Biochimica et Biophysica Acta, 325 (1973) 558–564

BBA 46642

C-550 IN PHOTOSYSTEM II SUBCHLOROPLAST PARTICLES

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(Received June 26th, 1973)

SUMMARY

The photoreactions mediated by Photosystem II at low temperatures were examined in subchloroplast particles enriched in Photosystem II to determine if the Photosystem II activity was independent of C-550 in such particle preparations. Two types of Photosystem II particle preparations were tested, one enriched in chlorophyll *b* and the other purified further to eliminate the chlorophyll *b*. The Photosystem II-mediated photoreactions at low temperature were the same in both of the Photosystem II particle preparations as they were in normal chloroplasts. C-550 acted as if it were the primary electron acceptor of Photosystem II in all of the experiments performed

INTRODUCTION

Much evidence has accumulated showing that C-550 acts as if it were the primary electron acceptor of Photosystem II in chloroplasts[1]. The question of whether C-550 is the actual electron acceptor or a closely coupled redox indicator of the primary acceptor has been discussed[1,2], but has not been resolved because of the lack of experimental procedures to distinguish between these two possibilities. Recently, however, Boardman[3] presented evidence, from subchloroplast particles enriched in Photosystem II activity (DT-10 particles collected as a $10\,000 \times g$ pellet after consecutive detergent treatments with digitonin and Triton X-100), that the Photosystem II reaction centers could be separated functionally from C-550. Boardman reported that C-550 was fully reduced in the dark in DT-10 particles (in contrast to normal chloroplasts where it is fully oxidized), so that no further photoreduction occurred on irradiation at low temperature. Furthermore, he reported that cytochrome b_{559} could be photooxidized by the DT-10 particles at -196 °C without the concomitant photoreduction of C-550, presumably with another compound serving as the primary electron acceptor. Boardman[3] concluded, therefore, that C-550 was not the primary electron acceptor in the DT-10 particles. Our purpose in initiating the

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide.

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work reported here was to confirm the experimental results reported by Boardman.

The data which Boardman presented to support his conclusions about the redox states of C-550 and cytochrome b_{559} consisted of low temperature difference spectra between a chemically reduced sample and a $Fe(CN)_6^{3-}$ oxidized sample, both samples having had the same irradiation treatment at -196 °C The effects of light (*e.g.* the photooxidation of cytochrome b_{559} without the concomitant photoreduction of C-550) were inferred from differences between two such difference spectra, one measured with both samples irradiated at -196 °C, the other with both samples unirradiated. Our experience with these kinds of measurements has indicated that difference spectra obtained with two independent samples frozen to -196 °C are more subject to artifacts due to sample mismatch than are light-induced difference spectra measured on the same frozen sample. We, therefore, set out to examine DT-10 particles with the latter technique in an effort to confirm Boardman's report that the photooxidation of cytochrome b_{559} in these particles at -196 °C was independent of the photoreduction of C-550. We also extended these measurements to the TSF 2a particles of Vernon *et al.*[4].

METHODS

DT-10 subchloroplast particles were prepared according to the methods described by Boardman[3] with only slight modifications. These modifications included the use of commercial spinach instead of greenhouse grown spinach and slightly longer incubation times at the two detergents steps. (We incubated our chloroplasts preparations for 45-min periods during the 0.5 % digitonin and 0.18 % Triton X-100 treatments instead of the 30-min periods recommended by Boardman.) The longer incubation times markedly decreased the yield of DT-10 particles but resulted in particles with a lower P_{700} content.

TSF 2a subchloroplast particles were made according to the methods described by Vernon *et al.*[4] and our normal chloroplasts by methods used previously in our laboratory[5]. Chlorophyll *a* and chlorophyll *b* contents were calculated by Arnon's equations[6] from spectral measurements made at room temperature with a Unicam SP-800 spectrophotometer.

The photochemical activities of the chloroplast and particle preparations were measured during red actinic irradiation obtained with a tungsten lamp and Corning 2030 cutoff filter The reaction medium was a Tris–HCl buffer (pH 7 8, 3 ml) containing 15 mM Tris, 20 mM NaCl and 4 mM $MgCl_2$. 2,6-Dichlorophenolindophenol (DCIP) photoreduction was measured by the absorbance at 603 nm with an actinic intensity of $5 \cdot 10^4$ μW/cm^2 83 μM DCIP and 50 μM 1,5-diphenylcarbazide (DPC) and a chlorophyll concentration of 10 μg/ml were added to the reaction medium for measurements on DT-10 and TSF 2a particle With chloroplasts the DPC was omitted $NADP^+$ photoreduction was measured by the absorbance at 340 nm with $1.5 \cdot 10^4$ μW/cm^2 actinic light and with 83 μM $NaDP^+$, 2 mM NH_4Cl, 2 mM ascorbate, 83 μM DCIP, saturating amounts of plastocyanin, ferredoxin and ferredoxin–$NADP^+$ reductase and 10 μg chlorophyll/ml. Methylviologen photoreduction was measured as O_2 uptake (with a Clark-type electrode) with $5 \cdot 10^4$ μW/cm^2 actinic light and with 83 μM methylviologen, 1.8 mM NH_4Cl, 1 8 mM ascorbate, 83 μM DCIP, 33 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a saturating

amount of plastocyanin and 25 μg chlorophyll/ml.

Absorption spectra were measured at −196 °C with our single-beam spectrophotometer on line with a PDP-8/I computer as described previously[7,8]. The chloroplast preparations at the chlorophyll concentrations indicated in the figure legends were frozen to −196 °C in a vertical cuvette and Dewar system[8]; the 0.3-ml samples gave a thickness of about 2 mm. Chemical oxidants and reductants, as indicated in the figure legends, were added prior to freezing. The samples were irradiated at −196 °C with red (670 nm, 10^3 μW/cm^2) or far red (735 nm, $2 \cdot 10^3$ μW/cm^2) actinic light in the spectrophotometer sample compartment.

Spectra of each sample were measured before actinic irradiation, after irradiation with far-red light and after irradiation with red light. Each spectrum was the sum of two spectra, one measured immediately after the other. Such pairs of spectra were accepted as data only if they were identical within the limits of white noise. This procedure was adopted to prevent occasional spurious changes which might occur during a single spectral scan from appearing as data. Absolute spectra were plotted as the difference between the spectrum measured with the chloroplast sample and that measured with frozen buffer as a sample blank. Light-induced difference spectra between samples with different irradiation treatments at −196 °C were plotted at higher sensitivity. The spectral curves presented were read out directly from the computer to an X-Y recorder.

RESULTS

The photochemical activities of DT-10 and TSF 2a particles to photoreduce exogenous electron acceptor compounds are summarized in Table I. Both types of particles show Photosystem II activity in that they photoreduced DCIP with DPC as the electron donor at about one-third the rate that normal chloroplasts photoreduce the dye with water as the electron donor. Photosystem I activity, assayed by the photoreduction of $NADP^+$ or by the photoreduction of methylviologen (measured aerobically as O_2 uptake) with ascorbate–DCIP as the electron donor in the presence of 10^{-5} M DCMU, is low in both types of particles.

The absolute absorption spectrum at −196 °C and light-induced difference spectra measured at different redox states at −196 °C are shown in Fig. 1 for normal chloroplasts, in Fig. 2 for DT-10 particles and in Fig. 3 for TSF 2a particles It is apparent from the absolute spectra that the three preparations have different ratios of chlorophyll *a* to chlorophyll *b* as indicated in Table I and that the long wavelength

TABLE I

	Activities (μmoles/mg chlorophyll per h)		
	Chloroplast	*DT-10*	*TSF 2a*
Chlorophyll *a*/Chlorophyll *b*	3 5	2 3	6 3
DPC–DCIP	222★	81	75
DCIP–$NADP^+$	216	17	0
Methylviologen	385	60	23

★ H_2O was the electron donor rather than DPC.

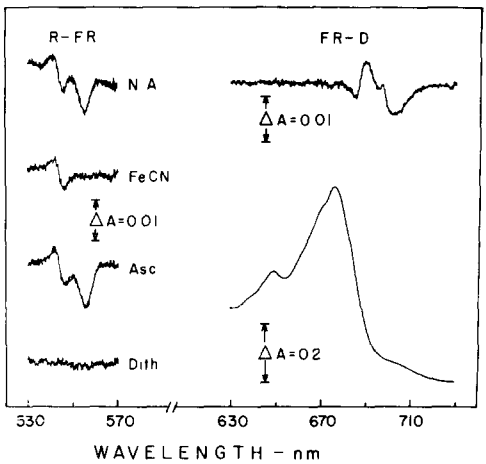

Fig 1 Absorption spectrum (lower right), far-red-irradiated *minus* dark difference spectrum (upper) right) and red *minus* far-red-irradiated difference spectra at different redox states (left) of chloroplasts at −196 °C. The chlorophyll concentration of the chloroplasts was 50 μg/ml for measurements in the red region (right side) and 150 μg/ml for measurements in the green (left side). 5 mM ascorbate, 5 mM $Fe(CN)_6^{3-}$ or a few grains of solid dithionite (Dith) were added prior to freezing where indicated.

form(s) of chlorophyll with the broad absorption band near 705 nm, which has been associated with Photosystem I particles[9], is absent from the Photosystem II particle preparations. It is also apparent that the chlorophyll a_{670} to chlorophyll a_{680} ratio is different in the TSF 2a particles. The amount of P_{700} present in the different fractions is indicated by the bleaching at 700 nm due to irradiation by far-red light at −196 °C (see the far-red *minus* dark difference spectra in Figs 1, 2 and 3). The P_{700} assay is enhanced relative to the chlorophyll absorbance by the light scatter in the frozen samples because the increase of absorbance due to the increased optical path

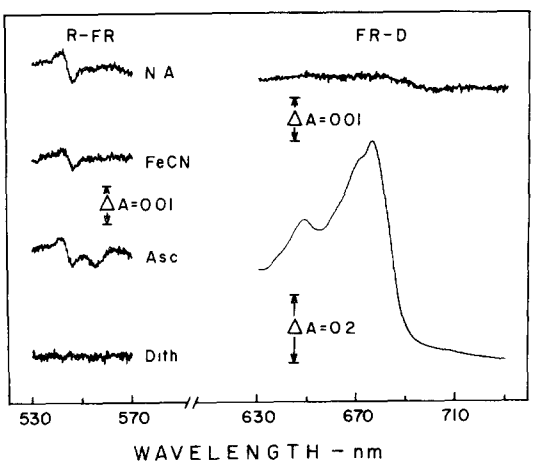

Fig 2 Same as Fig. 1 for DT-10 particles.

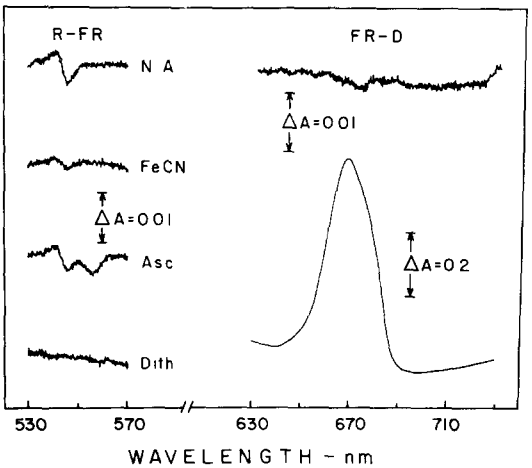

Fig. 3. Same as Fig. 1 for TSF 2a particles.

length is greatest in spectral regions of low absorbance[10]. It is apparent that the DT-10 and TSF 2a preparations are largely devoid of P_{700}.

The spectral changes induced by red light at −196 °C are shown in the green region of the spectrum. The normal chloroplasts (Fig. 1) show the typical absorbance increase at 543 nm and bleaching at 546 nm due to the photoreduction of C-550 and the bleaching at 556 nm due to the photooxidation of cytochrome b_{559}. Addition of $Fe(CN)_6^{3-}$ prior to freezing oxidizes cytochrome b_{559} and thus prevents its photooxidation. Addition of ascorbate prior to freezing has no effect on the light-induced difference spectra of the normal chloroplasts. The DT-10 and TSF 2a particles both show a photoreduction of C-550 (Figs 2 and 3) in the untreated samples. The magnitude of the C-550 change is not increased by addition of $Fe(CN)_6^{3-}$ prior to freezing. (Boardman reported that C-550 in DT-10 particles was fully reduced in the dark but could be photoreduced at −196 °C if $Fe(CN)_6^{3-}$ was added prior to freezing) In fact the extent of the C-550 absorbance change is somewhat less in the presence of $Fe(CN)_6^{3-}$. In the case of the TSF 2a particles we suspect that $Fe(CN)_6^{3-}$ caused an irreversible oxidative destruction of some of the C-550. It is known that detergent treatment of chloroplasts modifies the high potential cytochrome b_{559} to lower potential, autooxidizable forms[11]. Addition of ascorbate to the DT-10 and TSF 2a particles prior to freezing reduces some of the autooxidized cytochrome b_{559} so that it can be photooxidized. In the presence of dithionite no Photosystem II photoreaction is observed in any of the preparations because the C-550 is reduced and therefore cannot serve as the primary electron acceptor. Boardman[3] reported that cytochrome b_{559} could be photooxidized at −196 °C in the presence of dithionite.

The particle preparations

We experienced more variation in preparing the DT-10 particles than the TSF 2a particles, primarily from a standpoint of the residual amount of P_{700} and Photosystem I activity. In all cases, however, (including DT-10 particles made with 30-min incubation times instead of the 45-min periods described in Methods) the

cytochrome b_{559} and C-550 changes were the same regardless of the amount of P_{700} present.

It is apparent from the spectra that the amount of C-550 relative to the chlorophyll content is not enriched in the Photosystem II particle preparations as might be expected The lack of enrichment may be due to a partial destruction of C-550 in the particle preparations; incubation of normal chloroplasts with Triton X-100 causes a progressive destruction of C-550.

DISCUSSION

One of the main lines of evidence linking C-550 with the primary electron acceptor of Photosystem II came from experiments with chloroplasts showing that the photooxidation of cytochrome b_{559} at -196 °C required the concomitant photoreduction of C-550[7]. Boardman[3] reported, however, that this restriction did not hold in DT-10 particles and concluded therefore that C-550 was not the primary acceptor. That conclusion does not necessarily follow from the premise since it is possible that the detergent treatment might create an artifactual condition, whereby the reaction centers could photoreduce a non-physiological electron acceptor when C-550 was not available. In any event, it seemed likely from Boardman's report that the detergent treatment opened up the reaction centers and provided the opportunity of elucidating the primary photochemistry of Photosystem II in greater detail than had been done with normal chloroplasts. Unfortunately, we cannot confront the questions raised by Boardman directly because we could not confirm his results. In our work with DT-10 particles, C-550 functioned normally (as if it were the primary electron acceptor).

Having failed to separate Photosystem II from its dependence on C-550 in DT-10 particles, we extended the work to TSF 2a particles which are considered to represent more highly purified Photosystem II reaction centers. However, we obtained the same results with these particles as well. It should be noted that we observed a normal photooxidation of cytochrome b_{559} at low temperature with TSF 2a particles frozen in the presence of ascorbate while Ke *et al.*[12] looked for but failed to find such a reaction. We know that it is possible to destroy C-550 and the low temperature photoreactions by treatments such as lipase digestion[13] or detergent incubation which markedly modify the structural integrity of the chloroplast membranes. It may be that the TSF 2a particles used by Ke *et al.* [12] were in a greater state of disrepair than those used by us. Results essentially the same as ours were reported recently by Wessels *et al* [14] with their Photosystem II reaction center particles (F_{II}) which are also devoid of chlorophyll *b*. They observed a normal photoreduction of C-550 at -196 °C with F_{II} particles frozen in darkness without additions. When ascorbate was added prior to freezing to reduce some of the autooxidized cytochrome b_{559}, irradiation at -196 °C resulted in the photoreduction of C-550 and the photooxidation of cytochrome b_{559}.

We can offer no explanation for the discrepancies between the results reported here and those reported by Boardman except to suggest that our measurements of the light-induced absorbance changes afford a more reliable assay of the photochemical changes of C-550 and cytochrome b_{559} than the chemical difference spectra presented by Boardman[3]. However, we do not put this forward as the final explanation. It is quite possible that Boardman succeeded in opening up the Photosystem II

reaction center complexes and observed low temperature photoreaction which are not normally observed. If his results can be confirmed and the techniques of preparation regularized, such a system might provide valuable insights into the primary photochemical mechanisms of Photosystem II

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