BBA 45608

THE SITE OF ACTION OF PLASTOCYANIN IN CHLOROPLASTS TREATED WITH DETERGENT

GEOFFREY HIND

Biology Department, Brookhaven National Laboratory, Upton, New York (U.S.A.)

(Received July 11th, 1967)

SUMMARY

1. Triton concentrations of about 0.01 % uncouple photosynthetic processes and speed the light-induced reduction of cytochrome b_6 and oxidation of cytochrome *b*-559. Above 0.05 % Triton, electron flow rates rise to a maximum and a slow, almost complete, oxidation of cytochrome *b*-559 can be observed.

2. Chloroplasts treated with 0.1 % Triton are thereafter dependent on Triton for photochemical activity.

3. Kinetic evidence supports the following sequence of electron carriers in relation to Photosystem I:

cytochrome *b*-559–?–cytochrome *f*–plastocyanin–P700–Photosystem I–?–cytochrome b_6.

INTRODUCTION

The soluble copper protein plastocyanin is known to be necessary for the photochemical reduction of $NADP^+$ by sonicated chloroplast fragments[1,2] or by a Photosystem-I particle obtained upon resolution of chloroplasts with detergent[3]. Since the midpoint potential of plastocyanin is +0.37 V at pH 7.0 (see ref. 4), lying close to the value for cytochrome *f*, 0.365 V (see ref. 5), it is considered likely that these two heavy-metal catalysts interact. Evidence for their functioning in alternative parallel routes to the oxidizing end of Photosystem I has been presented by KOK, RURAINSKI AND HARMON[6]. However, FORK AND URBACH[7] and AVRON AND CHANCE[8], citing experiments using the inhibitor salicylaldoxime, propose that plastocyanin reduces cytochrome *f*. A third possibility, that plastocyanin mediates electron flow between cytochrome *f* and P700, is supported by data of GORMAN AND LEVINE[9] from a Chlamydomonas mutant lacking plastocyanin and data of WESSELS[10] using digitonin-resolved chloroplast particles.

This paper reports experiments in which the sensitive technique of double-beam spectrophotometry employed by other workers[8,9] is combined with detergent treatment of chloroplasts to render them deficient in plastocyanin. Restoration of purified plastocyanin should then allow determination of its specific interactions with other chloroplast components.

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves by grinding in (mM): NaCl 300, Tris–HCl 20, $MgCl_2$ 4, sodium ascorbate 2 (pH 7.4). After filtration and sedimentation the chloroplasts were washed once in (mM): sucrose 200, Tricine 30, $MgCl_2$ 3, sodium ascorbate 2 (pH 7.4). They were then suspended for 10 min at 0° in (mM): NaCl 20, Tricine 20, $MgCl_2$ 2, sodium ascorbate 2 (pH 7.0) to which was added 1.0 mg Triton X-100 per mg chlorophyll. After sedimentation by spinning at 30000 × *g* for 7 min, the green supernatant containing some liberated Photosystem-I particles[11] was discarded and the pellet resuspended in the same medium *minus* Triton and ascorbate.

Crude spinach ferredoxin was prepared by acetone fractionation as described by SAN PIETRO AND LANG[12]. Purified ferredoxin was obtained by 2 subsequent passages through DEAE-cellulose[13]. Plastocyanin was prepared by the procedure of KATOH, SHIRATORI AND TAKAMIYA[4], but starting with isolated chloroplasts and omitting the third chromatographic separation.

Difference spectra were obtained using a Chance-type double-beam spectrophotometer. The cell compartment was cooled to +2°. Actinic light was passed through a 705-nm interference filter, half bandwidth 12 nm, and a Corning CS 2-64 blocking filter, giving an incident energy of $2 \cdot 10^4$ erg·cm^{-2}·sec^{-1} (below satn.). The composition of reaction mixtures was adjusted to maximize the absorbance change due to a given chloroplast component, and minimize overlapping absorbance shifts of other components. For this reason several different reaction mixtures were used, though variations were kept to the necessary minimum. All on- and off-responses ascribed to a specific pigment were verified by plotting a difference spectrum[16]. The faster absorbance changes were also recorded on an oscillographic recorder to obtain the quoted response rates.

RESULTS

The effect of Triton was first studied using chloroplasts which had not been subjected to previous incubation with the detergent. For this purpose a chloroplast sample was withdrawn from the final suspending medium (NaCl–Tricine–$MgCl_2$–ascorbate) prior to the Triton addition. Light-induced absorbance changes at the cytochrome b_6 and *b*-559 α-band maxima were monitored[11], as well as the $NADP^+$ reduction rate.

Fig. 1 shows that the initial $NADP^+$ reduction rate was more than doubled by 0.01 % Triton, while above this concentration a strong inhibition set in. Beyond 0.04 % the rate increased gradually again to a new maximum. This behavior was noted earlier by VERNON AND SHAW[14]. Concomitant with the enhancement of reduction rate by 0.01 % Triton is an increase in the illuminated steady state extent and initial rate of the cytochrome b_6 absorbance change[15]. The peak enhancement is observed at a higher Triton concentration than that found for $NADP^+$ reduction presumably because a higher chlorophyll concentration is employed for observation of cytochrome responses. There is no secondary rise in the extent of the cytochrome b_6 response.

Cytochrome *b*-559 is known to be reduced by ascorbate and oxidized by Photosystem-I light in uncoupled chloroplasts[16]. Increasing Triton concentrations promote

an initial small rise and fall followed by a gradual rise in the extent and on-response rate of the cytochrome *b*-559 absorbance change (Fig. 1).

Treatment of chloroplasts with Triton as described in MATERIALS AND METHODS led to a depletion of plastocyanin and ferredoxin, and loss of the ability to evolve oxygen. With such a low detergent concentration, significant resolution of the two photochemical pigment systems did not occur. If plastocyanin and purified ferredoxin were added, the chloroplast fragments were capable of photoreducing $NADP^+$ in the presence of ascorbate and 2,6-dichlorophenolindophenol (DCIP) with rates of about 250 μmoles/mg chlorophyll/h in saturating red light. The control rate of $NADP^+$ reduction in the absence of plastocyanin and ferredoxin was between 10 and 20% of this value.

When Triton-treated chloroplasts are washed free of Triton and placed in a Triton-free reaction mixture, no photochemical activity is detectable (Fig. 2). Upon addition of Triton, activity is rapidly restored in proportion to the amount of added detergent. The Triton concentration dependence of $NADP^+$ photoreducing activity shown in Fig. 2 is similar to the secondary rise of $NADP^+$ reduction rate shown in Fig. 1. So also is the Triton requirement of the cytochrome *b*-559 oxidation rate. Since removal of Triton from treated chloroplasts leads to clumping of particles, it seems probable that the high-Triton effect is due to some gross physical change in lamellar structure caused by the detergent, resulting in exposure of photochemically active components such as P700.

Fig. 3 (top) demonstrates that plastocyanin accelerates the rate of cytochrome *b*-559 oxidation in Triton-treated chloroplasts, with methyl viologen as the electron

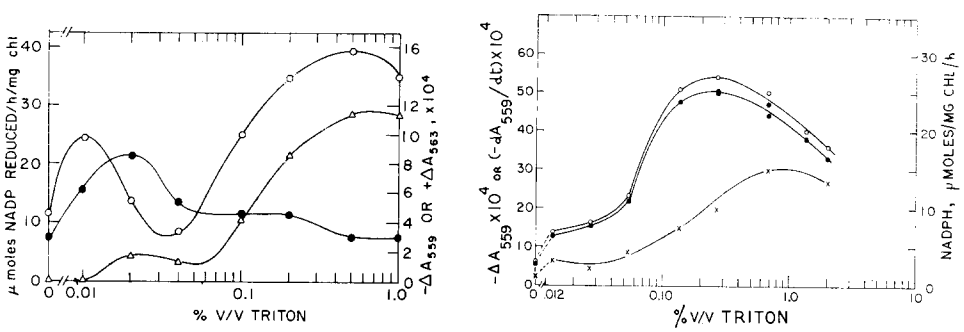

Fig. 1. The effect of increasing Triton X-100 concentration on $NADP^+$ reduction and cytochrome responses in illuminated untreated chloroplasts. $NADP^+$ reduction rate, O—O; cytochrome b_6 on-response (reduction), ●—●; cytochrome *b*-559 on-response (oxidation), △—△. The relative extents of the cytochrome b_6 and *b*-559 absorbance changes were also indicative of the relative initial on-response rates for the same cytochrome. The half-response times were for cytochrome b_6, 0.25 sec; for cytochrome *b*-559, 8 sec, at 0.02% Triton. The reaction mixture contained in 1.5 ml, 1.1 ml of chloroplast suspension (360 μg chlorophyll), sodium ascorbate 0.5 μmole, DCIP 0.35 μmole, $NADP^+$ 1.0 μmole, crude ferredoxin 0.24 mg. For $NADP^+$ reduction assays only 120 μg chlorophyll were present. NADPH was determined at 380 nm using $\varepsilon = 1.4\ mM^{-1} \cdot cm^{-1}$.

Fig. 2. The dependence of $NADP^+$ reduction and cytochrome *b*-559 oxidation on added Triton, in chloroplasts pretreated with Triton. $NADP^+$ reduction rate, O—O; cytochrome *b*-559 oxidation extent, ×—×, and initial rate per min, ●—●. Chloroplasts were washed twice after incubation with Triton (see MATERIALS AND METHODS). The reaction mixtures, chlorophyll concentrations and NADPH assay were as described for Fig. 1.

acceptor. The slow back-reduction by ascorbate has a half-time of about 12 sec at pH 7.0. Plastocyanin has a similar effect when ferredoxin and $NADP^+$ replace viologen as the acceptor (Fig. 3, bottom, right). The initial cytochrome oxidation rate corresponds with transfer of 2 electron pair μequiv per mg chlorophyll per h. The biphasic reduction of cytochrome *b*-559 is not understood. In the same suspension the observed $NADP^+$ reduction rate was initially 40 and then after 10 sec or so, 20 μmoles reduced per mg chlorophyll per h (Fig. 3, bottom, left). It follows that cytochrome

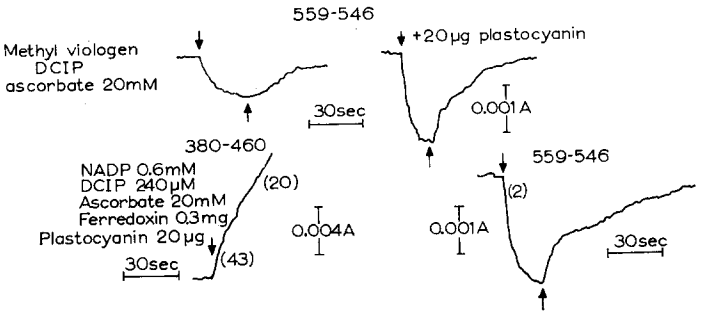

Fig. 3. The effect of plastocyanin on cytochrome *b*-559 kinetics. In addition to the components listed, the reaction mixtures contained in 1.5 ml, 1.0 ml of Triton-treated chloroplast suspension (400 μg chlorophyll) and 1.3% Triton X-100. Downward arrow: actinic light on. Upward arrow: actinic light off. Downward trace deflection: decrease in absorbance. Numbers in parentheses are equivalent μmoles NADPH per mg chlorophyll per h. '559–546' denotes $\Delta A_{559\text{ nm}}-\Delta A_{546\text{ nm}}$, and so on.

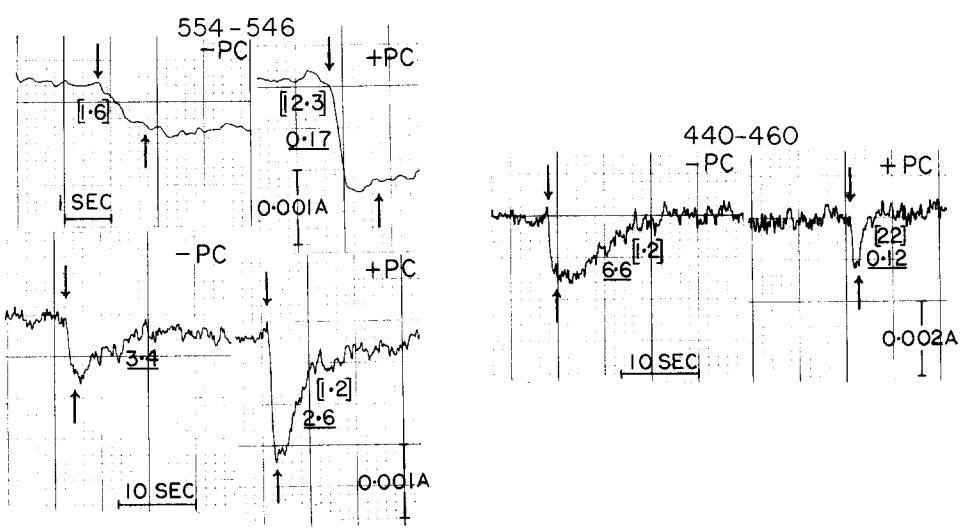

Fig. 4. The effect of plastocyanin (PC) on P700 and cytochrome *f* responses. The reaction mixtures (1.5 ml) contained methyl viologen 125 μM, ascorbate 3 mM, chlorophyll 400 μg (top and bottom curve) or 200 μg (right), 20 μg plastocyanin where indicated and 1.3% Triton X-100. Downward and upward arrows mark the beginning and end of a 1.0-sec flash of actinic light. Brief illumination minimized interference from slow cytochrome *b*-559 changes. Downward trace deflections signify a decrease in absorbance. Figures in parentheses are equivalent μmoles NADPH per mg chlorophyll per h. Underlined figures are half-response times (sec).

b-559 plays no significant role in the mediation of electron transfer between ascorbate and $NADP^+$.

In contrast, the cytochrome *f* oxidation rate is enhanced 7- or 8-fold by plastocyanin, giving a value of 12.3 equivalent μmoles $NADP^+$ per mg chlorophyll per h (Fig. 4, top) and the absorbance change induced by a 1-sec flash is correspondingly larger. Both with and without plastocyanin, ascorbate fully reduced the cytochrome *f* as determined by the absorbance decrease at 554 nm upon addition of an excess of potassium ferricyanide.

Fig. 4 (bottom) shows on a longer time scale the reduction of cytochrome *f* by ascorbate following an illumination period. Plastocyanin accelerates the reduction slightly, from a half-time of 3.4 to 2.6 sec, but the maximum initial reduction rate is only 1.2 equivalent μmoles $NADP^+$ per mg chlorophyll per h.

The effect of plastocyanin on P700 reduction was studied at 440 nm (Fig. 4, right). This is 4 nm higher than the wavelength maximum of the P700 Soret band, but is chosen because interference from cytochrome changes is here minimal[15]. The P700 oxidation rates, being faster than the machine response time, are not calculated. The reduction rate was found to be increased from 1.2 to about 22 equivalent μmoles $NADP^+$ per mg chlorophyll per h. Among the pigment systems investigated this is the greatest observed enhancement due to restoration of plastocyanin. The ΔA at 440 nm per 1-sec flash was consistently less in the presence of plastocyanin.

In the restored system, the cytochrome *f* oxidation rate is about half the P700 reduction rate, suggesting that these pigments and plastocyanin interact with reasonable efficiency. However, the observed slow rate of cytochrome *f* reduction indicates that this pigment cannot participate significantly in continuous electron flow between ascorbate and viologen.

DISCUSSION

The complex response of untreated chloroplasts to Triton X-100 (Fig. 1) has been interpreted by VERNON AND SHAW[14] as follows. The stimulation of electron transfer by low concentrations of detergent is due to an uncoupling of rate-limiting steps concerned with photophosphorylation[17]. The inhibitory phase at slightly higher concentration is associated with the loss of soluble chloroplast components as evidenced by the ability of added plastocyanin to enhance electron flow rates in this range. The increase in the $NADP^+$ reduction rate at high Triton levels is ascribed to chloroplast fragmentation and exposure of P700 to artificial electron donors.

The increase of the cytochrome b_6 reduction rate and extent concomitant with the low-Triton uncoupling effect supports the view that this cytochrome is reduced by Photosystem I rather than by Photosystem II. The rise in the cytochrome *b*-559 oxidation rate at this same concentration suggests that this pigment may be on the reducing side of the phosphorylation site. Since the oxidation rate of cytochrome *b*-559 at high Triton levels (Fig. 3) is too slow for this cytochrome to play a significant role in ascorbate photooxidation and since its reduction rate is even slower, the interaction of cytochrome *b*-559 with Photosystem I may be entirely unphysiological under these experimental conditions, depending on chance contact of resolved Photosystem I and II fragments[11].

Cytochrome *f* clearly contributes little to the overall flow of electrons to P700

on account of its slow reduction by ascorbate, yet its oxidation rate is markedly increased by addition of plastocyanin (Fig. 4). In untreated spinach chloroplasts the cytochrome *f* oxidation half-time is about 1.3 msec (see ref. 18), while in Fig. 4 (top, *plus* plastocyanin) it is 170 msec. This rather long half-time might be decreased 5-fold by increasing the light intensity to saturation but the remaining difference must be ascribed to structural derangements resulting from detergent treatment.

The great effect of plastocyanin on P700 reduction rates (Fig. 4) supports earlier claims[19] that these pigments form a photochemically active complex. Our data lend no support to the hypothesis, based on experiments with added cytochrome *f*, that plastocyanin and cytochrome *f* are both directly oxidized by P700 (see ref. 6). Nor is there any evidence that plastocyanin may mediate to a significant degree in cytochrome *f* reduction[7,8].

The major source of electrons during P700 turnover in Triton-treated chloroplasts is presumably plastocyanin, which is directly reduced by ascorbate[4]. The sequence of electron carriers relative to Photosystem I is deduced to be:

cytochrome *b*-559–?–cytochrome *f*–plastocyanin–P700–Photosystem I–?–cytochrome b_6.

ACKNOWLEDGEMENTS

The author thanks Dr. J. M. OLSON for many helpful comments, and HERBERT Y. NAKATANI for technical assistance. This investigation was carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

REFERENCES

1 S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 99 (1965) 156.
2 S. KATOH AND A. SAN PIETRO, *J. Biol. Chem.*, 241 (1966) 3575.
3 L. P. VERNON, E. R. SHAW AND B. KE, *J. Biol. Chem.*, 241 (1966) 4101.
4 S. KATOH, I. SHIRATORI AND A. TAKAMIYA, *J. Biochem. Tokyo*, 51 (1962) 32.
5 H. E. DAVENPORT AND R. HILL, *Proc. Roy. Soc. London, Ser. B*, 139 (1952) 327.
6 B. KOK, H. J. RURAINSKI AND E. A. HARMON, *Plant. Physiol.*, 39 (1964) 513.
7 D. C. FORK AND W. URBACH, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1307.
8 M. AVRON AND B. CHANCE, *Brookhaven Symp. Biol.*, 19 (1966) 149.
9 D. S. GORMAN AND R. P. LEVINE, *Plant Physiol.*, 41 (1966) 1648.
10 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 126 (1966) 581.
11 G. HIND AND H. Y. NAKATANI, in preparation.
12 A. SAN PIETRO AND H. M. LANG, *J. Biol. Chem.*, 231 (1958) 211.
13 K. TAGAWA AND D. I. ARNON, *Nature*, 195 (1962) 537.
14 L. P. VERNON AND E. SHAW, *Plant Physiol.*, 40 (1965) 1269.
15 G. HIND AND J. M. OLSON, *Brookhaven Symp. Biol.*, 19 (1966) 188.
16 G. HIND, *Photochem. Photobiol.*, submitted.
17 J. NEUMANN AND A. T. JAGENDORF, *Biochim. Biophys. Acta*, 109 (1965) 382.
18 B. CHANCE, D. DEVAULT, W. W. HILDRETH, W. W. PARSON AND M. NISHIMURA, *Brookhaven Symp. Biol.*, 19 (1966) 115.
19 B. KOK AND H. J. RURAINSKI, *Biochim. Biophys. Acta*, 94 (1965) 588.