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LIGHT-INDUCED ABSORBANCE CHANGES OF P518 IN INTACT CHLOROPLASTS

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SUMMARY

The light-induced absorbance change centred at 518 nm (P518 response) is 20–30-fold greater in intact chloroplasts than in swollen chloroplasts. The various characteristics of this large P518 response, including induction effects and chromatic transients, were studied. 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) (2 μM) strongly inhibited the response and the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, abolished it. Other uncouplers such as NH_4Cl, methylamine, atebrin and nigericin were without effect. Valinomycin inhibited the response and valinomycin in combination with NH_4Cl or nigericin abolished it.

In the presence of DCMU the P518 response could be restored with 2,6-dichlorophenolindophenol (+ ascorbate) but not with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (+ ascorbate).

The results support a correlation between the P518 response and a membrane potential across the functional photosynthetic membrane, which may be produced by two mechanisms, one fast and one slow. It is suggested that an oxidation–reduction loop between the two photosystems, forms a significant component of the slower mechanism.

INTRODUCTION

Large light-induced spectral shifts in the region 515–525 nm, which have variously been attributed to carotenoids, chlorophyll *b*, quinones, *etc.*, have been described frequently in many types of photosynthetic organisms ever since the first observations by DUYSENS[1]. Recently there has been growing interest in the relationship of these shifts to the energised state of the photosynthetic apparatus[2–4]. JUNGE AND WITT[5] claimed that the P518 response (*i.e.* an absorbance increase with a peak at 518 nm (ref. 6) in chloroplasts is related to the potential difference across the

Abbreviations: MES, 2-(*N*-morpholino)ethane sulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-ethane sulphonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; diquat, 1,1′-ethylene-2,2′-bipyridylium dibromide; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

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functional photosynthetic membrane, and JACKSON AND CROFTS[4] have presented evidence to support a direct correlation between the carotenoid signal in chromatophores of *Rhodopseudomonas spheroides* and the membrane potential which was estimated by other means.

One of the great difficulties with work on chloroplasts or even subchloroplast particles is the great variability of the P518 response induced by light. In the present investigation we have used intact chloroplasts which we have found to show very large and constant P518 responses induced by light. It is possible with such chloroplasts to show that the P518 response is susceptible to uncoupling combinations of nigericin and valinomycin in much the same way as in chromatophores. In addition, by the use of other inhibitors and of electron donor couples, it has been possible to define more precisely the sites on the photosynthetic electron-transport chain where the response is generated.

MATERIALS AND METHODS

The material and methods used in the present investigation were exactly as previously described for work on cytochrome *f* absorbance changes[7]. The following is a brief description of the techniques. Chloroplasts were isolated from pea leaves obtained from 10 to 14 days cultured plants. The isolation of intact chloroplasts involved the isolation of high-salt chloroplasts by a modification of the method of HARVEY AND BROWN[8] employing a sucrose density centrifugation and an isolation medium of 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 1 mM ascorbic acid, 10 mM morpholinopropane sulphonic acid buffer (pH 6.5) and 0.01 % bovine serum albumin (Grade A). The final precipitate of chloroplasts was taken up in the reaction medium of 330 mM sorbitol, 10 mM *N*-2-hydroxyethylpiperazine-*N*-ethane sulphonic acid (HEPES) (pH 7.5, adjusted with KOH) and 0.01 % bovine serum albumin. These chloroplasts were of the Class I type with outer membranes intact. High-salt chloroplasts were obtained by adding a denser sucrose layer (1.5 M) to the gradient, removing the chloroplasts from the interface after centrifugation and suspending them in a hyperosmolar solution at approx. 0.8 M sorbitol and 10 mM HEPES (pH 7.5).

Light-induced absorbance changes were recorded in a dual-wavelength difference spectrophotometer with access to the chloroplast suspension from the side for illumination with actinic light. The 1 cm × 1 cm cuvette was kept routinely at a constant temperature of 10° in order to preserve the chloroplasts intact for longer periods; comparative tests at 25° showed no qualitatively different responses. Actinic illumination at the wavelengths 680 and 720 nm was obtained using Baird Atomic interference filters with 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 glass filter (Corning CS 4-96) screened the photomultiplier from actinic light. Rapid responses were recorded with a Tetronix storage oscilloscope with a Polaroid camera attachment.

Biochemicals were of the highest purity obtainable from Calbiochem Co., Los Angeles, Calif. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and diquat were gifts of Dr. P. G. Heytler, Dupont Nemours Co., Experimental Field Station, Wilmington, Dela., U.S.A. Nigericin and valinomycin were gifts of Dr. B. C. Pressman.

RESULTS

The pattern and kinetics of the P518 response

The form of the light-induced P518 response in high-salt chloroplasts[7], intact chloroplasts and swollen chloroplasts is shown in Fig. 1. Red actinic light (680 nm) was used to stimulate both Photosystems I and II and far-red light (720 nm) to stimulate Photosystem I (refs. 7, 9, 10), although it is probable that at the high intensity used some turnover of Photosystem II also took place[7]. In intact chloroplasts the steady-state response was 20–30-fold greater than in swollen chloroplasts. The initial rapid phase of the response had a maximum half-rise time of 150–200 msec (Table I) but this rate may well have been instrument-limited (*cf.* 15 msec for bacterial chromatophores under continuous illumination[11]). The fall to a steady-state level had a half-time of 5–15 sec dependent upon light intensity (Fig. 3).

As shown in Fig. 2 the P518 response was centred broadly at 518 nm in intact

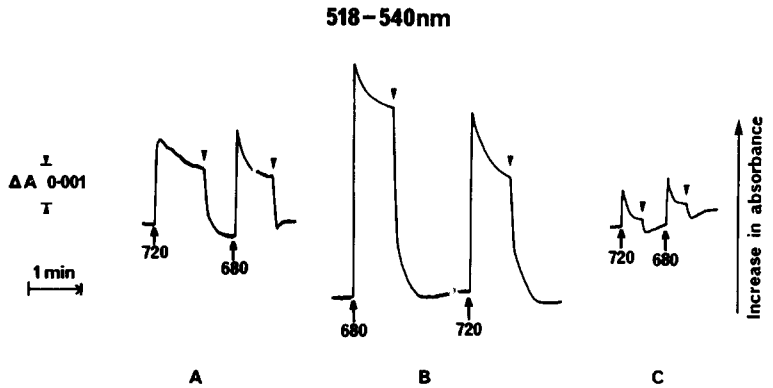

Fig. 1. The light-induced P518 response in high-salt (A), Class I (B) and swollen Class II (C) chloroplasts from pea leaves. The chlorophyll concentrations were (μg/ml): A, 110; B, 60; C, 90. Actinic light intensities: 680 nm, $4 \cdot 10^4$ ergs/cm^2 per sec; 720 nm, $2 \cdot 10^4$ ergs/cm^2 per sec. Temperature, 10°. Chloroplasts for (C) swollen in 10 mM morpholinopropane sulphonic acid buffer (pH 7.2) and resuspended in 0.3 M sorbitol solution + 20 mM NaCl.

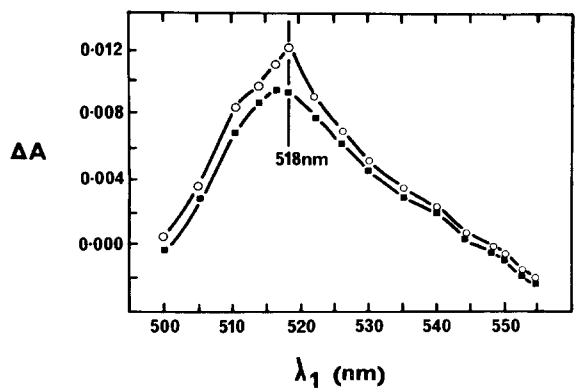

Fig. 2. The light *minus* dark difference spectrum of the light-induced P518 response to red light (680 nm, ○ - ○) and to far-red light (720 nm, ■ — ■) in Class I chloroplasts. Chlorophyll concentration, 71 μg/ml. Other conditions were as in Fig. 1.

chloroplasts and as shown in Fig. 3 and Table I it was at or near to light-saturation at the highest light intensities (the intensities routinely used). The most marked effect of increasing light intensity was on the initial spike, which was both increased in extent and rate of decay.

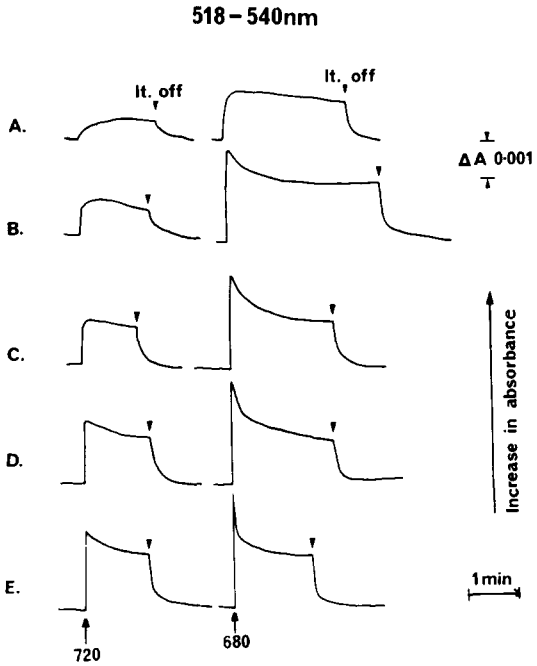

Fig. 3. The effect of light intensity on the light-induced P518 response in Class I chloroplasts. Light intensities (percentage of maximum illumination, which was as given in Fig. 1): A, 2.5; B, 10; C, 25; D, 50; E, 100. Chlorophyll concentration, 49 μg/ml. Temperature, 10°.

TABLE I

THE RELATIONSHIP OF LIGHT INTENSITY TO THE HALF-TIME OF THE P518 RESPONSE INDUCED BY RED LIGHT (680 nm) AND FAR-RED LIGHT (720 nm) IN CLASS I CHLOROPLASTS

The half-times (sec) of the light "on" and the light "off" responses are shown. Half-times are also given for a treatment with valinomycin (0.3 μg/ml) at the highest light intensity. Chlorophyll concentration, 48 μg/ml. Temperature, 10°. The light intensity was varied using Kodak Wratten neutral density filters. Maximum light intensities were: 680 nm, $4 \cdot 10^4$ ergs/cm² per sec; 720 nm, $2 \cdot 10^4$ ergs/cm² per sec.

Light intensity (% *of max.*)	*Half-time (sec)*			
	Far-red light		*Red light*	
	on	*off*	*on*	*off*
2.5	5.0	10.0	1.0	5.0
10.0	1.0	6.0	0.5	3.0
25.0	0.5	4.0	0.25	2.0
50.0	0.4	3.0	0.20	2.0
100.0	0.2	2.0	0.15	1.5
Valinomycin	1.7	40.0	0.4	10.0

The typical P518 response could become modified by certain induction effects, as shown in Fig. 4 (*cf.* ref. 9). Such effects were accentuated in slightly uncoupled chloroplasts and in aged or poor preparations (of which the preparation used for Fig. 4 was probably an example since the far-red light response was comparatively

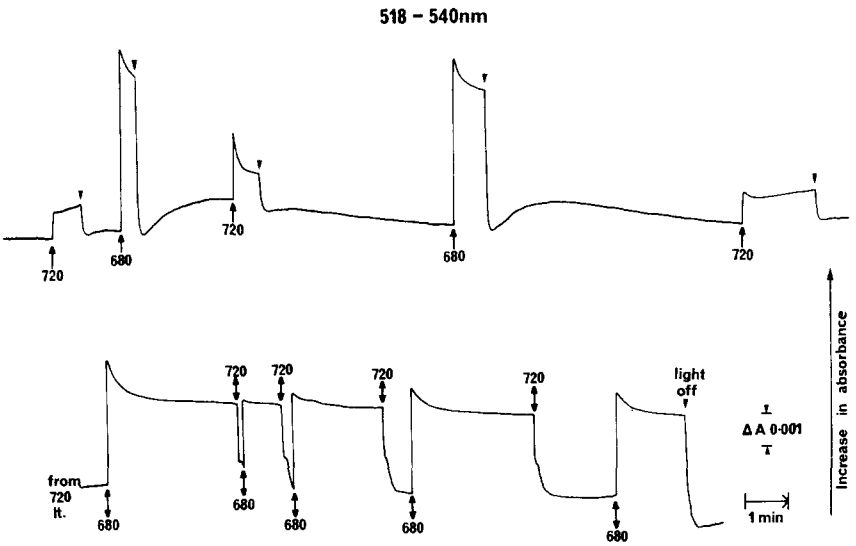

Fig. 4. Induction and chromatic transient effects in the light-induced P518 response in Class I chloroplasts. Chlorophyll concentration, 40 μg/ml. Other conditions as in Fig. 1.

so small). As shown, the spike of the far-red response could be elicited only in the first 2 min or so of darkness following red illumination, during which time there was a small absorbance increase. In contrast the spike of the red response required about 1 min of darkness for complete regeneration (*cf.* refs. 12 and 13).

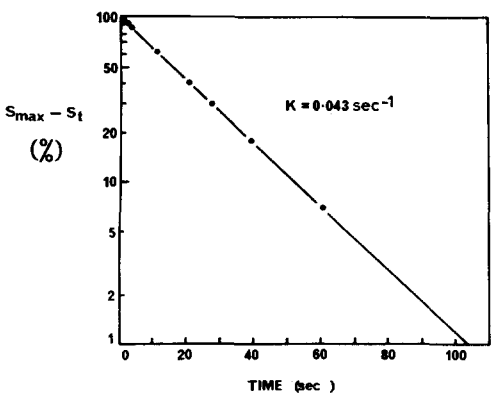

Fig. 5. The relationship of spike height (S) induced by red light (680 nm) to time of pre-illumination in far-red light (720 nm) for the P518 response in intact chloroplasts. The spike height (S) was measured against the final steady level in red light. The value of S_{max} *minus* S_t is plotted on a logarithmic scale against time, t. $S_{max} = 7.2 \cdot 10^{-4} \Delta A$. Experimental conditions as in Fig. 2. Chlorophyll concentration, 57 μg/ml.

Chromatic transients of the type first observed by BLINKS[14] were observed for the P518 response (Fig. 4). Following the change from red to far-red light an initial phase of rapid decay (half-time < 1 sec) was followed by a slower phase (half-time about 15 sec) of either a further fall or a rise in absorbance depending upon the steady-state level of the far-red response. After the onset of this second phase it was possible to elicit a spike of increased absorbance on changing back to red light (Fig. 4). This spike had a generation half-time of about 17 sec (Fig. 5) matching that of the slower phase itself; this is comparable with the half-times of other transients[15]. Transients were not found with artificial electron donor systems (of the type described later) with Photosystem II blocked by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU).

The effect of photosynthetic inhibitors and cations

Only inhibitors of non-cyclic electron transport were found to have a marked effect on the P518 response. DCMU (2 μM) abolished the response in swollen chloroplasts (Fig. 6B) and strongly inhibited it in intact chloroplasts (Fig. 6A). The effect of DCMU was much less in high-salt chloroplasts (not shown), suggesting the presence of a larger component of cyclic electron flow. Inhibitors of *b*-type cytochromes, antimycin A (up to 15 μg/ml) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) (up to 20 μM), had little effect except at high concentrations, where at least antimycin A probably promoted uncoupling[16]. (*N.B.* Fig. 6 is a composite of severa

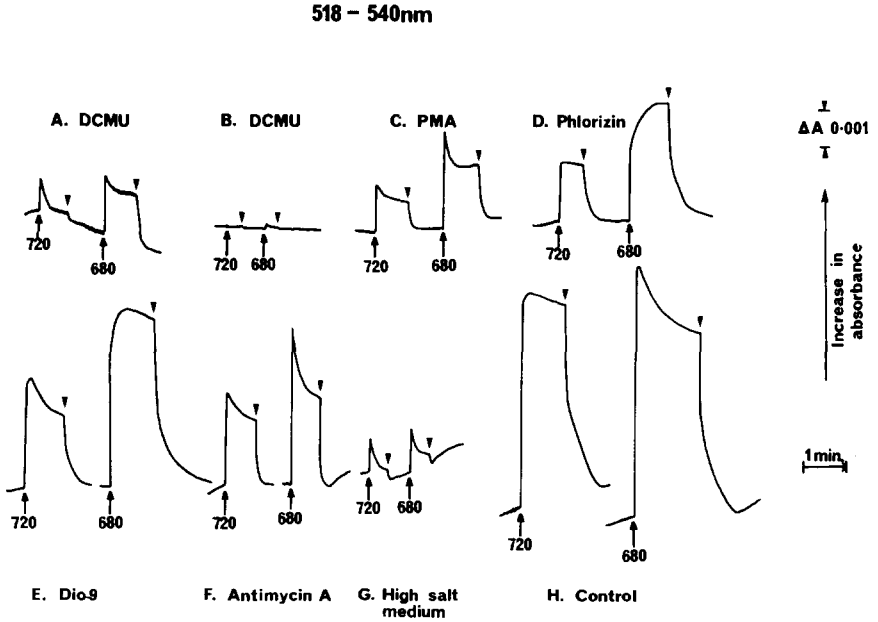

Fig. 6. The effect of various inhibitors on the light-induced P518 response in intact and swollen chloroplasts. A, DCMU (2 μM), intact; B, DCMU (2 μM), swollen; C, phenylmercuric acetate (50 μM), intact; D, phlorizin (1 mM), intact; E, Dio-9 (15 μg/ml), intact; F, antimycin A (15 μg/ml), intact; G, high-salt medium (see ref. 7), intact; H, a typical control response, intact chloroplasts. Experimental conditions as in Fig. 2. Chlorophyll concentrations (μg/ml): A, 60.5; B, 71.0; C, 65.5; D, 71.0; E, 68.0; F, 45.0; G, 90.0; H, 60.5.

experiments and the control treatment shown is not from the antimycin A experiment.) Both inhibitors abolished the DCMU-resistant response in intact chloroplasts, indicating a contribution from a small component of cyclic electron transport. This supports the view that both photosystems may contribute to the response[10,13,17,18]. It is not clear, however, why the DCMU-resistant response was so much greater in red light.

Phenylmercuric acetate, which has recently been postulated to inhibit a second cycle of electron transport around Photosystem I (ref. 19), had little effect up to 50 μM (Fig. 6, control shown not comparable); above this concentration swelling effects occurred. The energy-transfer inhibitors, Dio-9 and phlorizin, also had no great effect (Fig. 6D and E, control not comparable), as would be expected if the P518 response is related to the high-energy state (see INTRODUCTION). However, the initial spike of the response was inhibited and this effect remains unexplained.

Certain cations, such as Na^+ (> 5 mM), Ca^{2+} (> 50 mM) and Mg^{2+} (> 50 mM) were found to severely inhibit the steady-state response in intact chloroplasts, a phenomenon which seems to be linked to their effect on cytochrome *f* photooxidation[7]. The effect may be related to light-induced swelling but a similar phenomenon has recently been noted in already-swollen chloroplasts[20].

The effect of uncouplers

The effect of FCCP at 1 and 5 μM on the pattern of the P518 response in intact chloroplasts is shown in Fig. 7, and the effects of increasing concentration of FCCP on the steady-state response and the decay rate in the dark are shown in Fig. 8. The strong inhibitory effect of FCCP is in accord with an effect on a high-energy state (or intermediate) but the negative-absorbance response above about 0.5 μM was unexpected and is discussed later. The dark decay rate was increased about 10-fold by 1 μM FCCP (*cf.* ref. 21) and the decay rate of the spike was similarly increased. However, the "light-on" rate was unaffected.

The nitrogen base group of uncouplers—NH_4Cl, methylammonium chloride and atebrin—had little effect (*e.g.* Fig. 9E) on the response in intact chloroplasts (*cf.* ref. 21 swollen chloroplasts). Such a lack of effect is predictable from the chemi-

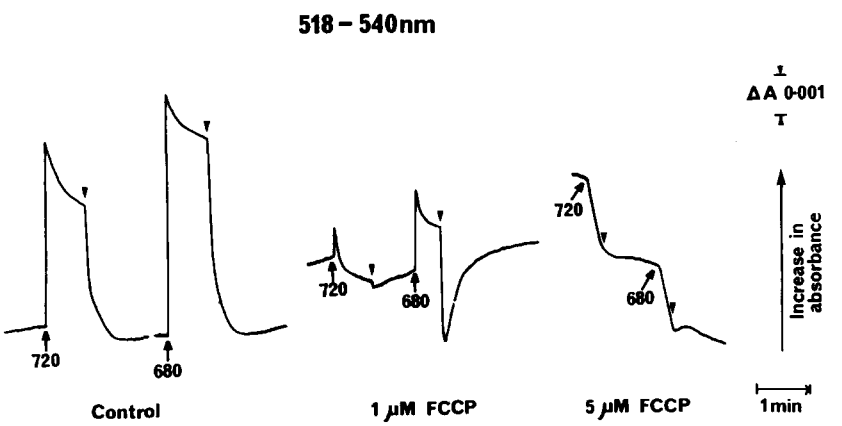

Fig. 7. The effect of FCCP on the light-induced P518 response in intact chloroplasts. Experimental conditions as in Fig. 2. Chlorophyll concentration, 70 μg/ml.

osmotic hypothesis of MITCHELL[21] if it is assumed that in intact chloroplasts phosphorylation is driven by a membrane potential component (ΔE) as well as a pH gradient[20,22]. Furthermore, the complete lack of effect of these uncouplers supports the proposal of JUNGE AND WITT[5] and JACKSON AND CROFTS[4] that the P518 response

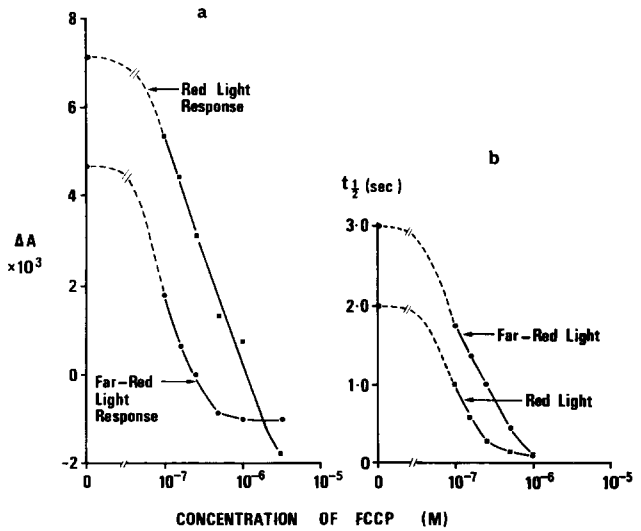

Fig. 8. The relationship of (a) the extent of light-induced P518 response, and (b) the half-time ($t_{½}$) of the "off" response to FCCP concentration in Class I chloroplasts. Experimental conditions as in Fig. 2. Chlorophyll concentration, 70 μg/ml. Datum from the same experiment as Fig. 7.

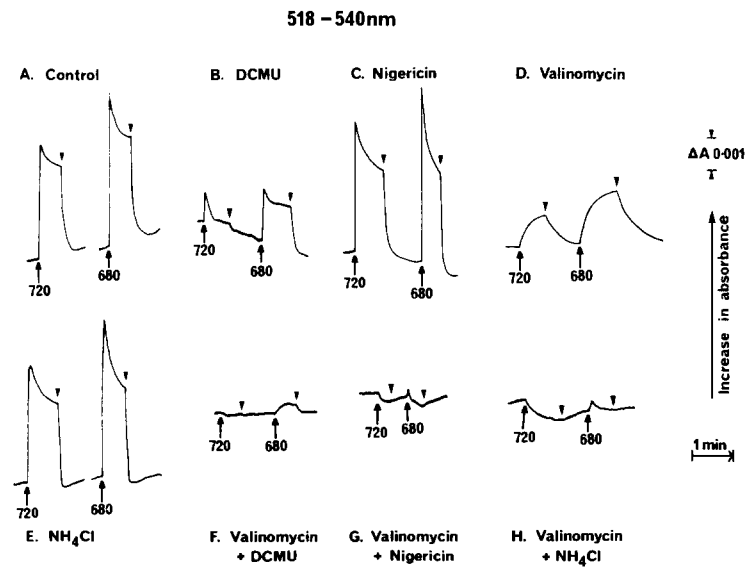

Fig. 9. The effect of DCMU and uncoupling agents on the light-induced P518 response in Class I chloroplasts. A, control; B, DCMU (2 μM); C, nigericin (0.3 μg/ml); D, valinomycin (0.3 μg/ml); E, NH_4Cl (20 mM); F, valinomycin (0.3 μg/ml) + DCMU (2 μM); G, valinomycin (0.3 μg/ml) + nigericin (0.3 μg/ml); H, valinomycin (0.3 μg/ml) + NH_4Cl (2 mM). Experimental conditions as in Fig. 1. Chlorophyll concentrations (μg/ml): A–D, F, G, 51.2; E, H, 58.0.

(and the related response in chromatophores) is an indicator of ΔE itself This hypothesis was tested further by the use of ion-transport antibiotics.

The effect of ion transporting antibiotics

Nigericin, at concentrations which have been shown to uncouple swollen chloroplasts[24], was without any inhibitory effect (in the presence of external K^+) on the P518 response of intact chloroplasts (Fig. 9C). Since there is good evidence that nigericin causes an electrically neutral (or near neutral) exchange of H^+ for K^+ (or to a lesser extent Na^+) across biological membranes[25,26], this agent would be expected to act like the nitrogen base uncouplers in dissipating a pH gradient. The complete resistance of the response therefore supports a relationship with ΔE.

The effect of valinomycin is shown in Fig. 9D; the rapid response was completely inhibited and there remained only a slow response with a diminished steady-state. Since valinomycin increases the permeability of biological membranes to K^+ ions[25] and since high-salt and, to a lesser extent, intact chloroplasts have large amounts of internal K^+, the depolarisation of the membrane potential would be expected and accords with the strong inhibition of the P518 response. The slow response remaining would be the result of a light-driven proton pump contributing to the ΔE term (see DISCUSSION), in agreement with its complete inhibition by the nigericin (Fig. 9G). Likewise, valinomycin and NH_4Cl have the same effect (Fig. 9H). Valinomycin also inhibited the DCMU-resistant response (Fig 9F) whereas nigericin did not (not shown); this may imply a different type of generation mechanism, perhaps by the $\Delta E'$ mechanism discussed later.

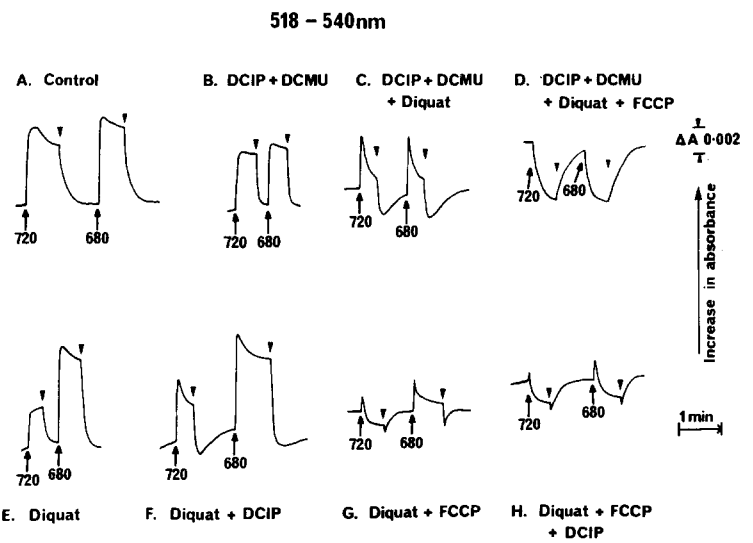

Fig. 10. The effect of an artificial electron donor system (DCIP + ascorbate) and an artificial electron acceptor (diquat) in the presence or absence of DCMU and FCCP on the light-induced P518 response in Class I chloroplasts. Concentrations (μM): DCIP, 100; diquat, 10; FCCP, 2; DCMU, 2. DCIP was accompanied with 2 mM ascorbate (potassium salt). Experimental conditions as in Fig. 1. Chlorophyll concentrations (μg/ml): A, C–G, 57.0; B, 55.0; H, 75.1.

The effect of artificial electron donor systems

Following inhibition by DCMU, the P518 response can be restored by the reducing couple of DCIP (10–100 μM) and ascorbate (2 mM) as shown in Fig. 10B. In contrast, the addition of TMPD (at low concentrations *viz* 30 μM) and ascorbate led not only to the complete inhibition of the steady-state response but also to the appearance of a large negative response (Fig. 11C). The P518 response with the DCIP system was inhibited by FCCP (Fig. 10D) or nigericin *plus* valinomycin, giving rise to large negative responses than those with the TMPD system. The use of diquat in these experiments was necessitated by the inhibition by FCCP of electron flow in intact chloroplasts[17,18], probably by an effect on a non-cyclic or pseudocyclic system. Under these conditions, diquat stimulates a pseudocyclic electron flow[19]. In the absence of FCCP, diquat had only a small inhibitory effect (Fig. 10C), and only in the presence of FCCP did the P518 response give way to the negative response. In comparison, FCCP and diquat had little effect on the TMPD system other than on inhibition by FCCP of the transient positive responses (Figs. 11E and 11F).

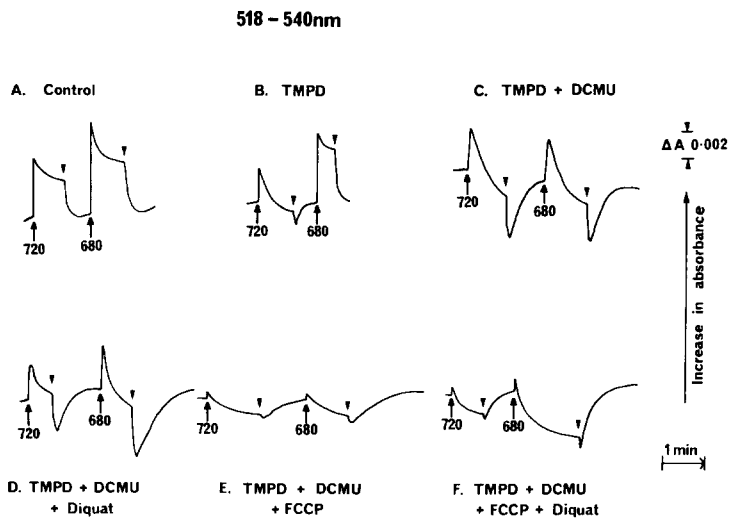

Fig. 11. The effect of an artificial electron donor system (TMPD + ascorbate) and an artificial electron acceptor (diquat) in the presence or absence of DCMU and FCCP on the light-induced P518 response in Class I chloroplasts. Concentrations (μM): TMPD, 30; diquat, 10; FCCP, 2; DCMU, 2. TMPD was accompanied with 2 mM ascorbate (potassium salt). Experimental conditions as in Fig. 1. Chlorophyll concentrations (μg/ml): A, B, 44; C, 51; D–F, 55.

DISCUSSION

The nature of the P518 response

The present results accord with the hypothesis that the light-induced P518 response is a manifestation of changes in membrane potential across the functional photosynthetic membrane[4,5]. The possible explanations for such an electrochromic relationship have recently been discussed by JACKSON AND CROFTS[11]. Accordingly, the light-induced membrane potential in intact chloroplasts, *i.e.* chloroplasts retaining the stroma phase and outer membranes, would be 20–30-fold greater than in swollen chloroplasts. However, this evidence relates to the steady P518 response.

Evidence from rapid responses of P518, and related responses, to laser pulses[1-6,30] suggests that these are too rapid to be generated by a conventional electron transport process[1] on which the arguments for the electrochromic relationship of the steady-state responses are based. The evidence of the fast responses suggests, rather, that a field is generated by charge separation between reaction centres of the photosystems and primary acceptors[2,5,11]. This is further supported by the insensitivity of the laser-induced "on" response to electron transport inhibitors[30] and uncouplers (unpublished results) (*cf.* ref. 20 for xenon flash results) and other arguments[11].

Thus, according to the electrochromic hypothesis, it must be considered that a fast and a slower electrogenic mechanism contribute to the P518 response. In intact chloroplasts under continuous illumination it is probable that the slower mechanism makes the major contribution, even to the initial rapid response since these are eliminated by valinomycin and FCCP. We assume that the slower electrogenic mechanism is linked to the light-driven proton pump[31,32], generated by an oxidation–reduction loop of the electron transport chain as proposed by MITCHELL[22]. Thus the difference between the initial change and the final steady-state response could result from secondary ionic readjustments through the reversible ATPase and ion-exchange systems[22].

The existence of two electrogenic mechanisms contributing to the P518 response would provide an explanation for the previously puzzling results concerning the contributions of Photosystems I and II (refs. 10, 13, 17, 18). It would also mean a reappraisal of previous work where conclusions have been drawn from comparisons of the fast response and steady-state phosphorylation rates[20].

According to the hypothesis of MITCHELL[22], the driving force on phosphorylation is the electrochemical potential difference for H^+ ($\Delta\bar{\mu}_{H^+}$) across the functional membrane, which will have terms for the membrane potential set up by the fast electrogenic mechanism (ΔE_f), and the membrane potential (ΔE_s) and pH differential (ΔpH) set up more slowly by the proton pump. Initially then, $\Delta\bar{\mu}_{H^+} = RT\Delta\text{pH} + zF\Delta E_f + zF\Delta E_s$ (where the symbols R, T, z and F have their usual meaning). However, in chloroplasts two other events, at least, seem likely to alter these terms: (i) an electrically neutral exchange of H^+ for K^+ leading to a decrease in the ΔpH term (ΔpH_1), (ii) a depolarisation of the membrane potential by electrophoretic movement of Cl^- and other anions with the result that in the extreme $\Delta\bar{\mu}_{H^+} = RT\Delta\text{pH}_1$. Such a treatment is probably a gross oversimplification because it neglects, for example, contributions due to the passive permeability of the membrane to various ions (see, for example, the equation of GOLDMAN[33]). Nevertheless it is a useful model and is a logical development of earlier proposals by WITT and co-workers[2,5].

In terms of the above treatment swollen chloroplasts with their small P518 responses may be regarded as having a relatively high permeability to Cl^- (refs. 34, 35) resulting mainly in the ΔpH term (*i.e.* $\Delta\bar{\mu}_{H^+} = RT\ \text{pH}_1$). Intact chloroplasts (and chromatophores[4], and to some extent subchloroplast particles[36]), having large P518 and related responses, may be regarded as having the membrane potential terms as well. Therefore, nigericin (in the presence of K^+) and the nitrogen base uncouplers which dissipate ΔpH (refs. 26, 37) and are therefore strong uncouplers in swollen chloroplasts would only partially inhibit phosphorylation in intact chloroplasts and would not affect the P518 response. In the special case recently put forward for an ammonium salt with an impermeant anion[35] in swollen chloroplasts, it would be

predicted that uncoupling would occur together with a small P518 response, but swelling would be prevented. Valinomycin, while having no effect on phosphorylation in swollen chloroplasts[38], should have a pronounced inhibitory effect in intact chloroplasts, since from the P518 results it would seem that the ΔE terms make a large contribution towards $\Delta\bar{\mu}_{H^+}$. However, inhibition will be largely dependent upon the levels of internal and external K^+ and the permeability properties of the functional membrane. Furthermore, the demonstration of phosphorylation in intact chloroplasts will be difficult due to (i) the controlled permeability of the outer membranes to ATP[39] and (ii) the high turnover of ATP due to CO_2 fixation[40].

FCCP presumably dissipates $\Delta\bar{\mu}_{H^+}$ completely by increasing the proton permeability of the membrane[22,41,42] and NH_4Cl *plus* valinomycin, or nigericin *plus* valinomycin, in the presence of K^+, would similarly dissipate $\Delta\bar{\mu}_{H^+}$.

The electron donor systems

The present results provide further support for the interaction of DCIP–ascorbate before a coupling site on an intermediate pathway between the two photosystems[27,43,44]. According to the treatment outlined above, the coupling site would consist of an oxidation–reduction loop generating a proton pump and evidence elsewhere[28] would locate cytochrome *f* on this loop. Under these circumstances, the P518 response would be dependent upon ΔE_s (and whatever component of ΔE_f remained after DCMU treatment) and would be inhibited by FCCP, nigericin *plus* valinomycin, *etc.* The interaction of TMPD–ascorbate (at low concentration of TMPD[45]) after cytochrome *f* and the coupling site[28,46,47] is supported by the absence of a P518 response with this couple.

As indicated by the effects of electron transport inhibitors (Fig. 6), it seems likely that, in the absence of the DCIP couple, a similar coupling site contributes predominantly to the P518 response in intact chloroplasts.

The large negative absorbance changes observed with both donor couples under some conditions may be related to P518. Similar negative absorbance changes have been induced in spinach chloroplasts (G. P. STRICHARTZ, personal communication) and chromatophores[4] in the dark by KOH pulses. It is therefore possible that the responses result from charge transfer in the opposite direction to normal[27]. The smaller negative responses induced by high concentration of FCCP and uncoupling combinations of nigericin *plus* valinomycin, *etc.*, may have a similar explanation.

The chromatic transient response

According to the electrochromic explanation of P518, the transients reflect changes in membrane potential and since it is unlikely that pools of electron transport intermediates would change so rapidly at the changeover from red to far-red light, or *vice-versa*, it seems probable that ΔE_r is affected, perhaps by a change in the quantum efficiency of the two photosystems[15]. The slower changes could more easily be accounted for by changes in an intermediate pool, perhaps of the reducing pool, R, previously predicted[7]. Changes in pools of intermediates could also account for some of the induction phenomena.

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