SPECTRAL EVIDENCE FOR A NEW PHOTOREACTIVE COMPONENT OF THE OXYGEN-EVOLVING SYSTEM IN PHOTOSYNTHESIS

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Abstract.—On illuminating chloroplasts with "short-wavelength" monochromatic light that supports oxygen evolution, spectral evidence was obtained for a new photoreactive chloroplast component, provisionally designated C550, which shows a reversible decrease of absorbance with a maximum at 550 mμ. The light-induced absorbance changes in C550 have been separated from those due to cytochromes in the same spectral region.

The light-induced decrease of absorbance in C550 appears to be independent of temperature, persisting even at −189° and is therefore likely to be linked to the primary light reaction associated with oxygen evolution in photosynthesis.

One of the least understood parts of photosynthesis in chloroplasts is the light-induced transfer of electrons from water and the concomitant evolution of oxygen.[1] Especially meager is our knowledge of those chloroplast constituents which undergo changes during the primary light reactions (independent of temperature) involved in these events.

We have recently reported[2] that cytochrome b_{559} of chloroplasts is photooxidized even at −189° and that this temperature-independent photooxidation can be induced only by "short-wavelength" monochromatic light which activates the oxygen-evolving system (photosystem II) in chloroplasts. We now wish to present evidence for a new chloroplast component (which we have provisionally named C550) that undergoes spectral changes induced also by monochromatic light characteristic of photosystem II. The light-induced change in C550 appears to be independent of temperature—it occurred even at −189°.

Methods.—"Broken" chloroplasts and washed "broken" chloroplasts from spinach and romaine lettuce leaves were prepared according to the method of Whatley and Arnon.[3] Chloroplasts from leaves of *Amaranthus edulis* Speg. were prepared according to Kalberer, Buchanan, and Arnon[4] and hypotonically disrupted by placement in a 1:10 dilution of the blending medium. Digitonin-treated chloroplast particles were prepared by a modification of the procedure of Anderson and Boardman.[5] Tris-treated chloroplasts were prepared by a modification of the procedure of Yamashita and Butler.[6] Chlorophyll was determined as described by Arnon.[7]

Absorbance changes at room and liquid nitrogen temperature were measured with a dual-wavelength spectrophotometer (Phoenix Precision Instrument Co.) as described previously,[2] except that for measuring absorbance changes below 440 mμ, Corning 5-56 and 4-96 filters were used to shield the spectrophotometer phototube.

Monochromatic illumination, as previously described,[2, 8] was introduced through a hole in the side of the spectrophotometer. Balzer and Baird-Atomic interference filters, supplemented by a Schott RG-1 filter, were used to isolate the monochromatic light beams. The intensity of each light beam was measured with an YSI-Kettering model 65 radiometer.

Results and Discussion.—We set out to explore possible spectral changes that

may occur in illuminated chloroplasts during photoreduction of ferricyanide. Ferricyanide was chosen because its photoreduction by chloroplasts is accompanied by a stoichiometric evolution of oxygen,[9] and second, because it chemically oxidizes, independently of light, a number of chloroplast constituents, including cytochromes that can also undergo oxidation by the action of light. By pretreating chloroplasts with excess ferricyanide these constituents would become chemically oxidized in the dark, thereby simplifying the search for those chloroplast constituents whose oxidation (or reduction) can only be induced by light, specifically, by the action of "short-wavelength" monochromatic light (below 700 mμ) that supports the photoreduction of ferricyanide with a concomitant evolution of oxygen.[8]

Figure 1 shows that addition of potassium ferricyanide to chloroplasts in the dark gave a decrease in absorbance at 550 mμ. This absorbance change was caused by the chemical oxidation of cytochrome *f*.[10-13] A second addition of ferricyanide in the dark produced no further decrease in absorbance, indicating that cytochrome *f* was already fully oxidized. When a monochromatic light beam (647 mμ) was turned on next, a marked decrease in absorbance occurred. This change was completely reversed when the light was turned off.

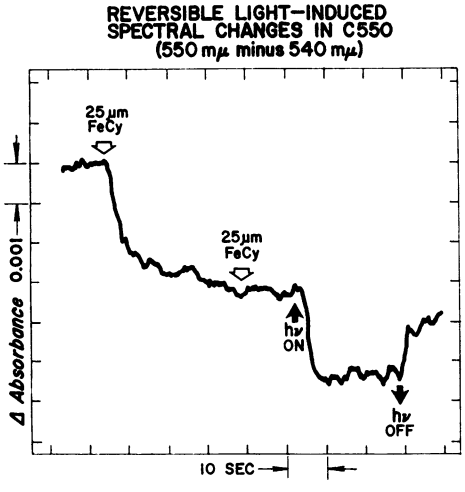

FIG. 1.—Light-induced reversible absorbance changes in C550 (550 mμ minus 540 mμ). The reaction mixture contained (per 1.0 ml) spinach chloroplasts (equivalent to 75 μg chlorophyll) and 33.3 μmoles Tricine [N-tris(hydroxymethyl)methylglycine] buffer (pH 8.2). At the indicated times 50 μl of 0.5 *M* potassium ferricyanide were added to the 5.0-ml sample. The 647-mμ actinic light had an intensity of 4.8×10^3 ergs/cm^2/sec.

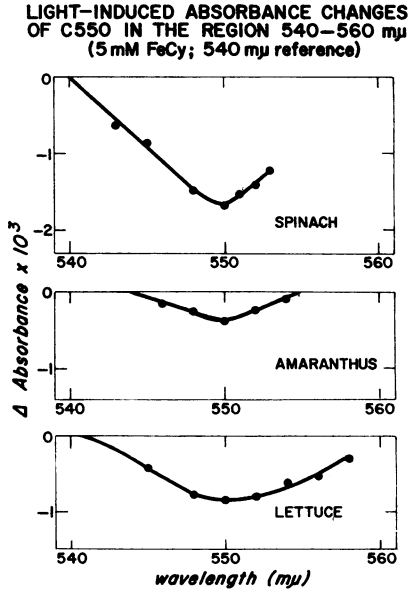

FIG. 2.—Light-induced absorbance changes of C550 in the region 540–560 mμ (540-mμ reference). Experimental conditions were as described in Fig. 1, except that 5 μmoles of potassium ferricyanide (per 1.0 ml) were present in the reaction mixture.

The reversible light-induced decrease in absorbance could not be attributed to a photooxidation of chloroplast cytochromes which are known to have their absorption peaks in the neighborhood of 550 mμ. One of the three known chloroplast cytochromes, cytochrome b_6, is known to be in an oxidized state[12-14] when chloroplasts are isolated from leaves. The other two cytochromes (f and b_{559}) were oxidized by the ferricyanide pretreatment in the dark.[10-13] The light-induced decrease in absorbance must therefore have been caused by another chloroplast component.

To help characterize the photoreactive component, we investigated at what wavelengths, other than 550 mμ, could its photoinduced absorbance change be measured. We were able to measure it only in the region from 540 to 560 mμ. As shown in Figure 2 this apparent spectrum of the decrease in absorbance for spinach chloroplasts had a maximum at 550 mμ. Similar patterns of absorbance changes were also found in romaine lettuce and *Amaranthus* leaf chloroplasts. The similarity of the absorbance changes in chloroplasts from three different species of plants suggests that these changes are due to the same chloroplast component. Since the decrease in absorbance had a maximum at 550 mμ, we have provisionally designated this component C550.

Having observed the light-induced absorbance changes in C550 in the presence of ferricyanide, a nonphysiological electron acceptor for chloroplasts, we next investigated the occurrence of similar changes in the presence of ferredoxin and NADP, the physiological acceptors of electrons released by the photooxidation of water.[15-17] Here, however, parallel light-induced absorbance changes in cytochromes could not be set aside by chemical oxidation since, unlike ferricyanide, ferredoxin and NADP do not react with chloroplast cytochromes in the dark. We therefore took advantage of the fact that "long-wavelength" (photosystem I) monochromatic light (714 mμ) is capable of inducing the complete oxidation of cytochrome f,[18] the one chloroplast component whose spectral properties were most likely to obscure changes in C550. Photosystem I is identified with chloroplast light reactions that are independent of oxygen evolution,[8, 24] i.e., cyclic photophosphorylation and NADP reduction with artificial electron donors.[19]

Our procedure then was to measure light-induced changes in C550, in the presence of ferredoxin and NADP, in two parallel cuvettes: (1) one illuminated by a 714-mμ light beam, which completely oxidized cytochrome f but gave no significant NADP reduction by water,[8, 19] and (2) another cuvette illuminated by a 647-mμ light beam, which also completely oxidized cytochrome f[18] but which, in addition, was capable of supporting NADP reduction by water.

Figure 3 shows absorbance changes in spinach chloroplasts induced by 647-mμ (A) and 714-mμ (B) light beams, in the presence of ferredoxin and NADP. Subtraction of curve B from curve A eliminates the contribution of cytochrome f to the absorbance changes induced by 647-mμ light. As shown in Figure 3, the resultant difference ($A - B$) curve shows an absorbance change peak at 550 mμ, characteristic of C550.

The light-induced absorbance changes in C550, accompanying the photooxidation of water, were not limited to the use of ferricyanide or NADP as terminal electron acceptors. Similar results were obtained with p-benzoquinone and

benzyl viologen. We conclude, therefore, that C550 absorbance changes always accompany the noncyclic electron transfer from water. Significantly, no C550 absorbance changes were observed when ferredoxin and NADP were photoreduced with a 714-mμ light and an artificial electron donor (reduced dichlorophenol indophenol) to replace water.

A question of great interest was whether the light-induced absorbance change in C550 would also take place at temperatures low enough to preclude thermochemical reactions. That this was the case is indicated by Figure 4, which shows the time course of a light-induced spectral change in C550 at −189° in the presence of ferricyanide. At this low temperature, the light-induced decrease in ab-

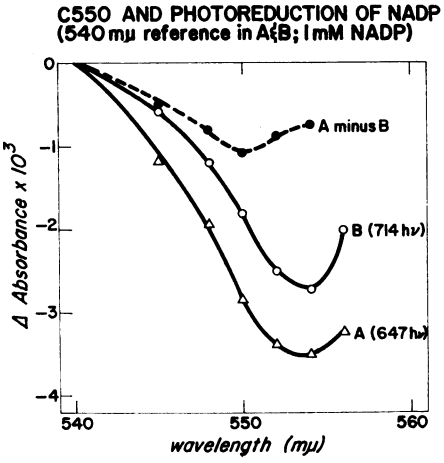

FIG. 3.—Absorbance changes in C550 accompanying NADP reduction by spinach chloroplasts. The reaction mixture contained (per 1.0 ml) spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following in μmoles: Tricine buffer (pH 8.2), 33.3; ferredoxin, 0.0056; NADP, 1.0. The 647-mμ actinic light had an intensity of 4.5 × 10³ ergs/cm²/sec and the 714-mμ actinic light had an intensity of 1.28 × 10⁴ ergs/cm²/sec. 540-mμ reference. For other details, see text.

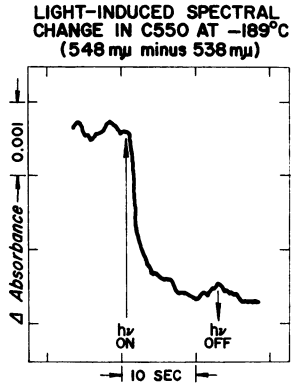

FIG. 4.—Light-induced spectral change in C550 at −189°C (548 mμ minus 538 mμ). The reaction mixture contained (per 1.0 ml) spinach chloroplasts (equivalent to 200 μg chlorophyll), 0.5 ml glycerol, and the following in μmoles: Tricine buffer (pH 8.2), 33.3; potassium ferricyanide, 5. The 664-mμ actinic light had an intensity of 7.8 ×10³ ergs/cm²/sec.

sorbance was not reversed in the dark. A similar irreversibility of spectral changes at low temperature has been reported for cytochrome *f*[20, 21] and cytochrome b_{559}.[2]

Figure 5 shows the spectra of the photoinduced irreversible absorbance changes at −189° in chloroplasts from spinach, romaine lettuce, and *Amaranthus* leaves. In comparison with room temperature, absorbance changes at −189° show a slight shift of the peaks toward the violet end of the spectrum—a shift that also occurs in the absorption peaks of all the chloroplast cytochromes.[2, 12, 20, 22, 23]

The conclusion drawn from these experiments is that the light-induced absorbance changes in C550 are closely linked to the events surrounding photon capture by photosystem II. To test this conclusion further, we compared the effects of

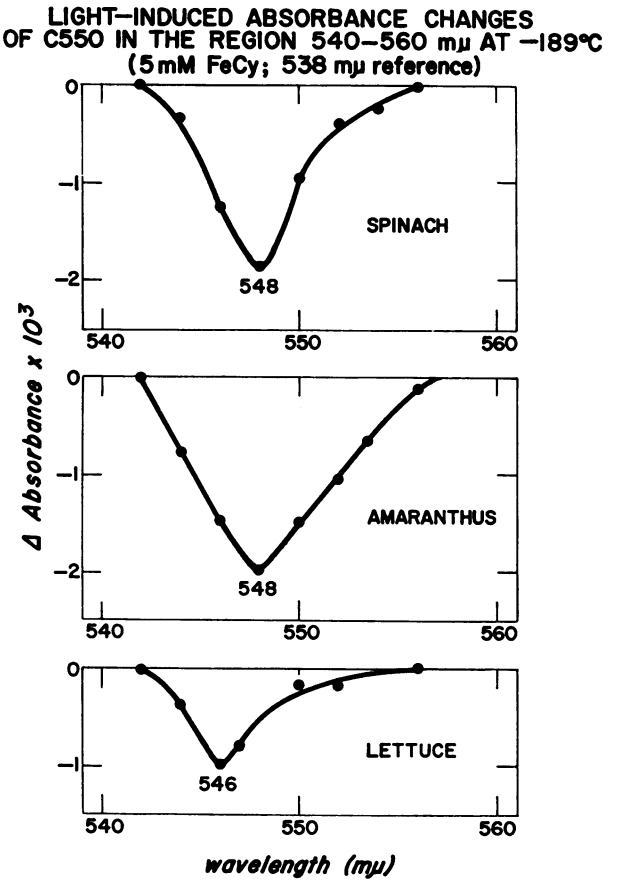

FIG. 5.—Light-induced absorbance changes of C550 in the region 540 to 560 mμ at −189°C (538-mμ reference). Experimental conditions were as described in Fig. 4.

photosystem II (650 mμ) and photosystem I (715 mμ) light when each was supplied at a saturating intensity. The absorbance change induced by 715-mμ light was much smaller than that induced by 650-mμ light (Fig. 6).

The association of C550 with photosystem II was further tested in chloroplasts fractionated with digitonin. This procedure yields a heavy chloroplast fraction (D-10) enriched in photosystem II and a light fraction (D-144) which has only photosystem I activity.[5, 12, 13] The light-induced C550 absorbance change was markedly greater in the D-10 chloroplast fraction than in the control chloroplasts and was completely absent in the D-144 fraction (Fig. 7). (One anomaly of undetermined significance followed the digitonin treatment. The D-10 fraction used 714-mμ light more effectively than the control chloroplasts in producing the C550 absorbance change. However, 647-mμ light was still much more effective than 714-mμ light.)

Since the association of C550 with photosystem II seemed well established, it became of interest to investigate how C550 is affected by such well-known inhibitors of photosystem II as 3-(3,4-dichlorophenol)-1,1-dimethyl urea (DCMU) and *o*-phenanthroline (cf. ref. 24), Figure 8 shows that DCMU, at concentrations which markedly inhibit oxygen evolution, had little or no effect on the

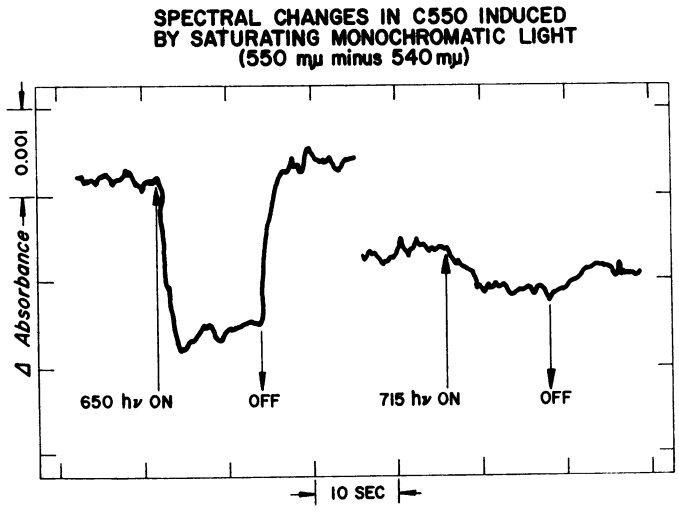

FIG. 6.—Spectral changes in C550 induced by saturating monochromatic light at 650 and 715 mμ (550 mμ minus 540 mμ). Experimental conditions were as described in Fig. 2. The 650-mμ actinic light had an intensity of 2.1×10^4 ergs/cm²/sec, and the 715-mμ actinic light had an intensity of 8.5×10^3 ergs/cm²/sec.

magnitude of the light-induced decrease in absorbance but markedly inhibited the rate of the dark reversal. Similar results were obtained with *o*-phenanthroline.

An analysis of the dark reversal showed it to be a first-order reaction. The apparent first-order rate constants decrease with increasing inhibitor concentrations in a manner that parallels the inhibition of oxygen evolution. At certain inhibitor concentrations, the decrease in the rate of dark reversal was accompanied by an increase in the rate of the light-induced absorbance change.

Several additional experiments were undertaken in an attempt further to characterize C550. Washing the chloroplasts twice did not affect the size of the C550 change, either at room or liquid nitrogen temperatures—an indication that

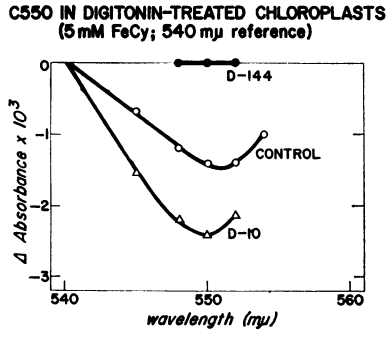

FIG. 7.—C550 in digitonin-treated spinach chloroplasts (540-mμ reference) Experimental conditions were as described in Fig. 2.

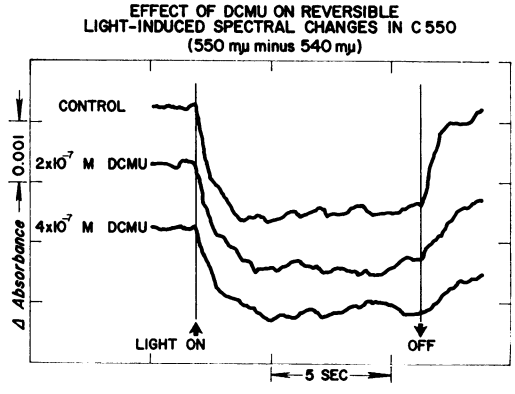

FIG. 8.—Effect of DCMU on reversible light-induced spectral changes in C550 (550 mμ minus 540 mμ). Experimental conditions were as described in Fig. 2. The 664-mμ actinic light had an intensity of 2.5×10^4 ergs/cm²/sec.

C550 is not a soluble component but one bound to the chloroplast membrane structure. Heating the chloroplasts at 55° for two minutes completely eliminated the C550 change. Treating the chloroplasts with 0.8 *M* tris, a treatment which eliminates oxygen evolution but not the photoreactions of system II with artificial electron donors,[6] does not affect the C550 change at −189°.

Concluding Remarks.—The characteristics of C550 such as its absorption peak at 550 mμ and the reversible absorbance change on illumination, suggest that it might be a chloroplast cytochrome of a *c* type with an α peak at 550 mμ. However, we cannot draw this conclusion because we have so far been unable to observe a spectral change in C550 in the Soret region, characteristic of cytochromes. Should C550 prove to be a cytochrome, then the observed decrease of absorbance at 550 mμ would be diagnostic of a light-induced oxidation. Such a cytochrome, not oxidized by ferricyanide in the dark, would thus have a redox potential more positive than that of any other known cytochrome or any known chloroplast component (cf. refs. 25, 26). On the other hand, it is possible that C550 is a hitherto unrecognized intermediate electron acceptor in a primary light reaction of photosystem II. The light-induced and temperature-insensitive decrease of absorbance at 550 mμ would then represent a reduction brought about by a transfer of electrons from excited chlorophyll to C550. The relative merits of these two interpretations are now under investigation.

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