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LIGHT-INDUCED ABSORBANCE CHANGES IN CHLOROPLASTS MEDIATED BY PHOTOSYSTEM I AND PHOTOSYSTEM II AT LOW TEMPERATURE

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SUMMARY

Stable light-induced absorbance changes in chloroplasts at −196 °C were measured across the visible spectrum from 370 to 730 nm in an effort to find previously undiscovered absorbance changes that could be related to the primary photochemical activity of Photosystem I or Photosystem II. A Photosystem I mediated absorbance increase of a band at 690 nm and a Photosystem II mediated absorbance increase of a band at 683 nm were found. The 690-nm change accompanied the oxidation of P_{700} and the 683-nm increase accompanied the reduction of C-550. No Soret band was detected for P_{700}.

A specific effort was made to measure the difference spectrum for the photooxidation of P_{680} under conditions (chloroplasts frozen to −196 °C in the presence of ferricyanide) where a stable, Photosystem II mediated EPR signal, attributed to $P_{680}{}^{+}$ has been reported. The difference spectra, however, did not show that $P_{680}{}^{+}$ was stable at −196 °C under any conditions tested. Absorbance measurements induced by saturating flashes at −196 °C (in the presence or absence of ferricyanide) indicated that all of the $P_{680}{}^{+}$ formed by the flash was reduced in the dark either by a secondary electron donor or by a backreaction with the primary electron acceptor. We conclude that $P_{680}{}^{+}$ is not stable in the dark at −196 °C: if the normal secondary donor at −196 °C is oxidized by ferricyanide prior to freezing, $P_{680}{}^{+}$ will oxidize other substances.

INTRODUCTION

Significant progress has been made in recent years to elucidate the primary photochemical reactions by which photosynthetic organisms convert light energy to

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Abbreviation: DBMIB, dibromothymoquinone.

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chemical free energy. These reactions appear to be light-driven electron transfer reactions between a reaction center chlorophyll, P, which can harvest excitation energy from a large number of chlorophyll molecules and an electron acceptor. A, closely associated with the reaction center [1, 2]. Excitation energy in the excited reaction center chlorophyll is used to effect the electron transfer and create a primary oxidant, P^+, and a primary reductant, A^-, which represent the chemical free energy used in subsequent dark reactions.

The primary photochemical reactions of photosynthesis can be distinguished from the secondary thermochemical reactions by using low temperatures to selectively inhibit the thermochemical reactions or by using short pulse irradiation and rapid kinetic measurements to temporally isolate the primary photochemical reactions. Assay methods based on optical absorbance or EPR measurements have been described to measure redox changes of all four of the primary reactants of the two photochemical reactions of plant photosynthesis. Thus, in principle, it should be possible to study the primary photochemical reactions of Photosystem I or Photosystem II by measuring the light-induced changes of a given pair of primary reactants under the same experimental conditions. In practice, however, the assay methods tend to focus on, or be applicable to, only one of the primary reactants. For example, the primary electron donor of Photosystem II, P_{680}, has been studied by repetitive flash experiments at room temperature [3] or by single flash experiments at low temperature [4] but the primary electron acceptor of Photosystem II, C-550, has not been measured in the same type of experiment. On the other hand, C-550 has been studied in steady illumination experiments both at room temperature [5–8] and at low temperature [5, 9–11] but P_{680} has not been measured under these conditions. One specific purpose of the work reported here was to attempt to measure the bleaching of P_{680} in light-minus-dark difference spectra of chloroplasts at −196 °C under conditions where C-550 reduction could also be observed. If such measurements were successful it should be possible to study the kinetics of P_{680} changes during irradiation at low temperature under the same conditions that have been used to study the kinetics of the changes of C-550, cytochrome b_{559} and fluorescence yield [12]. Such measurements would also permit correlations between the optical measurements of P_{680} at low temperature and the light-induced EPR signal which Malkin and Bearden [13] attributed to the oxidized form of P_{680}.

The primary photochemical activity of Photosystem I and Photosystem II can be distinguished readily at low temperatures and examined separately. Far-red light activates only Photosystem I so that a difference spectrum at −196 °C between a far-red irradiated sample and a dark sample should reveal only changes in reactants associated with the Photosystem I reaction centers. Likewise, a difference spectrum at −196 °C between a red irradiated sample and a far-red irradiated sample should indicate only those changes directly associated with Photosystem II. These techniques of difference spectroscopy at low temperature have been used to look throughout the entire visible spectrum under different redox conditions for any undiscovered light-induced absorbance changes which are stable at −196 °C and to relate these changes to the primary photochemistry of either Photosystem I or Photosystem II.

MATERIALS AND METHODS

Spinach chloroplasts were prepared by methods described by Yamashita and Butler [14] and Photosytem I subchloroplast particles (sedimented by centrifugation at 160000 × g for 1 h) by the procedure of Sane et al. [15]. Absorption spectra were measured with a single beam spectrophotometer on line with a PDP8/I computer [16]. Chloroplasts, at the chlorophyll concentration indicated in the figure legends, were suspended in a vertical cuvette in 0.5 ml of a 50% glycerol–50% 0.1 M phosphate buffer (pH 8.0) mixture and frozen, after a dark incubation period of a few minutes, to -196 °C as a clear glass. The vertical cuvette and Dewar have been described previously [17]; 0.5 ml samples gave an optical thickness of about 3 mm. Absorption spectra of a given sample were measured at -196 °C before and after one or more irradiation treatments. Samples were irradiated at -196 °C with far-red light (735 nm, 2.0 mW · cm^{-2} for 2 min), with red light (630 nm, 1.5 mW · cm^{-2}, for 1 min) or with saturating flashes of white light from a xenon lamp (16 μs duration). Difference spectra showing the absorbance changes induced by a given irradiation treatment or the difference between two different irradiation treatments were obtained from individual frozen samples. Chemical difference spectra between unirradiated samples frozen under different redox conditions required pairs of frozen samples. The spectra presented were read out directly from the computer to an X–Y recorder and include no human errors due to hand plotting.

EPR spectra were measured with a Varian E-3 spectrometer fitted with a slotted cavity which permitted actinic irradiation of the sample. A Dewar cooled with cold nitrogen gas kept the sample in the cavity at about -180 °C.

RESULTS

A. Light-induced absorbance changes in the red region of the spectrum

The absorption spectrum of spinach chloroplasts at -196 °C and the light-induced difference spectra due to irradiation of the sample with red and far-red light at -196 °C are shown in Fig. 1A. Irradiation of the frozen chloroplasts with far-red light (see FR–D difference spectrum in Fig. 1A) causes the photooxidation of P_{700}, as indicated by the bleaching at 702 nm. An absorbance increase is also apparent at 690 nm but there are no changes associated with Photosystem II activity. A subsequent irradiation of the same sample with red light (see R–FR difference spectrum) induces the Photosystem II mediated changes which include the photoreduction of C-550 (absorbance increase at 543 nm and bleaching at 546 nm), the photooxidation of cytochrome b_{559} (bleaching at 556 nm) and an increase of fluorescence yield which causes the large apparent bleaching of the main chlorophyll absorption band at 677 nm*. Another light-induced absorbance change is apparent in the R–FR differ-

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* The light reaching the phototube consists of the measuring beam transmitted by the sample and the chlorophyll fluorescence excited by the measuring beam. With relatively dense chloroplast samples the fluorescence can be an appreciable part of the light signal at the phototube. The increase of the fluorescence yield due to irradiation with red light results in an increase of fluorescence excited by the measuring beam and, therefore, an apparent bleaching in the red-minus-far-red (R–FR) or red-minus-dark (R–D) difference spectra of all absorption bands that excite chlorophyll fluorescence. The possible influence of this fluorescence yield artifact on previous measurements of P_{680} has been discussed in greater detail elsewhere [18].

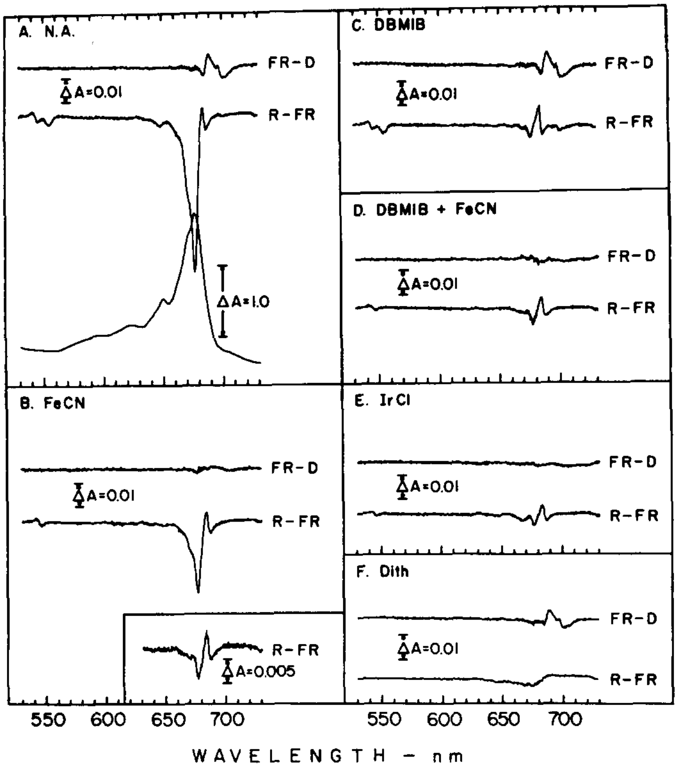

Fig. 1. Light-induced absorbance changes in spinach chloroplasts at −196 °C. Chloroplasts were suspended at 140 μg chlorophyll/ml in a 50 % glycerol buffer and frozen in complete darkness to −196 °C as a clear glass. Absorption spectra were measured before irradiation (D) and after sequential irradiations with far-red (FR) and red (R) light and difference spectra between the far-red irradiated and the dark spectra (FR–D) and between the red irradiated and the far-red irradiated spectra (R–FR) are presented. A, no addition made. The absolute absorption spectrum of the dark sample is also shown. B, 20 mM potassium ferricyanide (FeCN) was added to the chloroplasts before freezing. Inset shows R–FR difference spectrum at a chlorophyll concentration of 72 μg chlorophyll/ml. C, 100 μM dibromothymoquinone (DBMIB) added prior to freezing. D, 100 μM DBMIB and 20 mM FeCN added prior to freezing. E, 1 mM hexachloroiridate (IrCl) added prior to freezing. F, a few grains of dithionite (Dith) added prior to freezing.

ence spectrum near 683 nm but absorbance changes in the spectral region of the main chlorophyll band are difficult to characterize because of the large fluorescence yield artifact.

Most of the data presented in this paper represent some compromise in order to obtain difference spectra over an extended wavelength interval encompassing both C-550 and P_{700} changes from a given sample. We would generally use a chlorophyll concentration of about 200–250 μg/ml for chloroplasts frozen in glycerol to measure absorbance changes in the green region at an optimum signal-to-noise ratio. However, a lower chlorophyll concentration, 70–100 μg/ml, would generally be used for measurements in the red to decrease the strong absorbance of chloroplast samples in this region and the resulting fluorescence yield artifact. Most of the measurements made over the extended spectral region were made at a chlorophyll concentration of 140

μg/ml so that the fluorescence yield artifact is larger than we would like but the C-550 and cytochrome b_{559} absorbance changes are large enough to be characterized. In a few cases, measurements at other concentrations over a limited spectral range are presented as insets to the figures.

The fluorescence yield artifact which presents a severe limitation to measuring absorbance changes in the red region can be reduced or eliminated by using chemical reagents which quench fluorescence. It was noted previously [19] that light-induced fluorescence yield changes at −196 °C were considerably less with chloroplasts which had been frozen in the presence of ferricyanide. Fig. 1B shows the far-red light-induced and the red light-induced absorbance changes with such chloroplasts. The ferricyanide (20 mM) oxidized P_{700} prior to freezing so that no primary electron donor was available for Photosystem I mediated reactions. Thus, no Photosystem I reactions should be expected and none are observed in the FR–D difference spectrum. A subsequent irradiation of the ferricyanide-treated chloroplasts at −196 °C with red light caused the photoreduction of C-550 without the concomitant photooxidation of cytochrome b_{559} (cytochrome b_{559} was oxidized by the ferricyanide), a smaller fluorescence yield artifact than in Fig. 1A because of the smaller fluorescence yield increase and the absorbance increase at 683 nm that is also apparent in Fig. 1A. The red light-induced changes in the red region of the spectrum are shown in the inset of Fig. 1B for a ferricyanide-treated chloroplast sample of lower chlorophyll concentration (72 rather than 140 μg/ml) where the fluorescence yield artifact is still less prominent. The light-induced absorbance increase at 683 nm is observed both in the presence and in the absence of ferricyanide but the absorbance change is more readily characterized in the presence of ferricyanide because the fluorescence yield artifact is less.

The oxidized form of the electron transport inhibitor dibromothymoquinone (DBMIB) is a much more effective quencher of fluorescence than ferricyanide. It was shown previously [20] that 5 μM DBMIB quenched over 50% of all chlorophyll fluorescence emission at room temperature and at −196 °C. In experiments reported here 100 μM DBMIB was used. This concentration decreased the fluorescence yield at 692 nm by approx. 100-fold and eliminated the fluorescence yield artifact. The fluorescence quenching appears to be a general property of oxidized quinones [21] but DBMIB is a much more effective quencher than any other quinone tested.

The far-red and red light-induced absorbance changes of chloroplasts frozen in the presence of 100 μM DBMIB are shown in Fig. 1C. Far-red irradiation causes the typical Photosystem I mediated absorbance changes and red light the typical Photosystem II mediated absorbance changes but without any fluorescence yield artifact. The R–FR difference spectrum in Fig. 1D measured in the presence of DBMIB plus ferricyanide shows the same 683-nm change as that measured without ferricyanide; the only difference due to ferricyanide is that cytochrome b_{559} was not photooxidized because of the prior chemical oxidation.

The light-induced absorbance changes measured in the presence of 1 mM hexachloroiridate are shown in Fig. 1E. The hexachloroiridate ($E^0 > 1.0$ V) is a much stronger oxidant than ferricyanide and can cause a chemical destruction of C-550 and bleaching of the bulk forms of chlorophyll*. In the sample used for Fig. 1E,

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* We had hoped at one time to be able to titrate the redox potential of the primary electron donor to Photosystem II by determining the potentials at which the photoreduction of C-550 at −196 °C would be blocked by the chemical oxidation of the primary donor prior to freezing. This experiment was not feasible, however, because of the oxidative destruction of the C-550 at high potentials.

approx. 30% of the C-550 and approximately the same fraction of the bulk chlorophyll were destroyed during a 10-min incubation period at room temperature. No far-red light-induced changes are apparent in the FR–D difference spectrum because P_{700} was chemically oxidized prior to freezing but those Photosystem II reaction centers which are still functional show the normal photoreduction of C-550 and the absorbance increase of 683 nm. As in the case of ferricyanide, however, the presence of hexachloroiridate did not result in any new Photosystem II mediated light-induced absorbance changes.

Fig. 1F shows the light-induced difference spectra of chloroplasts frozen in the presence of dithionite. In the absence of a mediator, such as one of the viologen dyes, dithionite does not readily reduce the primary electron acceptor of Photosystem I so the primary photoreactions induced by far-red light still occur. (In the presence of a viologen dye the photooxidation of P_{700} at −196 °C is blocked by the chemical reduction of the primary acceptor at potentials below −550 mV. This technique has been used to titrate the midpoint potential of the primary electron acceptor of Photosystem I [22].) Dithionite does reduce the C-550, however, so that the Photosystem II mediated reactions do not occur on irradiation with red light because of the absence of a primary electron acceptor. Thus, it is possible to selectively block Photosystem II activity by freezing chloroplasts in the presence of dithionite or Photosystem I activity by freezing in the presence of ferricyanide.

B. Light-induced absorbance changes in the blue region of the spectrum

The absorption spectrum and light-induced difference spectra of chloroplasts in the blue and green regions of the spectrum are shown in Fig. 2A. The FR–D difference spectrum shows surprisingly little detail in this spectral region. There is a small absorbance increase at 445 nm, possibly a just-detectable bleaching at 430 nm and small absorbance changes at about the noise level in the carotenoid region. We had expected to observe the bleaching of a well developed Soret band of P_{700} comparable to the bleaching (0.005) of the 702-nm band observed in Fig. 1.

A subsequent irradiation with red light (see R–FR difference spectrum in Fig. 2A) causes the photoreduction of C-550 and the photooxidation of cytochrome b_{559} and in this difference spectrum the bleaching of the cytochrome b_{559} Soret band at 427 nm is readily apparent. In addition, the irradiation with red light at −196 °C caused small absorbance changes, primarily a bleaching of bands at 492 and 487 nm, which are probably due to carotenoids. The Photosystem II mediated fluorescence yield artifacts have been eliminated from the measurements in the blue region by using a phototube (EMI 6225) which is not sensitive to the wavelengths of chlorophyll fluorescence and by using blocking filters to prevent the fluorescence from reaching the phototube.

The light-induced difference spectra for chloroplasts frozen in the presence of dithionite are shown in Fig. 2B. It is apparent from Fig. 1F that dithionite does not block the photooxidation of P_{700} by far-red light but no changes are observed in the FR–D difference spectrum because the photobleaching of the P_{700} Soret band is not expressed in these low temperature difference spectra. No Photosystem II changes should be expected because the primary electron acceptor should be reduced by dithionite and none are observed in the R–FR difference spectrum.

The light-induced difference spectra for chloroplasts frozen in the presence

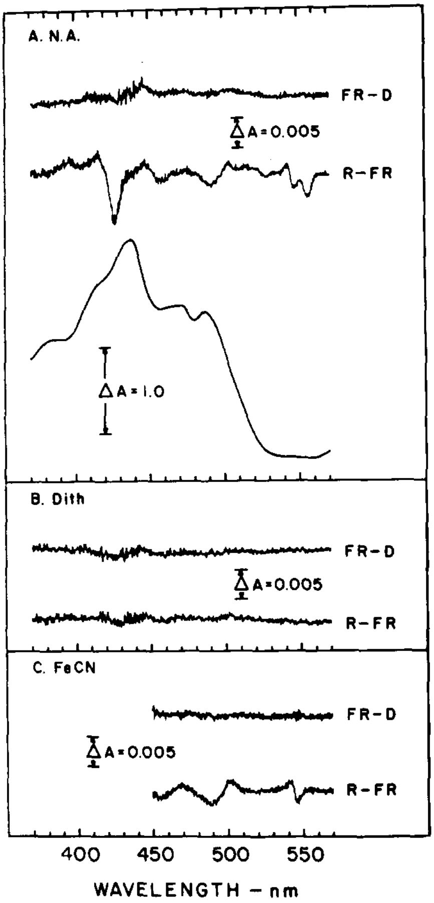

Fig. 2. The same sample preparation and irradiation treatments as indicated in the legend of Fig. 1. The absorption spectra were measured with an EMI 6255 phototube blocked with a Corning 9788 colored glass filter. A, no addition; B, dithionite; C, ferricyanide added prior to freezing.

of 20 mM ferricyanide are shown in Fig. 2C. The measurements were not carried below 450 nm because of the strong absorbance by the ferricyanide. The measurements do not show any light-induced absorbance changes that require the presence of ferricyanide. (The significance of the requirement for ferricyanide will be discussed vide infra.)

C. Flash-induced changes

It was noted previously [23, 24] that a single saturating flash of white light at −196 °C was relatively ineffective in inducing Photosystem II photoreactions. Similar observations have been repeated here because they bear on the stability of the primary reaction products. Absorption spectra of the chloroplast samples were measured before irradiation (D), after a single saturating flash (F1), after a second flash, after a total of 10 flashes, and after 30 s of irradiation with continuous red light (R). The fraction of the total change induced by the flash is indicated by comparing the F1−D

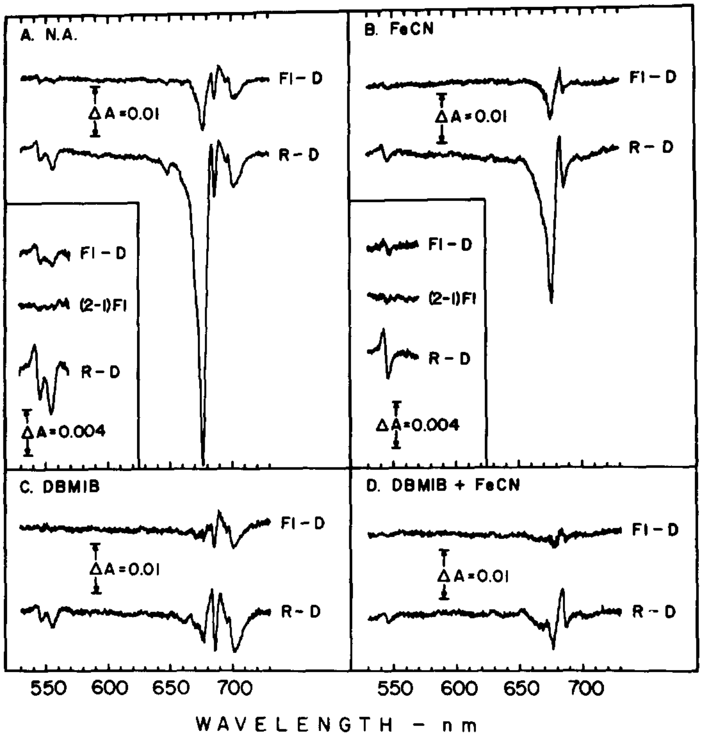

Fig. 3. The same sample preparation as indicated in the legend of Fig. 1. Absorption spectra were measured before irradiation, after sequential irradiations with one and with two flashes from a xenon lamp and finally after a 30-s irradiation with red light. Fl–D is the difference spectrum due to one flash. (2–1)Fl is the difference between two flashes and one flash, R–D is the total of the light-induced changes. A, B. C, D, additions as indicated prior to freezing. Insets in A and B are for chloroplasts at a concentration of 200 μg chlorophyll/ml rather than 140 μg/ml for the rest of the figure.

difference spectrum with the R–D difference spectrum. Measurements in the green region of the spectrum on chloroplast samples with a higher chlorophyll content (200 instead of 140 μg/ml) are presented in the insets in Figs 3A and 3B to better distinguish the action of the flash on C-550 and cytochrome b_{559}. The first saturating flash transformed about 30% of the total C-550. (There is some variability in the fraction of the C-550 transformed by a single saturating flash among different chloroplast preparations; the value of 30% is toward the upper end of that range of variability.) The effect of the second flash at −196 °C, however, is much less than that of the first and is scarcely detectable in the difference spectrum between the twice flashed and once flashed sample, (2–1)Fl. If the second flash had been equally effective it would have transformed an additional 20% of the C-550. The cumulative effect of ten supersaturating flashes was to transform only 70% of the total C-550 (data not shown).

The difference spectra induced by the flashes of white light or by the continuous irradiation with red light show both Photosystem I and Photosystem II mediated changes. Photosystem I causes the bleaching at 702 nm and the absorbance increase at 690 nm; Photosystem II causes the absorbance increase at 683 nm, the apparent bleaching at 677 nm due to the fluorescence yield artifact and the absorbance changes in the green region. It is apparent that the flash is more effective in mediating Photosystem I changes than Photosystem II changes: approx. 70% of the maximum light-induced change of P_{700} was effected by a single saturating flash at −196 °C while only 30% of the C-550 was transformed.

A question relevant to this work which will be discussed in greater detail in Discussion is whether the presence of ferricyanide alters the effectiveness of the flash to induce Photosystem II mediated changes. The flash-induced changes with chloroplasts frozen in the presence of 20 mM ferricyanide are shown in Fig. 3B. Photosystem I mediated changes do not occur under these conditions because the P_{700} is oxidized. The fraction of the C-550 found in the reduced state after the saturating flash is essentially the same (30%) with or without ferricyanide. The flash induced changes are also shown in the presence of DBMIB (Fig. 3C) and DBMIB plus ferricyanide (Fig. 3D) to eliminate the fluorescence yield artifact and better elucidate the 683-nm change. The extent of the 683-nm change appears to be related to the extent of the C-550 change and independent of the presence of ferricyanide.

D. Chemical difference spectra

Chemical difference spectra resulting from oxidation of the chloroplasts by ferricyanide and reduction by dithionite were measured to determine whether the Photosystem I mediated absorbance increase at 690 nm and the Photosytem II medi-

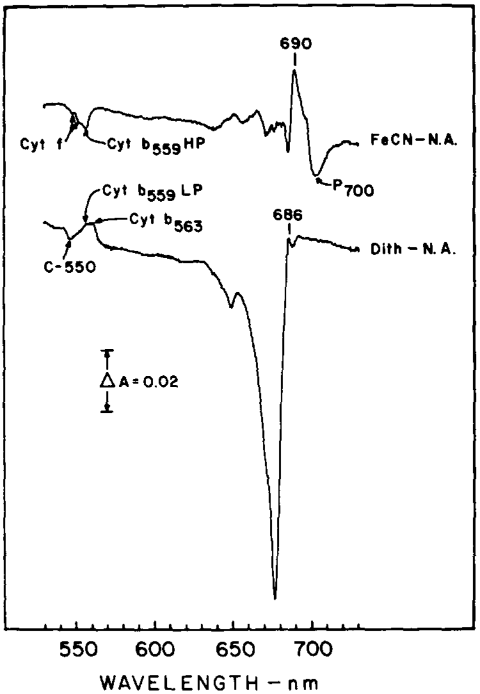

Fig. 4. Chemical difference spectra between a sample frozen in the presence of 20 mM ferricyanide and a sample frozen without addition (FeCN-N.A.) and between a sample frozen with a few grains of dithionite added and a sample without addition (Dith-N.A.)

ated absorbance increase at 683 nm were linked to redox changes of the primary electron donors or acceptors of those photosystems. It seemed possible that the absorbance increase at 690 nm might reflect the reduction of the primary electron acceptor of Photosystem I while the bleaching at 702 nm was due to the oxidation of the primary donor. Likewise, it was not certain whether the absorbance increase at 683 nm was linked to the primary electron donor or acceptor of Photosystem II. These possibilities should be distinguishable in chemical difference spectra. The FeCN–N.A. difference spectrum in Fig. 4 shows that chemical oxidation by ferricyanide causes both the absorbance increase at 690 nm and the typical bleaching of the P_{700} absorption band. Thus, the 690-nm absorbance change is linked to the oxidation of P_{700} and not to the primary acceptor because the latter should not be affected by ferricyanide. The measurements in the green region also show the chemical oxidation of cytochrome *f* and cytochrome b_{559}. The Dith–N.A. difference spectrum in Fig. 4 shows a large fluorescence yield artifact due to the higher fluorescence yield after reduction by dithionite (DBMIB cannot be used to eliminate the fluorescence yield artifact from such an experiment because only the oxidized form of the inhibitor quenches fluorescence) and an apparent absorbance maximum at 686 nm. This latter band is the 683-nm absorbance increase, minimized in appearance and shifted to longer wavelength by the steep fluorescence yield artifact. The fact that this band is observed in the chemical difference spectrum, however, indicates that the absorbance increase at 683 nm is linked to the reduction of the primary electron acceptor of Photosystem II and not to the oxidation of the primary electron donor. In all of the data presented, the absorbance increase at 683 nm accompanies the reduction of C-550. The Dith–N.A. difference spectrum also shows the reduction of C-550 and probably both a low potential cytochrome b_{559} and cytochrome b_{563}.

E. Reversibility of P_{700} at −196 °C.

The absence of the Soret band of P_{700} in the FR–D difference spectrum of chloroplasts led us to make similar measurements with Photosystem I subchloroplast particles. It could be supposed that the Soret band of P_{700} was not observed because the P_{700} in whole chloroplasts was heavily screened by other blue absorbing pigments. If that were the case, such screening should be appreciably less in the small Photosystem I particles. The absorption spectrum and the light-induced difference spectrum of the Photosystem I particles are shown in Fig. 5A. The Soret band of P_{700} is also absent from the light-minus-dark difference spectrum of Photosystem I particles. We have no explanation for the absence of the Soret band of P_{700} from these low temperature difference spectra.

In the course of making measurements of P_{700} it was found both in chloroplasts and in Photosystem I particles that only about half of the P_{700} was in the oxidized state after an irradiation at −196 °C. Fig. 5B shows two difference spectra; one is the chemically induced difference spectrum, FeCN–Asc, between a sample frozen in the presence of ferricyanide and a sample frozen in the presence of ascorbate; the other is a light-induced difference spectrum, L′–D, between a sample which was irradiated throughout the freezing process and another sample frozen in darkness. It is apparent that the two difference spectra of Fig. 5B indicate at least twice as much P_{700} as is indicated by the L–D difference spectrum of Figure 5A. EPR measurements of P_{700} (Signal I) were made to study P_{700} during irradiation at −196 °C.

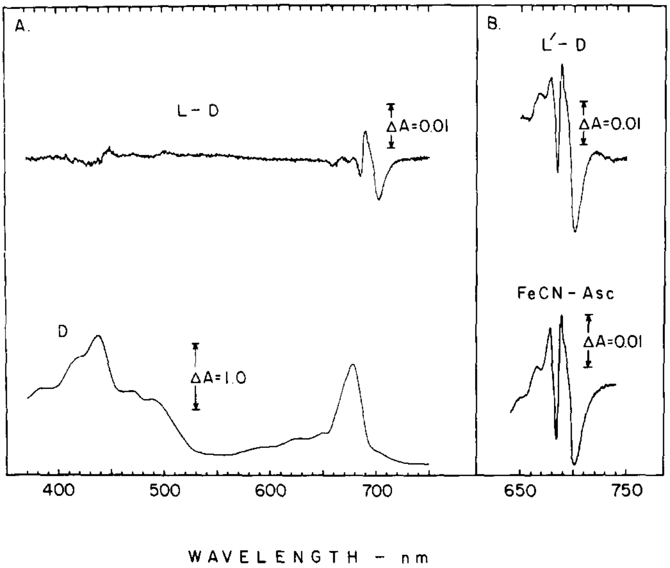

Fig. 5. Absorption spectra of Photosystem I subchloroplast particles frozen to −196 °C in a 50 % glycerol buffer with 1 mM sodium ascorbate. A, absolute absorption spectrum of the sample before irradiation (D) and the difference spectrum due to irradiation with far-red light for 2 min (L–D). B, difference spectrum between a sample frozen in the presence of 20 mM ferricyanide and a sample frozen in the presence of 1 mM ascorbate (FeCN–Asc) and the difference spectrum between a sample frozen during irradiation with far-red light and a sample frozen in darkness (L′–D).

Fig. 6A shows the EPR Signal I spectrum of the Photosystem I prepration before, during, and after actinic irradiation. It is apparent that a part of the P_{700} oxidized by light at −196 °C is reduced in the dark and that this reversible part remains reversible in subsequent irradiations (Fig. 6A′). If the sample is frozen during irradiation (Fig. 6B), all of the P_{700} is frozen in the oxidized state and no further change is observed in subsequent irradiation at −196 °C. Chemical oxidation of P_{700} by ferricyanide (Fig. 6C) gives essentially the same extent of Signal I as is shown in Fig. 6B. The additional signal seen on the first irradiation of the ferricyanide-treated sample with white light is probably the Photosystem II generated EPR signal reported by Malkin and Bearden [13] and indicates some contamination of the Photosystem I particles. The reversibility of approximately half of the light-induced P_{700} oxidation at −196° C was seen previously in blue-green algae by Mayne and Rubinstein [25].

DISCUSSION

The photoreduction of C-550 and the photooxidation of cytochrome b_{559} by Photosystem II at low temperature, first noted by Knaff and Arnon [5], was taken to indicate a reaction sequence for the Photosystem II reaction center complexes [26]:

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

where A is C-550, P is the reaction center chlorophyll P_{680} and D is cytochrome b_{559}. This scheme gained support from redox titration experiments [11] on the photo-

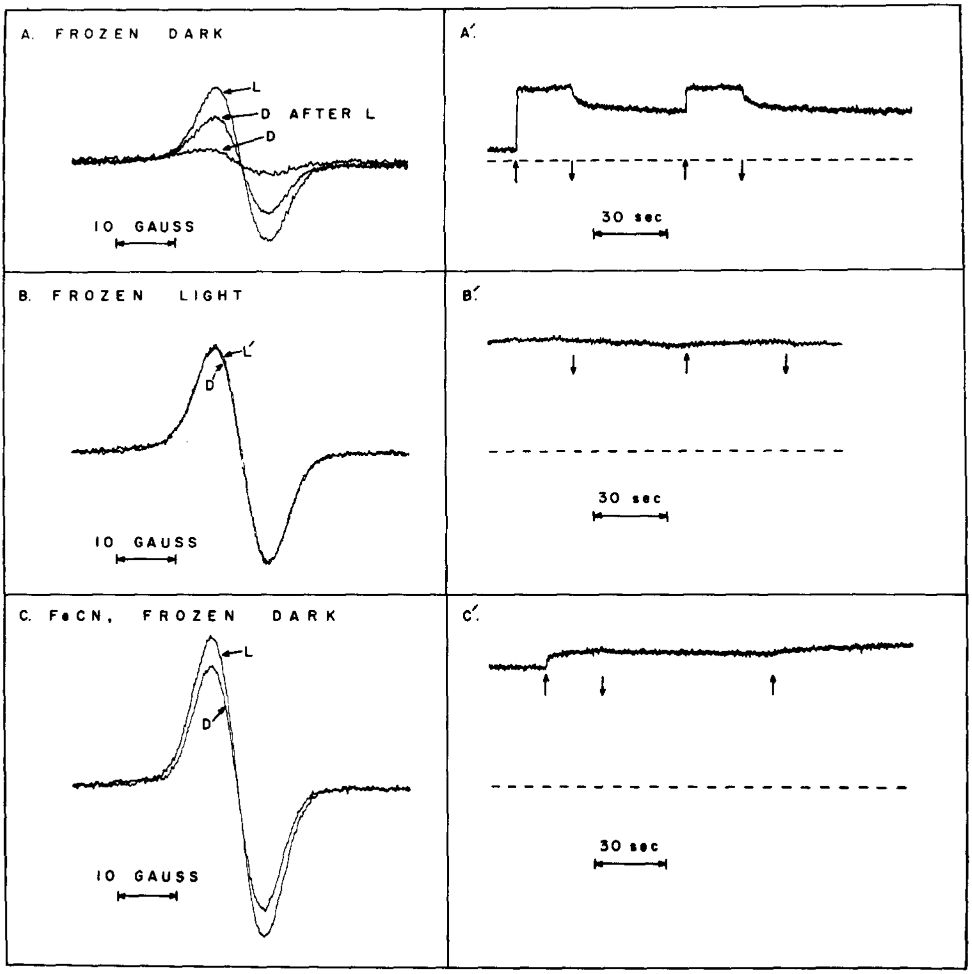

Fig. 6. EPR spectra of Photosystem I subchloroplast particles frozen to −180 °C in a 50 % glycerol buffer with 1 mM sodium ascorbate. A, EPR spectrum of the sample frozen in the dark (D), during irradiation with white light, 10 mW/cm^2 (L) and in the dark after the light (D after L). A', kinetics of the signal measured at the low field maximum. White light on at upward arrows, off at downward arrows. B, EPR spectrum of sample frozen during irradiation and measured during irradiation (L') and of the sample in the dark after the irradiation (D). B', same as A'. C, EPR signal of sample frozen in the presence of 20 mM ferricyanide (D) and during irradiation with wihte light (L). C' same as A'.

chemical reactions at low temperature and from kinetic measurements [12] of the rates of the photoreduction of C-550, the photooxidation of cytochrome b_{559} and the fluorescence yield increase in nonsaturating actinic light at low temperatures. The latter experiments also showed that cytochrome b_{559} was not the only compound which could function as a secondary electron donor at low temperature.

In the previous work [19], where it was found that the maximum level of the fluorescence yield at −196 °C was considerably lower if chloroplasts were frozen in the presence of ferricyanide, two hypotheses were proposed: (1) P^+ might be stabilized at low temperature if the secondary electron donor were oxidized prior to freezing:

$$D^+ \cdot P \cdot A \xrightarrow{h\upsilon} D^+ \cdot P^+ \cdot A^-$$

and the P^+ might act as a quencher or; (2) another compound might act as a secondary donor when the normal donor was oxidized by ferricyanide and the oxidized form of the alternative secondary donor quenched fluorescence. The first of these two hypotheses appeared to be confirmed by the work of Malkin and Bearden [13] which showed a stable, Photosystem II mediated, light-induced EPR signal at low temperatures which required the presence of ferricyanide. They suggested [13, 27] that this new EPR signal, which was similar in g value, linewidth and number of spins to the EPR signal of oxidized P_{700}, was due to the oxidized form of P_{680} which was stabilized at low temperature in the presence of ferricyanide because of the lack of a secondary electron donor to reduce $P_{680}{}^+$.

However, measurements [24] of absorbance and fluorescence yield changes induced by a single saturating flash of light indicated the operation of a relatively efficient backreaction between P^+ and A^- at low temperatures. It had been expected that a saturating flash at −196 °C would reduce all of the C-550 and increase the fluorescence yield to the maximum level; it was found that such a flash reduced only a small fraction of the C-550 and increased the fluorescence yield to only a small extent. To account for these observations a backreaction was introduced into the photochemical reaction sequence [2, 24]:

$$D \cdot P \cdot A \underset{k_{-1}}{\overset{h\upsilon}{\rightleftharpoons}} D \cdot P^+ \cdot A^- \xrightarrow{k_2} D^+ \cdot P \cdot A^-$$

According to this scheme the saturating flash produces the primary charge separation ($D \cdot P^+ \cdot A^-$) in all of the reaction centers but the backreaction, k_{-1}, is faster than the charge stabilization reaction, k_2, so that relatively little of charge separation is preserved in A^- and D^+ after the flash.

The efficient operation of the backreaction at −196 °C cast doubt on the proposed stability of P^+ in the $D^+ \cdot P^+ \cdot A^-$ state obtained by irradiating ferricyanide-treated chloroplasts. It could be argued that the presence of D^+ in the reaction center complex might influence the electron tunnelling time between P^+ and A^- so that the backreaction would be less likely. However, two independent experimental tests were available to determine if the $D^+ \cdot P^+ \cdot A^-$ state were stable: (1) if $D^+ \cdot P^+ \cdot A^-$ were stable a saturating flash should be more effective in reducing C-550 in ferricyanide-treated chloroplasts at −196 °C than in control chloroplasts without ferricyanide and, (2) the difference spectrum of P^+ should be apparent in a light ($D^+ \cdot P^+ \cdot A^-$)-minus-dark ($D^+ \cdot P \cdot A$) difference spectrum of ferricyanide-treated chloroplasts at −196 °C. Furthermore, the difference spectrum for P^+, if it were found, should require the presence of ferricyanide.

Both experimental tests for the presence of $P_{680}{}^+$ after irradiation of ferricyanide-treated chloroplasts at −196 °C were negative. Fig. 3 showed that the effectiveness of the saturating flash to reduce C-550 is the same in the presence or absence of ferricyanide. The light-minus-dark difference spectra in Figs 1 and 2 fail to reveal any absorbance changes expected for P_{680} bleaching and no absorbance changes were found which required the presence of ferricyanide. We conclude that the backreaction functions in the $D^+ \cdot P^+ \cdot A^-$ state and that an unknown secondary donor (not oxidized by ferricyanide) reduces P^+ in competition with the backreaction.

The Photosystem II mediated light-induced absorbance increase at 683 nm does not appear to be related to P_{680}. The observation that the 683-nm band appears after chemical reduction by dithionite suggests it is linked to the primary electron acceptor of Photosystem II. The 683-nm band is not related to the species that gives rise to the EPR signal measured by Malkin and Bearden since the 683-nm absorbance change showed no dependence on ferricyanide and the EPR signal appears only in chloroplasts frozen in the presence of ferricyanide.

The assignment by Malkin and Bearden of the EPR signal to ${P_{680}}^+$ is attractive because of the similarities to the EPR signal of oxidized P_{700}. However, such an EPR signal is not a very specific criterion for chemical identification so that, in the absence of the expected absorbance changes for P_{680}, we suggest that the light-induced EPR signal measured in the presence of ferricyanide is due to the oxidized form of an alternative secondary donor which is oxidized by ${P_{680}}^+$ when the normal secondary donor has been previously oxidized by ferricyanide.

The light-induced increase of absorbance at 683 nm with the slight bleaching on both the long and short wavelength sides looks like an increase of extinction accompanied by a narrowing of an absorption band at 683 nm. The increase of absorbance at 690 nm which accompanies the oxidation of P_{700} could also be due to a similar narrowing and extinction increase of a band at 690 nm. Such chlorophyll absorption bands are present. A fourth derivative analysis of the absorption spectrum of chloroplasts at −196 °C showed absorption bands with maxima at both 684 and 690 nm [16, 28]. These bands had been observed earlier [29] in a first derivative analysis of the low temperature absorption spectra of chloroplasts and of Photosystem I and Photosystem II subchloroplast particles: the 684-nm band was shown to be associated with Photosystem II and the 690-nm band with Photosystem I. The absorbance changes ascribed to the 683- and 690-nm bands (hyperchromicity and band narrowing and/or possibly small band shifts) appear more like indicator responses rather than direct redox changes; the 683-nm band indicates the redox state of the primary electron acceptor of Photosystem II and the 690-nm band indicates the redox state of the primary electron donor to Photosystem I. By analogy, the P_{800} pigment of bacterial reaction centers appears to indicate the redox state of P_{870} by shifting its absorption maximum to slightly shorter wavelength when the P_{870} is oxidized, either chemically or photochemically.

The Photosystem II mediated absorbance changes noted in the carotenoid region, i.e. the bleaching at 492 and 457 nm, are probably also indicator responses. However, we did not observe the 520-nm change in the low temperature difference spectra induced by red or by far-red irradiation.

The flash results in Fig. 3 showing that the first flash at −196 °C is considerably more effective than subsequent flashes in reducing C-550 indicate a heterogeneous population of reaction center complexes at −196 °C. If the reaction center complexes froze with slight variations in the electron tunnelling distances between D and P and/or between P and A, different reaction centers should have different probabilities for the reactions k_2 and k_{-1}. Since the quantum yield for the production of stable charge separation should be proportional to the ratio $k_2/(k_{-1}+k_2)$ some reaction centers should be more transformable than others. The more transformable reaction centers would be more likely to be converted by the first flash. The reaction center complexes which remained unconverted after 10 saturating flashes at −196 °C (approx. 30%) must represent a fraction of the population with a low quantum yield for transformation, presumably because of a low $k_2/(k_{-1}+k_2)$ ratio: these reaction centers have to be excited many times before the forward reaction k_2 finally occurs. This heterogeneity of reaction center complexes should be kept in mind when interpreting low temperature photoreactions. For instance, such a heterogeneity of reaction centers at low temperature predicts that the fluorescence yield increase which occurs during steady irradiation at −196 °C should not be a simple first order reaction. Careful measurements [12] show that there is a long tail as the fluorescence yield approaches the maximal level consistent with the prediction that the quantum yield for transformation is less for those reaction centers which are transformed last.

The observations of P_{700} at low temperature also suggest a heterogeneity of Photosystem I reaction center complexes. About half of the P_{700} participates in a reversible photooxidation at −196 °C and half in an irreversible photooxidation. The dark reduction of the reversible P_{700} is probably due to a backreaction with the primary electron acceptor, X, rather than to reduction by a secondary electron donor since the reversible part is repeatedly reversible.

$$P \cdot X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P^+ \cdot X^-$$

The irreversible photooxidation could be due to the close proximity of a secondary electron acceptor, Y, to some of the reaction centers.

$$P \cdot X \cdot Y \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P^+ \cdot X^- \cdot Y \xrightarrow{k_2} P^+ \cdot X \cdot Y^-$$

The observation that 50% of the P_{700} is reversible at −196 °C, however, suggests two types of reaction centers rather than a broad range of heterogeneity.

An interesting model emerges, which lends itself to a 1:1 reversible: irreversible ratio, if we let X be another chlorophyll molecule. We must also assume that the secondary electron acceptor Y is in contact with (or accepts electrons from) only one of the two chlorophyll molecules and that, on freezing, a polarity is frozen in to the dimer so that one chlorophyll is the potential donor and the other is the potential acceptor.

$$\begin{tikzcd}
 & & {[P^+ \cdot P^-] \cdot Y} \arrow[r] & {[P^+ \cdot P] \cdot Y^-} \\
{[P \cdot P] \cdot Y} \arrow[r, shift left, "h\nu"] & {[P \cdot P]^* \cdot Y} \arrow[l, shift left] \arrow[ur, shift left] \arrow[dr, shift left] & & \\
 & & {[P^- \cdot P^+] \cdot Y} \arrow[ul, shift left] &
\end{tikzcd}$$

The observation that the same fraction of the P_{700} is repeatedly reversible requires that the polarity between the two chlorophyll molecules be maintained at low temperatures. The P · P model provides a candidate for the 690-nm absorbing form of chlorophyll that senses the redox state of P_{700}, i.e. the P in the $(P^+ \cdot P] \cdot Y^-$ state of reaction center complex.

Comparison of the models P · X and P · P points out an experimental test to verify whether the primary electron acceptor of Photosystem I is a nonheme iron compound as has been suggested by EPR [30, 31] and optical absorbance [32] measurements. The measurements reported do not prove that the nonheme iron is a primary acceptor, X, rather than a secondary acceptor, Y, that can be reduced in the dark at low temperature. If the nonheme iron is the primary acceptor it should show the same ratio of reversible to irreversible change at low temperature as the P_{700}. If it is a secondary acceptor, it should show only the irreversible change. Measurements of the photoreversibility of P_{700} (Signal I) and the nonheme iron should be feasible at the deep low temperatures (12–25 °K) that are required to measure the nonheme iron EPR signal.

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