addition of ferricyanide did not significantly affect the high steady level showing that direct

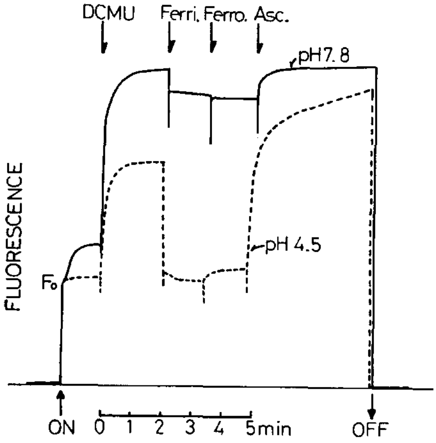

Fig. 1. Changes in the fluorescence due to various additions at pH 7.8 and 4.5. Solid line, pH 7.8. Dashed line, pH 4.5. At times indicated by arrows, 10 μM DCMU, 0.25 mM potassium ferricyanide, 12.5 mM potassium ferrocyanide and a few crystals of sodium ascorbate were added successively. Excitation intensity, 7.8 ergs/cm^2.

oxidation of Q^- by ferricyanide was quite slow in the presence of DCMU at pH 7.8. A slight decrease in fluorescence upon addition of ferricyanide is probably largely due to a decrease in the intensity of excitation by absorption of light by ferricyanide. Further addition of ferrocyanide had almost no effect. On addition of ascorbate, which reduced ferricyanide, the fluorescence intensity recovered to the level with DCMU alone.

At pH 4.5, the slow increase after the onset of excitation light was much less than that at pH 7.8. Upon addition of DCMU, a rapid increase in the intensity was observed, suggesting an accumulation of Q^-. The new level thus attained was somewhat lower than that at pH 7.8. This seems to be due to the inhibition on the oxidizing side of System II [13, 14] caused by acidification, which can also be suspected from the slow increase without DCMU. Addition of ferricyanide induced a rapid and almost complete abolition of the enhancement caused by DCMU. The fluorescence intensity was decreased almost to F_0. A further addition of ferrocyanide did not restore the fluorescence intensity. Reduction of ferricyanide by addition of ascorbate restored the fluorescence yield to a high level, similar to that at pH 7.8. The level attained was higher than that with DCMU alone. This effect of ascorbate can be interpreted to be due to its activity as an artificial electron donor to System II.

From these results, it can be concluded that the inhibitory effect of DCMU is similar at pH 4.5 and at pH 7.8, since DCMU elevated the fluorescence to similar high levels at both pH values if the slow electron donation at the low pH was supplemented by the addition of ascorbate. It can also be concluded that at pH 4.5 ferricyanide caused a reoxidation of Q^- even in the presence of DCMU. The extent of the ferricyanide-induced decrease in the fluorescence yield in the presence of DCMU at low pH was dependent on the intensity of excitation light and on the concentration of ferricyanide added. The higher the excitation intensity the smaller the extent of the decrease due to ferricyanide addition. The higher the concentration of ferricyanide the more the extent of decrease. These results suggest that the reactivity of Q^- to added ferricyanide was increased at pH 4.5.

Effect of ferricyanide on the dark oxidation rate of Q^-

In order to analyze the reaction rate between Q^- and added ferricyanide, fluorescence induction was studied on a faster time scale under moderate intensity of excitation light. Fig. 2 shows a typical example of the effect of ferricyanide on the induction and its dark recovery in the presence of DCMU at pH 4.5. The fluorescence increase during illumination at this pH in the presence of DCMU was somewhat slower than that at pH 7.8, probably due to a partial inhibition at the oxidizing side. The initial fluorescence level, F_i, observed upon illumination 1 min after the first light period was higher than F_0, showing that a significant amount of Q^- was still present. In the presence of both DCMU and ferricyanide (8 μM), the induction of fluorescence during the first illumination was similar to that without ferricyanide. The steady level of fluorescence yield attained was slightly lower than that without ferricyanide. However, upon the second illumination given after a dark period of 1 min, the initial F_i level was found to be almost the same as that of the first illumination, indicating that ferricyanide accelerated the dark oxidation of Q^-, in spite of the presence of DCMU. By varying the dark time the kinetics of this oxidation were studied. The ratio of Q^- to the total amount of Q was calculated according to Malkin

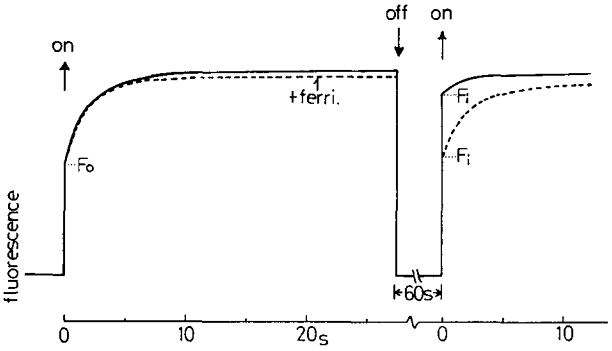

Fig. 2. Effect of ferricyanide on the induction of fluorescence and its dark recovery in the presence of DCMU at pH 4.5. Solid line, in the presence of 20 μM DCMU. Dashed lines, in the presence of 20 μM DCMU and 8 μM ferricyanide.

and Kok [12] by the following equation:

$$\frac{[Q^-]}{[Q]_{total}} = \frac{F_\infty - F_i}{F_\infty - F_0}$$

For the value of F_∞, steady-state level of fluorescence during illumination in the presence of DCMU alone was used in the calculation. Fig. 3A shows the effect of varied concentrations of ferricyanide on the dark oxidation of Q^- in the presence of DCMU at pH 7.8. Without ferricyanide, a biphasic dark oxidation of Q^- was observed. About 10 % of the total Q^- was oxidized within 10 s after the cessation of excitation while the rest of Q^- was oxidized quite slowly. It took about 500 s for the half recovery of Q. Ferricyanide seemed to accelerate the slow phase of dark oxidation. The oxidation rate increased with the ferricyanide concentration. In the presence of 960 μM ferricyanide the half recovery time of Q was shortened to 5 s

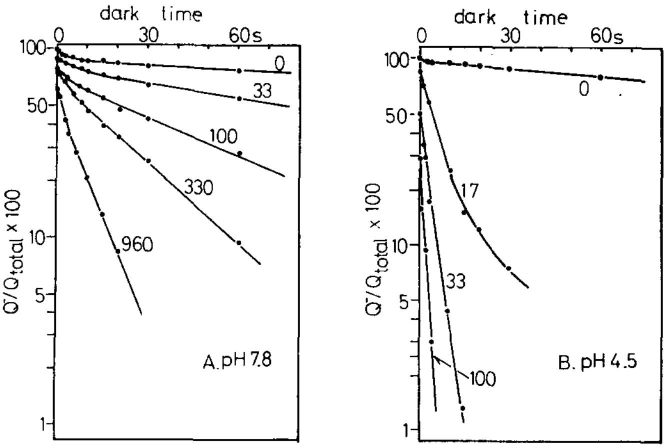

Fig. 3. Effect of ferricyanide on the rate of dark oxidation of Q^- in the presence of 20 μM DCMU. The dark oxidation of Q^- was calculated from the F_i level observed in the second excitation after varied lengths of dark time. The concentration of ferricyanide added is shown in the figure in μM.

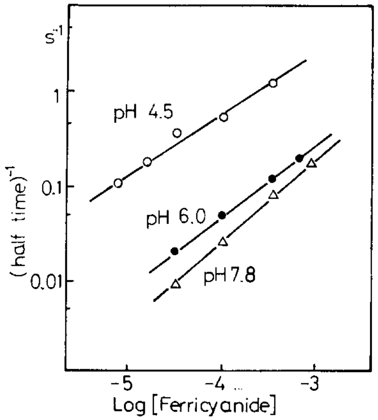

Fig. 4. Dependence of the rate of dark oxidation of Q^- on the concentration of ferricyanide in the presence of 20 μM DCMU at various pH values. Logarithms of the reciprocals of half-oxidation time of Q^-, calculated from the measurements as in Fig. 3, were plotted against logarithms of the concentrations of ferricyanide at various pH values.

(about one hundredth of that without ferricyanide). A decrease in the light level of Q^- (the level at time zero in the figure) was observed at higher concentrations of ferricyanide even at this pH.

Also at pH 4.5 a quite slow dark oxidation of Q^- in the presence of DCMU alone was observed (half time of 300 s, Fig. 3B). This slow oxidation was accelerated markedly by ferricyanide at very low concentrations. The half time for the oxidation became shorter with increasing ferricyanide concentration. It was 0.5 s in the presence of only 100 μM ferricyanide. The lowering of the light level of Q^- by ferricyanide was more pronounced than at pH 7.8. The half time for the oxidation of Q^- at pH 4.5 was about 23 times shorter than at pH 7.8 (both with 100 μM ferricyanide).

To analyze the concentration dependence of the dark oxidation rate, logarithms of the rate constants, approximated by the reciprocals of half-oxidation times, were plotted against logarithms of ferricyanide concentrations at pH 4.5, 6.0 and 7.8 (Fig. 4). The plots gave straight lines with inclinations of 0.74, 0.82 and 0.85 at pH 4.5, 6.0 and 7.8, respectively. In spite of the large difference among the absolute values of rate constants at these pH values, similar inclinations (dependence on ferricyanide concentration) were obtained at extreme pH. It is likely that the mode of oxidation of Q^- by ferricyanide was not different at various pH values. From the data of Figs. 3 and 4, the rate of dark oxidation of Q^- may in first approximation be written as follows,

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

In the equation above, *k* represents the rate constant. Apparently the pH affects the *k* value only.

Fig. 5 shows the pH dependency of the dark oxidation rate of Q^- in the presence of 50 μM ferricyanide and 20 μM DCMU. The rates with and without ferricyanide were plotted on logarithmic scale against pH. Without ferricyanide the

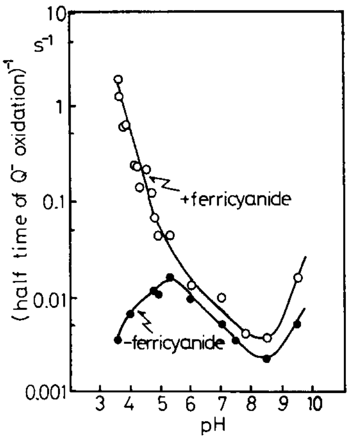

Fig. 5. pH-dependent changes in the rate constant for the dark oxidation of Q^- in the presence of 50 μM ferricyanide and 20 μM DCMU. The reciprocal of the half time of dark oxidation of Q^- was plotted against pH on a logarithmic scale. ●, 20 μM DCMU added. ○, 20 μM DCMU and 50 μM ferricyanide added.

rate was quite low throughout the pH range tested, with a small maximum at pH 5.3. It shows that DCMU was effective in the whole pH range. In the presence of ferricyanide the rate increased with decreasing pH, except at pH 9.5. No maximum or plateau was observed in the pH range between 3.6 and 9.5. The curve had a quite sharp rise below pH 5. At pH 3.6 the half time was 0.5 s, which suggests that if the concentration of ferricyanide was increased to 5 mM, the half time would be about 5 ms. The ratio of the rate constant at pH 3.6 to that at pH 8.5 was about 500. These results indicated that a large increase in the reactivity of Q^- towards ferricyanide took place at low pH.

Reversibility of the effects of low pH

Fig. 6 shows the fluorescence induction of the chloroplasts at pH 7.8 which were once washed in succinate buffer, pH 3.5. The fluorescence increase in acid-washed chloroplasts was slower than that in untreated chloroplasts even in the presence of DCMU, probably due to the irreversible inhibition on the oxidizing side of System II [13, 14]. However, the steady level during the illumination and the rate of dark oxidation of Q^- were only affected a little. Time courses of the dark oxidation of Q^- in the acid-washed chloroplasts with and without ferricyanide are shown in Fig. 7. At pH 7.8, half times for the dark oxidation were 140 and 400 s in the presence and absence of ferricyanide, 300 μM, respectively. These half times were somewhat longer than the values in untreated chloroplasts (Fig. 3A). On the other hand, the rate of dark oxidation of Q^- at pH 4.5 was accelerated significantly on addition of ferricyanide.

These results clearly show that the acceleration of the oxidation of Q^- at low pH is completely reversible and that this low pH-induced acceleration is a different phenomenon from the irreversible low pH-induced inhibition of electron donation to System II.

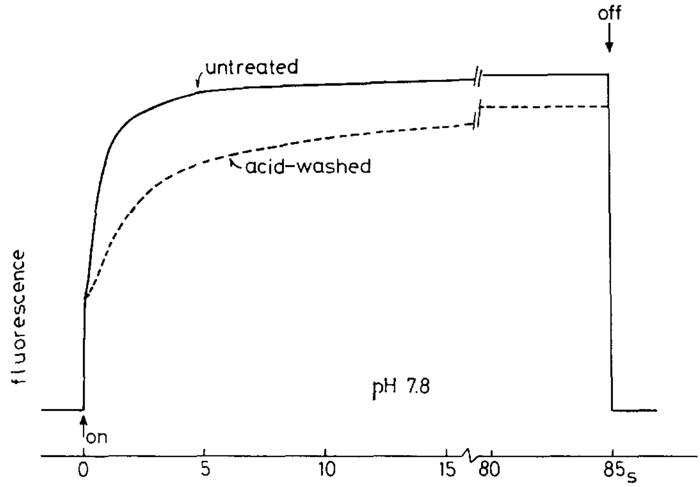

Fig. 6. Effect of acid wash of chloroplasts on the induction of fluorescence at pH 7.8. Measurements were done in the presence of 20 μM DCMU.

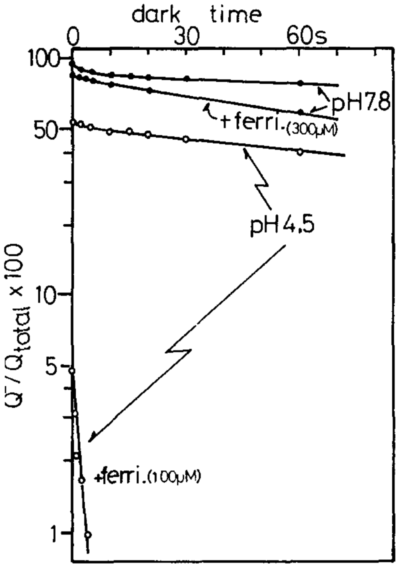

Fig. 7. Effect of ferricyanide on the rate of dark oxidation of Q^- in the acid-washed chloroplasts at pH 7.8 and 4.5 in the presence of 20 μM DCMU. The dark oxidation of Q^- was calculated from the dark recovery of fluorescence induction as in Fig. 3. Chloroplasts were washed with 0.5 M succinate (pH 3.5) and resuspended in the measurement medium at pH 7.8 or at pH 4.5 after being washed in the medium used for the preparation of chloroplasts. Ferricyanide was added in concentrations indicated.

Effect of pH on the dark reduction of Q by dithionite

On addition of a strong reductant, sodium dithionite, Q is known to be reduced in the dark. According to Ikegami and Katoh [2] the dark reduction by dithionite was quite slow if the reductant was added after the addition of DCMU.

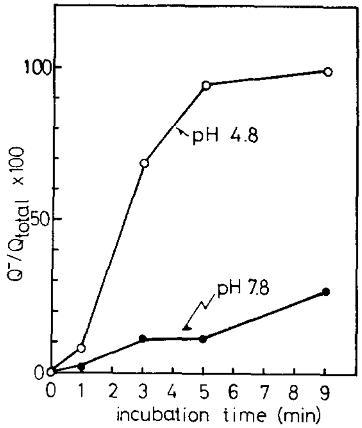

Fig. 8. Time courses of dark reduction of Q by sodium dithionite in the presence of DCMU. The dark reduction of Q by dithionite was calculated from the F_i level after varied lengths of dark preincubation with 2 mM dithionite which was added in crystal 2 min after the addition of 20 μM DCMU.

We studied the effect of pH on the dark reduction of Q by dithionite in the presence of DCMU (Fig. 8). The extent of the F_i level at the onset of illumination was measured to follow the dark reduction of Q after varied length of dark incubation of chloroplasts with 2 mM dithionite which was added 2 min after adding DCMU. At pH 7.8, the rate of dark reduction was quite low as has been reported [2]. At pH 4.8, the rate was much higher. More than 90 % of Q was reduced by dithionite within 5 min at pH 4.8 while it was only 11 % at pH 7.8. The half time for the dark reduction at pH 4.8 was one-seventh of that at pH 7.8.

Effect of CCCP

CCCP (carbonylcyanide *m*-chlorophenylhydrazone) is known to be an inhibitor of System II reactions on the oxidizing side [15] as well as an uncoupler of photophosphorylation. It is known to inhibit the oxidation of Q^- by the component on the oxidizing side of System II in the reverse reaction [16]. We found almost no effect of 10 μM CCCP on the rate of dark oxidation of Q^- by ferricyanide at pH 4.5 in the presence of 20 μM DCMU. This suggests that the observed dark oxidation of Q^- by ferricyanide has almost no relation to the changes on the oxidizing side of System II.

Effect of chelators

On addition of heavy metal chelators such as *o*-phenanthroline, bathocuproine or neocuproine, which were reported to inhibit DCMU-insensitive reduction of silicomolybdate [3], the oxidation rate of Q^- in the presence of DCMU and ferricyanide at pH 4.5 was hardly suppressed. This result indicates that the rapid oxidation of Q^- by ferricyanide at low pH in the presence of DCMU observed in the present study was not due to the electron leak from the site where silicomolybdate accepts electrons. It seems that ferricyanide is directly reduced by Q^- in the reaction observed in the present study.

Effects of trypsin digestion of chloroplasts on the rate of dark oxidation of Q^- by ferricyanide

Trypsin digestion of chloroplasts was recently shown to eliminate the DCMU-sensitivity of the oxygen evolution reaction supported by ferricyanide [5]. It is also known to cause a lowering of the fluorescence yield [4, 17]. Effects of mild trypsin digestion on the extent of the fluorescence induction and on the rate of oxidation of Q^- by ferricyanide in the presence of DCMU were studied (Table I). After 2 min of digestion, a 40 % decrease in the extent of the variable part of fluorescence, expressed as F_v, took place, while the dark oxidation rate of Q^- in the presence of DCMU alone was not affected. In the presence of 300 μM ferricyanide, a half time slightly shorter than that of untreated chloroplasts was observed. In 3 min-treated chloroplasts in which F_v was about one third of that of the untreated chloroplasts, a little enhancement in the oxidation rate took place without ferricyanide. On the other hand, the rate with ferricyanide was found to be much more accelerated. This is consistent with the conclusion of Renger et al. [4, 5] that trypsin digestion of chloroplasts enhances the reactivity of Q^- to ferricyanide. However, the observed acceleration of the dark oxidation is rather low compared to that due to acidification.

DISCUSSION

The results obtained in the present study showed that the reactivity of the primary electron acceptor of System II, Q, to added ferricyanide or dithionite in the presence of DCMU is highly dependent on the pH of the medium. The reactivity was much higher at lower pH values. This confirms results recently reported concerning the existence of a DCMU-insensitive electron flow to ferricyanide at low pH [8, 13, 14]. The results obtained in the present work cannot be interpreted as DCMU becoming less effective at a lower pH, since DCMU added at low pH was effective enough to elevate the level of light-induced Q^- accumulation and to inhibit the reoxidation of Q^- in the absence of ferricyanide. DCMU inhibits electron flow between Q and the secondary electron acceptor almost completely throughout the pH range tested, while Q becomes more accessible to the added hydrophilic reagents

TABLE I

Changes in the extent of fluorescence induction and the half time for the dark oxidation of Q^- with and without ferricyanide in the presence of 20 μM DCMU in trypsin-treated chloroplasts. Chloroplasts were incubated with trypsin (1.7 μg/ml) at room temperature at pH 7.8. The trypsin digestion was stopped by adding trypsin inhibitor. F_v represents the variable part of the fluorescence, observed in dark-adapted chloroplasts. The rate of O_2 evolution in the presence of 1 mM ferricyanide and 1 mM methylamine at pH 7.8 was 85 and 47 μmol O_2/mg chlorophyll/h before and after the 3-min trypsin treatment, respectively.

Incubation time	Ferricyanide concentration			
	0		300 μM	
	F_v	Half time (s)	F_v	Half time (s)
0	100	450	75	90
2 min	57	440	42	62
3 min	26	300	12	20

at decreasing pH. The rate of oxidation of Q^- depended on approximately the 0.8th power of ferricyanide concentration. It suggests that Q^- is oxidized in a near first-order reaction as for the concentration of ferricyanide. It was also shown that Q^- was oxidized at a significant rate by a high concentration of ferricyanide even at high pH in the presence of DCMU.

Ikegami and Katoh [2] suggested that Q is in a special hydrophobic region which is inaccessible to hydrophilic reagents such as dithionite. Recently, Renger et al. [4, 5] showed that trypsin treatment made Q accessible to ferricyanide in the presence of DCMU and concluded that Q is protected by a proteinaceous shield which hinders the access of hydrophilic reagents.

The effect of low pH observed in the present study seems to be in line with these earlier works. By some mechanism, hydrophilic reagents in the aqueous phase may react directly with Q at low pH. A change in the extent of protonation of Q^- cannot explain our results, since the reactivity of both the oxidized and reduced form of Q to the added reagents was increased at low pH. Some change in the nature of the microenvironment of Q from hydrophobic to hydrophilic may occur at low pH. However, the almost complete reversibility of the low pH effect suggests that it is not due to the loss of some component which shields Q from the aqueous phase as was suggested in the case of trypsin digestion of chloroplasts [4, 5]. The rather small enhancement of Q^- reactivity to the added ferricyanide by the trypsin digestion of chloroplasts, compared to that at low pH, suggests that this treatment only partially exposed the primary acceptor to the aqueous phase. A decrease of the electrostatic barrier allowing access of negatively charged hydrophilic redox reagents will also explain the enhanced reactivity at low pH. According to Deamer et al. [18] and Murakami and Packer [19], chloroplast membranes take up 4.5 μmol proton/mg chlorophyll during acidification from pH 7 to pH 4.5. This suggests that a quite significant decrease in the number of negative charges occurs on the surface of the membranes by the acidification, which will strongly increase the accessibility of negatively charged hydrophilic reagents to the membrane surface. It was also shown [19] that acidification caused a reversible decrease in the thickness and the spacing of grana membranes by more than 20 % probably increasing the hydrophobicity of the membranes. These results seem to favor the possibility that the low pH effect seen in the present study is mainly due to the elimination of the electrostatic barrier by protonation of negatively charged groups. It seems reasonable to conclude that the primary electron acceptor molecules are situated on the outer surface of the thylakoid membranes and are densely surrounded by negatively charged groups which hinder the penetration of negatively charged hydrophilic reagents at neutral or high pH, but not at low pH. It may also be suggested that the trypsin treatment partially removes these negative charges in the vicinity of the primary acceptor. However, a possibility still remains that changes in the association between protein molecules or between proteins and lipids in the microenvironment of the primary electron acceptor, due to changes in the net charge of proteins or lipids by protonation, expose the primary acceptor to the aqueous phase [18, 19]. The fact that the pH dependence of the oxidation rate of Q^- by ferricyanide did not show any plateau at low pH side down to pH 3.6 (Fig. 5) suggests that a drastic change in the microenvironment is required to expose completely the primary acceptor to the aqueous phase.

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