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LASER-INDUCED REACTIONS OF P700 AND CYTOCHROME *f* IN A BLUE-GREEN ALGA, *PLECTONEMA BORYANUM**

TETSUO HIYAMA AND BACON KE

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387 (U.S.A.)

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SUMMARY

Kinetics of the absorption change of P700 (blue band) and cytochrome *f* in whole cells of a blue-green alga, *Plectonema boryanum*, have been studied by Q-switched ruby-laser flash excitation (694 nm; approx. 20 nsec) to elucidate the sequential relationship of these two components in photosynthetic electron transport. "P700" was photooxidized within 2 μsec and recovered in two phases ($t_{1/2} \cong$ 10 μsec and 200 μsec). Under the same conditions cytochrome *f* was oxidized with a half time of 15 μsec. The magnitude of the fast phase of "P700" recovery, however, diminished at lower laser intensity while the cytochrome *f* change remained unaffected. The result suggests that cytochrome *f* and P700 may not be on the same electron-transport chain.

INTRODUCTION

The reaction-center chlorophyll of Photosystem I of green plants and algae was first proposed by KOK[1] to be a specialized form of chlorophyll, which was designated as P700. The spectral and photochemical properties of this pigment complex have been extensively studied since then (see ref. 2 for a review). According to the current hypothesis of two photosystems and the "Z" scheme of electron transport, the photooxidized P700 is supposed to accept electrons from other redox components such as cytochrome *f* and plastocyanin situated on the electron-transfer chain between the two photosystems.

A number of studies have shown that cytochrome *f*[3–5], plastocyanin[3–5] and mammalian cytochrome *c*[3,4,6–8] as well as a number of artificial electron donors can donate electrons to P700 *in vitro*. From studies with mutant cells of *Chlamydomonas reinhardi*, LEVINE and coworkers[9] suggested that the electron transfer sequence is in the order of cytochrome *f* to plastocyanin to P700.

The directly coupled reaction between a *c*-type cytochrome and the reaction-center chlorophyll, namely the oxidation of cytochrome by the photooxidized reaction-center, has been demonstrated in photosynthetic bacteria[10,11] as well as in chloroplast systems[8] only recently. By direct spectroscopic examination, PARSON showed that by using Q-switched ruby-laser flashes, the onset of cytochrome-555

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oxidation and the decay of P890 recovery in *Chromatium* chromatophores have an identical halftime of 2–3 μsec[10]. The exact kinetic correspondence has also been found for the oxidation of exogenous cytochrome *c* by the reaction-center particles prepared from the carotenoidless mutant strain of *Rhodopseudomonas spheroides*[11].

Recently we found that the green-plant reaction-center chlorophyll, P700, can directly oxidize mammalian cytochrome *c in vitro*. Using a P700-enriched particle prepared from a blue-green alga, Anabaena, the corresponding reaction half times were 10 msec[8]. The P700-enriched particles prepared from spinach cannot directly oxidize mammalian cytochrome *c*, but can extract electrons directly from cytochrome-555 of Euglena[8].

WITT and co-workers[12] earlier suggested a correspondence between the rise time of cytochrome *f* oxidation to the decay time of P700 in spinach chloroplasts. More recently, KOK[13] also briefly reported such a kinetic correspondence in chloroplasts. On the other hand, CHANCE AND BONNER[14] studied the photooxidation of cytochrome *f* and P700 in green-plant leaves and chloroplasts at liquid nitrogen temperature, and concluded that neither cytochrome *f* nor P700 appears to be the rate-limiting intermediate in the oxidation of the other. They further suggested the possibility that cytochrome *f* may be oxidized by a reaction center other than P700. More recently, RURAINSKI *et al.*[15] reported that their observation of relaxation times of cytochrome *f* and P700 in whole cells of algae is inconsistent with sequential electron transport. They also pointed out certain differences between whole cells and isolated chloroplasts.

Thus, in green-plant photosynthesis, results from the various biochemical and kinetic studies on P700 and the immediately involved components cannot yet be integrated into a generally accepted scheme. The present work is an attempt to re-examine the kinetic relationship between P700 and cytochrome *f* in whole cells and a possible direct interaction between them by means of laser-flash excitation. We have used the blue-green alga, *Pl. boryanum*, because earlier work showed that light-induced absorption changes in both P700 and cytochrome *f* can readily be detected spectrophotometrically in this alga[16]. Due to difficulties in separating the actinic ruby-laser flash at 694 nm from the red band of P700 at 703 nm, the blue band at 435 nm has been employed for following the P700 response. The results will be discussed in terms of the role of P700 relative to the oxidation of cytochrome *f*.

MATERIALS AND METHODS

The cells of *Pl. boryanum* were cultivated in modified Detmer's medium[17] in 500 ft candles light by shaking and bubbling with air containing 5 % CO_2. The cells were harvested after 4 days, washed once with the growth medium and resuspended in the same medium. Most of the spectrophotometric measurements were carried out with this suspension without further treatment. In order to avoid fluctuations in transmission due to sedimentation of the cells during measurement, a modified moist chamber described by CHANCE AND STREHLER[18] was constructed and used throughout this study.

The basic set-up of flash kinetic spectrophotometry utilizing a Q-switched ruby laser as the actinic source has been described briefly earlier[19]. A rotating prism was used as a Q-spoiler to produce the 20-nsec laser pulse (20 MW, maximum). The ruby rod and the pumping xenon flash lamp were water cooled so that the flash was

repeatable every 5 sec. The laser beam was attenuated by means of negative lenses and neutral density filters. Average incident intensity was measured by a calibrated photodiode (Lite-Mike, EG and G) to be 1 $mJ \cdot cm^{-2}$ on the front face of the sample cuvette.

The measuring light (less than 200 $erg \cdot cm^{-2}$) was provided by a quartz-iodine lamp through a grating monochromator (B and L, 500 mm). The photocurrent from the photomultiplier (EMI 9558B) was fed to an analog-to-digital converter (Fabritek 952) after proper amplification. It should be noted that, though the response time of the amplifier was down to 50 nsec, the actual time response was limited to 1 μsec due to the time resolution of the digitizer. Digitized signal for each flash cycle was memorized and averaged in an instrument computer (Fabritek 1062). The averaged signal was displayed on an oscilloscope or transcribed on an X-Y recorder. Usually 32-128 repetitive flashes were necessary to obtain a reasonable signal to noise ratio.

RESULTS

Typical kinetics of the absorption changes induced by a 20-nsec ruby-laser flash are shown in Fig. 1. At 405 nm, where the band shift due to cytochrome *f* oxidation is expected, an absorption increase of intermediate speed ($t_{1/2}$ = 15 μsec) followed by a small amount of slow increase was observed. This indicates that the absorption change at 405 nm is not only caused by cytochrome *f* but also by some other components. At 420 nm, a bi-phasic absorption decrease occurred, with one component

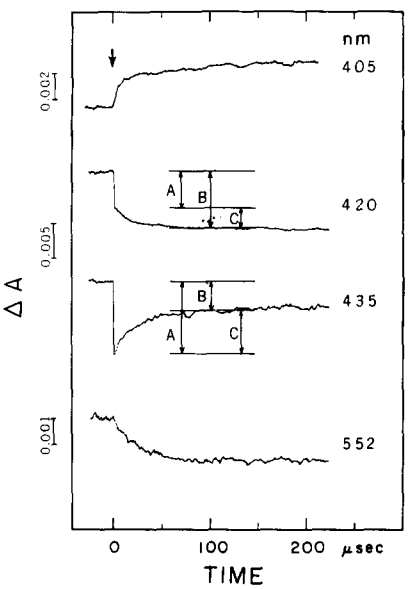

Fig. 1. Absorption-change transients induced in *Pl. boryanum* cells by 20-nsec Q-switched ruby-laser flashes at 694 nm. Measurements were made after the cells were settled in the moist chamber. Arrow indicates moment the flash was applied. The transients were obtained by averaging either 16 (for 405, 420 and 435 nm) or 64 flashes (for 552 nm). Chlorophyll concentration, 16 μM. Optical path length, 6.5 mm. Average incident intensity, approx. 1 $mJ \cdot cm^{-2}$. The magnitude of absorption changes designated by A and B represent those occurring 2 and 100 μsec after the flash. Segment C represents the absorption change occurring between 2 and 100 μsec. These segments are to be plotted into difference spectra in Fig. 4.

rising at a rate similar to that at 405 nm and another component rising faster than 1 μsec. Because of the overlapping rapid and slow absorption changes, a precise determination of the rise time of cytochrome *f* oxidation is difficult from absorption changes in the Soret region.

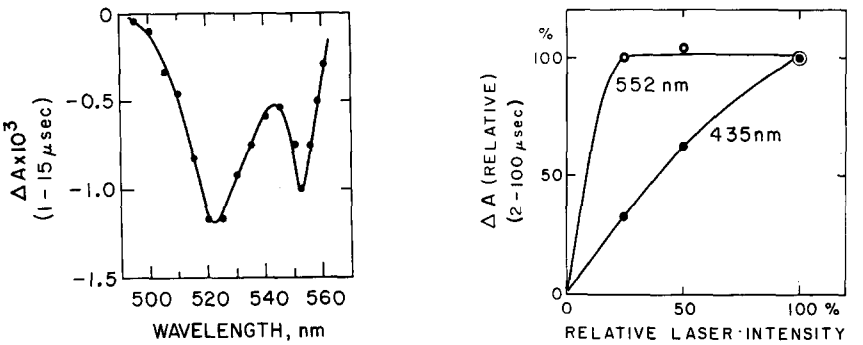

Fig. 2. Absorption-spectrum changes in the cytochrome α-band region between 1 and 15 μsec after the laser flash. ΔA values were obtained by averaging with 32 repetitive excitation flashes. Experimental conditions were the same as in Fig. 1.

Fig. 3. Excitation-intensity dependence of the absorption changes at 552 and 435 nm between 2 and 100 μsec after the flash. Experimental conditions were the same as in Fig. 1. Intensity was attenuated by means of neutral density filters. Absolute laser intensity at 100% was 1 mJ·cm^{-2}.

At 552 nm, the α-band of cytochrome *f*, the kinetics of absorption changes were found to be relatively simple (see Fig. 1, bottom). The absorption decrease had an intermediate rise time of approx. 15 μsec. It should be noted here that in some blue-green algae there is always an accompanying rapid absorption increase of an unknown nature (rise time less than 1 μsec) in this wavelength region, which interferes with observations of cytochrome *f* kinetics. In Plectonema, although there exists a similar change around 530 nm, it becomes negligible at 552 nm. The difference spectrum for changes occurring between 1 and 15 μsec in this spectral region is shown in Fig. 2. The absorption decrease at 552 nm with a half rise time of approx. 15 μsec as shown in Fig. 1 can thus be attributed to the oxidation of cytochrome *f*. The absorption changes in the 520-nm region might be partly from the unknown change mentioned above and partly from the β-band of the cytochrome. When the cytochrome-oxidation kinetics were fitted to a first-order plot, the half rise time was estimated to be 15 μsec. From this rise time value, one may conclude that the rise portions with an intermediate speed at 405 nm and 420 nm are also due to cytochrome *f* oxidation.

At 435 nm (see Fig. 1), where the Soret-band of P700 is supposed to be located in this alga[16], a fast decrease occurred within 2 μsec and was followed by a biphasic recovery which consisted of an intermediate ($t_{1/2}$ = 10–15 μsec) and a slow ($t_{1/2}$ > 200 μsec) phase.

The absorption changes at 435 and 552 nm responded differently to the excitation intensity as shown by the light saturation curves in Fig. 3, in which the net absorbance changes occurring between 2 and 100 μsec after the flash were plotted against light intensity. At lower intensity of excitation, where neither the rate nor the magnitude of cytochrome changes at 552 nm was affected, the magnitude of the intermediate

recovery phase at 435 nm became much smaller than at higher intensity excitation. Technical difficulties caused by laser artifacts at higher excitation intensities prevented us from accurately measuring the light saturation curve for the 15 μsec decay component at 435 nm. However, assuming that the 15 μsec component belongs to P700 and using the molar differential extinction coefficient value of $8.6 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 435 nm[8], even at the highest intensity, P700 molecules equivalent to only half of the cytochrome *f* could have participated in the reaction. At lower actinic intensity (about 25 % of the highest) this ratio was only 1:8.

In order to further analyze the nature of the components involved in the blue band relative to the excitation intensity, the magnitude of absorption changes taking place during a certain time period after the flash was applied, is plotted against wavelength in Fig. 4. The 2-μsec rapid components (see the initial rapid rise represented by "A" at 420 and 435 nm in Fig. 1) are plotted in Fig. 4A. The absorption changes occurring at 100 μsec after the flash (see the portion represented by "B" at 410 and 435 nm in Fig. 1) are plotted in Fig. 4B. The absorption changes occurring between 2 and 100 μsec are plotted in Fig. 4C. The periods of 2 and 100 μsec after the flash were selected because cytochrome *f* oxidation is practically complete within 100 μsec. During this time period, changes due to cytochrome *f* oxidation and/or responses by other chemical components with similar kinetics would be represented.

The difference spectrum of the absorption changes within 2 μsec after the flash (see Fig. 4A) shows a considerably broader band which apparently consists of three separate bands at 420, 428 and 435 nm. The 420-nm band does not seem to be caused by cytochrome *f* since it has a rapid rise time. In terms of the kinetics, it is more

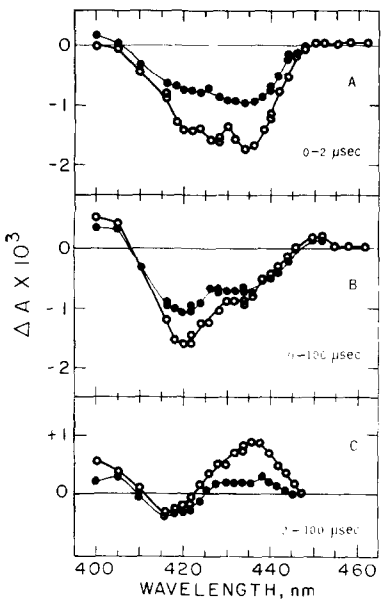

Fig. 4. Absorption-spectrum changes in the Soret region during different time periods after the laser flash. See Fig. 1 for an illustration on the magnitudes of absorption changes occurring during different time periods. Experimental conditions were the same as in Fig. 1 except the chlorophyll concentration was 6.6 μM. Data points obtained with a laser intensity of 1 $mJ \cdot cm^{-2}$ (○) and at 25 % of this intensity (●).

likely a part of the main peak at 435 nm. The similarity in the response of the absorption changes at 435 and 420 nm to the two excitation intensities further supports this suggestion.

The plot in Fig. 4B shows that the 0–100 μsec absorption change at 420 nm was more sensitive to excitation intensity than that at 435 nm. It suggests that there may be another slowly decaying component absorbing at 420 nm besides cytochrome *f*. This slowly decaying component might be related to the 420 nm shoulder in Fig. 4A. The relatively intensity-insensitive, slowly decaying component at 435 nm actually took more than 200 μsec to decay completely (*cf.* also Fig. 1).

It appears from Fig. 4C (*cf.* also Fig. 1) that at high-intensity excitation, both the "P700"-reaction at 435 nm and cytochrome *f* reaction at 420 nm took place with apparently corresponding kinetics. However, at lower excitation intensity, while the magnitude of the cytochrome *f* changes remained nearly unchanged, the magnitude of the "P700"-change decreased to approx. 1/4 (*cf.* also Fig. 3).

DISCUSSION

In spite of previous reports of a kinetic correspondence between oxidation of cytochrome *f* and re-reduction of P700 (refs. 12, 13), and in spite of the fact that in photosynthetic bacteria a direct interaction between cytochrome-555 and reaction-center P890 has been demonstrated in *Chromatium* chromatophores[10] and between mammalian cytochrome *c* and the Spheroides reaction-center P870 (ref. 8) and that in subchloroplast preparations direct interactions have been found between mammalian cytochrome *c* or Euglena cytochrome-552 and P700 (ref. 8), experimental results from this work have not confirmed such an interaction between P700 and cytochrome *f* in Plectonema cells. The kinetics of absorption changes at 435 and 552 nm induced by high-intensity laser flashes indicate an apparent correlation between the oxidation of cytochrome *f* at 552 nm and the recovery of a portion of the signal at 435 nm presumably due to P700. However, when the excitation intensity was lowered, the rapid decaying phase at 435 nm diminished much faster than the 552-nm signal which was relatively unaffected by excitation intensity. If the signals observed at 435 and 552 nm at high-intensity excitation truly represent a direct interaction between P700 and cytochrome *f*, then they should be affected to an equal extent under changing conditions. For instance, in the case of *Chromatium* chromatophores, the kinetic correlation between the signals due to cytochrome-555 and P890 could be observed over a wide range of excitation intensities[10].

The validity of using the absorption change at 435 nm for monitoring the P700-change appears to have been established by several previous studies which showed that both the blue and the red band are involved in P700-changes[16,20,21]. More recent studies on the coupled reaction between photooxidized P700 in subchloroplast particles from spinach, Anabaena and Plectonema with mammalian ferrocytochrome *c* or Euglena cytochrome-552 as well as other electron donors (*e.g.* phenazine methosulfate and ascorbate) have conclusively demonstrated the exact kinetic correlation for the changes in the blue band and the red band[8]. Thus the lack of a correlation between the 435-nm and 552-nm signals at low excitation intensities suggests the following possibilities:

(a) An intermediate or intermediates may be present between cytochrome *f* and

P700. This intermediate could react very rapidly with P700, thus competing with the photochemical oxidation of the latter. This component, which is oxidized by P700, is able to oxidize cytochrome *f* in about 15 μsec. In this case, no direct correlation is expected between cytochrome *f* and P700. This intermediate could be plastocyanin, as suggested by the mutant studies[9]. In this case, the re-reduction of P700 would be very fast and escape detection by our present instrument.

(b) Since the slow (approx. 100 μsec) decay at 435 nm is likely to be associated with the reduction of P700, and is taking place while cytochrome *f* is already completely oxidized, it is reasonable to suggest that P700 and cytochrome *f* are not located on the same electron-transfer chain.

(c) One other possibility is that both situations (a) and (b) are operative. Through a lack of homogeneity, only part of the P700 can react with cytochrome *f* through an intermediate as suggested in (a) above. The remainder of the P700 represented by the slow decay (approx. 100 μsec) does not have any topological relation with cytochrome *f*.

Since the 435 nm absorption change with a decay time of 15 μsec occurred only under high excitation intensities, they may not represent changes due to P700 at all. These rapidly decaying signals bear some resemblance to those which WITT and coworkers[22,23] attributed to the formation of metastable states of chlorophyll and/or carotenoids. These changes have very rapid rise (< 1 μsec) and decay (approx. 10 μsec) kinetics[22,23]. The slowly decaying component observed at 435 nm is most likely associated with P700, although the nature of the coupled reaction is unknown. The nature of the 428-nm band is also unclear at the present moment. In Plectonema cell fragments obtained by sonication, we have recently observed that the absorption change at 428 nm has a different kinetics from that of P700 at 435 nm. The relationship between the 428-nm changes in intact cells to those in fragments remains to be elucidated. On the other hand, in the presence of different electron donors, the absorption changes at 420 and 435 nm in the fragments have identical re-reduction kinetics. Therefore, it is quite likely that the 420-nm shoulder is also a part of the absorption band of P700 in whole cells. Details on these experiments will be reported elsewhere.

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REFERENCES

1 B. KOK, *Biochim. Biophys. Acta*, 22 (1956) 399.
2 L. P. VERNON AND B. KE, in L. P. VERNON AND G. R. SEELY, *The Chlorophylls*, Academic Press, New York, 1966, p. 596.
3 J. H. LIGHTBODY AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 131 (1967) 508.
4 B. KOK, H. J. RURAINSKI AND E. A. HARMON, *Plant Physiol.*, 39 (1964) 513.
5 L. P. VERNON, B. KE AND E. R. SHAW, *Biochemistry*, 6 (1967) 2210.
6 S. KATOH AND A. TAKAMIYA, in *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci.–Natl. Res. Council Publ.*, 1145 (1963) 262.
7 M. MURANO AND T. FUJITA, *Plant Cell Physiol.*, 8 (1967) 673.
8 B. KE, T. OGAWA, T. HIYAMA AND L. P. VERNON, *Biochim. Biophys. Acta*, 226 (1971) 53.
9 D. S. GORMAN AND R. P. LEVINE, *Plant Physiol.*, 41 (1966) 1648.

10 W. W. PARSON, *Biochim. Biophys. Acta*, 153 (1968) 248.
11 B. KE, T. H. CHANEY AND D. W. REED, *Biochim. Biophys. Acta*, 216 (1970) 373.
12 H. T. WITT, A. MÜLLER AND B. RUMBERG, *Nature*, 192 (1961) 967.
13 B. KOK, in A. SAN PIETRO, F. A. GREEN AND T. J. ARMY, *Harvesting the Sun*, Academic Press, New York, 1967, p. 29.
14 B. CHANCE AND W. D. BONNER, JR., in *Photosynthetic Mechanism in Green Plants, Natl. Acad. Sci.–Natl. Res. Council Publ.*, 1145 (1963) 66.
15 H. J. RURAINSKI, J. RANDLES AND G. HOCH, *Biochim. Biophys. Acta*, 205 (1970) 254.
16 B. KE AND E. NGO, *Biochim. Biophys. Acta*, 109 (1965) 431.
17 A. WATANABE, *Gen. Appl. Microbiol., Tokyo*, 6 (1965) 283.
18 B. CHANCE AND B. STREHLER, *Plant Physiol.*, 32 (1957) 536.
19 B. KE, *Biochim. Biophys. Acta*, 172 (1969) 583.
20 B. KOK, *Acta Botan. Neerl.*, 6 (1957) 316.
21 J. WEIKARD, Doctoral Dissertation, Tech. Univ. Berlin, 1968.
22 H. T. WITT, in S. CLAESSON, *Fast Reactions and Primary Processes in Chemical Kinetics*, Almqvist and Wiksell, Stockholm, 1968, p. 216.
23 C. WOLFF AND H. T. WITT, *Z. Naturforsch.*, 24B (1969) 1031.