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# THE EFFECT OF TEMPERATURE ON THE PRIMARY REACTION OF CHLOROPLAST PHOTOSYSTEM II

## EVIDENCE FOR A TEMPERATURE-DEPENDENT BACK REACTION

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## SUMMARY

The primary reaction of Photosystem II has been studied over the temperature range from −196 to −20 °C. The photooxidation of the reaction-center chlorophyll (P680) was followed by the free-radical electron paramagnetic resonance signal of $P680^+$, and the photoreduction of the Photosystem II primary electron acceptor was monitored by the C-550 absorbance change.

At temperatures below −100 °C, the primary reaction of Photosystem II is irreversible. However, at temperatures between −100 and −20 °C a back reaction that is insensitive to 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea (DCMU) occurs between $P680^+$ and the reduced acceptor.

The amount of reduced acceptor and $P680^+$ present under steady-state illumination at temperatures between −100 and −20 °C is small unless high light intensity is used to overcome the competing back reaction. The amount of reduced acceptor present at low light intensity can be increased by adjusting the oxidation–reduction potential so that $P680^+$ is reduced by a secondary electron donor (cytochrome $b_{559}$) before $P680^+$ can reoxidize the reduced primary acceptor. The photooxidation of cytochrome $b_{559}$ and the accompanying photoreduction of C-550 are inhibited by DCMU. The inhibition of C-550 photoreduction by DCMU, the dependence of P680 photooxidation and C-550 photoreduction on light intensity, and the effect of the availability of reduced cytochrome $b_{559}$ on C-550 photoreduction are unique to the temperature range where the Photosystem II primary reaction is reversible and are not observed at lower temperatures.

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## INTRODUCTION

Two light-induced changes have been detected in chloroplasts at low temperatures (77 °K or lower) and have been related to the primary reactants of Photosystem II. Knaff and Arnon [1, 2] discovered a light-induced absorbance change near

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

550 nm and attributed it to the photoreduction of an electron-transfer component which they designated C-550. Evidence from several laboratories suggested that C-550 was related to the primary electron acceptor of Photosystem II [2–4]. The C-550 absorbance change has been shown to result from a band shift [5] and was associated with the presence of $\beta$-carotene in the chloroplast membrane [6]. Recent data from this laboratory have shown that it is possible to prepare chloroplast fragments which have Photosystem II activity but lack C-550 [7]. These results suggest that C-550 is not the actual primary electron acceptor molecule but rather may be a membrane-bound chromophore, probably $\beta$-carotene, that indicates the oxidation–reduction state of the primary acceptor.

A second light-induced change, an electron paramagnetic resonance (EPR) free-radical signal associated with Photosystem II, was observed by Malkin and Bearden [8]. This signal was identified with the oxidized reaction-center chlorophyll ($P680^+$) of Photosystem II on the basis of its EPR parameters [8] and response to oxidation–reduction potential [9].

In a model developed by Butler and coworkers for the behavior of Photosystem II reactions at low temperatures, it was suggested that a back reaction can occur between $P680^+$ and the reduced primary acceptor, thereby reversing the primary charge separation [4, 10, 11]. The evidence for such a back reaction was based primarily on the behavior of C-550 at low temperatures after a short saturating flash [4, 10, 11].

This investigation of the reactions of C-550, P680, and cytochrome $b_{559}$ (a chloroplast cytochrome that can be oxidized by $P680^+$ at cryogenic temperatures [3–5, 10, 12]) provides evidence that such a back reaction does occur at temperatures between $-20$ and $-100$ °C but only in that temperature range and that at lower temperatures the primary reactants are formed in an irreversible reaction. These results will be considered in terms of current models for the primary reaction of Photosystem II.

## METHODS

Chloroplasts were prepared from spinach as described previously [13] and were resuspended in 50 mM potassium phosphate buffer (pH 7.6) plus 20 mM NaCl. Chlorophyll concentrations were determined according to the method of Arnon [14].

Cytochrome $b_{559}$ photooxidation and C-550 photoreduction were measured as described previously [1, 7, 12], using a cuvette with a 2-mm optical path. The temperature of the sample was adjusted using a slurry of dry ice in methanol and the sample temperature was monitored directly with a plantium resistance thermometer (United Systems Corp.) inserted in the cuvette. The 664-nm actinic light was defined by an interference filter with a half-band width of 20 nm.

EPR measurements were made as previously described [8, 9]. Samples were cooled to the desired temperature by a stream of $N_2$ and allowed to equilibrate for at least 5 min. Sample temperature was monitored by a carbon resistance thermometer and temperatures could be controlled to ±5° C. Oxidation–reduction potentials were determined as described previously [8].

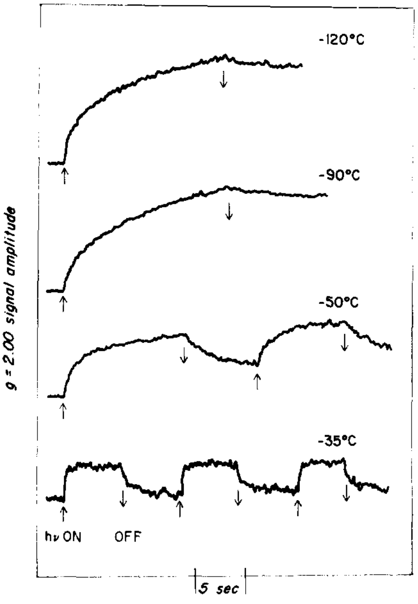
## RESULTS

Fig. 1 shows the time course for the appearance of the Photosystem II free-radical signal on illumination and its disappearance on cessation of illumination as a function of temperature. The reaction is irreversible at temperatures of $-120$ °C and lower [8]. As the temperature is increased, the reaction becomes reversible, with the rate of both the forward and the reverse reactions increasing with increasing temperature. The increase in the rate of the forward reaction as a function of temperature may be related to changes in the optical properties of the EPR sample. At temperatures between $-50$ and $-35$ °C, the light–dark cycles can be repeated many times. The light-induced EPR signal cannot be detected under steady-state illumination at temperatures greater than approx. $-20$ °C. The Photosystem II inhibitor 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea (DCMU) at a concentration of $5 \cdot 10^{-6}$ M had no effect on either the light-induced appearance of the signal or its subsequent decay in the dark.

The experiments shown in Fig. 1 were performed in the presence of $Fe(CN)_6{}^{3-}$ (the light-induced free-radical is observed only at oxidation–reduction potentials greater than $+420$ mV and maximal extent is observed at potentials greater than $+540$ mV [9]) and at an actinic light intensity of $2.5 \cdot 10^5$ ergs/cm$^2$/s. In the temperature range where the reaction is reversible, the extent of the light-induced free-radical signal depends on the actinic light intensity. No signal could be observed at intensities less than $1.0 \cdot 10^4$ ergs/cm$^2$/s. At temperatures where the reaction was irreversible, the extent of P680 photooxidation was not dependent on light intensity.

The dark decay of the light-induced EPR signal in a DCMU-insensitive reaction at temperatures near $-50$ °C is consistent with the existence of a back reaction between P680$^+$ and the reduced primary acceptor. At low actinic light intensities, the rate of the back reaction becomes greater than that of the forward reaction, resulting in a low steady-state level of P680$^+$ (and reduced acceptor). The temperature dependence shown in Fig. 1 indicates that such a back reaction does not operate at temperatures below $-100$ °C.

If a back reaction between P680$^+$ and the reduced acceptor does occur, it should be detectable by observations of the primary acceptor of Photosystem II monitored by the C-550 absorbance change. To avoid illumination artifacts, it was necessary to use actinic light intensities of less than $1.0 \cdot 10^4$ ergs/cm$^2$/s. Fig. 2 shows the extent of the C-550 absorbance change induced at $-50$ °C by actinic light with an intensity of $5.0 \cdot 10^3$ ergs/cm$^2$/s. In the presence of ascorbate, a decrease of absorbance at 548 nm minus 543 nm (these wavelengths were chosen to avoid interference from cytochrome $b_{559}$) characteristic of the reduction of the Photosystem II primary acceptor was observed and there was no reversal on cessation of illumination. In the presence of $Fe(CN)_6{}^{3-}$ the extent of the absorbance change was considerably smaller (approximately 15 % of that observed in the presence of ascorbate) and appeared to be reversible. The small amount of reduced acceptor present in the steady-state during illumination in the presence of $Fe(CN)_6{}^{3-}$ is similar to the small amount of P680$^+$ observed under similar conditions with actinic light of low intensity described above. These results differ from those observed previously at either $-196$ °C or $+20$ °C, where the extent and reversibility of the C-550 absorbance change were unaffected by the replacement of ascorbate with $Fe(CN)_6{}^{3-}$ [1, 2].



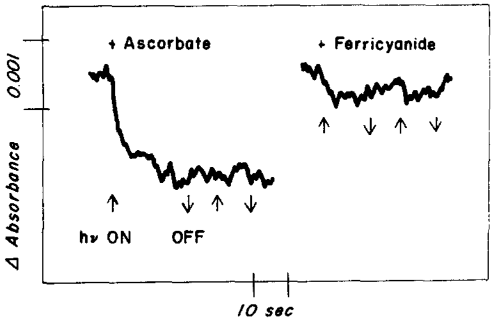


Fig. 1. Effect of temperature on photooxidation and dark reduction of P680. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 20 mM NaCl, 50 mM $K_3Fe(CN)_6$, and chloroplast fragments (0.25 mg chlorophyll/ml). Samples were illuminated at the indicated temperature with 664-nm light (intensity: $2.5 \cdot 10^5$ ergs/cm$^2$/s) and the time course of the reaction was followed at the low-field peak of the $g = 2.0026$ free-radical signal of P680$^+$.

Fig. 2. C-550 photoreduction at −50 °C (548 nm minus 543 nm). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 20 mM NaCl, chloroplast fragments (0.25 mg chlorophyll/ml), and, where indicated, 10 mM sodium ascorbate or 10 mM $K_3Fe(CN)_6$. Intensity of actinic light (664 nm): $5.0 \cdot 10^3$ ergs/cm$^2$/s.

The spectra of the light-induced absorbance changes produced by illumination at −50 °C in the presence of either ascorbate or $Fe(CN)_6{}^{3-}$ are shown in Fig. 3. In the presence of ascorbate a minimum at 550 nm and the maximum at 543 nm (the extent of the absorbance increase at 543 nm varied somewhat; that shown in Fig. 3 is at the lower limit of the observed range) were observed and may be attributed to the photoreduction of C-550 [1, 6, 15–17]. The minimum at 556 nm is caused by the photooxidation of cytochrome $b_{559}$ (the α band of the cytochrome shifts to 556 nm at cryogenic temperatures [2–6, 12, 15–17]). The observed spectrum shows no contribution that can be attributed to the photooxidation of cytochrome *f*, which has an absorbance maximum at 554 nm (room temperature) or 552 nm (−196 °C). No cytochrome $b_{559}$ photooxidation could be observed in the presence of ferricyanide because cytochrome $b_{559}$ was oxidized by the ferricyanide prior to illumination [12].

These results are consistent with the operation of a back reaction between P680$^+$ and the reduced acceptor at −50 °C. At the relatively low light intensities used, the back reaction prevents accumulation of a substantial amount of reduced Photosystem II primary acceptor unless a secondary donor (cytochrome $b_{559}$) is available to reduce P680$^+$ and to prevent the back reaction.

Experiments at −50 °C in which samples were poised at defined oxidation–

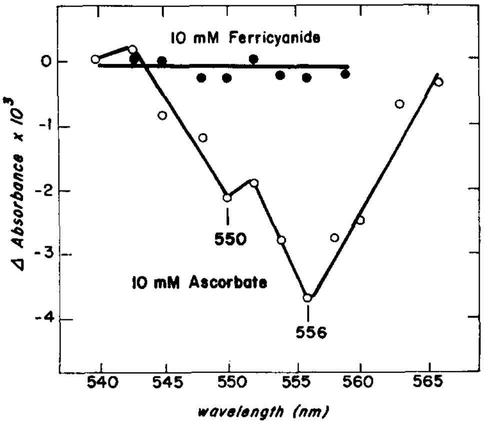
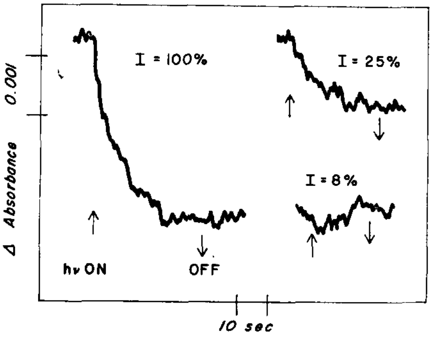


Fig. 3. Spectrum of C-550 photoreduction and cytochrome $b_{559}$ photooxidation at −50 °C (540-nm reference wavelength). Reaction mixture and illumination as in Fig. 2.

reduction potentials prior to freezing showed no C-550 photoreduction at potentials above +400 mV. Titrations of the extent of C-550 photoreduction at −50 °C gave a midpoint potential of approx. +350 mV for the disappearance of C-550 reduction, the same value as that found for the midpoint potential of cytochrome $b_{559}$ in the chloroplasts(data not shown). These results differ from those found at lower temperatures, where the extent of C-550 photoreduction did not depend on the potential over the range from +150 mV to +600 mV. The observation that C-550 photoreduction at the temperature of liquid $N_2$ is not reversed in a subsequent dark period [1, 3] and does not depend on the availability of cytochrome $b_{559}$ as a secondary donor [1, 16, 17] is consistent with the absence of a back reaction at this temperature.

The dependence of the extent of cytochrome $b_{559}$ photooxidation at −50 °C on the actinic light intensity is shown in Fig. 4. No cytochrome $b_{559}$ photooxidation



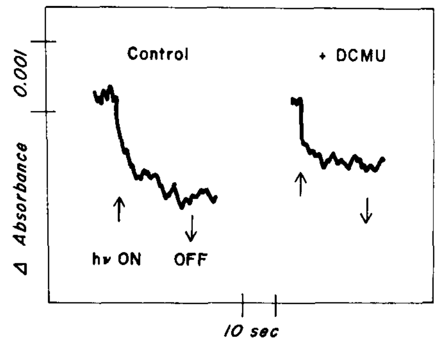


Fig. 4. Effect of light intensity on cytochrome $b_{559}$ photooxidation at −50 °C (556 nm minus 540 nm). Reaction mixture as in Fig. 2 with 10 mM sodium ascorbate present. 100 % intensity corresponds to $2.0 \cdot 10^3$ ergs/cm²/s.

Fig. 5. Effect of DCMU on C-550 photoreduction at −50 °C (548 nm minus 543 nm). Reaction mixture as in Fig. 2 with 10 mM sodium ascorbate present. DCMU was present at a concentration of 1.0 μM where indicated. Illumination as in Fig. 2.

could be observed at this temperature with actinic light intensities of less than $2.5 \cdot 10^2$ ergs/cm$^2$/s. The extent of reduction of the Photosystem II primary acceptor, monitored by C-550, showed the same dependence on light intensity as did cytochrome $b_{559}$ photooxidation, again demonstrating the linkage between the two reactions at −50 °C. At −196 °C the extent of cytochrome $b_{559}$ photooxidation and C-550 photoreduction did not depend on light intensity over a comparable range of intensities.

Fig. 5 shows that the photoreduction of C-550 at −50 °C in the presence of ascorbate is inhibited by DCMU. The average of several experiments gave a value of 35 % inhibition at a DCMU concentration of 1 μM. In contrast, DCMU had no effect on the reversible C-550 reduction observed in the presence of $Fe(CN)_6^{3-}$ at −50 °C. These results differ from those obtained at lower temperatures or at room temperature: at −196 °C, DCMU does not inhibit C-550 reduction [1, 16, 17], while at +20 °C DCMU stimulates C-550 reduction [2, 18].

Cytochrome $b_{559}$ photooxidation at −50 °C is also inhibited by DCMU (data not shown) with the average inhibition being 70 % at 1 μM DCMU. Inhibition of cytochrome $b_{559}$ photooxidation at −50 °C by DCMU would be expected to result in an inhibition of C-550 reduction by eliminating the ability of the cytochrome to reduce P680$^+$ and to prevent the back reaction.

The temperature dependence of the photoreduction of the primary acceptor in the presence of $Fe(CN)_6^{3-}$ monitored by the C-550 absorbance change is shown in Fig. 6. The extent of C-550 photoreduction is small at temperatures between −50 °C and −10 °C. At temperatures above −10 °C, the extent increases and reaches a maximum at temperatures above +15 °C. As the temperature is lowered below −50 °C, the extent of C-550 photoreduction increases and the reversal in the dark becomes slow, suggesting that the rate of the back reaction decreases as the temperature is lowered below −50 °C.

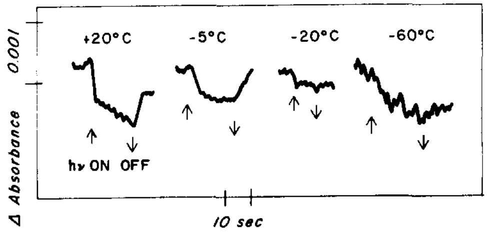


Fig. 6. Effect of temperature on photoreduction and dark oxidation of C-550 (548 nm minus 543 nm). Reaction mixture as in Fig. 2 with 10 mM $K_3Fe(CN)_6$ present. Illumination as in Fig. 2.

## DISCUSSION

The photoreduction of C-550 and the photooxidation of cytochrome $b_{559}$ by Photosystem II at low temperature have been analyzed by Butler and coworkers [4, 11] in terms of the following sequence of events in the reaction-center complex of Photosystem II.

$$D \cdot P \cdot A \xrightarrow{h\nu} D \cdot P^+ \cdot A^- \longrightarrow D^+ \cdot P \cdot A^- \qquad (1)$$

where A is the primary electron acceptor which can be monitored by the state of

C-550, P is the reaction-center chlorophyll P680, and D is a secondary donor which can be cytochrome $b_{559}$. $P^+$ should be observable after illumination at low temperature if all of the secondary electron donors are oxidized prior to freezing.

$$D^+ \cdot P \cdot A \xrightarrow{h\nu} D^+ \cdot P^+ \cdot A^- \tag{2}$$

Malkin and Bearden [8, 9] observed a light-induced free-radical signal when chloroplast fragments enriched in Photosystem II poised at potentials greater than +450 mV were illuminated at −196 °C. They suggested that this EPR signal, which had EPR parameters similar to those observed for other oxidized chlorophyll free radicals, was due to the oxidized form of P680 [8, 9]. Titration results suggested that an unknown component with a midpoint potential of +475 mV could serve as a secondary donor to reduce $P^+$ (ref. 9), in addition to the previously known reaction of cytochrome $b_{559}$ ($E_m = +350$ mV) with $P^+$.

In recent experiments [4, 11], Butler and coworkers hypothesized a back reaction between P680$^+$ and the reduced primary electron acceptor to explain the small amount of C-550 photoreduced in a single short saturating flash at −196 °C. The reaction sequence would then be as shown in Eqn 3.

$$D \cdot P \cdot A \underset{k_{-1}}{\overset{h\nu}{\rightleftarrows}} D \cdot P^+ \cdot A^- \xrightarrow{k_2} D^+ \cdot P \cdot A^- \tag{3}$$

The results of the present investigation reported above seem most compatible with the scheme shown in Eqn 3 with the modification that the back reaction (characterized by the rate constant, $k_{-1}$) does not occur at temperatures below −100 °C. At temperatures above −20 °C, reoxidation of $A^-$ by other carriers of the electron transport chain and reduction of P680$^+$ by components of the water-oxidizing apparatus come into play and the model of Eqn 3 is no longer adequate.

The observation that the light-induced free-radical reaction and the photoreduction of C-550 both became reversible in the same range of temperature is consistent with a back reaction between $P^+$ (as monitored by the EPR signal) and $A^-$ (as monitored by C-550). This reaction is not observed at temperatures below −100 °C. Accumulation of $P^+$ and $A^-$ in the steady state at this temperature range (near −50 °C) should depend on the actinic light intensity being high enough to allow the rate of the forward reaction to exceed that of the back reaction. As shown above, the extent of the free-radical signal and C-550 reduction depend on light intensity in the expected manner at −50 °C but do not depend on intensity at lower temperatures where no reverse reaction occurs.

The observation that complete photoreduction of the acceptor at −50 °C with low light intensity depends on the availability of cytochrome $b_{559}$ as a secondary donor is also consistent with the existence of a back reaction at −50 °C. Reduction of $P^+$ by cytochrome $b_{559}$ prevents the reoxidation of $A^-$ by $P^+$ ($k_2 > k_{-1}$ in Eqn 3). The finding that C-550 reduction at −50 °C was not observed at potentials above +400 mV suggests that the +475 mV component detected by Malkin and Bearden [9] cannot compete with the back reaction at −50 °C. This dependence of C-550 reduction on the presence of reduced cytochrome $b_{559}$ is unique to the temperature range in which the free-radical and C-550 reactions are reversible. At −196 °C, C-550 can be photoreduced to the same extent whether cytochrome $b_{559}$ is oxidized or reduced [1, 16, 17]. This may be explained if one assumes that no back reaction occurs at −196 °C so that $P^+$ reduction by cytochrome $b_{559}$ is not required to stabilize $A^-$. At +20 °C, the oxidation–reduction state of cytochrome $b_{559}$ does not affect the extent of C-550 reduction because $P^+$ is reduced by water with a rate that is characteristically faster than that of the back reaction.

There have been conflicting reports as to whether cytochrome $b_{559}$ is photooxidized at temperatures near −50 °C. Butler et al. [10] reported that the extent of cytochrome $b_{559}$ photooxidation decreased as the temperature was raised above −160 °C with no photooxidation observed at −100 °C (no measurements at temperatures above −100 °C were reported). Vermeglio and Mathis [19, 20] reported that little cytochrome $b_{559}$ photooxidation (0–30 % of the amount observed at −196 °C) occurred at −50 °C in the absence of flash preillumination prior to freezing. Floyd et al. [21] reported cytochrome $b_{559}$ photooxidation at −78 °C with a half-time of 5 ms after a 30-ns laser flash. Our results (see Figs 3 and 4) indicate that cytochrome $b_{559}$ photooxidation does occur at −50 °C and that the extent of the reaction depends on the actinic light intensity.

The inhibition of C-550 photoreduction at −50 °C by DCMU (see Fig. 5) is unique to the temperature range around −50 °C where the C-550 and P680 reactions are reversible. No such inhibition was observed at −196 or +20 °C [1, 2, 16–18]. The inhibition of C-550 reduction by DCMU may be explained as a secondary effect caused by the DCMU inhibition of cytochrome $b_{559}$ oxidation which prevents reduction of $P^+$ by the cytochrome from competing with the back reaction ($k_2 < k_{-1}$ in the presence of DCMU). It is not yet clear why the photooxidation of cytochrome $b_{559}$ is inhibited to a greater extent than is the photoreduction of C-550.

Inhibition of the Photosystem II photooxidation of cytochrome $b_{559}$ by DCMU also occurs at +20 °C [2] but not at −196 °C [16, 17]. The inhibition by DCMU of the reaction between $P680^+$ and cytochrome $b_{559}$ may be related to its inhibition of reactions on the oxidizing side of Photosystem II as was recently reported by Renger [22].

In conclusion, the results reported above indicate the existence of a back reaction between the reduced primary acceptor of Photosystem II (as monitored by C-550) and an oxidized species that appears to be the primary donor of Photosystem II (monitored by the EPR free-radical signal). This back reaction operates over the range from −20 to −100 °C but not at lower temperatures.

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