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LIGHT-INDUCED ABSORBANCE CHANGES DUE TO PHOTOSYSTEMS 1 AND 2 IN SPINACH CHLOROPLASTS AT −50 °C

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SUMMARY

Absorbance changes in the region 500–565 nm and at 702 nm, brought about by excitation of Photosystems 1 and 2, respectively, were measured in spinach chloroplasts at −50 °C. Either dark-adapted chloroplasts were used or chloroplasts preilluminated with a number of short saturating flashes just before cooling.

Both photosystems were found to cause a light-induced increase of absorbance at 518 nm (due to "P518"). The System 1-induced change was not affected by preillumination. It decayed within 1 s in the dark and showed similar kinetics as P700. Experiments in the presence of external electron acceptors (methylviologen or $Fe(CN)_6^{3-}$) suggested that P518 was not affected by the redox state of the primary electron acceptor of System 1. The absorbance increase at 518 nm due to System 2 decayed in the dark with a half-time of several min. The kinetics were similar to those of C-550, the presumed indicator of the primary electron acceptor of System 2. After two flashes preillumination the changes due to P518 and C-550 were reduced by about 40 %, and a relatively slow, System 2-induced oxidation of cytochrome b_{559} occurred which proceeded at a similar rate as the increase in yield of chlorophyll *a* fluorescence. The results indicate that at −50° C two different photoreactions of System 2 occur. One consists of a photoreduction of the primary electron acceptor associated with C-550, accompanied by the oxidation of an unknown electron donor; the other is less efficient and results in the photooxidation of cytochrome b_{559}.

INTRODUCTION

Some years ago an important step towards the elucidation of the reaction sequence involved in the electron transfer from water to the primary electron donor of System 2 of photosynthesis was made by the discovery [1] that different reaction centers do not cooperate in the oxidation of water to oxygen and that four con-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; C-550, P518, pigments showing light-induced absorbance changes near 550 and 518 nm, respectively; P700, P680, reaction-center chlorophylls, primary electron donors of Photosystems 1 and 2, respectively.

secutive electron transfers at one reaction center are needed for the production of oxygen. More recent studies [2–5] have shown that the number of positive charges generated in this way (the so-called "S-state" of the reaction center [6]) has a marked effect upon the light-induced reactions that occur in the temperature range between about −40 and −90 °C. Spinach chloroplasts converted to States S_2 and S_3 by preillumination with two short, saturating, light flashes before cooling, showed a slower increase in the yield of chlorophyll fluorescence upon illumination than those that had been preilluminated with four flashes or not at all, and were in States S_0 and S_1 mainly.

Measurements of absorbance changes in the region around 518 nm, reported in a previous paper [4] indicated that in States S_2 and S_3 also a rapid reaction occurred, in addition to the slow increase in fluorescence. The amplitude of the absorbance changes at 518 nm, however, was found to be preillumination-dependent and varied with the number of flashes in a similar fashion as the rate of fluorescence increase. Difference spectra indicated a more extensive phototransformation of C-550 at −40 °C without than with preillumination with two flashes. These results suggested a fairly complicated reaction mechanism at low temperature which is only poorly understood.

Since it is known that at room temperature the pigment change causing the so-called 518-nm change (P518) is brought about by both photosystems [7, 8] it was desirable to further analyse the absorbance changes below −40 °C in terms of the contribution by light reactions 1 and 2. The results of these experiments are reported in this paper, together with a tentative interpretation of the phenomena observed.

MATERIAL AND METHODS

Chloroplasts were obtained as described earlier [4] and stored on ice in the dark before use in a solution containing 0.05 M *N*-tris-(hydroxymethyl)-methylglycine (Tricine), 0.01 M KCl, 0.002 M $MgCl_2$ and 0.4 M sucrose, pH 7.8. The chlorophyll concentration, determined by the method of Whatley and Arnon [9], was 10^{-3} M.

Just before each measurement, the chloroplast suspension was mixed with glycol to a final concentration of 53 % (v/v), transferred in darkness to the spectrophotometer vessels and cooled to the desired temperature. When indicated a number of saturating, 8-μs xenon flashes were given at 1-s intervals when the temperature reached 3 °C. Cooling to −40 °C took about 2 min. The experiments were done in a "split-beam" spectrophotometer, earlier used in our laboratory [10]. The apparatus enabled simultaneous measurements of absorbance changes at two different wavelengths, or, alternatively, simultaneous measurement of fluorescence and absorbance changes. The chloroplast suspension was kept at the desired temperature by means of a thermostated vessel holder [11]; it was illuminated by red (630 nm) or far-red (727 nm) actinic light. Fluorescence excited by the red actinic beam was measured at 692 nm. The far-red actinic light was obtained by means of a Schott AL 727 interference and RG 715 glass filter. Other filter combinations used were described before [4].

RESULTS

Fig. 1 shows recordings of absorbance changes at 518 and 702 nm, obtained at −50 °C. Recording a was made with dark-adapted chloroplasts that were first illuminated with far-red (727 nm) and subsequently with red (630 nm) light. Both illuminations gave a rapid absorbance increase at 518 nm. With two flashes preillumination, the absorbance change induced by far-red light was almost the same (Fig. 1b) but the change induced by red light was smaller. The absorbance change induced by 630-nm light was strongly reduced in chloroplasts which, before cooling,

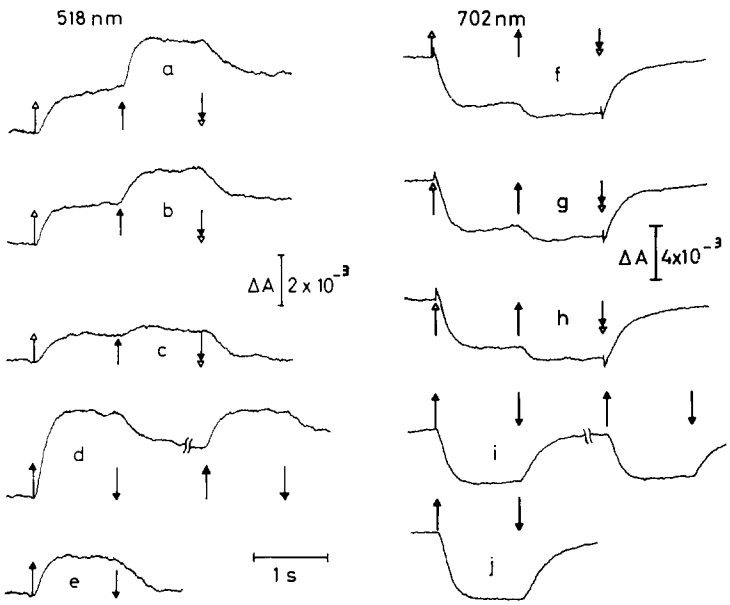

Fig. 1. Absorbance changes of chloroplasts at −50 °C at 518 nm (a–e) and 702 nm (f–j). Upward and downward pointing arrows mark the beginning and the end of illumination, respectively. Open arrows, 727 nm (17 nEinstein · $cm^{-2} \cdot s^{-1}$); solid arrows, 630 nm (3.5 nEinstein · $cm^{-2} \cdot s^{-1}$). An upward moving trace indicates an increase in absorbance. Conditions: a, d, f and i, dark-adapted chloroplasts; b and g, preillumination two flashes at 3 °C; c, e, h and j, 20 μM DCMU, 1 mM hydroxylamine, preillumination 12 flashes, given at room temperature. For d and i, the second recording was made after 8 s darkness. For each experiment, corresponding recordings at 518 and 702 nm were made simultaneously. The transients in recordings f–h are artifacts due to false light.

had been illuminated in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and hydroxylamine [12, 13], whereas the absorbance change brought about by far-red light was hardly affected (Fig. 1c). This indicates that the absorbance changes induced by System 1 and System 2 were largely separated kinetically, and that the far-red light had only little System 2 activity.

Recordings f–h show the corresponding kinetics of P700 photooxidation. They were not affected by preillumination or by hydroxylamine and DCMU and were similar to those of the System 1 component at 518 nm. A relatively large absorbance change due to oxidation of P700 was brought about by the far-red illumination;

additional illumination with red light gave only a small decrease in absorbance at 702 nm. In contrast to the System 2 component at 518 nm, the System 1 absorbance changes reversed rapidly in the dark (Recordings d, e, i and j).

The yield of chlorophyll *a* fluorescence was only little affected by far-red light. In chloroplasts preilluminated with two flashes, the fluorescence increase in red light was relatively slow, as observed earlier [2–4], independent of the preceding far-red illumination.

Kinetics and difference spectrum of System 1-induced absorbance changes

Fig. 2 shows difference spectra at −50 °C of the absorbance changes induced by far-red light (with and without preillumination) and by red light in the presence of DCMU and hydroxylamine (after preillumination). The spectra are all very similar, and show a maximum near 518 nm, which indicates that they are due to photoconversion of P518. This was supported by measurements with a more dilute suspension

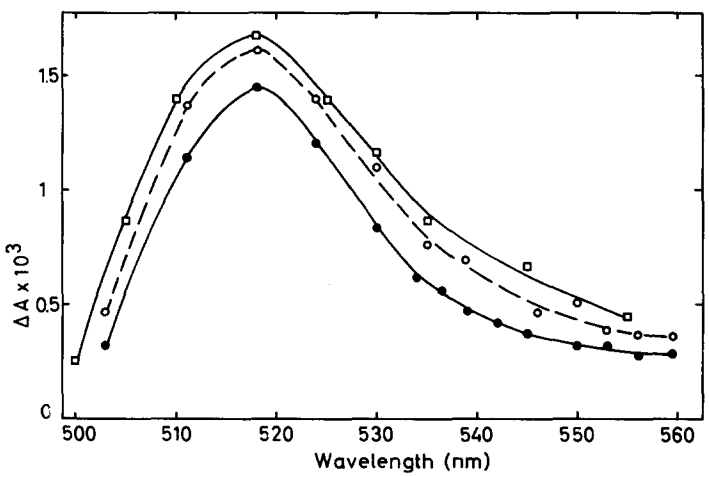

Fig. 2. Spectra of System 1-induced absorbance changes (light minus dark) at −50 °C. ●—●, dark-adapted chloroplasts; ○- - -○, preillumination two flashes; illumination 727 nm. □—□, chloroplasts preilluminated with 12 flashes in the presence of DCMU and hydroxylamine; illumination 630 nm. The spectra were obtained with three different batches of chloroplasts. Conditions as for Fig. 1.

at shorter wavelengths, which showed a negative band in the region of 480 nm, of about one-third of the size of the positive band. The relatively small absorbance increase in the region near 560 nm is probably partly due to photooxidation of P700 [14, 15]. The spectra gave no evidence for a band shift of C-550 in agreement with the notion that the shift is caused by System 2 only [16]. Photooxidation of cytochrome *f* was not observed either, confirming results obtained by Vermeglio and Mathis [5] by a somewhat different technique. Apparently the oxidation is inhibited at −50 °C.

The kinetics of P700 and of the System 1-driven change of P518 were almost irreversible in the presence of $K_3Fe(CN)_6$ or methylviologen (Fig. 3). This is most simply explained by assuming that these oxidants are able to react with the reduced primary acceptor, presumably ferredoxin [17, 11], thus preventing a back reaction

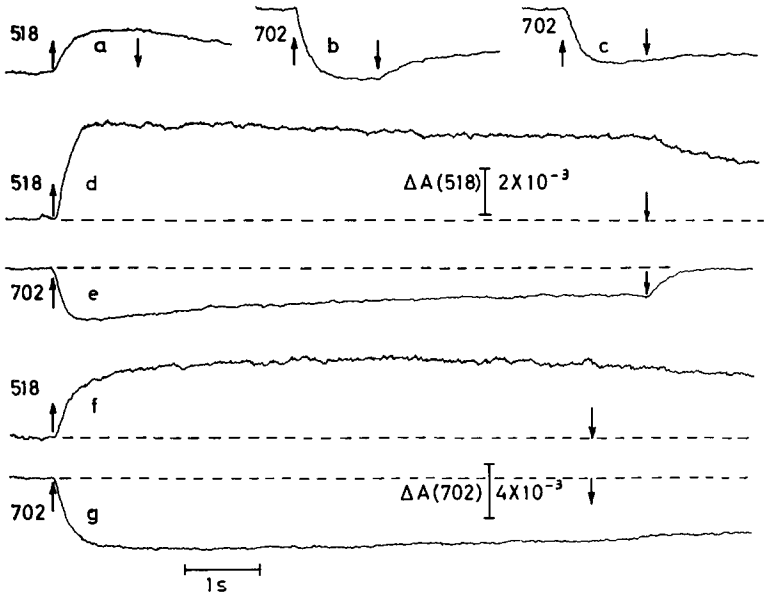

Fig. 3. Absorbance changes at 702 and 518 nm. a, b, f and g: in the presence of 20 μM DCMU, 1.0 mM hydroxylamine and 0.1 mM methylviologen. Preillumination: 12 flashes. c: in the presence of 2 mM $Fe(CN)_6^{3-}$. d and e: no additions. Illumination: 630 nm. For further conditions and explanation: see Fig. 1.

of reduced ferredoxin with oxidized P700. Upon prolonged illumination, the steady-state deflection at both 702 and 518 nm decreased slowly (Fig. 3d, e). This may be explained by a slow reaction of $P700^+$ with another electron donor, leaving the reaction centers in the state P700-ferredoxin$^-$, unable to again photooxidize P700. These experiments suggest that at -50 °C the System 1 518-nm change is not affected by the redox level of the primary acceptor, as can be expected if the "charge separation" [8, 18] over the membrane is delocalized.

Absorbance changes induced by System 2

Absorption difference spectra induced by illumination with red light upon a far-red background are shown in Figs 4 and 5. The spectra were obtained under conditions as used for Recordings a and b of Fig. 1. The spectrum obtained without preillumination (Fig. 4, solid circles) shows clearly the band of P518 and the band shift due to C-550, discernable on the slope of the P518 band. The kinetics of the absorbance changes were similar at each wavelength (Figs 1 and 6). After two flashes preillumination, the difference spectrum (Fig. 5) showed a smaller absorbance change of P518, and in addition there was now a negative band at 558 nm, presumably due to photooxidation of cytochrome b_{559}. This photooxidation was 2–3 times slower than the absorbance change due to P518. On basis of a differential molar extinction coefficient of 16 $mM^{-1} \cdot cm^{-1}$ at 558 nm [19], the amount of cytochrome oxidized was estimated to be one per 650 chlorophyll molecules. Analysis of the spectrum in the region 530–550 nm was difficult because of overlapping absorbance changes of the two compounds mentioned above. The shallow minimum near 530 nm

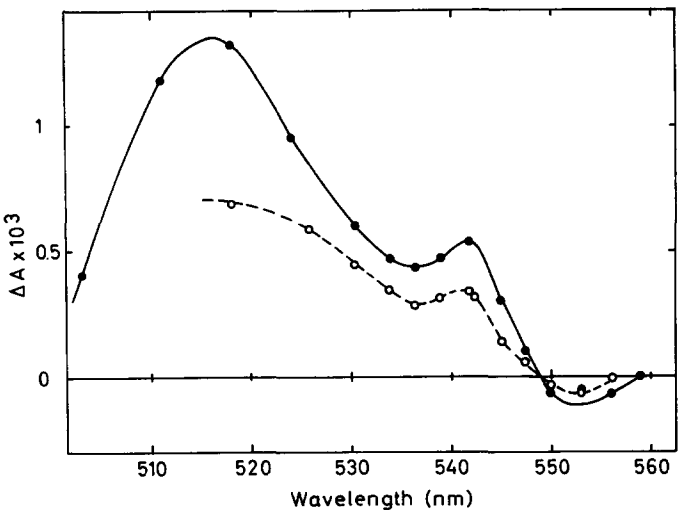

Fig. 4. Spectra of absorbance changes caused by System 2. ● — ●, absorbance changes brought about by 630-nm light upon a background of far-red light (conditions as for Fig. 1, Curve a). No preillumination. ○ - - - ○, conditions as for the other spectrum, but in the presence of 3 mM $Fe(CN)_6^{3-}$ and after two flashes preillumination.

is probably due to the β-band of cytochrome b_{559} [19], but it was not possible to decide from the spectrum whether photoconversion of C-550 occurred, not even when only the short-time absorbance changes were plotted.

The spectrum of Fig. 4 (open circles) was obtained in the presence of 3 mM $K_3Fe(CN)_6$, under otherwise identical conditions. In this case, no photooxidation of

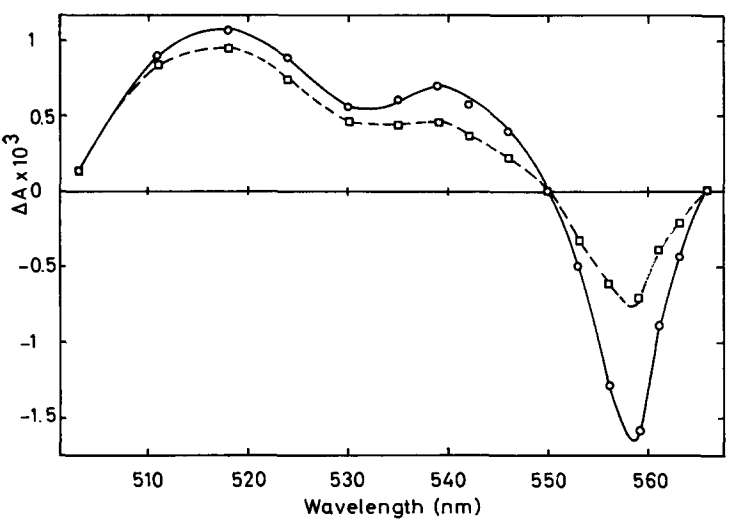

Fig. 5. Absorbance difference spectra obtained by illumination with 630-nm light upon a background of far-red light (conditions as for Fig. 1, Curve b). The absorbance changes are plotted after 300 ms (□ . . . □) and 1.5 s (○ — ○) illumination, respectively.

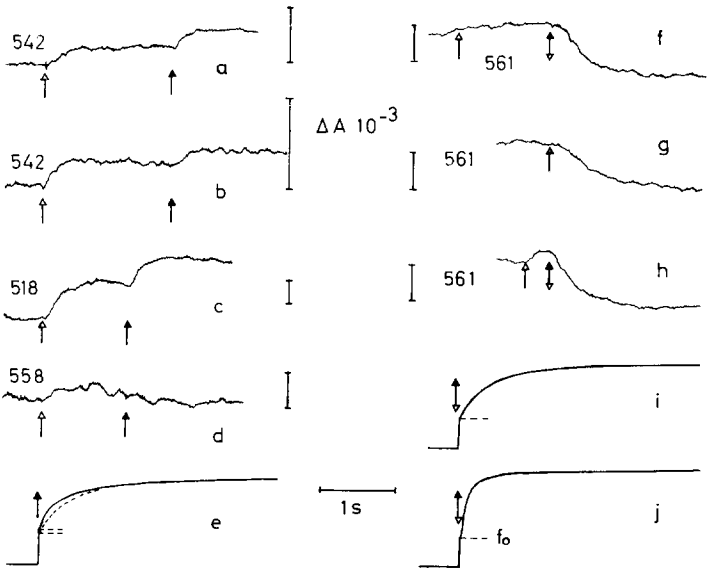

Fig. 6. Absorbance changes at the wavelength indicated (Recordings a–d, f–h) and changes in chlorophyll fluorescence (e, i, j). Illumination 727- (open arrows) and 630-nm light (solid arrows) as for Fig. 1 (except for Recording e). The recordings were made with different sensitivities; the vertical bars denote an absorbance difference of 10^{-3}. For recordings e, i and j, the horizontal broken line gives the level of the initial fluorescence (f_0). Conditions: Recording a, dark-adapted chloroplasts; b, preillumination two flashes, 3 mM $Fe(CN)_6^{3-}$; c and d, as a, but obtained upon a second illumination, 14 min after the first one; e, as c, but with 630-nm illumination only (broken line: obtained upon the first illumination); f–i, 2 flashes preillumination, 0.1 mM methylviologen. The far-red light was turned off less than 20 ms before the onset of the 630-nm light. A shutter in front of the photomultiplier recording the fluorescence was opened immediately after the far-red light was turned off. Recording h was obtained with five times lower paper speed and intensity of 630-nm light than the other recordings. Recording g, 727-nm light omitted; j, as i, but no preillumination.

cytochrome b_{559} was observed, presumably because it was already oxidized in the dark and the shape of the spectrum in the region 540–550 nm now showed the bands of C-550. These bands were about 40 % smaller than without preillumination. This was also true for P518.

Fig. 6 and Recording b of Fig. 1 allow a comparison of the kinetics of the various System 2-induced reactions observed in chloroplasts preilluminated with two flashes of light. As mentioned already, the kinetics of cytochrome b_{559} photooxidation were clearly slower than those of P518. Recordings f and i of Fig. 6 show that the photooxidation of cytochrome b_{559} proceeded at a similar rate as the light-induced increase in the yield of chlorophyll *a* fluorescence. With some preparations, the absorbance increase caused by System 1 was larger than shown here, which may explain why the cytochrome change was not clearly seen in earlier experiments [4]. As reported earlier for the fluorescence increase [4], the kinetics of the photooxidation of cytochrome b_{559} were independent of the intensity when plotted as function of the total amount of light absorbed, indicating that the rate of the reaction is not determined by a slow intermediary dark reaction. Measurements at 518 and 542 nm in the presence of $Fe(CN)_6^{3-}$ indicated that the kinetics of P518 and C-550 were similar.

The photooxidation of cytochrome b_{559} was completely or nearly completely irreversible at −50 °C. After several min of darkness, a second illumination with far-red and red light showed again a System 2-induced absorbance change of P518 accompanied by a slow increase in fluorescence, but no evidence for cytochrome b_{559} oxidation (Fig. 6, Recordings c, d and e). Apparently, the chloroplasts now behaved in the same way as if the cytochrome had been oxidized chemically before cooling.

DISCUSSION

The results reported in this paper show that at −50 °C, the light-induced absorbance change at 518 nm consists of two different components, associated with the primary photochemical reaction of System 1 and of System 2, respectively. The spectra of both components are similar, suggesting that both are due to a band shift of P518, which is presumably a carotenoid [20, 18]. Fig. 1 shows that the two phenomena can be largely separated kinetically; by comparing the kinetics with those of P700 and from the experiment with DCMU and hydroxylamine it can be estimated that, with dark-adapted chloroplasts under the conditions applied, of the total absorbance change of P518, 40 % was due to System 1 and 60 % to System 2. The System 1-induced absorbance change of P518 appears to reflect the oxidation state of P700; the experiments with prolonged illumination or added oxidants suggest that the absorbance change is not or little affected by the redox level of the primary acceptor.

The System 2 absorbance change of P518 showed the same kinetics as C-550 within the limits of accuracy of the measurements. With chloroplasts preilluminated with two flashes of light before cooling, the absorbance changes of C-550 and of the System 2 component of P518 were about 40 % smaller than with dark-adapted chloroplasts, as shown by the difference spectrum obtained in the presence of $Fe(CN)_6^{-3}$ (Fig. 4). The same effect was observed in measurements of absorbance changes in the ultraviolet region [21]. Without $Fe(CN)_6^{3-}$, the spectrum of preilluminated chloroplasts was obscured in the region near 550 nm by cytochrome b_{559}. The bands of C-550 are probably more clearly visible in difference spectra obtained at −196 °C [5, 16] than at −50 °C, because the differential absorption coefficient of the band shift increases upon lowering the temperature [15].

Our results confirm and strengthen the conclusion obtained earlier [4] that reaction centers of System 2 converted to States S_2 and S_3 by preillumination, perform two different photoreactions with different photochemical efficiencies. At temperatures between −40 and −90 °C these reactions are most easily observable, because secondary reactions are inhibited at this temperature, but fast measurements of fluorescence [22] suggest that they may also occur at room temperature. One reaction, apparently occurring in about 60 % of the reaction centers, consists of an efficient photoreduction of the electron acceptor associated with C-550, which acceptor is probably plastoquinone [21, 23]. The reduction is accompanied by a band shift of P518, and coupled to the oxidation of an unidentified electron donor. The second reaction, which is less efficient results in the photooxidation of cytochrome b_{559}. Comparison of the kinetics of cytochrome oxidation and of fluorescence suggests that the fluorescence yield, at least to a large extent is controlled by this reaction. The amount of cyto-

chrome photooxidized appeared to be somewhat less than one molecule per reaction center. A slow increase in fluorescence occurred also when cytochrome b_{559} had already been oxidized, either by $Fe(CN)_6^{3-}$ [3, 4] or by previous illumination at −50 °C (Fig. 6) indicating the photooxidation of an alternative donor under these conditions. Recently, Knaff and Malkin [24], from measurements with chloroplasts that had probably not been dark-adapted before cooling, concluded that at −50 °C cytochrome b_{559} is the only secondary electron donor to System 2. This conclusion does not agree with our results, as discussed above (see also ref. 4). It should be noted, however, that their conclusion was based upon measurements of C-550 with a "double-beam" spectrophotometer set at 543 and 548 nm; as Fig. 5 shows such measurements will be strongly affected by cytochrome b_{559}, and cannot be ascribed to C-550 only.

The electron acceptor for the reaction that causes oxidation of cytochrome b_{559} is not known. The kinetics at 518 or 540 nm gave no evidence for a slow conversion of C-550 after the first, rapid phase had been completed. This suggests that at least in part of the reaction centers the electron acceptor is different from the compound associated with C-550, as is also indicated by measurements of the kinetics of cytochrome b_{559} and C-550 at −196 °C [25, 26]. Vermeglio and Mathis [5] reported that a 10-s illumination with strong light eventually transformed all the available C-550, and oxidized an equivalent amount of cytochrome b_{559} per reaction center but the time courses of these reactions were not compared in these experiments.

One might speculate that in States S_2 and S_3 in some reaction centers at −50 °C the electron acceptor is an oxidized donor produced by a previous photoact, and that in addition to the normal Reaction 1 the Reaction 2 would also occur:

$$D_1^+ D_2^+ D_3 D_4 PQ\ (a) \xrightarrow{h\nu} D_1^+ D_2^+ D_3 D_4 P^+ Q^-\ (b) \longrightarrow D_1^+ D_2^+ D_3 D_4^+ PQ^-\ (c) \qquad (1)$$

$$D_1^+ D_2^+ D_3 D_4 PQ\ (a) \xrightarrow{h\nu} D_1^+ D_2 D_3 D_4 P^+ Q\ (d) \longrightarrow D_1^+ D_2 D_3 D_4^+ PQ\ (e) \qquad (2)$$

P and Q denote the primary electron donor, P680, and the primary electron acceptor (plastoquinone) associated with C-550, respectively; D_1–D_4 the various electron donors on the pathway to water; (a) then symbolizes a reaction center in State S_2. In Reaction 2, D_2^+, only present in States S_2 and S_3 would act as electron acceptor for the light reaction. Reaction 1 produces reduced Q, Reaction 2 does not, which would explain why less C-550 is converted in States S_2 and S_3 than in States S_0 and S_1. In some reaction centers P^+ might oxidize cytochrome b_{559} instead of D_4. The photoconverted reaction centers (e) would according to similar reasoning be able to react again photochemically, resulting in the oxidation of more cytochrome b_{559}. Such a reaction might also occur by photochemical conversion of reaction centers (c) if it is assumed that D_4^+ can also act as electron acceptor.

A detailed scheme of the various photochemical reactions that might be postulated to account for the observed phenomena would be too speculative at present. More precise kinetic data and especially more specific information about the nature of the reactants involved will be needed before the construction of a more detailed model will be profitable.

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