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LIGHT-INDUCED OXIDATION OF CYTOCHROME *f* IN ISOLATED CHLOROPLASTS OF *PISUM SATIVUM*

A. W. D. LARKUM* AND W. D. BONNER

Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)

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SUMMARY

Photooxidation of cytochrome *f* has been studied in a number of types of chloroplast preparations from pea leaves. On the basis of the dark reversibility after far-red illumination, the effect of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea and other criteria, it is possible to distinguish three basic types of response according to the type of preparation: (a) high-salt chloroplasts; (b) intact chloroplasts and unswollen chloroplast fragments, and (c) swollen chloroplasts and swollen chloroplast fragments. Types a and b were further characterised by relatively high oxidation of cytochrome *f* in red light.

It is suggested that in type b preparations photooxidation of cytochrome *f* can be explained by (i) a large component of electron flow through an intermediate pathway between the two photosystems in which a large redox pool is linked through a coupling site to cytochrome *f*, and (ii) a small component of endogenous cyclic electron flow involving cytochrome *f*. In high-salt chloroplasts there may be in addition a pool of low molecular weight substance capable of reducing cytochrome *f* in the dark.

INTRODUCTION

It is now well established that cytochrome *f* is oxidised in green tissues in the light[1]. The ability of far-red light to induce oxidation of cytochrome *f* in algae and higher plant chloroplasts[2,3] placed this cytochrome close to photosystem I. Furthermore, the occurrence of photooxidation at 77°K (ref. 4), indicated that cytochrome *f* interacted directly with the primary acceptor of Photosystem I, P700. Such a position became somewhat doubtful with the evidence favouring a position of plastocyanin between cytochrome *f* and P700 (refs. 5–8), and, with the more recent evidence that the photooxidation at 77°K can be attributed to cytochrome *b*-559 (refs. 9 and 10), the position and role of cytochrome *f* have again become open to question.

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N*-ethane sulphonic acid; MES, 2-(*N*-morpholino)ethane sulphonic acid; MOPS, morpholinopropane sulphonic acid.

* Present address: Plant Physiology Unit, School of Biological Sciences, University of Sydney, N.S.W. 2006, Australia.

The light-induced oxidation of cytochrome *f* in swollen Class II chloroplasts has been studied in some detail by AVRON AND CHANCE[3]. It was concluded that (a) electron transport was limited in the basal system by the turnover of Photosystem I resulting in slow oxidation of cytochrome *f* in far-red light, and (b) cytochrome *f* was connected to Photosystem II through a pool of intermediates and a coupling site with the result that there was little oxidation of cytochrome *f* in red light. No redox changes attributable to cytochrome *b* were observed and no evidence was found for a cyclic electron transport system around Photosystem I. However, other workers have obtained evidence on both points[7,8,11–12].

In the present work an attempt has been made to compare the cytochrome *f* photo-oxidation in a wide variety of chloroplast preparations. It has been confirmed that the type of cytochrome *f* and cytochrome *b* responses found depends very much on the type of chloroplast preparation. In this paper we report only on the cytochrome *f* responses and attempt to relate these to a working hypothesis for electron transport in these various types of chloroplasts. A part of this work was reported briefly elsewhere[13].

MATERIALS AND METHODS

Chloroplasts were isolated from pea leaves (*Pisum sativum* var. Alaska) from plants grown in growth chambers, or from spinach (*Spinaceae oleracea* var. Virofly) collected from a market garden in New Jersey. The peas were grown under low light intensity (100 ft candles; tungsten lamps) at a temperature of 20° in damp vermiculite.

The isolation procedure was similar to that of HARVEY AND BROWN[14] using media similar to those of WALKER *et al.*[15]. About 100 g of chilled leaves were placed in a Braun Blender together with 200 ml of 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 1 mM ascorbic acid, 10 mM morpholinopropane sulphonic acid (MOPS) (pH 6.5) and 0.1 % bovine serum albumin (Grade A), and blended for 5–7 sec at full speed. The resultant slurry was passed quickly through 8 layers of cheesecloth. The suspension was run on to the top of a sucrose gradient consisting of 2 layers, each of 50 ml, of 0.75 and 1.0 M sucrose *plus* 5 mM MOPS (pH 7.2), and centrifuged for 5 min at 200 × *g* and 10 min at 500 × *g*. The chloroplast precipitate was gently resuspended in 2 ml of a reaction medium combining 330 mM sorbitol, 0.01 % bovine serum albumin and 10 mM *N*-2-hydroxyethylpiperazine-*N*-ethane sulphonic acid (HEPES) (pH 7.5, adjusted with KOH). These chloroplasts were intact and of the Class I type[16].

High-salt chloroplasts were obtained by adding a third layer of 1.5 M sucrose to the density gradient and harvesting the chloroplasts at the interface between the 1.0- and 1.5-M layers and adding approx. 50 % by volume a solution containing 0.8 M sorbitol, 10 mM HEPES (pH 7.5) and 0.01 % bovine serum albumin, which was also used as the reaction medium for these chloroplasts.

The high-salt medium used to resuspend chloroplasts and subchloroplast particles in some treatments contained (mM): sorbitol, 100; KCl, 100; NaCl, 20; $MgCl_2$, 5; $CaCl_2$, 10; KH_2PO_4, 1; HEPES (pH 7.5), 50 and in addition 0.40 mg/ml bovine serum albumin.

Chloroplast fragments were prepared from intact chloroplasts by osmotic shock. The chloroplasts were suspended in 10 ml undiluted cold glycerol for 15 min and then

were passed through a gauge-22 hypodermic needle into 1 l of rapidly stirred dilute buffer (1 mM MOPS, pH 7.2). The resultant chloroplast fragments were collected by centrifugation at 8000 rev./min for 20 min (6 × 250 ml angle head, Servall RC 2 centrifuge). The precipitate was resuspended in a small amount of supernatant and placed on top of a sucrose gradient with concentrations of sucrose, in 0.1 M steps, from 1.1 to 1.6 M sucrose (buffered with 5 mM MOPS, pH 7.2) as shown in Fig. 1. The gradient was centrifuged with an SW 25/1 swinging bucket rotor in a Spinco Model L centrifuge at 25000 rev./min for 2.5 h. Particles and membranes were found mainly in layers a, b and c with much less material in a_1 and c_1 (see Fig. 1). The ratio of chlorophyll *a*/*b* was found to be the same in all fractions. The fractions were resuspended in the normal reaction medium.

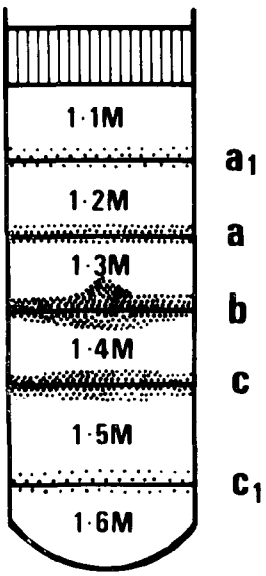

Fig. 1. The sucrose density gradient used in separating chloroplast fragments. The layers of sucrose indicated (in molar concentration) were buffered with 5.0 mM MOPS (pH 7.2). a and a_1, particles; b, small vesicles; c, large vesicles; c_1, large vesicles and lamellar sheets.

Light-induced absorbance changes were recorded in a dual-wavelength difference spectrophotometer of the type described by CHANCE[17] using a cuvette held at a constant temperature of 10 or 25°. Side illumination from a tungsten lamp was screened by 20 cm water, a broad-band red filter and an interference filter (Baird Atomic interference filters: T_{max} at 680 nm and 720 nm and a 95 % transmission between ± 5 nm). The light intensity at the cuvette was $4 \cdot 10^4$ ergs/cm² per sec at 680 nm and $2 \cdot 10^4$ ergs/cm² per sec at 720 nm. A combination of a glass filter (Corning CS 4-96) and a Kodak Wratten filter screened the photomultiplier from red light. The response time of the integrating circuit was set at 10 msec. Rapid responses were recorded on a Tetronix storage oscilloscope. The actinic light was switched on and off by a hand operated shutter.

Chlorophyll was determined by the method of MACKINNEY[18]. Cation content was determined using an atomic absorption spectrophotometer (Techtron Pty., Melbourne).

3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea (DCMU) was a gift of Dr. P. G. Heytler of E.I. DuPont Nemours and Co., Experimental Station, Wilmington, Delaware 19898. It was used in an ethanol solution, to give a final concentration of ethanol of less than 0.1 %. HEPES, 2-(*N*-morpholino)ethane sulphonic acid (MES) and MOPS buffers and bovine serum albumin, Grade A, were obtained from Calbiochem Co., Los Angeles, Calif. Valinomycin was a gift of Dr. B. C. Pressman.

RESULTS

Intact chloroplasts

The light-dark difference spectra of intact chloroplasts are shown in Fig. 2. A peak of cytochrome *f* oxidation occurred at 554 nm, for both wavelengths of actinic light (red light was used to activate both photosystems I and II and far-red light was used to activate Photosystem I alone[3,11]). A subsidiary peak or shoulder at 548–550 nm was often observed (as in Fig. 2) which may be related to the P550 component recently described[19]. A further large peak was always found at approx. 572 nm (refs. 20, 21) which obscured the cytochrome *b* region.

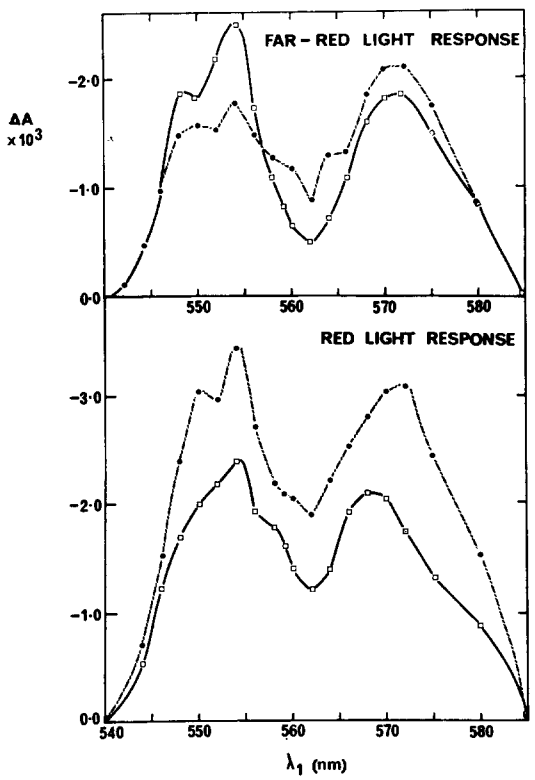

Fig. 2. Light–dark difference spectra of Class I chloroplasts for far-red light and for red light. The solid curve is the final light-on response, and the dashed curve is the rapid light-on response (<2 sec). The reference wavelength was 540 nm in each case. Chlorophyll concentration, 55 μg/ml. Temperature, 10°. Actinic far-red light 720 nm of $2 \cdot 10^4$ ergs/cm² per sec; red light 680 nm of $4 \cdot 10^4$ ergs/cm² per sec. Chloroplasts suspended in routine sorbitol medium. Temperature, 10°.

The pattern of cytochrome *f* photooxidation is shown in Fig. 3A. This differed from the pattern previously described for swollen, Class II chloroplasts[3,11] principally in the fast responses to both red and far-red light, the partial reversibility of the far-red light response and the high degree of oxidation in red light.

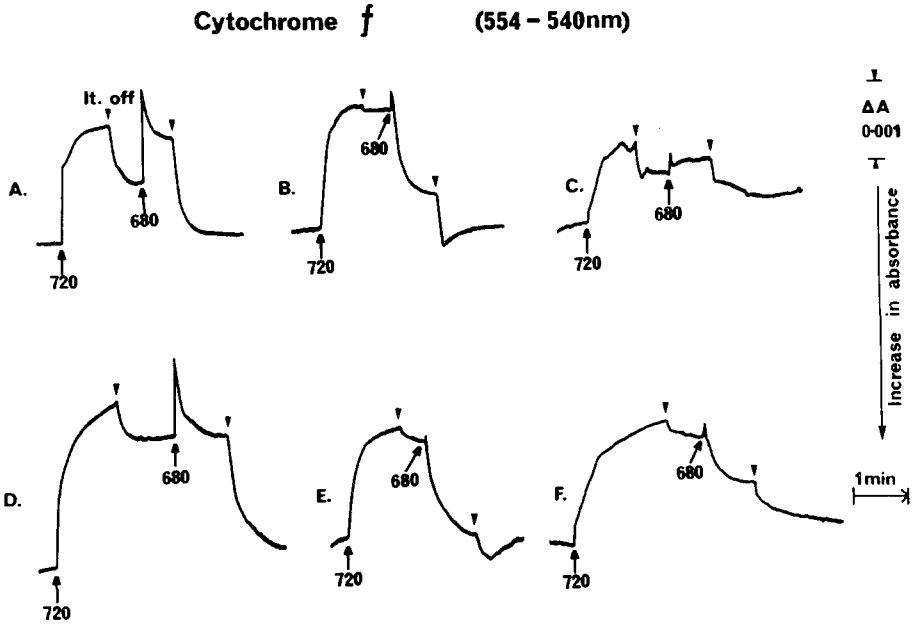

Fig. 3. Light-induced responses of cytochrome *f*. A, Class I chloroplasts; B, swollen, Class II chloroplasts; C, Class I chloroplasts, reference wavelength 564 nm instead of 540 nm; D, chloroplast fragments, Band a; E, chloroplast fragments, Band c; F, chloroplast fragments from Band a suspended in a high-salt medium. Chlorophyll concentrations (μg/ml): A, 61; B, 76; C, 61; D, 98; E, 50; F, 98. Apart from cytochrome *f*, the chloroplasts were suspended in routine sorbitol medium. Conditions as in Fig. 2.

Increasing light intensity increased the rate of both the red and far-red responses and increased the reversibility of the far-red responses (Fig. 4). From measurements of the rates of the rapid responses it appeared that in red light saturation was reached before the highest intensity (the half-time at saturation approx. 200 msec) and that far-red light was near to saturation at the highest intensity (half-time, approx. 500 msec). The routine light intensity for other experiments was the highest intensity used in these experiments. The increased reversibility of the far-red response with increased intensity may indicate that some Photosystem II activity occurred even in far-red light[11,12].

To test the possibility that the characteristic rapid response of intact chloroplasts was an artifact caused by the long tail of the accompanying P518 response[22,23] the reference wavelength was changed from 540 to 564 nm in one experiment (Fig. 3C). Although there was much interference from other spectral responses in this region (see Fig. 2) it is clear that the results were qualitatively similar to those for the normal reference wavelength. An artifactual origin of these fast responses is also excluded by the evidence that (a) the responses to both red and far-red light were found to

have peaks at 554 nm in difference spectra (not shown) and (b) the responses were inhibited by DCMU, valinomycin and other inhibitors[20,24]. The fast responses therefore appear to be an integral part of the cytochrome *f* responses of intact chloroplasts.

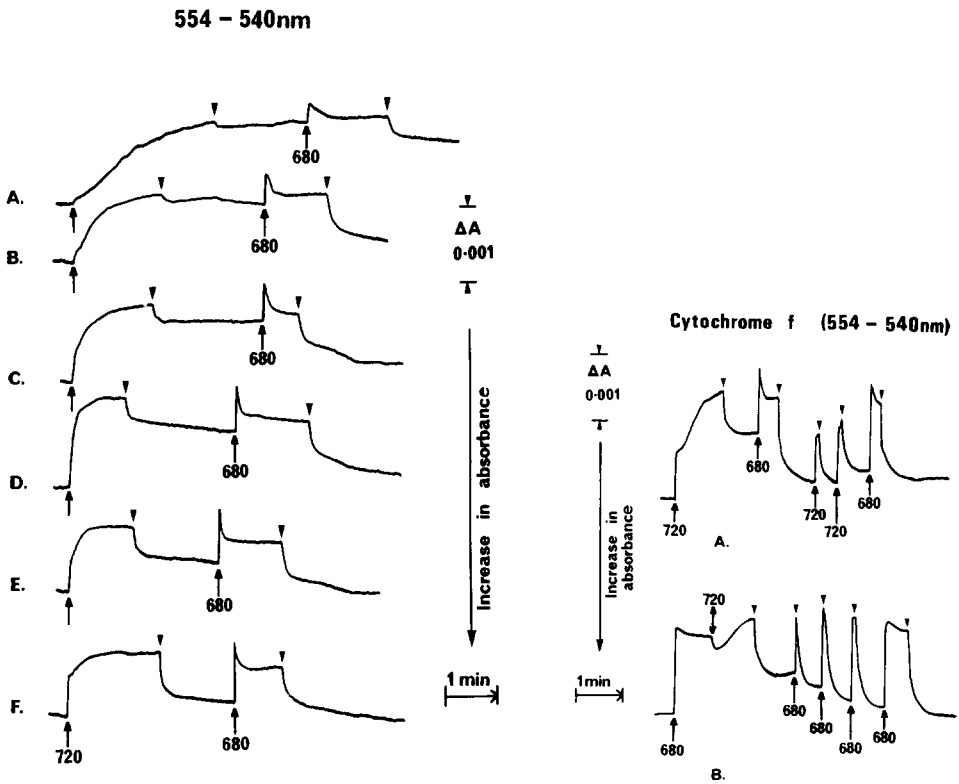

Fig. 4. The effect of light intensity on the cytochrome *f* response in Class I chloroplasts. The light intensity, as a percentage of the highest intensity used (far-red light of $2 \cdot 10^4$ ergs/cm^2 per sec and red light of $4 \cdot 10^4$ ergs/cm^2 per sec) was: A, 2.5; B, 10; C, 2.5; D, 50; E, 63; F, 100. The light intensity was altered using neutral density filters. Chlorophyll concentration, 47 μg/ml.

Fig. 5. Induction effects in the responses of cytochrome *f*. A and B, same preparation of Class I chloroplasts (chlorophyll concentration, 59 μg/ml); C and D, separate preparations of Class I chloroplasts (chlorophyll concentration, C 54 μg/ml; D 57 μg/ml); E, Class I chloroplasts suspended in high-salt medium (see MATERIALS AND METHODS). Chlorophyll concentration, 45 μg/ml. Chloroplasts of A–D were suspended in routine sorbitol medium. Experimental conditions as in Fig. 2.

Both the biphasic oxidation in far-red light and the spike of the cytochrome *f* response in red light (Figs. 3–5) are indicative of complex reactions of cytochrome *f* in intact chloroplasts. As shown in Fig. 6A the fast phase of the far-red response was completely reversible if illumination was stopped at this stage. However, the reversible part of the steady-state response was not found to be equal in extent to the fast phase. The spike of the red response, following a cycle of far-red light and darkness, was also related to the mechanism of reversibility; as shown in Fig. 5B, if the light is cut off before the end of the spike, the red light response is not fully reversible.

Swollen Class II chloroplasts and chloroplast fragments

Swollen, Class II chloroplasts were found to have a similar pattern of cytochrome *f* response (Figs. 3B and 6) to that described elsewhere[3,11]. It therefore seemed

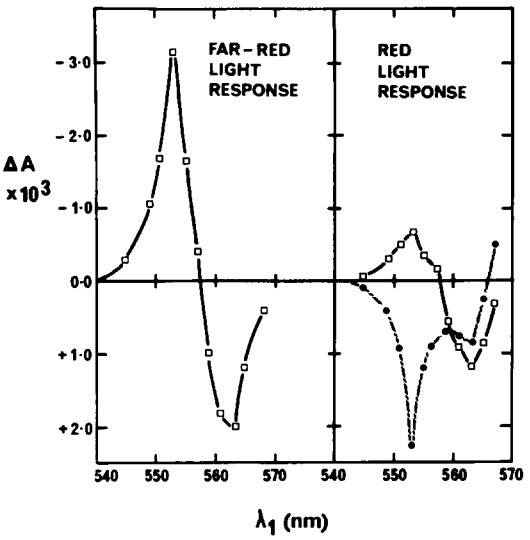

Fig. 6. Light–dark difference spectra of Class I chloroplasts suspended in high-salt medium (see MATERIALS AND METHODS). The solid curves represent the steady-state redox level elicited by far-red (720 nm) or red (680 nm) light, with reference to the dark state, following red illumination. The hatched curve represents the change elicited by red light, with reference to the dark state following far-red illumination (*i.e.* although red light causes a reduction of cytochrome *f*, the redox state remains oxidised relative to the final dark state). Experimental conditions as in Fig. 2. Chlorophyll concentration, 59 μg/ml.

possible that the differences between the responses of intact and swollen chloroplasts were due to changes in the integrity of the chloroplasts. However, further investigation showed that similar differences could be obtained between unswollen and swollen chloroplast fragments (Figs. 3D and 3E).

The unswollen and swollen chloroplast fragments were prepared by osmotic shock, as described in MATERIALS AND METHODS. Unswollen fragments (rods or particles of 0.2–0.4 μm) were collected in Band a (Fig. 1) after density gradient separation. Swollen fragments (large vesicles or membrane aggregates of up to 5 μm) were collected in Band c and to a lesser extent in Band b.

As shown in Table I the unswollen fragments contained higher levels of cations than swollen fragments on a chlorophyll basis and in terms of concentration the differences would be much greater. This indicated that permeability changes, occurring upon swelling, could be the critical factor behind the different responses of the intact chloroplasts and unswollen fragments on the one hand and the swollen chloroplasts and swollen fragments on the other (*cf.* ref. 25). In support of this it was found that preparations of Class II chloroplasts, which were not deliberately swollen by dilution or addition of NaCl but which probably contained some swollen chloroplasts, showed a response intermediate between the intact chloroplast response and the swollen, Class II response. Furthermore, unswollen particles resuspended in a high-salt medium

TABLE I

THE CATION CONTENT OF WHOLE CHLOROPLASTS AND FRAGMENTS

The high-salt chloroplasts were centrifuged at low speed (200–500 × *g*) on a two-layered sucrose density gradient and collected as the precipitate. The subchloroplast fragments and vesicles were prepared from high-salt chloroplasts by osmotic shock and were fractionated on a sucrose density gradient by centrifugation at high speed (100 000 × *g*). Cation content was determined by atomic absorption spectroscopy. The standard error of the mean is given for high-salt chloroplasts ($n = 6$). The values for the chloroplast fragments and vesicles (as described in MATERIALS AND METHODS) are the means of two preparations. The contribution of chlorophyll Mg^{2+} has been deducted from the Mg^{2+} values.

	Content (μmoles/mg chlorophyll)			
	K^+	Na^+	Ca^{2+}	Mg^{2+}
High-salt chloroplasts	4.1 ± 0.2	0.01 ± 0.001	0.03 ± 0.005	1.55 ± 0.07
Chloroplast fragments (Band a)	2.17	0.06	0.10	0.18
Chloroplast vesicles (Band c)	1.09	0.05	0.10	0.14

were observed to swell and exhibited cytochrome *f* responses similar to swollen, Class II chloroplasts (Fig. 3F).

High-salt chloroplasts

Chloroplasts *in vivo* have a high salt content[26] and recently it has become possible to isolate high-salt chloroplasts *in vitro*[14]. The cation content of a typical preparation of high-salt chloroplasts is shown in Table I. (The major anion is probably Cl^- (refs. 26 and 27).) These chloroplasts, resuspended in the 0.8 M sorbitol reaction medium described in MATERIALS AND METHODS exhibited responses which differed markedly from those described for intact chloroplasts (Fig. 7C). The far-red light

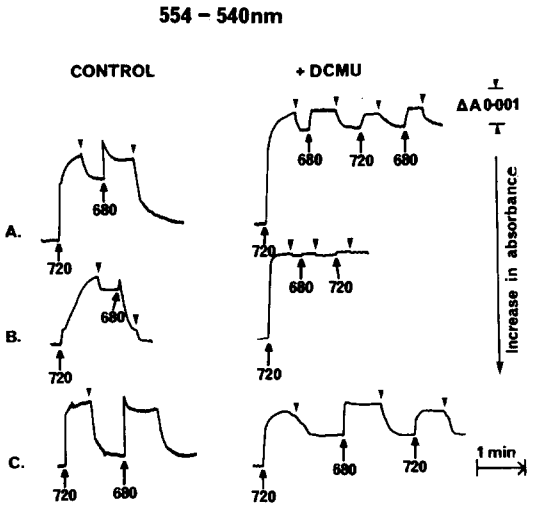

Fig. 7. The effect of DCMU (1 μM) on the light-induced responses of cytochrome *f* in A, swollen, Class II; B, Class I; C, high-salt chloroplasts. The experimental conditions were the same in Fig. 2. Chlorophyll concentrations (μg/ml): A, 71; B, 45; C, 110. The three types of chloroplasts were suspended in the same media as indicated in Figs. 3 and 4.

response was fully reversible and the responses generally were similar to those of intact algae[2,28,29]. Attempts at resuspending the chloroplasts in the normal 330-mM sorbitol reaction medium were not successful in retaining this pattern of response. Resuspension in a high-salt medium, similar to the concentration of salts inside the chloroplasts (see MATERIALS AND METHODS) gave rise to responses characteristic of swollen chloroplasts. In fact, it was found that low levels of Na^+ (concn. 10 mM), Mg^{2+} (concn. 50 mM) and Ca^{2+} (concn. 50 mM) can cause such a changeover[21].

Inhibition by DCMU

In Fig. 7 are shown the effects of DCMU (2 μM) which was used to inhibit electron flow from Photosystem II. DCMU was added in the dark with cytochrome *f* in the reduced state. In swollen Class II chloroplasts (Fig. 7B) and swollen chloroplast fragments far-red light induced an oxidation of greater extent and at a faster rate compared with the control. However, a subsequent illumination caused almost no change at all. In intact chloroplasts (Fig. 7A) and unswollen chloroplast fragments, DCMU resulted in similar photooxidation in far-red light. However, there was some reduction in the dark and subsequently illumination produced a small amount of reversible oxidation. In high-salt chloroplasts the reversible light-induced component was much larger (Fig. 7C) amounting to approx. 50 % of the control response.

DISCUSSION

The results described deal with chloroplasts from pea leaves. However, similar results were obtained with spinach chloroplasts. Perhaps the most striking result to emerge from the present investigation is the very different pattern of response of cytochrome *f* to red and far-red light in intact chloroplasts and unswollen fragments to that in swollen chloroplasts or fragments. This difference is clearly related to the state of the photosynthetic membranes, which is not necessarily linked to the integrity of the chloroplasts although integrity does seem to have some influence on the light-induced cytochrome *f* response.

Many of the observations on the swollen, Class II, the intact and the high-salt chloroplasts can be explained by the scheme presented in Fig. 8.

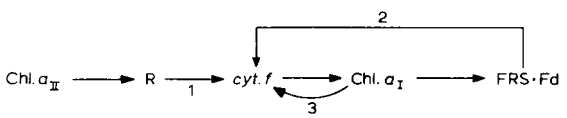

Fig. 8. Hypothetical scheme of electron mediation between cytochrome *f*, Photosystem I and Photosystem II. Fd, ferredoxin; FRS, ferredoxin reducing substance; cyt. *f*, cytochrome *f*; R, intermediate redox pool; Chl. a_I, chlorophyll a_I of Photosystem I; Chl. a_{II}, chlorophyll a_{II} of Photosystem II.

The swollen chloroplast responses suggest a simple pathway of electron flow involving Pathway 1 of the scheme in Fig. 8 but not Pathways 2 or 3. This simple scheme is very similar to that envisaged by AVRON AND CHANCE[3]. The action of DCMU in increasing the photo-oxidation rate and its extent in far-red light clearly demonstrates a contribution of Photosystem II in the control treatment. The lack of any dark reduction of the cytochrome *f*, photo-oxidised by far-red light, indicates that

the size of the reducing pool, R, is small, possibly much smaller than in unswollen chloroplasts. The poor rate of far-red photo-oxidation indicates a slow flux of electrons out of the cytochrome *f* pool, which is probably due to a restricted flow of electrons to $NADP^+$ or to O_2, through a pseudocyclic pathway, since the rate of photooxidation is greatly enhanced by the addition of 1,1′-ethylene-2,2′-bipyridylium dibromide (diquat)[21], an autoxidisable electron acceptor.

In intact chloroplasts and unswollen fragments explanations are required for the biphasic and partially reversible oxidation in far-red light, the spike in red light and the subsequent high level of steady-state oxidation and a more complex scheme involving at least two of the pathways of Fig. 8 seems necessary. The resistance to DCMU inhibition by the reversible far-red component also indicates a role for Pathway 2 or 3. Moreover, since this component has been found to be sensitive to inhibitors of cytochrome *b* reactions[24] a cyclic electron transport around Photosystem I (Pathway 2) is favoured. Similar evidence[24] suggests that a cyclic pathway is also important in high-salt chloroplasts.

Many of the differences between intact and swollen chloroplasts can therefore be explained in terms of intact chloroplasts having (a) a cyclic electron transport system; (b) a greater flux of electrons from cytochrome *f* to Photosystem I and (c) a larger reducing pool, R. It is also possible that the coupling site acts as a greater resistance to electron flow in intact chloroplasts, which together with the increased flux of electrons to Photosystem I, would explain the high level of red photo-oxidation. The action of uncouplers, such as carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and nigericin *plus* valinomycin is consistent with this explanation[24].

The major difference between cytochrome *f* photooxidation in intact and high-salt chloroplasts was the degree of reversibility following far-red illumination. Since the most obvious difference between the two types of chloroplasts is their ability to retain ions, it seems possible that the greater degree of reversibility in high-salt chloroplasts is due to the presence of a low molecular weight substance which reduces cytochrome *f* in the dark and which is leached out during the preparation of intact chloroplasts. An alternative explanation is that in the special ionic environment of high-salt chloroplasts a cyclic electron pathway around Photosystem I is activated. However, the attempts to simulate this by resuspension in similar salt concentrations were not successful (see also ref. 20).

We assume that the high-salt chloroplast response which is very similar to the response found in algal cells[2,28,29], may be close to the *in vivo* response. However, it has recently been shown that in intact pea leaves the level of cytochrome *f* oxidation in red light is much less than in far-red light[30]. Thus there are probably still differences between the high-salt and *in vivo* chloroplasts and their cytochrome *f* responses, and this is not surprising since the experiments with high-salt chloroplasts were carried out in solutions of high osmolarity (0.8–1.0 M).

Further work is clearly necessary to investigate the photosynthetic electron transport pathways more fully and especially to correlate the findings with the CO_2 fixation and phosphorylative capacity in the various types of chloroplasts. At present it is only possible to say that conditions conducive to CO_2 fixation, *e.g.* the presence of bicarbonate, 3-phosphoglyceric acid, ADP or even ATP, did not alter the pattern of the light-induced cytochrome *f* responses in intact chloroplasts.

Finally, the work described here and in the succeeding papers[20,21,24] lends no

support to the presence of any cytochrome on the intermediate pathway other than cytochrome *f* which is considered to be separated by a coupling site from a large reducing pool on the Photosystem II side.

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