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LIGHT-INDUCED ABSORBANCE CHANGES IN FRACTION I PARTICLES PREPARED FROM SPINACH CHLOROPLASTS BY FRENCH-PRESS TREATMENT*

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SUMMARY

Light-induced changes of absorbance were measured in Fraction I particles prepared from spinach chloroplasts according to a method developed by MICHEL AND MICHEL-WOLWERTZ[6] that employs breakage of chloroplasts in the French pressure cell and centrifugation of the resulting fragments on a sucrose density gradient. Absorbance changes were measured in the presence of ascorbate and 2,3,5,6-tetramethyl-1,4-phenylenediamine as an electron donating system. In addition to the oxidation of P_{700} and cytochrome *f*, that was investigated in our previous study[11], three other light-induced absorbance changes were observed; reduction of cytochrome *b* with a peak at 562 nm, the so-called 515-nm change showing an absorbance increase at 515 nm and a decrease at 480 nm, and a broad band of unknown absorbance increase covering wavelengths from 490 to 570 nm. Uncouplers such as carbonylcyanide-*m*-chlorophenylhydrazone and gramicidin D inhibited the 515-nm change under continuous light and accelerated the dark decay of flash-light-induced change. The other broad absorbance change was insensitive to these inhibitors.

INTRODUCTION

Methods have been discovered for physical separation of System I and II of the photosynthetic electron transport system. Detergent was first used by WESSELS[1], BOARDMAN AND ANDERSON[2,3], and sonic oscillation by JACOBI AND LEHMANN[4,5]. MICHEL AND MICHEL-WOLWERTZ[6,7] produced a separation by breaking spinach chloroplasts in a French pressure cell followed by centrifugation on a sucrose density gradient. Investigations of activities of the separated particles showed that those in the top band of the sucrose gradient had only System I activity and those in the bottom band had System II activity and weak System I activity[6–9]. In these preparations addition of plastocyanin was not necessary to sustain good rates of a System I reaction as measured by $NADP^+$ reduction[8]; but the requirement for added plasto-

Abbreviations: DAD, 2,3,5,6,-tetramethyl-1,4-phenylenediamine; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone

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cyanin is strict in System I particles prepared by sonic oscillation[4,5] or detergent treatment[3,10].

In our previous study on light-induced absorbance changes in the Fraction I particles prepared with French-pressure treatment[11], it was found that P_{700} and cytochrome *f* were oxidized by light and reduced by an artificial electron donor system such as ascorbate and 2,3,5,6-tetramethyl-1,4-phenylenediamine (DAD), but not by reduced plastocyanin. The oxidation of cytochrome *f* occurred in less than 1 msec. Treatment of these Fraction I particles with the detergent Triton X-100 changed them in such a way that the artificial electron donor system was no longer effective, but plastocyanin or algal *c*-type cytochromes with low isoelectric points became electron donors to P_{700} (ref. 11). The rate of cytochrome *f* oxidation was remarkably retarded by the detergent treatment. These findings lead us to conclude that the electron transport system remains more intact in the Fraction I particles prepared with the French pressure cell than in those particles prepared with detergent or sonic treatment[11].

JACOBI AND LEHMANN[4,5], as well as, SANE *et al.*[9] studying ultra-structure of chloroplasts and separated particles, concluded that small particles having only System I activity come from stroma lamellae and that large particles having both System I and II activities come from grana. A further study by these latter authors on the contents of electron carriers in the preparations showed that the Fraction I particles originating from stroma lamellae contained P_{700}, cytochrome *f* and cytochrome b_6.

In the present study, we found evidence for a light-induced reduction of cytochrome b_6. In addition, absorbance changes with a minimum at 480 and a maximum at 515 nm in the difference spectrum were sensitive to the uncouplers carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP) and gramicidin D. Another component was found producing absorbance changes with a broad positive band covering the region from 500 to 570 nm.

METHODS

Fraction I particles were prepared from spinach chloroplasts as described previously[11].

For measurement of light-induced absorbance changes the single-beam spectrophotometer constructed in this laboratory was used[12]. A 0.5-cm path length cuvette was positioned horizontally so that the measuring and actinic beams were both incident on the same side of the cuvette. For use with flash illumination the apparatus was modified so that measuring and actinic beams were incident on a 1-cm path length cuvette at right angles to each other.

A continuous actinic beam was obtained by filtering light from a 650-W quartz-iodine lamp through 2.7 cm of water, a Balzers heat-reflecting filter (Calflex C) and a red cut-off (Schott RG-5) or two blue filters (Corning 9782). The red actinic light consisted of a broad band covering wavelengths extending from 650 to 750 nm and having an intensity of $3.9 \cdot 10^5$ ergs/cm^2/sec. The blue actinic light had a band of wavelengths extending from about 390 to 590 nm and had an intensity of $1.1 \cdot 10^5$ ergs/cm^2/sec. When absorbance changes in the blue region were measured, the red actinic light was used and two blue filters (Corning 9782) were placed between a photomultiplier

(EMI 9558B) and the sample. Blue actinic light was used when absorbance changes at 703 nm were measured. A red cut-off filter (Schott RG 5, 3 mm) and an interference filter (Balzers) with a transmission peak at 704 nm were placed between the photomultiplier and the sample.

A second photomultiplier (RCA 6217) was placed below the sample but out of the measuring beam for detection of chlorophyll *a* fluorescence excited by the blue actinic light. The output signals of both photomultipliers were fed into a differential amplifier (Hewlett Packard 2470A). By selecting suitable operating voltages for the photomultipliers, the signal due to the fluorescence was cancelled. The output of the amplifier was recorded on a Massa oscillographic recorder (Model BSA 250A) or displayed on a Hewlett Packard oscilloscope (Model 130C) and photographed.

For flash illumination a xenon flash lamp (General Radio Stroboslave, Type 1539A) having a flash duration of about 8 μsec at 10 % intensity of the maximum was used in conjunction with the red cut-off and heat-reflecting filters described above.

RESULTS

Illumination of Fraction I particles from spinach chloroplasts by flash as well as by continuous light produced changes of their absorption spectrum. Although the changes showed different time courses with different wavelengths, they reached a steady level in the light at all the wavelengths after continuous illumination of 0.5 sec. The absorbance difference between the light and dark steady states was taken in constructing a light-induced difference spectrum (Fig. 1). The measurements were performed in the presence of 0.05 mM DAD and 1 mM ascorbate.

Judging from the difference spectra in Fraction I particles of spinach chloroplasts shown in Fig. 1a, the negative peaks at 423 and 553 nm can be attributed to a light-induced oxidation of cytochrome *f*, and the positive peak at 562 nm to photoreduction of cytochrome b_6. The positive peak at 512 nm and the negative one at

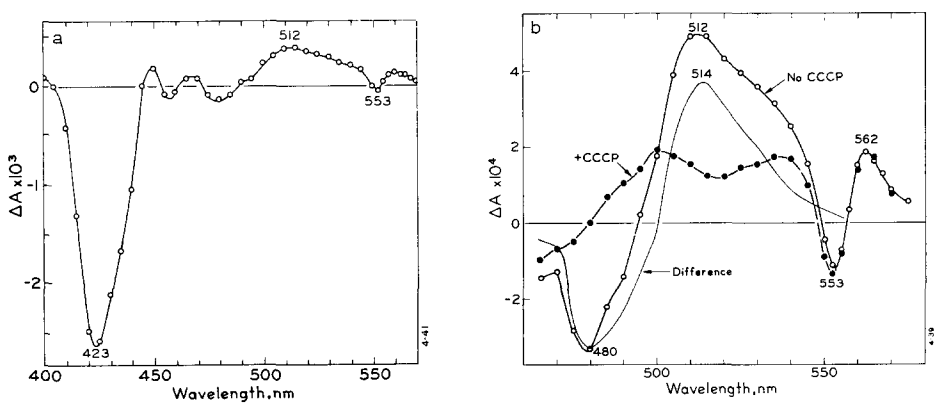

Fig. 1. Light-induced difference spectrum in Fraction I particles of spinach chloroplasts. The steady-state levels in the light and dark were taken. Bandwidth of measuring light was 2 nm. Thickness of cuvette was 0.5 cm. Reaction mixture: 250 mM sucrose, 150 mM KCl, 50 mM Tricine–KOH buffer (pH 7.8), 1 mM ascorbate and 0.05 mM DAD. The chlorophyll concn. was 34 μM for a and 50 μM for b. Open circles: no CCCP. Closed circles: 4 μM CCCP. Thin line: difference between them.

480 nm can be ascribed to the 515-nm change. Oxidation of P_{700} must contribute to the absorbance decreases around 430–435 nm.

In Fig. 1b the absorbance changes, measured with a different sample, were plotted on an expanded wavelength scale from 460 to 575 nm. In the absence of CCCP (open circles in Fig. 1b) there are positive peaks at 512 and 562 nm, a shoulder at 540 nm and negative peaks at 479 and 553 nm.

With the addition of an uncoupler of photophosphorylation, CCCP, (closed circles in Fig. 1b) the positive peak at 512 nm and the negative peak at 479 nm disappeared and new positive peaks appeared at 500 and 537 nm. The changes of cytochromes *f* and b_6 were not influenced by this uncoupler. The spectrum of the difference between the two curves with and without CCCP (thin line in Fig. 1b) had a maximum at 514 nm and a minimum at 480 nm and an isosbestic point at 500 nm.

The remaining absorbance changes with peaks at 500 and 537 nm were resistant to this uncoupler even at 12 μM. Judging from the absorbance decrease of the α-band of cytochrome *f* at 553 nm, cytochrome *f* was oxidized by the actinic light. This oxidation also gives rise to an absorbance decrease at 520 nm and increases at 537 and 480 nm (see Fig. 7). These changes of cytochrome *f* overlapping a broad flat absorbance increase may be responsible for peaks at 500 and 537 nm and a trough at 520 nm in the difference spectrum in the presence of CCCP. As will be shown later, the broad absorption increase appears, in turn, to overlap the changes of cytochromes *f* and b_6 at 553 and 562 nm (see DISCUSSION for details).

Fig. 2a shows time courses of absorbance changes at several wavelengths induced by continuous red actinic light. At 552.5 nm where cytochrome *f* shows the maximum absorbance change in its α-band upon oxidation–reduction, a small positive transient change appeared upon turning on the actinic light that was followed by a

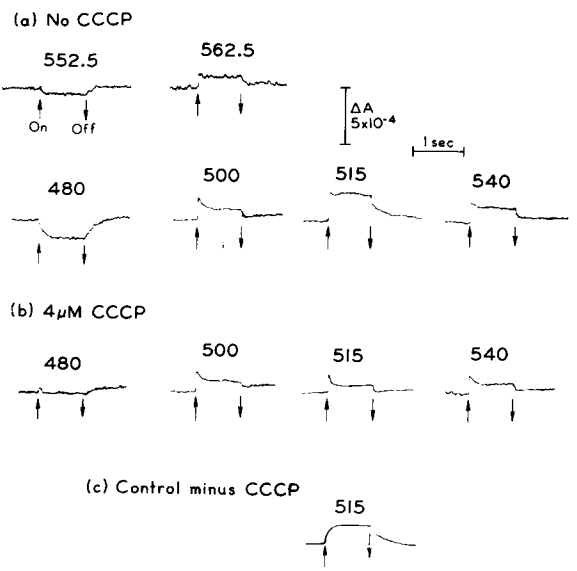

Fig. 2. Time courses of light-induced absorbance changes at 480, 500, 515, 540, 552.5 and 562.5 nm in Fraction I particles. Experimental conditions were the same as in Fig. 1. Chlorophyll concn., 50 μM. Time constant of measuring system was 5 msec. a. No CCCP. b. 4 μM CCCP. c. Difference at 515 nm.

slow decrease of absorbance. The steady level of the change was negative. A light-induced absorbance increase was observed at 562.5 nm, a wavelength near which cytochrome b_6 shows the maximum absorbance change. The positive change at the onset of illumination was a little higher than the steady-state level.

At 480 nm a slow absorbance decrease was seen upon actinic illumination that was slowly recovered after darkening. At 500 nm a rapid absorbance increase was observed upon turning on the actinic light, that was followed by a slow decline to a steady level slightly higher than the dark level. At 515 nm where the largest change was observed in the green region, the absorbance increased rapidly upon illumination and stayed at about the same level. The time course at 540 nm was similar to that at 500 nm, although the decline after the initial increase was not so steep.

With addition of 4 μM CCCP (Fig. 2b), the absorbance change at 480 nm disappeared, and the time courses of changes at 515 and 540 nm were somewhat modified. CCCP had no influence on the change at 500 nm where there is an isosbestic point of the 515-nm change. As a result the absorbance changes at 500, 515 and 540 nm in the presence of 4 μM CCCP showed similar time courses. The absorption at these wavelengths rapidly increased upon turning on the actinic light and then slowly decreased to a steady level. Upon plotting the difference between the time courses of absorbance changes at 515 nm with and without CCCP (Fig. 2c) a time course was obtained that had a slow increase and decrease upon turning on and off the actinic light. This time course was the same as that seen at 480 nm without CCCP (but with reversed direction). Therefore, it can be concluded that the 515-nm change which is sensitive to the uncoupler responds slowly to continuous actinic light and that the uncoupler-resistant change responds quickly to the light. At 540 nm where the overlap of the 515-nm change is small the effect of CCCP on the time course was only slight.

Gramicidin D, another potent uncoupler of photophosphorylation, was also found to suppress the 515-nm change. Fig. 3 shows the dependence of the light-induced absorbance changes at 480 and 515 nm upon the concentration of Gramicidin D. The concentration for half inhibition was about 10 nM for both changes. At concentrations higher than 0.3 μM, there was no steady-state change at 480 nm while 17 % of the total change remained at 515 nm. The latter change is most likely due to the uncoupler-resistant change which overlaps the 515-nm change.

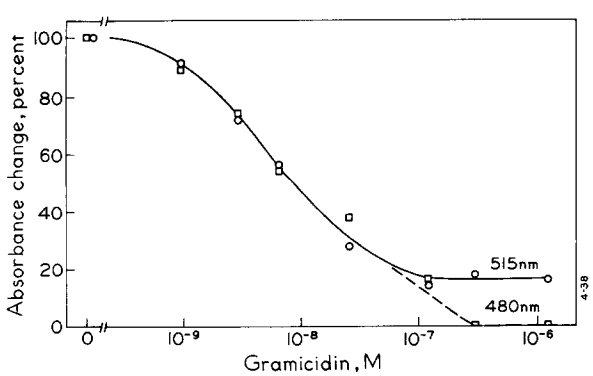

Fig. 3. Effect of Gramidicin D concentration on light-induced absorbance changes at 515 and 480 nm. Experimental conditions were the same as in Fig. 1 except for bandwidth of measuring light which was 3 nm. Chlorophyll concn., 36 μM.

The absorbance changes were measured at other concentrations of DAD. The changes of cytochrome *f*, cytochrome b_6 and the uncoupler-resistant change with peaks at 500 and 540 nm were observed at 0–50 μM DAD and were smaller at concentrations higher than 50 μM. The 515-nm change was observed at 50–150 μM DAD and was diminished when the DAD concentration was less than 5 μM.

The broad positive absorbance change which is resistant to uncoupler seems to contribute to the absorbance changes measured at 552.5 and 562.5 nm (Fig. 2a). The overlap of this change on the cytochrome *f* change would explain the transient absorbance increase at the onset time and the following slow decline to the steady-state level observed in the time course at 552.5 nm. Also at 562.5 nm a slight decline from the change at onset time to the steady state could be attributed to the overlap of the uncoupler-resistant change on the cytochrome b_6 change. It can be presumed that cytochromes *f* and b_6 quickly attained their steady-state levels which are kept constant during actinic illumination. From this point of view the contribution of the overlap seems to be much larger at 552.5 nm than at 562.5 nm.

The time courses of the absorbance changes at 435, 505 and 703 nm were com-

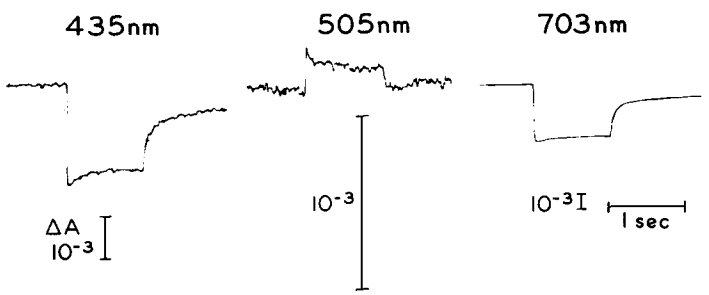

Fig. 4. Time courses of absorbance changes at 435, 505 and 703 nm induced by continuous illumination of Fraction I particles. Experimental conditions were the same as in Fig. 2. Chlorophyll concn., 34 μM. Four μM CCCP was added.

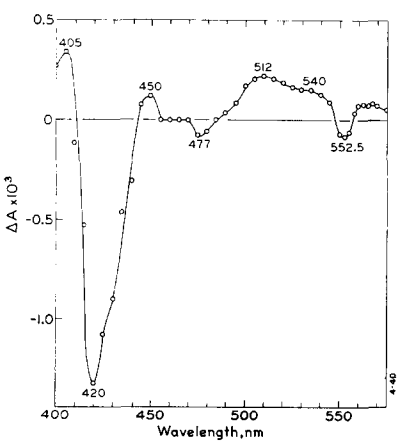

Fig. 5. Difference spectrum induced by flash illumination of Fraction I particles. Bandwidth of measuring light, 3 nm. Duration of the actinic flash was 8 μsec for 10% intensity of the maximum. Thickness of cuvette, 1 cm. Reaction mixture: 200 mM sucrose, 150 mM KCl, 50 mM Tricine–KOH buffer (pH 7.8), 1.7 mM ascorbate, 8 μM DAD. Chlorophyll concentration was 12 μM. Time constant of measuring system was 1 msec.

pared (Fig. 4). Upon illumination rapid changes were seen that were followed by slower changes prior to attaining steady levels in the light. Thus, the time courses were qualitatively similar in shape except for their reversed relationships. However, the decline during the actinic illumination was about 60 % of the change at the onset time at 505 nm, while it was only 15 % at 703 nm.

Absorbance changes were also induced by brief flashes of light (flash duration, 8 μsec for 10 % level). Fig. 5 shows a difference spectrum produced by flash illumination. The peaks at 405, 420 and 553 nm are attributed to the oxidation of cytochrome *f*, the peak at 563 nm to the reduction of cytochrome b_6, the peaks at 477 and 512 nm to the 515–480-nm change. P_{700} appeared to be oxidized as judged from the negative change at 430–435 nm. The flash-induced difference spectrum was similar to that produced by the continuous actinic light except that the amounts of the 515-nm change and P_{700} oxidation were small compared to the cytochrome *f* changes.

The time courses of absorbance changes at 500, 515 and 535 nm induced by flash illumination are shown in Fig. 6. The uncoupler CCCP accelerated the dark decay of the absorbance change at 515 nm, suggesting that the decay of the 515-nm change is stimulated by this substance. On the other hand, the changes at 500 and 535 nm were little influenced.

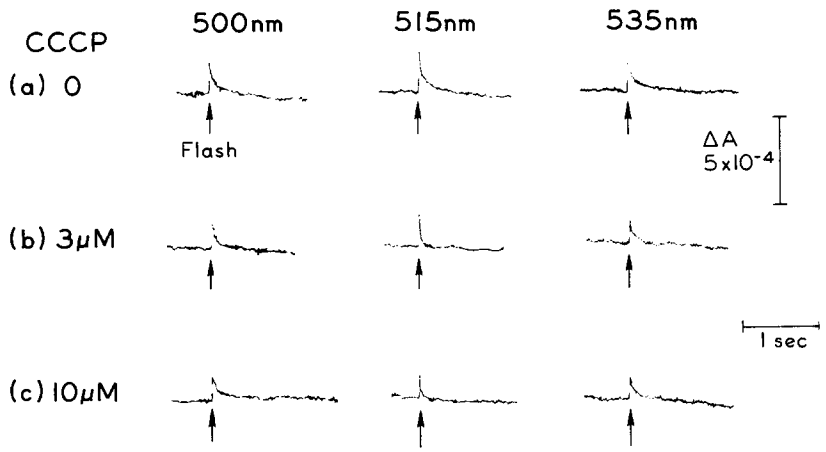

Fig. 6. Effect of CCCP on flash-induced absorbance changes at 500, 515 and 535 nm in Fraction I particles. Experimental conditions were the same as described for Fig. 5 except for addition of CCCP.

DISCUSSION

Illumination of Fraction I particles induced several different absorbance changes, one of which was inhibited by uncouplers of phosphorylation. The shape of the light-induced difference spectrum with its positive peak at 515 nm and negative peak at 480 nm as well as its sensitivity to uncouplers shows that these changes are the same as the so-called 515-nm change observed in other materials.

This change was first seen in Chlorella by DUYSENS[13]. Similar changes have been found in many photosynthetic organisms including higher plants, algae and photosynthetic bacteria[14–18]. A characteristic feature of the change is its rapid time response

to light (less than 1 μsec (refs. 19 and 20)) and high sensitivity to uncouplers of phosphorylation[21–24]. In higher plants the change is called the 515 or 475–515 change. It consists of an absorbance increase at 515–520 nm and a decrease at 480 nm. It seems to be produced by the absorbance changes of chlorophyll *b* and carotenoids[25,26].

In Fraction I particles it was necessary to add electron carriers such as DAD in order to see this light-induced change. DAD presumably functions as a bridge for a cyclic electron transport. A recent current concept of the 515-nm change is that it is produced by some conformational change that is related to phosphorylation[27,28].

After the addition of CCCP the 515-nm change disappeared. The difference spectrum remaining consisted of an absorbance decrease at 553 nm that can be attributed to the oxidation of cytochrome *f* and the absorbance increase at 562 nm which can be ascribed to cytochrome b_6 reduction. In addition, an unknown broad absorbance increase persisted after CCCP addition that has a difference spectrum with peaks at 500 and 537 nm and a trough in between. We attempted to separate the difference spectrum into its components (Fig. 7). The oxidized-*minus*-reduced difference spectrum of Porphyra cytochrome *c*-553 (supplied by courtesy of Dr. A. MITSUI, Department of Botany, Indiana University) was used for the light-induced oxidation of cytochrome *f*. Fig. 7 shows the analysis of the spectrum considered most reasonable.

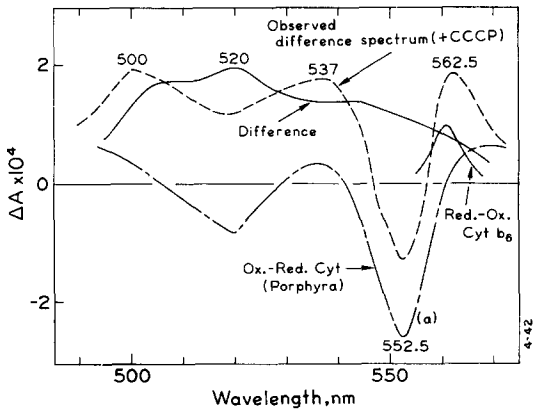

Fig. 7. The analysis of the difference spectrum observed in the presence of CCCP (see Fig. 1b). The contribution of cytochrome *f* to this spectrum was estimated by using the oxidized-*minus*-reduced difference spectrum for the *c*-type cytochrome from Porphyra. The difference spectrum remaining after accounting for the contribution of the two cytochromes is labelled "difference" and represents the absorption change that is insensitive to CCCP and has a broad absorption spectrum in this region.

The three components used were cytochrome *f*, cytochrome b_6 and the unknown broad absorption change. When the estimated contributions of cytochrome *f* and cytochrome b_6 were subtracted from the measured difference spectrum a broad difference spectrum remained as is shown in Fig. 7. The peak at 537 nm and the trough at 520 nm in the measured difference spectrum appears to be due to the oxidation of cytochrome *f* overlapping the broad absorbance increase. The positive band at 562 nm ascribable to cytochrome b_6 remained as a single component.

Cytochrome b_6 has been found in the System I particles prepared with digitonin treatment[3]. CRAMER AND BUTLER[29] observed a reduction of this cytochrome upon

illumination with long wavelength light. It was clearly seen here in Fraction I particles that cytochrome b_6 is reduced by System I if an electron donor system is present.

The origin of the broad and uncoupler-insensitive absorbance change (see Fig. 7) is not definite. However, P_{700} seems to be the most plausable substance producing this change. T. HIYAMA (personal communication) observed a similar light-induced absorbance increase in System I particles prepared with detergent treatment and ascribed it to the oxidation of P_{700}.

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