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LIGHT-INDUCED ABSORBANCE CHANGES OF TWO CYTOCHROME *b* COMPONENTS IN THE ELECTRON-TRANSPORT SYSTEM OF SPINACH CHLOROPLASTS

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SUMMARY

Light-induced absorbance changes of cytochromes *b* and *f* in chloroplasts have been measured in the cytochrome α-band region with a dual-wavelength spectrophotometer. The data indicate that there are *b*-type cytochromes at two different places in the electron-transport system. Whereas the cytochrome *f* changes induced by red (645 mμ) and far-red (715 mμ) light are typical of the two pigment system antagonism, the cytochrome *b* changes are more complex. With an actinic light intensity of $2–3 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} and no electron acceptor, the cytochrome *b* changes show two components, with difference spectra peaking at 563 and 560 mμ, respectively. Cytochrome *b*-563 is associated with a fast transient reduction upon illumination with far-red light. On turning off the far-red, cytochrome *b*-560 shows an oxidation which is reversible by red light. Cytochrome *b*-563 is oxidized in the dark and is not reduced by ascorbate, whereas cytochrome *b*-560, when oxidized, is ascorbate reducible. Both components are reduced by NADPH *via* a diaphorase. The cytochrome *b*-560 shows red, far-red reversibility at lower actinic light intensities and in the presence of carbonylcyanide *m*-chlorophenylhydrazone. It is concluded that cytochrome *b*-563 is reduced by Photosystem I and that cytochrome *b*-560 is in the electron transport chain between Photosystems I and 2.

INTRODUCTION

The existence of *b*-type cytochrome in the chloroplasts of green plants was first demonstrated by HILL and co-workers[1,2]. The participation of cytochrome *b* in the two light reactions of photosynthesis has been inferred from studies on the green alga, *Chlamydomonas reinhardi*, and various mutants of it by CHANCE, SCHLEYER AND LEGALLAIS[3], LEVINE *et al.*[4], and LEVINE AND GORMAN[5]; on Euglena and the blue-green alga Anacystis by OLSON AND SMILLIE[6]; on red algae by NISHIMURA[7]; and on spinach chloroplasts by RUMBERG[8], HIND AND OLSON[9], and BUTLER[10]. Although the existence of cytochrome *b* in the photosynthetic electron transport chain is well

Abbreviations: cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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established by these studies, its position relative to the known components of electron transport is the subject of controversy. Using the current concept of two pigment systems[11], which will be the framework for discussion in this paper, it has been suggested[4,5,8] that cytochrome *b* is in series with cytochrome *f* in the electron transport chain linking Photosystem 1 and Photosystem 2, possibly lying between plastoquinone and cytochrome *f* in the chain[8]. Other experiments[9,12] suggest that cytochrome *b* is closely linked to Photosystem 1 and cyclic phosphorylation. From our preliminary experiments[10] where we observed red, far-red reversibility of a cytochrome *b* like component, we inferred that at least part of the cytochrome *b* complement is in the electron transport chain joining Photosystem 1 and Photosystem 2. Based on further study with the dual-wavelength spectrophotometer we find that there are in fact two cytochrome *b* components present, one in the chain joining Photosystem 1 and Photosystem 2, and the other on the electron-accepting side of Photosystem 1. LUNDEGÅRDH[13] also proposed two cytochrome *b* components, but considered their role in a different theoretical context.

EXPERIMENTAL PROCEDURE

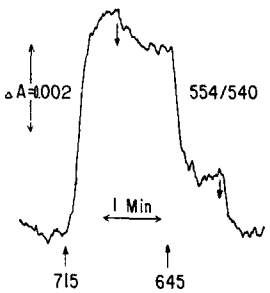
Chloroplasts were prepared from market spinach according to the following procedure: 50 g of leaves with mid-ribs removed were ground in 150 ml of ice-cold mixture of 0.4 M sucrose, 0.05 M Tris–HCl, 0.01 M NaCl and 0.25 g ascorbic acid (pH 7.6) for 20 sec at low speed in a model PB-5A Waring Blendor. The preparation was then filtered through a layer of muslin and the resulting filtrate was subjected to two cycles of low-speed–high-speed centrifugation. The total chlorophyll concentration of the final chloroplast pellet was usually 5–10 mg/ml. Such preparations can reduce $NADP^+$ in the light without added ferredoxin, though at a reduced rate, implying that the chloroplasts contain the flavoprotein, $NADP^+$ reductase, and some ferredoxin.

Cytochrome absorbance changes were followed in an Aminco–Chance dual-wavelength spectrophotometer in which the chloroplasts at a concentration of about 200 μg/ml were stirred continuously in a medium containing 15 mM Tris-HCl, 20 mM NaCl, 5 mM phosphate, and 4 mM $MgCl_2$, pH 7.5–7.8. The absorbance changes were measured in the α-band region with the reference wavelength at 540 mμ. The intensity of the modulated measuring beams (1.2 ± 0.2 ergs$\cdot$cm$^{-2}\cdot$sec^{-1} with 3.3 mμ half-band width) was sufficiently weak that they had no observable effect on the sample. The light signal was measured with an EMI 9524 phototube, blocked with a Corning 9788 filter and an Optics Technology 600 mμ short-pass cut-off filter. The red and far-red actinic light was obtained with a Unitron LKR microscope illuminator and Baird Atomic B-1 645 and 715 mμ interference filters (approx. 10 mμ half-band widths), with additional infrared blocking filters. The temperature of the sample was regulated at 24°.

RESULTS AND DISCUSSION

Fig. 1 shows the light-induced absorbance changes at 554 *vs.* 540 mμ in the absence of an electron acceptor. Far-red (715 mμ) light of intensity $3\cdot10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1} causes a decrease in absorption at 554 relative to 540 mμ which is reversed by

red (645 mμ) light of about the same intensity. From the known spectral properties of purified cytochromes and the difference spectra for these light-induced changes, the absorption changes induced by far-red and red light in Fig. 1 are interpreted to represent, respectively, oxidation and reduction of cytochrome *f*. There is generally some reduction in the dark after both far-red and red illumination, although the redox level in the dark after far-red light is considerably more oxidized than the dark level after red light. AVRON AND CHANCE[14] have reported similar changes for cytochrome *f* in spinach chloroplasts.



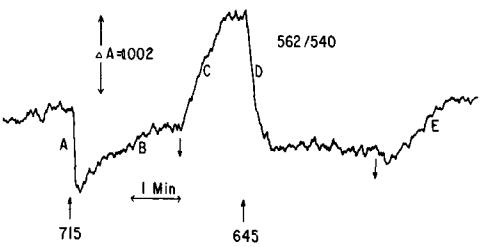


Fig. 1. Light-induced absorbance changes at 554 *vs.* 540 mμ. No electron acceptor. Chlorophyll concentration 200 μg/ml. Actinic light turned on at upward arrows and off at downward arrows. Intensities: $3.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 715 mμ; $2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ. Temperature, 24°.

Fig. 2. Light-induced absorbance changes at 562 *vs.* 540 mμ. Conditions as in Fig. 1. See text for description of changes A–E.

The absorbance changes measured in the absence of an electron acceptor at 562 *vs.* 540 mμ are attributed to *b*-type cytochrome (Fig. 2). These changes are characteristic of a high intensity of actinic light ($3 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}). Results obtained with lower intensities will be described below. At the high intensity, irradiation with far-red light causes a transient reduction (change A in Fig. 2) followed by a slower oxidation (B) to a steady-state level which is approximately the same as the previous dark steady state. On turning off the far-red light, the measurement at 562 mμ indicates a large dark oxidation (C) to a steady state which is much more oxidized than the original dark level. The high level of oxidation found in the dark after far-red light can be reversed by irradiation with red light (D), the steady-state level in red light being more reduced than that in far-red light. On turning off the red light, there is a dark oxidation (E) back to the original baseline. The cytochrome *b* changes also show that the dark steady-state level after far-red light is much more oxidized than the dark steady state after red light. On prolonged incubation in the dark after far-red light the oxidation level of both cytochromes *b* and *f* will decrease slowly.

Difference spectra were determined for the various changes shown in Figs. 1 and 2 by making the double-beam measurements at λ *vs.* 540 mμ. The difference spectrum for the far-red-induced oxidation (absorbance difference between the steady state in far-red light and the dark steady state after red light) shows a peak at about 555 mμ indicating a dominant influence of cytochrome *f* (Fig. 3). The amplitude of this spectrum in the cytochrome *b* region is a function of actinic light intensity. At lower light intensity the cytochrome *b* change is more apparent.

The difference spectrum for the transient far-red-induced reduction (A in Fig. 2) shows a maximum at about 563 mμ (Fig. 3). The difference spectrum for the dark oxidation following far-red light (C in Fig. 2) shows a maximum at about 560 mμ (Fig. 3). These two difference spectra indicate the presence of two cytochrome *b* components, cytochrome *b*-563 and cytochrome *b*-560.

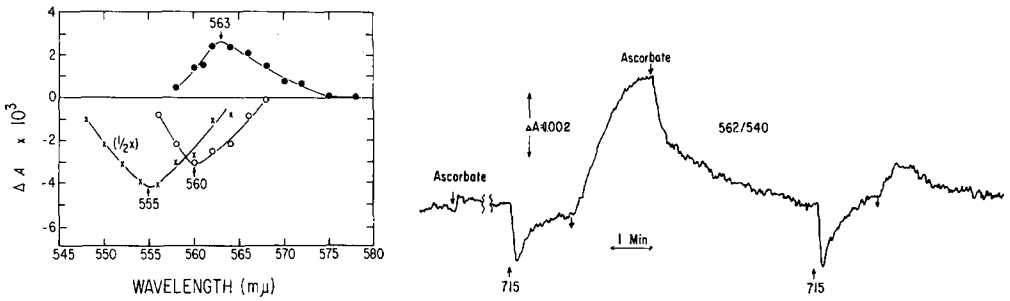


Fig. 3. Difference spectra for changes in Figs. 1 and 2. (X), far-red-induced oxidation (absorbance difference between the steady state in far-red light and the dark steady state after red light). Amplitudes shown for this spectrum are one-half of actual value; (●), transient far-red induced reduction (A in Fig. 2); (○), dark oxidation following far-red illumination (C in Fig. 2). Chlorophyll concentration 200 μg/ml. Actinic light intensities: $3.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 715 mμ; $2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ.

Fig. 4. Absorbance changes at 562 *vs.* 540 mμ. Effect of added ascorbate: ascorbate (1.5-mM) added at the dark steady-state level after red light (experiment to the left of the broken trace). In a second experiment (to the right of the broken trace), 1.5 mM ascorbate is added at the dark steady state after far-red light.

Further evidence for the existence of two cytochrome *b* components is provided by experiments using ascorbate and NADPH as reducing agents. Addition of 1 mM ascorbate in the dark prior to illumination with actinic light causes no change at either 562 mμ (Fig. 4) or at 554 mμ (not shown). Addition of ascorbate to the more oxidized dark steady state which follows far-red light, however, reduces the cytochrome *b* back to the baseline level (Fig. 4). Thus, cytochrome *b*-560 which is oxidized in the dark after far-red light is reducible by ascorbate. Experiments at 554 mμ show that oxidized cytochrome *f* is fully reduced by 1 mM ascorbate. Cytochrome *b*-563 which shows the transient reduction by far-red light, is not reduced by ascorbate, but it is reduced in the dark by NADPH (Fig. 5). NADPH will also reduce cytochrome *f* and cytochrome *b*-560, all presumably via diaphorase. The addition of ascorbate during the steady state in high-intensity far-red light does not cause a reduction of the cytochrome *b* components; if the far-red light is turned off immediately after the addition of ascorbate, there is a transient oxidation followed by a reduction to the

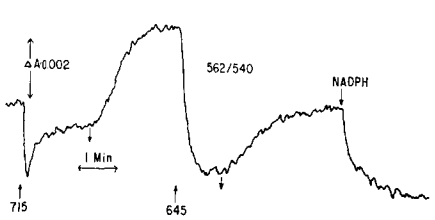


Fig. 5. Absorbance changes at 562 *vs.* 540 mμ. Effect of added NADPH: 0.15 mM NADPH added at the dark steady state after red light. Chlorophyll concentration 175 μg/ml.

baseline level (the same as the off response at the second irradiation period in Fig. 4). These results show that the oxidative response B in Fig. 2 is due to cytochrome *b*-563 and that the cytochrome *b*-560 is fully reduced during the steady state in high-intensity ($3 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}) far-red light. At lower intensity a part of the cytochrome *b*-560 is oxidized by far-red light.

TABLE I

CYTOCHROME REDOX LEVELS AT THE VARIOUS STEADY STATES OF FIGS. 1 AND 2

(— — — Reduced; + + +, Oxidized)

Steady state	*Cyt f*	*Cyt b-560*	*Cyt b-563*
Dark	— — —	— — —	+ + +
Far-red ($3 \cdot 10^4$ ergs·cm^{-2}·sec^{-1})	+ + +	— — —	+ + + (transient — — —)
Dark	+ + —	+ + +	+ + +
Red ($2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1})	+ — —	— — —	+ — —
Dark	— — —	— — —	+ + +

Table I summarizes the various steady-state conditions indicated in Figs. 1 and 2. The addition of ascorbate prior to illumination causes no reduction, showing that cytochrome *f* and cytochrome *b*-560 are fully reduced in this state. Cytochrome *b*-563 is at least partially oxidized (we assume largely oxidized) as shown by the chemical reduction by NADPH and the transient light-induced reduction at the onset of far-red irradiation. At the steady state in high-intensity far-red light cytochrome *f* is largely oxidized, cytochrome *b*-560 largely reduced and cytochrome *b*-563 largely oxidized. On turning off the far-red light, cytochrome *f* goes slightly reduced (the extent of this dark reduction is variable with different preparations and may be smaller or larger than that shown in Fig. 1), cytochrome *b*-560 goes largely oxidized and cytochrome *b*-563 presumably stays oxidized. In red light cytochrome *f* is largely but not completely reduced, cytochrome *b*-560 is largely or fully reduced, and cytochrome *b*-563 is partially reduced. In the dark after red light cytochrome *f* and cytochrome *b*-560 are fully reduced and cytochrome *b*-563 is oxidized.

The rapid transient reduction of cytochrome *b*-563 induced by far-red light suggests that cytochrome *b*-563 accepts electrons from Photosystem 1. Irradiation by red light (Fig. 6) causes a reduction which is slower than that observed initially in far-red light. The red-light-induced reduction of cytochrome *b*-563 is ascribed to Photosystem 1 which shows a lower efficiency in red light than in far-red light[15]. The subsequent oxidation of cytochrome *b*-563 in far-red light may reflect the accumulation of oxidized material which can oxidize cytochrome *b*-563. The cytochrome *b*-563 may also be a component in cyclic electron transport which transfers electrons from the primary reductant of Photosystem 1 back to the linear electron transport chain.

The light-induced reduction of cytochrome *b*-560 from the highly oxidized dark state is attributed to Photosystem 2. At high intensity ($3 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}) both red and far-red light cause a rapid reduction, but at lower intensity ($< 10^4$ ergs·cm^{-2}·sec^{-1}) the red light is much more effective in reducing cytochrome *b*-560 (Fig. 6).

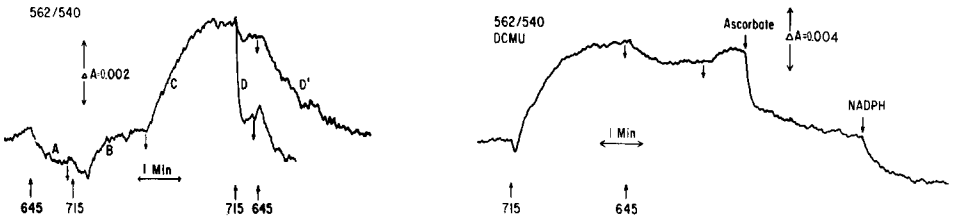


Fig. 6. Light-induced absorbance changes at 562 *vs.* 540 mμ. Initial irradiation with red light ($2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ). Subsequent illumination with far-red light ($3.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 715 mμ) and red light ($2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ). (D') Reduction in a different experiment by lower intensity far-red ($8.8 \cdot 10^3$ ergs·cm^{-2}·sec^{-1} at 715 mμ) and red light ($6.0 \cdot 10^3$ ergs·cm^{-2}·sec^{-1} at 645 mμ). Chlorophyll concentration 175 μg/ml.

Fig. 7. Light-induced absorbance changes at 562 *vs.* 540 mμ in the presence of 10 μM DCMU. Reduction by 1.5 mM ascorbate and 0.15 mM NADPH. Actinic light intensities: $3.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 715 mμ; $2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ. Chlorophyll concentration 200 μg/ml.

The reduced state of cytochrome *b*-560 during illumination with high-intensity far-red light in the absence of an electron acceptor is attributed to activity of Photosystem 2. Activation of Photosystem 2 with increasing intensity of far-red light was deduced previously from fluorescence-yield measurements[16]. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks the activity of Photosystem 2, causes cytochrome *b*-560 to go fully oxidized in far-red light (Fig. 7). (The transient reduction of cytochrome *b*-563 at the onset of irradiation is presumably less apparent because of the larger and faster cytochrome *b*-560 oxidation.) In a low intensity of far-red light, Photosystem 2 action is not sufficient to keep cytochrome *b*-560 fully reduced. At the steady state in $3 \cdot 10^3$ ergs·cm^{-2}·sec^{-1} of 715 mμ light, an appreciable part of the cytochrome *b*-560 is oxidized (Fig. 8).

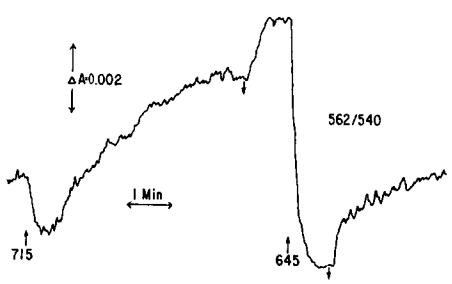


Fig. 8. Light-induced absorbance changes at 562 *vs.* 540 mμ with a lower intensity of far-red light. Actinic light intensities: $3.0 \cdot 10^3$ ergs·cm^{-2}·sec^{-1} at 715 mμ; $2.5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ. Chlorophyll concentration 225 μg/ml.

The observation that cytochrome *f* is fully oxidized while cytochrome *b*-560 is fully reduced at the steady state in high-intensity far-red light suggests that cytochrome *b*-560 is close to Photosystem 2 and that a relatively slow electron transfer step occurs between cytochrome *b*-560 and cytochrome *f*. This has been previously proposed by RUMBERG[8]. The slow step between cytochrome *b*-560 and cytochrome *f* might be associated with a pool of redox material. The dark oxidation of cytochrome *b*-560 following far-red irradiation suggests that a pool of oxidizing material accumulates during the far-red irradiation period.

In the presence of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at an uncoupling concentration of 2 to 10 μM, the cytochrome-*b* changes resemble those of cytochrome *f* (Fig. 9). These results suggest that CCCP bypasses the slow step between cytochrome *b*-560 and cytochrome *f*. The measurements are thus consistent with the slow step being associated with a site of phosphorylation. Other uncouplers, however, such as NH_4Cl, atebrin, and 2,4-dinitrophenol do not alter the light-induced absorbance changes shown in Fig. 2, indicating that they act at a different site or in a different manner than CCCP. The effect of CCCP on the absorbance changes is at least partially reversed by 1 mM cysteine (Fig. 10). The uncoupling effects of CCCP are also reversed by cysteine[17]. OLSON AND SMILLIE[6] previously demonstrated that CCCP caused the appearance of light-induced changes of a cytochrome *b*-560 in Euglena cells.

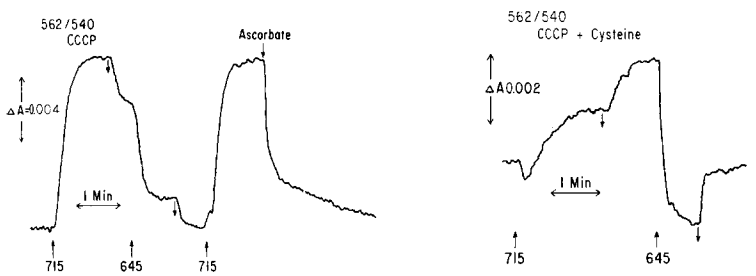


Fig. 9. Light-induced absorbance changes at 562 *vs.* 540 mμ in the presence of 10 μM CCCP. Reduction of oxidized cyt *b*-560 by 1.5 mM ascorbate. Actinic light intensities: $3.0 \cdot 10^4$ ergs·cm^{-2}sec^{-1} at 715 mμ; $7.1 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ. Chlorophyll concentration 200 μg/ml.

Fig. 10. Light-induced absorbance changes at 562 *vs.* 540 mμ in the presence of 10 μM CCCP and 1.5 mM cysteine. Actinic light intensities: $3.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 715 mμ; $7.1 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 645 mμ. Chlorophyll concentration 175 μg/ml.

The report that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone increases the proton conductance of mitochondrial membranes[18] suggests that the slow step in photosynthetic electron (or proton) transport between cytochrome *b*-560 and cytochrome *f* is due to a membrane impedance which is short-circuited by CCCP. It follows that Photosystem 2 and Photosystem 1 may be separated by a membrane associated with the phosphorylation process.

With either ferredoxin and $NADP^+$ or FMN as acceptor, the transient reduction of cytochrome *b*-563 is smaller and the net oxidation produced by the far-red light at 562 mμ is somewhat higher. There is no obvious effect on the changes at 562 mμ of added ferredoxin alone, probably because the chloroplasts themselves contain some ferredoxin, as well as flavoprotein reductase (see above in EXPERIMENTAL PROCEDURE). A more complete report on the effect of electron acceptors on the cytochrome *b* changes will be presented elsewhere.

It is concluded that the measurements at 562 mμ show two cytochrome-*b* components which act at different places in the photosynthetic electron transport system. Cytochrome *b*-560 is between Photosystem 2 and Photosystem 1 and is reducible by ascorbate. Cytochrome *b*-563 is reduced by Photosystem 1 and is reducible by NADPH, but not by ascorbate. The wavelength maxima of the α-bands and the reaction with ascorbate suggest that cytochrome *b*-560 and cytochrome *b*-563

are, respectively, the cytochrome b_3 and cytochrome b_6 which HILL AND SCARISBRICK[1] and HILL[2] found in green tissue.

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