BBA 46090

PHOTOOXIDATION OF CYTOCHROMES IN LEAVES AND CHLOROPLASTS AT LIQUID-NITROGEN TEMPERATURE

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(Received October 12th, 1970)

SUMMARY

1. Light *minus* dark difference spectra of leaves and chloroplasts at liquid-nitrogen temperatures were recorded in a Cary spectrophotometer. The spectra showed minima at 547 nm and 557 nm, but no band was observed at 552 nm, the position of the α-band of cytochrome *f* at low temperatures. Similar spectra were obtained with spinach and normal peas and with a pea mutant deficient in chlorophyll. It is concluded that cytochrome *b*-559, but not cytochrome *f* is photooxidized at liquid-nitrogen temperatures. Light of wavelength 650 nm was much more effective than 710-nm light, indicating that the oxidation of cytochrome *b*-559 at low temperatures is driven by light absorbed by Photosystem II.

2. The redox potential of the cytochrome which is photooxidized at low temperature was determined as 0.35 V at pH 7.2.

3. Chloroplasts were illuminated at room temperature, frozen rapidly to the temperature of liquid nitrogen, and light *minus* dark difference spectra were recorded. The spectrum of untreated chloroplasts showed only one small band at 547 nm. Chloroplasts treated with 3(3,4-dichlorophenyl)-1,1-dimethylurea gave sharp bands at 548 nm and 552 nm due to cytochrome *f*, but no band at 557 nm. 3(3,4-Dichlorophenyl)-1,1-dimethylurea inhibited the low temperature photooxidation of cytochrome *b*-559 provided the chloroplasts were illuminated at room temperature before freezing. Cytochrome *b*-559 was photooxidized to some extent at room temperature if the chloroplasts were treated with carbonyl cyanide *m*-chlorophenylhydrazone.

4. The results are difficult to reconcile with an electron transport scheme in which cytochrome *b*-559 is located on the main coupled pathway between the photoacts of Photosystems I and II. We suggest that cytochrome *b*-559 is on a side pathway connected to the reaction centre of Photosystem II.

INTRODUCTION

Light-induced decreases in absorbance of chloroplasts and green leaves in the cytochrome α-band region at low temperature were reported by WITT *et al.*[1], CHANCE

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

AND BONNER[2] and CHANCE *et al.*[3]. Light *minus* dark difference spectra[1-3] showed minima at 555 nm, and it was concluded that cytochrome *f* is photooxidized at liquid-nitrogen temperatures. The temperature independence of the light-induced change suggested that the oxidation of cytochrome *f* is a primary photochemical event, and it was concluded that cytochrome *f* is in close proximity to the reaction-centre chlorophyll of Photosystem I (*cf.* ref. 4).

The light *minus* dark difference spectra, however, did not coincide with an oxidized *minus* reduced difference spectrum of purified cytochrome *f* at 77°K (ref. 5). Cytochrome *f* showed minima at 552 nm and 548 nm in a ferricyanide oxidized *minus* ascorbate reduced difference spectrum. To explain the discrepancy, CHANCE AND BONNER[2] suggested that cytochrome *f* exists in a different state in the leaf, compared with the extracted pigment. BOARDMAN AND ANDERSON[6], however, observed bands at 548, 552 and 557 nm in an ascorbate reduced *minus* ferricyanide oxidized difference spectrum of chloroplasts at 77°K. They attributed the bands at 548 nm and 552 nm to cytochrome *f* and the band at 557 nm to cytochrome *b*-559.

Recently, KNAFF AND ARNON[7] reported that both cytochrome *f* and cytochrome *b*-559 were oxidized if spinach chloroplasts were illuminated at 84°K with 664 nm light, the light *minus* dark difference spectrum showing bands at 548, 552 and 556 nm. Light absorbed predominantly by Photosystem I (714 nm) was much less effective in photooxidizing cytochrome *b*-559 than 663-nm light, and it was concluded that the photooxidation of this cytochrome is driven by light absorbed by Photosystem II.

KNAFF AND ARNON[8] also observed an absorbance decrease at 548 nm on illumination of spinach chloroplasts at low temperatures in the presence of ferricyanide. Cytochrome *f* is chemically oxidized by ferricyanide, and KNAFF AND ARNON[8] attributed the absorbance decrease to the photoreduction of an unknown component, termed C-550. At room temperature, the absorbance decrease was maximal at 550 nm and it was reversible[8].

In the present work, we have recorded light *minus* dark difference spectra of leaves and chloroplasts at 77°K in a Cary spectrophotometer. We confirmed the finding of KNAFF AND ARNON[7] that cytochrome *b*-559 is photooxidized at low temperatures by light absorbed by Photosystem II, but we could not detect any photooxidation of cytochrome *f* at low temperature either in Photosystem I light or Photosystem II light. Conversely, cytochrome *f* but not cytochrome *b*-559 was photooxidized at room temperature in leaves or chloroplasts.

MATERIALS AND METHODS

Plant material

Leaves from spinach (*Spinacea oleracea L.*) and peas (*Pisum sativum*, var Greenfeast) and chloroplasts isolated from both plants were used in the present study. A mutant of pea[9], which contains little chlorophyll *b* and about half the amount of total chlorophyll of a normal pea, but the same amount of cytochromes was also used to advantage. On a chlorophyll basis, chloroplasts from the mutant plants gave larger light-induced absorbance changes than either spinach or normal pea chloroplasts.

The mutant pea was derived from the commercial variety as a spontaneous mutation, and it was distinguishable from normal plants by the pale-green colour

of its leaves[9]. Pea plants, both normal and mutant were grown in a controlled temperature glasshouse of the Commonwealth Scientific and Industrial Research Organization phytotron. Spinach plants were grown in nutrient solutions as described previously[10]. Chloroplasts were isolated from spinach or pea leaves in 0.05 M phosphate buffer (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl[10].

Determination of light minus dark difference spectra

Light *minus* dark difference spectra of leaves or chloroplasts at 77°K were recorded on a Cary Model 14R spectrophotometer, fitted with a scattered-transmission accessory. The cuvette assembly was as described previously[11]. Half pea leaves or small sections of spinach leaves were pressed gently between two Perspex windows mounted on the same side of the aluminium spacer of the cuvette assembly. Chloroplasts were suspended in a solution containing 62 parts by vol. of glycerol and 38 parts of 0.05 M phosphate buffer (pH 7.2). The optical path of chloroplast suspensions was 2 mm. The chlorophyll concentration was 0.2 mg/ml for the mutant pea chloroplasts and 0.45 mg/ml for spinach or normal pea chloroplasts. Leaves or chloroplasts were dark-adapted for several min before freezing to 77°K. In some experiments, chloroplasts or leaves were preilluminated at room temperature and frozen rapidly by plunging the cuvette assembly into liquid nitrogen.

Actinic light for illumination at 77°K was provided by a 650-W tungsten iodine lamp via the optical system of the Cary spectrophotometer. The lamp was operated at maximum voltage (110 V) and the slits of the spectrophotometer were fully open (3 mm). The reference cuvette was shaded from the actinic light by a piece of black cardboard. Following illumination, the slits were closed to normal operating width (*ca.* 0.1 mm), the cardboard removed and the light *minus* dark difference spectrum recorded.

Actinic light at room temperature was provided by a 650-W tungsten iodine lamp and interference filters (Balzers; half-band width, 10 nm). Corning filters 2-64 and 4-77 were used in conjunction with the interference filter at 714 nm. A 3-cm layer of water served as a heat absorbing filter. Light intensities for room temperature illuminations were measured with a YSI-Kettering Model 65 Radiometer.

3(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were donated by Dr. C. W. Todd and Dr. P. G. Heytler of Du Pont and Co.

Determination of oxidation–reduction potential

To determine the oxidation–reduction potential of the cytochrome which was photooxidized at 77°K, mutant pea chloroplasts were treated with various mixtures of potassium ferricyanide and potassium ferrocyanide at room temperature[12], and then dark-adapted before freezing to 77°K. The chloroplasts were suspended in a solution containing 62 parts by vol. of glycerol and 38 parts by vol. of 0.05 M phosphate buffer (pH 7.2). Potassium ferrocyanide was added at a final concentration of $8 \cdot 10^{-2}$ M, and appropriate concentrations of potassium ferricyanide were used to give the desired redox potentials. The sample cuvette was illuminated at 77°K in the Cary spectrophotometer with 650 nm light for 3 min, and the extent of the photooxidation of the cytochrome determined from the light *minus* dark difference spectrum. The redox potentials of various ferricyanide–ferrocyanide mixtures in the

presence of chloroplasts was checked by adding mammalian cytochrome *c* ($E'_0 = 0.255$) and recording difference spectra at room temperature against an ascorbate-reduced sample.

RESULTS

Light-induced absorbance changes at 77°K

Spinach and both normal and mutant pea leaves, after illumination for 3 min at 650 nm at 77°K showed similar light *minus* dark difference spectra at 77°K (Fig. 1). Minima were observed at 557 nm and 547 nm, but there was no evidence for a band at 552 nm, the position of the α-band of cytochrome *f* at 77°K. Illumination for longer periods did not increase the extent of the absorbance changes.

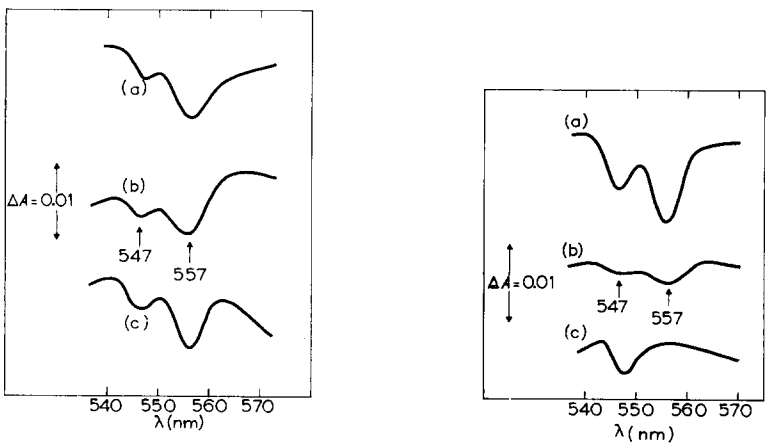


Fig. 1. Light *minus* dark difference spectra of leaves at liquid-nitrogen temperature. (a) Spinach; (b) normal pea; (c) mutant pea. Mutant pea leaves have an average chlorophyll content of 17.4 μg/cm² and normal pea leaves 45 μg/cm² (ref. 9). The leaves were illuminated for 3 min at 650 nm in a Cary spectrophotometer with 3-mm slits.

Fig. 2. Light *minus* dark difference spectra of mutant pea chloroplasts at liquid nitrogen temperature. The chlorophyll concentration was 0.31 mg/ml and the optical path length 2 mm. The chloroplasts were illuminated in the Cary spectrophotometer for 3 min at 650 nm (a and c) or 20 min at 710 nm (b). In (c) potassium ferricyanide was added at a final concentration of $1 \cdot 10^{-2}$ M to both sample and reference.

The light *minus* dark difference spectrum of chloroplasts from the mutant pea is shown in Fig. 2a. It differs from the spectrum of spinach chloroplasts reported by KNAFF AND ARNON[7] in not showing a band at 552 nm. The bands at 557 nm and 547 nm were observed by KNAFF AND ARNON[7] and attributed respectively to the photooxidation of cytochrome *b*-559 and cytochrome *f*. The difference spectra which we obtained with spinach and normal pea chloroplasts were similar to that obtained with the mutant pea chloroplasts, except that the extent of the absorbance changes were smaller. We conclude, therefore, that cytochrome *f* is not photooxidized by 650-nm light at 77°K either in leaves or isolated chloroplasts. The band observed at 547 nm in our experiments is apparently due to C-550 (*cf.* refs. 8 and 13).

Light of 710 nm wavelength, (Photosystem I) was much less effective in inducing

the absorbance changes than 650-nm light (Fig. 2b). Illumination for 40 min at 710 nm produced the same magnitude of absorbance change (64% of the maximum) as 30 sec at 650 nm, an 80-fold difference in effectiveness. The relative quanta absorbed at 710 nm compared with 650 nm were calculated from a low temperature absorption spectrum of mutant pea chloroplasts, corrected for light scattering and from the relative intensities of the incident beams at 650 nm and 710 nm. The absolute intensities of the beams from the Cary spectrophotometer at 650 nm and 710 nm were too low for accurate measurement by the light meter. Relative intensities were obtained in the following way. The slit widths of the Cary Spectrophotometer were observed at 650 nm and 710 nm in the absence of the sample and reference. The relative intensities were assumed to be proportional to the squares of the slit widths, and a correction was also made for the spectral response curve of the photomultiplier. The time required to photooxidize cytochrome *b*-559, even at 650 nm, is a reflection of the low intensity of the indicent light. The ratio of quanta absorbed at 650 nm compared to 710 nm was 8:1.

In agreement with the observations of KNAFF AND ARNON[7,8,13], ferricyanide abolished the band at 557 nm, but not that at 547 nm (Fig. 2c). Ascorbate at $1 \cdot 10^{-2}$ M had no effect on the light-induced spectrum. Since cytochrome b_6 is autoxidizable and is not reduced by ascorbate[14], we agree with the conclusion of KNAFF AND ARNON[7] that the *b*-type cytochrome which is photooxidized at liquid-nitrogen temperatures is cytochrome *b*-559. The magnitude of the absorbance decrease at 547 nm was not influenced either by ferricyanide or ascorbate. The band at 547 nm, therefore, cannot be attributed to cytochrome *f*, but it may be due to the photoreduction of a component, C-550 (*cf.* ref. 8).

Previously, we reported oxidized *minus* reduced difference spectra at 77°K for spinach chloroplasts and the subchloroplast fragments obtained by digitonin incubation[6]. At that time precautions were not taken to keep the samples in the dark at 77°K prior to recording the difference spectra. Fig. 3 compares ascorbate reduced *minus* ferricyanide oxidized difference spectra for spinach chloroplasts, frozen in the dark, and after illumination at 650 nm at 77°K. In this experiment, both reference and sample cuvettes were illuminated. It is apparent that the bands attributed to cytochrome *f* at 552 nm and 548 nm remain unchanged, but there is a decrease in the height of the band of cytochrome *b*-559 at 557 nm. The ascorbate reduced *minus* ferricyanide oxidized difference spectrum of spinach chloroplasts published previously[6] was intermediate between those reported in Fig. 3, the bands at 552 nm and 557 nm being of equal height. The spectra in Fig. 3 again demonstrate that cytochrome *f* is not photooxidized at liquid nitrogen temperature. It should be mentioned that the quantitative data for chloroplast cytochromes reported earlier[6] do not require correction since these were obtained from difference spectra determined at room temperature on dark-adapted chloroplasts.

Somewhat variable results were obtained on illumination of chloroplasts reduced with sodium dithionite. If the chloroplasts were frozen in the dark immediately after the addition of dithionite in the dark, then subsequent illumination at 650 nm at 77°K gave a decrease in absorbance at 557 nm (Fig. 4). On the other hand, if we followed our usual procedure[6] and allowed the chloroplasts to react with dithionite in the dark for 5 min prior to cooling, there was no decrease in absorbance at 557 nm on illumination.

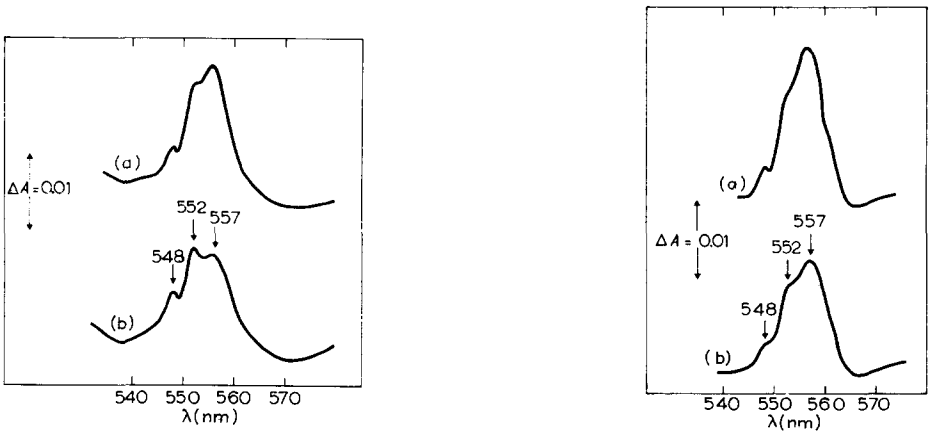


Fig. 3. Ascorbate reduced *minus* ferricyanide oxidized difference spectra of spinach chloroplasts at liquid-nitrogen temperature. The chlorophyll concentration was 0.46 mg/ml and the path length 2 mm. (a) Chloroplasts frozen in the dark; (b) after illumination of (a) in the Cary spectrophotometer at 650 nm for 3 min. Both reference and sample cuvettes were illuminated in this experiment.

Fig. 4. Dithionite reduced *minus* ferricyanide oxidized difference spectra of mutant pea chloroplasts at liquid-nitrogen temperature. The chlorophyll concentration was 0.32 mg/ml. The chloroplasts were frozen immediately after the addition of the dithionite. (a) Chloroplasts frozen in the dark; (b) after illumination of (a) in the Cary spectrophotometer at 650 nm for 3 min. Both reference and sample cuvettes were illuminated.

Illumination of chloroplasts at room temperature prior to freezing

When mutant pea chloroplasts were illuminated either at 703 nm or 650 nm and frozen rapidly to 77°K, the light *minus* dark difference spectra shown in Figs. 5a and 5c were obtained. A band is observed at 547 nm, but a band at 557 nm is barely detectable in Fig. 5a and no band is observed at 557 nm Fig. 5c. There is again no evidence for a band at 552 nm in either spectrum. It is well documented, however, that cytochrome *f* in isolated chloroplasts is oxidized at room temperature by Photosystem I light[15]. Apparently in our experiments the freezing was too slow to trap the cytochrome *f* in its oxidized state. Photooxidation of cytochrome *b*-559 was not observed in untreated chloroplasts, either in 650-nm or 703-nm light (*cf.* refs. 7 and 16–18). A slow reduction of cytochrome *b*-559 in 640-nm light has been reported by CRAMER AND BUTLER[16] and BEN HAYYIM AND AVRON[18], but in our experiments cytochrome *b*-559 was already mainly reduced in the isolated chloroplasts (*cf.* ref. 6).

Preillumination of chloroplasts at 703 nm at room temperature does not influence the subsequent photooxidation of cytochrome *b*-559 at low temperatures (Fig. 5b), but preillumination at 650 nm decreases slightly the extent of the change at 557 nm at 77°K (Fig. 5d). HIND[17] observed that cytochrome *b*-559 is photooxidized by 713 nm light at room temperature in chloroplasts treated with CCCP, an uncoupler of photophosphorylation. Fig. 5e shows the spectrum obtained when chloroplasts were illuminated at 703 nm at room temperature in the presence of $1 \cdot 10^{-5}$ M CCCP, and frozen rapidly to 77°K. A band is observed at 557 nm, but the extent of the absorbance change is only about one-third of the change in untreated chloroplasts illuminated at 77°K. Further illumination of the CCCP-treated chloroplasts at 77°K

with 650-nm light increased the extent of the absorbance decrease at 557 nm to the same level as in untreated chloroplasts (Fig. 5b).

If mutant pea leaves were soaked in $1 \cdot 10^{-4}$ M CCCP and illuminated at room temperature prior to freezing, the absorbance decrease at 557 nm was large, and only a slight increase was obtained on further illumination with 650-nm light at 77°K (Fig. 6).

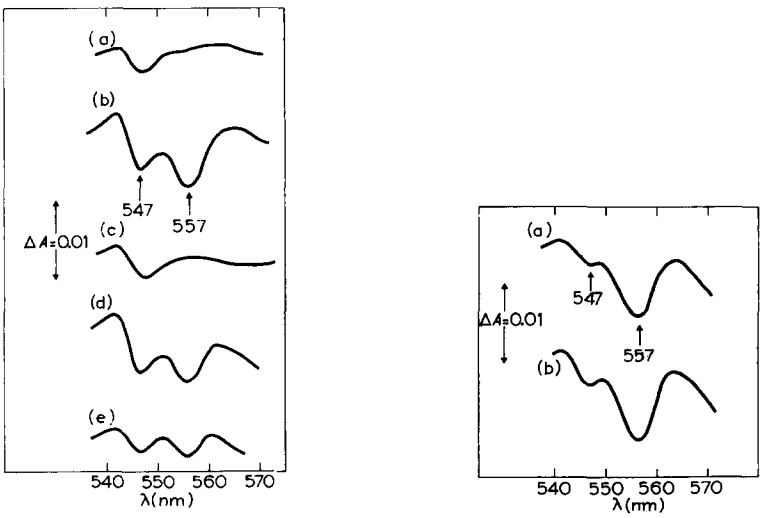
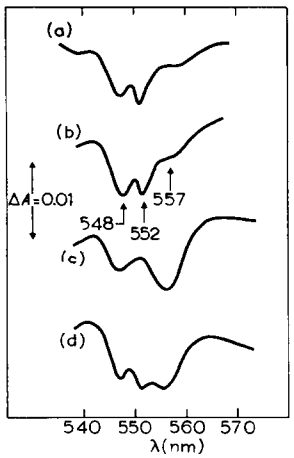


Fig. 5. Light *minus* dark difference spectra of mutant pea chloroplasts. The chlorophyll concentration was 0.33 mg/ml. (a) The chloroplasts were illuminated at room temperature for 60 sec at 703 nm (intensity, $6.5 \cdot 10^3$ erg·cm^{-2}·sec^{-1}) and frozen rapidly by plunging the cuvette holder into liquid nitrogen; (b) after illumination of (a) in the Cary spectrophotometer at 650 nm for 3 min at liquid nitrogen temperature; (c) chloroplasts illuminated at room temperature for 60 sec at 650 nm (intensity, $6.2 \cdot 10^3$ erg/sec) and frozen rapidly; (d) after illumination of (c) in the Cary spectrophotometer for 3 min at 650 nm at liquid nitrogen temperature; (e) chloroplasts were treated with $1 \cdot 10^{-5}$ M CCCP and illuminated at room temperature for 60 sec at 703 nm and frozen rapidly in liquid nitrogen. For all difference spectra in this figure, the reference cuvette remained in the dark continuously.

Fig. 6. Light *minus* dark difference spectra of mutant pea leaves. The surface of the leaves was damaged slightly by gentle pricking with a needle and the leaves soaked in $1 \cdot 10^{-4}$ M CCCP for 15 min. (a) A half leaf was illuminated at room temperature at 675 nm ($6.6 \cdot 10^3$ erg·cm^{-2}·sec^{-1}) for 30 sec and frozen rapidly; (b) after further illumination of (a) in the Cary spectrophotometer for 3 min at 650 nm. The reference leaf remained in the dark continuously.

The difference spectra shown in Fig. 7 demonstrate that cytochrome *f* is clearly resolvable from cytochrome *b*-559 at low temperature. Mutant pea chloroplasts were illuminated with 703 nm light at room temperature in the presence of $1 \cdot 10^{-5}$ M DCMU and frozen rapidly. A distinct band is observed at 552 nm (cytochrome *f*) in addition to a band at 547 nm (cytochrome *f* and C-550) (Fig. 7a). DCMU serves to inhibit the reduction of photooxidized cytochrome *f* in the interval between illumination at 703 nm and freezing to 77°K. Fig. 7b shows that cytochrome *b*-559 is not photooxidized on additional illumination at 650 nm at 77°K. However, DCMU does not inhibit the photooxidation of cytochrome *b*-559 at liquid-nitrogen temperatures provided the chloroplasts are kept in the dark prior to freezing (Fig. 7c).

Fig. 7d shows the light *minus* dark difference spectrum of mutant pea chloroplasts which had been illuminated at 714 nm at room temperature in the presence of $1 \cdot 10^{-5}$ M DCMU and $1 \cdot 10^{-5}$ M CCCP, and frozen rapidly. There is some photooxidation both of cytochrome *f* and cytochrome *b*-559. The band due to cytochrome *f* at 552 nm is clearly resolved from the band at 557 nm, due to cytochrome *b*-559. This experiment demonstrates that cytochrome *f* can be resolved from cytochrome *b*-559 at low temperature. There was no change in the difference spectrum on further illumination at 650 nm at 77°K.



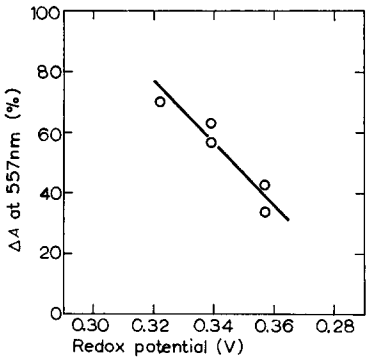


Fig. 7. Light *minus* dark difference spectra of mutant pea chloroplasts. Chlorophyll concentration, 0.33 mg/ml. (a) The chloroplasts were illuminated in the presence of $1 \cdot 10^{-5}$ M DCMU for 30 sec with 703 nm light ($3.5 \cdot 10^3$ erg·cm^{-2}·sec^{-1}) at room temperature, and frozen rapidly; (b) after a further illumination of (a) in the Cary spectrophotometer at liquid-nitrogen temperature for 3 min at 650 nm; (c) chloroplasts were treated with $1 \cdot 10^5$ M DCMU in the dark and frozen to liquid-nitrogen temperature. Illumination was in the Cary spectrophotometer at liquid-nitrogen temperature for 3 min at 650 nm; (d) chloroplasts were illuminated at room temperature in the presence of $1 \cdot 10^{-5}$ M DCMU and $1 \cdot 10^{-5}$ M CCCP for 2 min at 714 nm ($6.2 \cdot 10^3$ ergs·cm^{-2}·sec^{-1}), and frozen rapidly, There was no change in the spectrum on further illumination at 650 nm at liquid-nitrogen temperature. For all difference spectra the reference cuvette remained in the dark continuously.

Fig. 8. Relative absorbance decrease of mutant pea chloroplasts at 557 nm at liquid nitrogen temperature as a function of the redox potential of the mixture. The chloroplasts were treated with various mixtures of potassium ferrocyanide and potassium ferricyanide as indicated in MATERIALS AND METHODS. The chlorophyll concentration was 0.16 mg/ml and the optical path-length was 4 mm. The absorbance decrease of chloroplasts in $1 \cdot 10^{-2}$ M sodium ascorbate was taken as 100%.

Redox potential of cytochrome b-559

A plot of relative absorbance change at 557 nm at 77°K against the redox potential of mutant pea chloroplasts is shown in Fig. 8. A midpoint potential (E'_0) of 0.35 ± 0.015 V is obtained for the cytochrome which is photooxidized at low temperature. The slope of the line corresponds to a one-electron change.

DISCUSSION

The earlier studies on light-induced absorbance changes in green leaves and chloroplasts at liquid-nitrogen temperatures[1-3] in the cytochrome α-band region were

interpreted as a photooxidation of cytochrome *f*. The present work indicates that cytochrome *b*-559 and not cytochrome *f* is photooxidized at low temperature. We confirmed our earlier finding[6] with isolated chloroplasts that the absorbance bands of cytochrome *f* at 77°K (552 nm and 548 nm) are clearly resolvable from the low temperature band of cytochrome *b*-559 at 557 nm. The light *minus* dark difference spectrum reported by KNAFF AND ARNON[7] indicated that both cytochome *b*-559 and cytochrome *f* in spinach chloroplasts were photooxidized at low temperature. Their difference spectrum was plotted from measurements made at individual wavelengths in a dual-wavelength spectrophotometer. The half-band width of the measuring beams were not reported by KNAFF AND ARNON[7], but in our experiments the slit widths of the Cary spectrophotometer were < 0.1 mm (spectral bandwidth < 0.35 nm) over the cytochrome α-band region.

We confirmed the finding of KNAFF AND ARNON[7,13] that the oxidation of cytochrome *b*-559 at 77°K is driven by light absorbed by Photosystem II. CHANCE *et al.*[3] also showed a Photosystem II action spectrum for the light-induced absorbance decrease at 556 nm in chloroplasts, but they did not comment on their observation.

The redox potential which we have determined for the cytochrome which is photooxidized at low temperature agrees reasonably well with the earlier determinations by BENDALL[19] for cytochrome *b*-559 of pea chloroplasts ($E'_0 = 0.37$ V at pH 6.5 and pH 7.5), and by IKEGAMI *et al.*[20] for cytochrome *b*-559 of Euglena chloroplasts ($E'_0 = 0.32$ V at pH 6.5). KNAFF AND ARNON[13] metioned that they had obtained a redox potential of 0.33 V at pH 8.2 for cytochrome *b*-559 of spinach chloroplasts. On the other hand, FAN AND CRAMER[21] reported a potential of 0.080 V (pH 7.0) for cytochrome *b*-559 in spinach chloroplasts, and HIND[22] found 0.055 V (pH 7.0) for cytochrome *b*-559 in the Photosystem II subchloroplast fragments derived from spinach chloroplasts by incubation with Triton X-100.

From observations of the kinetics of cytochrome reduction in chloroplasts by dithionite, BENDALL[19] suggested that chloroplasts may contain 2 cytochromes with α-peaks at 559 nm, one having a redox potential of $+ 0.37$ V and the other with a redox potential in the vicinity of zero. Our present studies argue against the existence of two pools of chloroplast cytochrome *b*-559, at least in the leaf. The extent of the absorbance decrease at 557 nm at 77°K in a leaf accounts for most, if not all, of the chloroplast cytochrome *b*-559 in the leaf. With isolated chloroplasts, the extent of the absorbance change usually accounts for about 50% of the cytochrome *b*-559, although in one experiment we accounted for 90%.

It is possible that the binding of a ligand to the haem prosthetic group of cytochrome *b*-559 is readily modified in isolated chloroplasts, resulting in a shift in the redox potential to a more negative value. This may account for the variation in the reported values of the redox potential of cytochrome *b*-559. HIND[22] employed fragments from detergent-treated chloroplasts, and FAN AND CRAMER[21] used a bubbling technique to achieve an anaerobic condition. Our earlier studies[6] showed that cytochrome *b*-559 was no longer reducible with ascorbate after acetone extraction of lyophilized chloroplasts at $-20°$.

The present work does not support an electron transport scheme[4,23] in which cytochome *b*-559 is located on the main coupled pathway between the photoacts of System I and System II. We observed neither photoreduction nor photooxidation of cytochrome *b*-559 at room temperature in untreated spinach chloroplasts or in

freshly prepared mutant pea chloroplasts. Aged mutant chloroplasts showed some photoreduction of cytochrome *b*-559 with photosystem II light at room temperature (HILLER *et al*, in preparation) which agrees with the observations of CRAMER AND BUTLER[16] with spinach chloroplasts and BEN HAYYIM AND AVRON[18] with lettuce chloroplasts. GORMAN AND LEVINE[14] observed photoreduction of cytochrome *b*-559 by 650 nm light in chloroplast fragments from *Chlamydomonas reinhardi*.

To explain the present results, and further data on light-induced absorbance changes of chloroplasts at room teperature (HILLER *et al*, in preparation), we suggest that cytochrome *b*-559 is not a component of the main coupled electron transport pathway, but it is on a side pathway connected to the reaction centre of Photosystem II (Fig. 9). Excitation of the reaction centre chlorophyll of Photosystem II (chl a_{II}) at low temperature catalyses the flow of an electron from cytochrome *b*-559 to E, the primary acceptor of electrons from Photosystem II. If the chloroplasts are pre-illuminated at room temperature in the presence of DCMU prior to cooling to 77°K, E apparently remains in the reduced state and cytochrome *b*-559 is not oxidized by illumination at 77°K. It is known from fluorescence studies[25] that E is not reduced by ascorbate, and its reduction by dithionite may be slow.

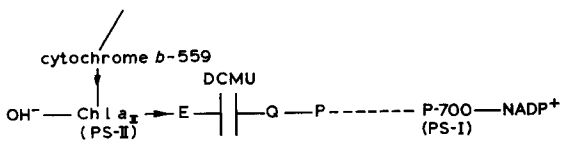


Fig. 9. Scheme showing possible location of cytochrome *b*-559 in relation to photosynthetic electron transport. DCMU acts to inhibit electron flow between E and Q. E, Q and P are hypothetical electron carriers in Photosystem II (*cf.* refs. 26, 27). PS, photosystem.

KNAFF AND ARNON[8,13] have suggested that the light-induced absorbance change at 547 nm at liquid nitrogen temperatures in ferricyanide-treated chloroplasts is due to the photoreduction of C-550 by Photosystem II. Our studies have shown that the absorbance change at 547 nm at 77°K is also observed with chloroplasts treated with sodium ascorbate. It is possible, therefore, that C-550 is identical with E.

Inhibition of electron flow from water to Photosystem II at low temperature may explain why the photooxidation of cytochrome *b*-559 is observed at low temperature and not at room temperature. KNAFF AND ARNON[7,13] found that cytochrome *b*-559 was photooxidized at room temperature if electron flow from water was inhibited by washing in Tris-buffer. An alternative explanation is that the quantum efficiency for the photooxidation of cytochrome *b*-559 is very low at room temperature. Perhaps there is a change in the structure of the chloroplast thylakoids on freezing, which brings cytochrome *b*-559 and chl a_{II} closer together.

The lack of photooxidation of cytochrome *f* at low temperature removes the need to place cytochrome *f* in close association with P-700. There is some evidence from studies with mutants of *Chlamydomonas*[23] and with detergent-treated spinach chloroplasts[15] for plastocyanin as the immediate electron donor to Photosystem I.

ACKNOWLEDGEMENTS

We wish to thank Mrs. S. Sapiets and Miss S. Muirhead for skilled technical assistance.

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