A Tris-induced change in the midpoint potential of Z, the donor to photosystem II, as determined by the kinetics of the back reaction

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A new method of measuring the rate of the back reaction from the state Z^+ P680 Q_A^- in Tris-washed chloroplasts is described. By using ratios of back reaction rates we demonstrate a Tris-induced change in the equilibrium between Z and P680 and attribute this change to an alteration of the midpoint potential of Z by Tris treatment. We also demonstrate that the previously observed inhibition of the back reaction by ADRY reagents can be localized at Z and understood in terms of electron donation to Z^+ by ADRY reagents.

Tris-washed chloroplasts *Photosystem II* *EPR signal IIf* *Back reaction* *pH effect* *ADRY reagent*

1. INTRODUCTION

Partial reactions of the donor chain of PS II have been extensively studied in chloroplasts in which the reactions of O_2 evolution have been inhibited. The initial donor to $P680^+$, Z, has been characterized indirectly through optical measurements [1,2] and by direct measurement as the component giving rise to EPR signal IIf in inhibited systems [3,4]. The kinetics of donation to $P680^+$ are modified in these preparations ($t_{1/2}$ 2–20 μs in Tris-washed or NH_2OH-treated chloroplasts [1,2,4], $t_{1/2}$ 50–200 ns in O_2-evolving preparations [5]) and the microwave saturation properties are also altered [6,7]. Here, we have studied the properties of Z by measuring the kinetics of the back reaction from the state Z^+ P680 Q_A^- in DCMU inhibited, Tris-washed chloroplasts. We conclude that in Tris-washed preparations the equilibrium constant for sharing an oxidizing equivalent between Z and P680 is much greater than in normal chloroplasts, and suggest that Tris-washing lowers the E_m of the Z^+/Z couple by at least 120 mV.

Abbreviations: ADRY, reagents which accelerate the deactivation reactions of the water-splitting enzyme, Y; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; H, magnetic field; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; MES, 2(*N*-morpholino)ethanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate; PS II, photosystem II; Q_A, primary stable PS II acceptor; Tricine, *N*-tris(hydroxymethyl)methylglycine; X'', magnetic susceptibility; Z, primary donor to P680 in non O_2-evolving chloroplasts

2. MATERIALS AND METHODS

All experiments were performed with Tris-washed chloroplasts prepared from market spinach as in [8]. The thylakoid membranes were suspended (3–5 mg chl/ml) in 0.4 M sucrose, 10 mM NaCl and 50 mM buffer [Tricine (pH 8.5 and 8.0), HEPES (pH 7.5) and MES (pH 6.5 and 5.5) with 20 μg spinach ferredoxin/ml and 5×10^{-4} M NADP (Sigma) as an electron acceptor system.

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Further additions are noted in the text and figure legends. All reagents were of analytical grade or of the highest grade commercially available and were purchased from standard commercial sources and used as received.

The light source was a xenon flash lamp described in [9]. The flash repetition rate is presented in the figure legends as are the number of passes accumulated and the experimental protocol. All experiments were performed at room temperature with a Bruker ER 200-D spectrometer fitted with a TM_{110} mode cavity (Varian E-238) which had been modified as in [7].

3. RESULTS

Fig. 1 shows the decay kinetics of EPR signal IIf upon addition of 10 mM $Fe(CN)_3^{3-}$ in the absence (a) and presence (b) of 100 μM DCMU. In the absence of DCMU, the decay corresponds to the slow reduction of the Z^+ radical ($t_{1/2}$ = 1 s) by a high potential endogenous reductant [10] or by low concentrations of the PS II donor, $Fe(CN)_6^{4-}$, formed by the reduction of $Fe(CN)_6^{3-}$ under signal averaging conditions. Addition of the acceptor side inhibitor DCMU leads to more rapid signal IIf decay kinetics ($t_{1/2}$ = 300 ms). In agreement with [11], we interpret this DCMU dependent decay

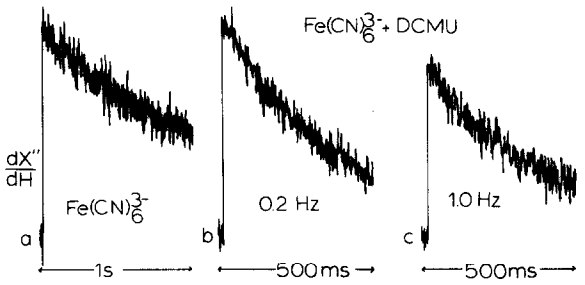

Fig. 1. Effect of DCMU and excitation flash frequency on the decay of EPR signal IIf in the presence of $Fe(CN)_6^{3-}$. Additions to Tris-washed chloroplasts: (a) 10 mM $Fe(CN)_6^{3-}$ and 50 mM $MgCl_2$; (b,c) 10 mM $Fe(CN)_6^{3-}$, 50 mM $MgCl_2$ and 100 μM DCMU. All at pH 8.0. Traces taken at field setting shown in [9]. Instrument time constant, 1 ms, power, 20 mW, 125 scans averaged. Flash frequency: (a) 0.2 Hz; (b) 0.2 Hz; (c) 1.0 Hz.

pathway as reflecting the rate of the back reaction from Q_A^- to Z^+ in Tris-inhibited systems. The $Fe(CN)_6^{3-}$ requirement can be understood in terms of the competition between the back reaction (fig. 1b) and slow electron donation (fig. 1a) as pathways for the decay of the Z^+ radical. A fraction of the centers will undergo reduction of Z^+ as in fig. 1a, rather than by back reaction. In those centers, Q_A will remain reduced after the flash, effectively blocking further photochemistry. The $Fe(CN)_6^{3-}$ is needed to re-oxidize this population of reduced acceptors between the flashes. This role is demonstrated in fig.1 (b,c) showing the effect of flash frequency on the amplitude of signal IIf under signal averaging conditions in DCMU inhibited systems. Trace (b) is an average of 125 scans accumulated at a flash frequency of 0.2 Hz. Trace (c) is an identical sample but the flash repetition rate has been increased to 1.0 Hz. The decay kinetics are unchanged, both reflecting the rate of the back reaction as shown above, but the signal loss in fig. 1c indicates that a fraction of the centers have been blocked from photochemistry by the inability of the $Fe(CN)_6^{3-}$ to re-oxidize the reduced acceptor at higher flash frequencies. These samples (fig. 1b,c) contain 50 mM Mg^{2+}, shown to accelerate the rate of Q_A^- oxidation by the anionic electron acceptor $Fe(CN)_6^{3-}$ through a diminution of the membrane surface potential [12]. A more complete study of the signal amplitude dependence of $Fe(CN)_6^{3-}$ concentration, the ionic strength of the medium and the flash frequency has been presented in [13].

The assignment of the DCMU dependent, rapid reduction of Z^+ as reflecting the back reaction from Q_A^- is strengthened by the results in fig. 2 which show the effect of the ADRY reagent CCCP on the decay kinetics and amplitude of EPR signal IIf under signal averaging conditions. Homann [14] reported that addition of ADRY reagents to DCMU poisoned systems blocks the re-oxidation of Q_A^-. Fig. 2a demonstrates that in the presence of the 8 μM CCCP, $Fe(CN)_6^{3-}$ and DCMU, there is almost no EPR signal IIf generated under signal averaging conditions. Addition of 20 mM Mg^{2+} (fig.2b) allows full signal development, presumably, as discussed above, because shielding of the surface potential in the vicinity of Q_A^- facilitates its re-oxidation by $Fe(CN)_6^{3-}$. However, the decay rate of signal IIf under these conditions ($t_{1/2} < 100$

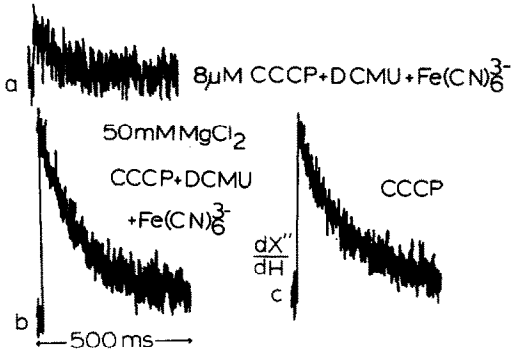

Fig. 2. Effect to ADRY reagents on the signal amplitude and decay kinetics of signal IIf. Additions to Tris-washed chloroplasts: (a) 8 μM CCCP, 100 μM DCMU and 10 mM $Fe(CN)_6^{3-}$; (b) same as (a) plus 50 mM $MgCl_2$; (c) 7 μM CCCP, all at pH 8.0. Instrumental conditions the same as in fig. 1.

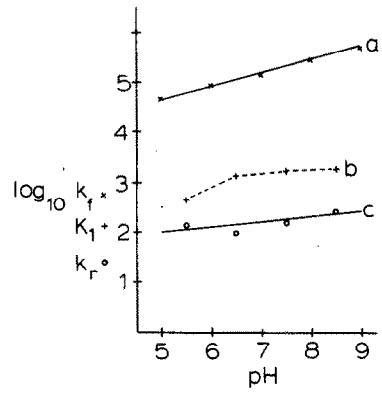

Fig. 4. Plot of log of rate and equilibrium constants *vs* pH as discussed in text. Solid lines are least squares fit to data points.

ms) is much faster than the rate attributed to the back reaction ($t_{1/2}$ = 300 ms). Fig. 2c demonstrates that this same decay rate and signal amplitude are seen when only CCCP (no DCMU, $Fe(CN)_6^{3-}$, Mg^{2+}) is added to the medium.

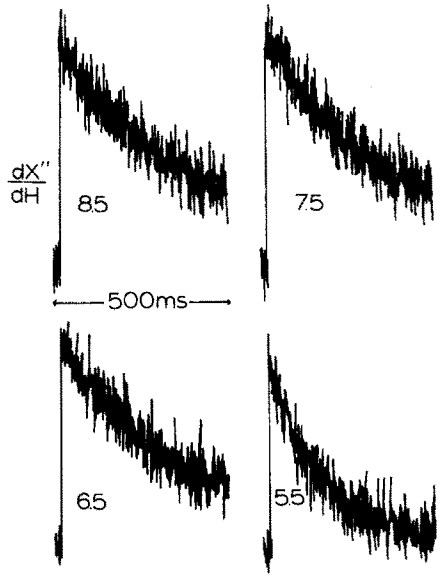

Fig. 3. Effect of pH on the rate of the back reaction in Tris-washed chloroplasts. All samples 10 mM $Fe(CN)_6^{3-}$, 100 μM DCMU, 50 mM $MgCl_2$. Buffers are described in the text and pH values indicated in the figure. Instrumental conditions the same as in fig. 1.

The rate of the back reaction is relatively independent of the medium pH at pH $\gtrsim$ 6.0 (fig. 3). However, there is a marked increase in the rate at pH 5.5.

4. DISCUSSION

These data are a direct measure of the rate of the back reaction between Z^+, the oxidized primary donor to $P680^+$, and Q_A^- in Tris-washed chloroplasts. A similar rate has been observed by Velthuys from the decay rate of Q_A^- measured spectrophotometrically at 313 nm in NH_2OH-treated chloroplasts [15], and we have measured the decay of Q_A^- in Tris-treated, EDTA-washed chloroplasts after 1 or 2 flashes in the absence of added donors or acceptors by a rapid fluorescence technique, and observed a set of decay constants as a function of pH (unpublished) similar to those measured from the decay of Z^+ reported here. Upon addition of the ADRY reagent, CCCP, the back reaction, and hence the re-oxidation of Q_A^- in DCMU poisoned systems, is inhibited. This can be interpreted in terms of the observation in [14] and the model proposed [16] in which it is suggested that ADRY reagents act as electron donors to PS II under signal averaging conditions in inhibited systems. In the presence of DCMU and a rapid PS II donor, CCCP ($k = 2\times10^6$ $M^{-1}.s^{-1}$, [15]), Q_A^- is unable to undergo back reaction, leading to the observed loss in signal amplitude

(fig. 2a). Addition of high concentrations of divalent cations (fig. 2b) allows Q_A^- to be re-oxidized by $Fe(CN)_6^{3-}$ between the flashes, establishing linear electron flow through PS II, from CCCP to $Fe(CN)_6^{3-}$. A comparison of fig. 2b and c demonstrates that the rate of Z^+ decay and the signal amplitude under averaging conditions are insensitive to the addition of DCMU if provision is made for the re-oxidation of Q_A^- between the flashes. These results localize the ADRY effect described by Homann [14] at Z and lend support to the hypothesis [16] that the ADRY effect, in inhibited systems, is to provide high potential reducing equivalents to the donor side of PS II under signal averaging conditions.

The rate of the back reaction is somewhat pH-dependent (fig. 3). There is little dependence seen between pH 8.5–6.5 At pH 5.5, there is a significant enhancement in the rate. The reaction measured in these experiments is a convolution of the two reactions shown below:

$$P680^+ + Z \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Z^+ + P680;\ K_1 = k_f/k_r \qquad (1)$$

$$P680^+ + Q_A^- \xrightarrow{k_b} P680 + Q_A \qquad (2)$$

Reaction (2) proceeded with a rate that shows little or no pH dependence and had an average half-time of 200 μs in Tris-inhibited systems [17]. Independent measurements [18] of the rate of decay of the EPR signal arising from $P680^+$ confirmed these results. The pH dependence of the rate of reduction of $P680^+$ (k_f in eq. (1)) by Z have been measured in [4,17,19]. The data from [17] are plotted in fig. 4a as log k_f *vs* pH. Under conditions in which Z is unable to reduce $P680^+$ [17,18], the initial rate of the back reaction (v_1) is given by:

$$-d[Q_A^-]/dt = k_b\,[P680^+Q_A^-] = v_1$$

By normalizing the total concentration of centers to 1, we get;

$$v_1 = k_b \times 1$$

Under conditions in which Z does reduce $P680^+$, the rate of the back reaction will depend on the concentration of centers in the state $Z\ P680^+\ Q_A^-$ which, in turn, is governed by the equilibrium in reaction (1). Letting $[Z^+\ P680\ Q_A^-] = x$ gives:

$$K_1 = x/(1-x)$$

Under these conditions:

$$-d[Q_A^-]/dt = k_b\,[Z\ P680^+\ Q_A^-]$$
$$= k_b\,(1-x) = v_2$$

This rate also corresponds to $-d[Z^+]/dt$ and can be determined from the data in fig. 3. Combining the two rates gives:

$$v_1/v_2 = k_b \times 1/(k_b(1-x)) = K_1/x$$
$$= K_1 + 1 \approx K_1 \text{ if } K_1 \text{ is large.}$$

These values of K_1 (k_f/k_r) are plotted in fig. 4b. It is now possible to calculate k_r as a function of pH, which is shown in fig. 4c. These data reveal that the rate of the reverse reaction (k_r) is essentially independent of pH and that the pH dependence seen in the EPR traces of fig. 3 is due to the previously measured pH dependence of the forward rate constant, k_f. The slope of the log k_f *vs* pH curve reveals that the rate change with pH unit is 1.8 rather than the 10-fold increase that would be expected if a proton were directly involved in the reaction.

Our observation that the value for K_1 is independent of pH over 6.5–8.5 (and relatively independent down to pH 5.5) is anomolous in the context of the results in [20]. They measured nearly stoichiometric release of protons with $t_{1/2} = 1$ ms on oxidation of D_1 (equivalent to ZH below), attributed to deprotonation of D_1^{ox} (ZH^+) in the following reactions:

$$P680^+ + ZH \rightleftharpoons P680 + ZH^+ \quad ;\ K_3 \qquad (3)$$

$$ZH^+ \rightleftharpoons Z + H^+ \quad ;\ pK_4 < 5 \qquad (4)$$

$$P680^+ + ZH \rightleftharpoons P680 + Z + H^+ \quad ;\ K_5 \qquad (5)$$

Since K_5 should be equivalent to our K_1, we would have expected the value to vary 10-fold with each pH unit, since the proton release occurs on a time scale which is rapid compared to the rate of the back reaction. The anomaly is particularly apparent if the reaction at pH 8 is considered. Here, K_4'(app), $K_4/[H^+]$, for reaction (4) would be $> 10^3$. Our measured value for K_1 ($= K_5$) is about 10^3. The reaction of eq. (3) would have $K_3 < 1$, so

that $P680^+$ reduction would be expected to occur only as reaction (4) proceeded, with $t_{1/2}$ 1 ms as compared to the measured value of 2.5 μs [19].

It is also unlikely that the pH dependence involves a deprotonation of the oxidized donor Z^+, since the lineshape of signal II has been attributed to splitting by protons on a quinone radical cation [21] and no lineshape change attributable to protonation is observed upon change in the medium pH (unpublished), over the pH range studied here (but see [22]). The pH dependence probably reflects the protonation and deprotonation of membrane groups in the vicinity of the PS II reaction center which influence the equilibrium of reaction (1).

An average value for K_1 in the Tris-treated system is 10^3. This is significantly different from the magnitude (< 10) of the value calculated for O_2 evolving systems [23]. We conclude, therefore, that Tris-washing alters the equilibrium constant between the couples $P680^+$, P680 and Z^+, Z, by a factor of > 100, equivalent to a change in E_m of > 120 mV. From the results in [24,25], demonstrating the insensitivity of the rate of back reaction between Q_A^- and $P680^+$ to the state of the donor side, it is likely that Tris-washing specifically affects Z. A Tris-induced change in the rise [4] and decay [3] kinetics of Z have been reported, as well as a change in the microwave saturation properties of the Z^+ radical [6,7]. To this list of effects we add a change in the midpoint potential of Z. The results from the NH_2OH-treated chloroplasts [15] suggest that a similar change is seen under those conditions.

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