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THE FUNCTIONS OF PLASTOQUINONE AND β-CAROTENE IN PHOTOSYSTEM II OF CHLOROPLASTS

RAYMOND P. COX* and DEREK S. BENDALL

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW (Great Britain)

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SUMMARY

1. We have investigated the role of plastoquinone and β-carotene in Photosystem II by examining their effects when added back to freeze-dried chloroplasts that had been extracted with heptane.

2. Under our conditions, extraction removed about 99.5% of the β-carotene and 90% of the plastoquinone. Photochemical activities associated with Photosystem II were reduced to a low level and 85–90% of the cytochrome b-559$_{HP}$ (E'_0 = +0.37 V) was converted into a low-potential form.

3. Plastoquinone alone restored the potential of the cytochrome to the original high value. The amount of cytochrome restored by plastoquinone was as great as by the heptane extract, and β-carotene was either ineffective or slightly inhibitory. Plastoquinone allowed a substantial rate of 2,6-dichlorophenolindophenol (DCIP) photoreduction, but a greater rate was observed when β-carotene was also present (in agreement with previous work). β-Carotene improved the efficiency of light utilisation and protected the chloroplasts from photoinactivation.

4. The Photosystem II-linked oxidation of cytochrome b-559$_{HP}$ and reduction of C550 (P546) at 77 °K could only be observed when both β-carotene and plastoquinone were present. A small effect with β-carotene alone could be attributed to unextracted plastoquinone.

5. The results support a model in which C550 (depending on β-carotene) indicates the redox state of the primary acceptor but is not essential for electron flow; β-carotene is needed to maintain the optimum conformation of membrane components for light utilisation and cytochrome photooxidation.

INTRODUCTION

The extraction of freeze-dried chloroplasts with non-polar solvents such as heptane or petroleum ether leaves most of the lipids in situ including nearly all the chlorophyll. Nevertheless, the ability to photooxidize water is lost, but can be re-

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Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

* Present address: Institut de Biologie Physico-chimique, 13 rue Pierre-et-Marie Curie, 75005 Paris, France.

covered by resuspending the chloroplasts in the heptane extract and evaporating off the solvent.

This reversible system was first investigated by Lynch and French [1] who considered the active component of the heptane extract to be β-carotene. Bishop [2] showed that the active factor was plastoquinone which was present as an impurity in the crude β-carotene of Lynch and French. Since then several groups have confirmed the requirement for plastoquinone [3–5]. Recently the role of β-carotene was reconsidered [5, 6, 12] and an enhanced activity was obtained when β-carotene was added back to the extracted chloroplasts in addition to plastoquinone.

Sofrová and Bendall [6] and Okayama and Butler [12] found that extraction with heptane also causes the potential of cytochrome b-559_{HP} to fall from +370 mV to a value about 300 mV lower. The light-induced oxidation of the cytochrome and the parallel photoreduction of C550 by Photosystem II at 77 °K [7–11] are also lost on extraction [6, 12]. All these changes can be reversed by re-addition of the extract to the chloroplasts.

The active components of the extract were shown to be plastoquinone and β-carotene, both of which were necessary for the two light-induced absorbance changes mentioned above [6]. The main aim of the present work has been to show how these two substances influence individual components of Photosystem II, that is cytochrome b-559_{HP} and C550.

The interpretation of experiments of this kind is complicated by the difficulty of ensuring complete extraction of β-carotene and plastoquinone and by the fact that both of these components might be present in more than one fraction [13]. A small amount of a tightly bound fraction would suffice to leave one molecule associated with each chain of electron acceptors.

METHODS

Chloroplast preparation

Peas (*Pisum sativum* var. Laxton's Superb) were grown in the laboratory in moist vermiculite for 3–4 weeks. A yellow variety of Orache (*Atriplex hortensis*) was grown in the garden of Dr R. Hill and covered for 24 h before harvesting to reduce the amount of starch. Pea chloroplasts were prepared essentially according to the method of Cockburn et al. [14]. 25–50 g of leaves were ground in a Polytron (Kinematica GmbH, Luzern) with 200 ml of a medium containing 0.33 M mannitol, 5 mM $MgCl_2$ and 10 mM sodium pyrophosphate adjusted to pH 6.5 at 0 °C with HCl. The chloroplasts were resuspended in a medium containing 10 g/l raffinose, 10 mM NaCl and 10 mM potassium phosphate buffer (pK 7.5) to a concentration of about 1 mg chlorophyll/ml. Orache chloroplasts were prepared similarly except for the addition of 2.5 mM sodium ascorbate to the grinding medium immediately before use, and the inclusion of a brief centrifugation after resuspension of the pellet in the hypotonic medium to remove starch from the broken chloroplasts.

Freeze-drying, extraction and reconstitution

The chloroplast suspension, containing about 1 g of glass beads (Ballotini No. 12)/mg chlorophyll, was frozen onto the walls of a 500-ml round-bottomed flask as a thin layer. Freeze-drying was continued until the flask had reached room temperature (about 3.5 h).

The freeze-dried chloroplasts were extracted at room temperature with heptane (British Drug Houses, IP specification) at a ratio of 30–40 ml of heptane/mg of chlorophyll. The extraction was done in a stoppered conical flask with continuous stirring. A single extraction of 3.5 h was used.

The extracted chloroplasts were separated by a low-speed centrifugation, washed with a small volume of fresh heptane and evaporated to dryness on a rotary evaporator at 25 °C until free-running beads were obtained.

The heptane extract was evaporated to dryness on a rotary evaporator. Pure β-carotene was from Koch-Light or British Drug Houses and plastoquinone-45 was a gift of Hoffmann-La Roche, Basel.

Reconstitution was achieved by resuspending the extracted chloroplasts in the relevant heptane solution and evaporating off the solvent in a rotary evaporator as before.

The amounts of the components of the heptane extract routinely added back were 100 μg β-carotene/mg chlorophyll and 250 μg plastoquinone/mg chlorophyll. This is about three times the amounts found to be present in normal chloroplasts, in agreement with previous results [15, 16]. When reconstitution was with the chloroplast extract an amount corresponding to three times the original ratio of extract to chloroplasts was used.

Dry preparations were resuspended by shaking them in aqueous media and the suspension of chloroplasts was then removed after the beads had settled out.

During freeze-drying and subsequent manipulations the chloroplasts were protected from the light as much as possible.

The use of glass beads made the material rather easier to handle. Raffinose was found to exert a protective effect on the high-potential character of cytochrome b-559_{HP} during freeze-drying.

Assay of β-carotene and plastoquinone

For the assay of β-carotene chloroplasts were extracted three times with acetone (50 ml/mg chlorophyll for each extraction) at room temperature. The acetone extract was filtered (Whatman No. 1) and evaporated to dryness in a rotary evaporator. The dry material was dissolved in a small volume of heptane and applied to a column prepared by suspending dry alumina (British Drug Houses, for chromatographic analysis) in heptane. The β-carotene was eluted with 2% (v/v) acetone in heptane and measured spectrophotometrically. The accuracy of this procedure was confirmed by checking the recovery of known amounts of added β-carotene.

Plastoquinone was measured by the method of Redfearn and Friend [16].

Other methods

Cytochromes were assayed by the methods of Bendall et al. [17]. Chloroplasts were resuspended in a medium containing 0.33 M mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM potassium phosphate buffer (pH 6.5). Light induced absorbance changes were measured as described by Bendall and Sofrová [7].

The photoreduction of 2,6-dichlorophenolindophenol (DCIP) was measured as described previously [18]. Oxygen evolution was measured with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge). The chloroplasts were suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaCl

to give a concentration equivalent to 30–50 μg chlorophyll/ml. The reaction medium also contained 3.3 mM NH_4Cl as an uncoupling agent and 20 μM DCIP as electron acceptor. 1.7 mM potassium ferricyanide was added to keep the DCIP in the oxidized state.

A slide projector was used as the source of actinic light. The beam was passed through a red filter (Schott RG 610) to give an intensity at the sample of about 90 mW/cm^2. The intensity of the beam was reduced with Balzers neutral density filters for the determination of light intensity curves.

Chlorophyll was assayed by the method of Arnon [19]. All reagents used were AR Grade where available.

RESULTS

The amounts of residual β-carotene and plastoquinone in freeze-dried chloroplasts after different times of extraction with heptane are shown in Fig. 1. After 200 min of extraction only about 0.5% of the original β-carotene was left. This amount corresponds to less than 1 molecule per System II reaction centre and may be regarded as negligible. On the other hand the quantity of plastoquinone remaining was significant, especially as it may exist in the chloroplast in more than one pool [13].

Previous results on the effect of plastoquinone and β-carotene on water oxidation were confirmed (Table I). Plastoquinone alone caused a substantial restoration of DCIP photoreduction. A greater restoration was obtained in the presence of plastoquinone and β-carotene together, although β-carotene alone had no effect.

A study of the effects of β-carotene and plastoquinone on the reconstitution of the high-potential character of cytochrome b-559_{HP} showed that plastoquinone is the active constituent of the extract (Table II). β-Carotene had no effect by itself and did not enhance the effect of plastoquinone.

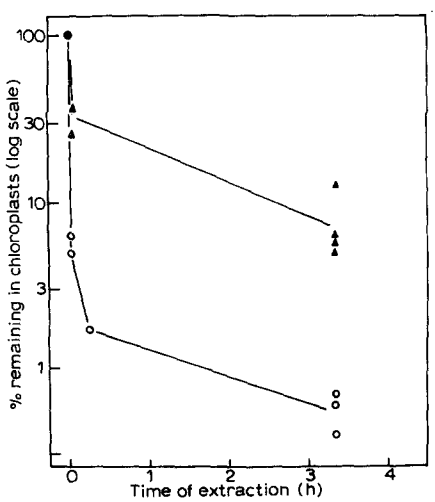

Fig. 1. Residual β-carotene (○) and plastoquinone (▲) in freeze-dried pea chloroplasts after different times of extraction with heptane. See Methods for details.

TABLE I

PHOTOREDUCTION OF DCIP IN EXTRACTED PEA CHLOROPLASTS RECONSTITUTED WITH DIFFERENT COMPONENTS OF THE HEPTANE EXTRACT

The absolute activity of DCIP photoreduction was 240 μmoles/mg chlorophyll per h in fresh chloroplasts and 180 μmoles/mg chlorophyll per h in chloroplasts immediately after freeze-drying. Freeze-drying, extraction, reconstitution and the measurement of DCIP photoreduction were as described in the Methods section. The two experiments represent different reconstitutions of the same batch of extracted chloroplasts, which contained 0.5 % of the β-carotene and 8 % of the plastoquinone present in the freeze-dried chloroplasts.

	Activity (% of freeze-dried control)	
	Expt 1	Expt 2
Extracted	0	0
Reconstituted with β-carotene	2	4
Reconstituted with plastoquinone	47	44
Reconstituted with β-carotene and plastoquinone	73	69

A more precise measurement of the potential of the cytochrome by redox titration (Fig. 2) confirmed that plastoquinone alone is able to restore the potential to its value in unextracted chloroplasts (+370 mV) [17].

By contrast we found that both plastoquinone and β-carotene were necessary for the maximum restoration of the photooxidation of cytochrome b-559_{HP} and the photoreduction of C550. The results of a series of experiments with orache chloroplasts early in the season, when the cytochrome/chlorophyll ratio was high, is shown in Fig. 3. Although a significant effect was observed with β-carotene alone this could be attributed to the influence of unextracted plastoquinone.

The results reported in Fig. 3 confirm the conclusions of Sofrová and Bendall

TABLE II

RECONSTITUTION OF THE HIGH-POTENTIAL CHARACTER OF CYTOCHROME b-559_{HP} WITH DIFFERENT COMPONENTS OF A HEPTANE EXTRACT

The residual β-carotene in extracted chloroplasts was 0.5 %. Experimental details are given under Methods.

	Relative amount of cytochrome b-559_{HP}			
	Pea		Orache	
	Expt 1	Expt 2	Expt 3	Expt 4
Freeze-dried chloroplasts	100	100	100	100
Extracted with heptane	12	16	10	–
Extracted+extract	47	54	54	66
Extracted+β-carotene	9	13	–	–
Extracted+plastoquinone	50	70	50	57
Extracted+β-carotene and plastoquinone	35	41	–	–

[6]. However, this type of experiment does not make it clear whether the effects of β-carotene and plastoquinone reflect a direct chemical relation with C550, or whether the light-induced electron transfer process is dependent on these substances. We therefore examined the effects of plastoquinone and β-carotene on the dark reduction of C550 by dithionite [7]. The results were essentially the same as had been obtained by photochemical reduction.

Both plastoquinone and β-carotene are present in chloroplasts in relatively large amounts and perform two kinds of function, one dependent on the presence of large quantities (electron transfer pool and accessory pigment respectively) and the other associated closely with the reaction centres of Photosystem II. The question arises whether the latter function is dependent on a small fraction which could be distinguished by the strength of binding. We therefore examined the effects of small quantities of β-carotene on the low-temperature light reactions in the presence of the

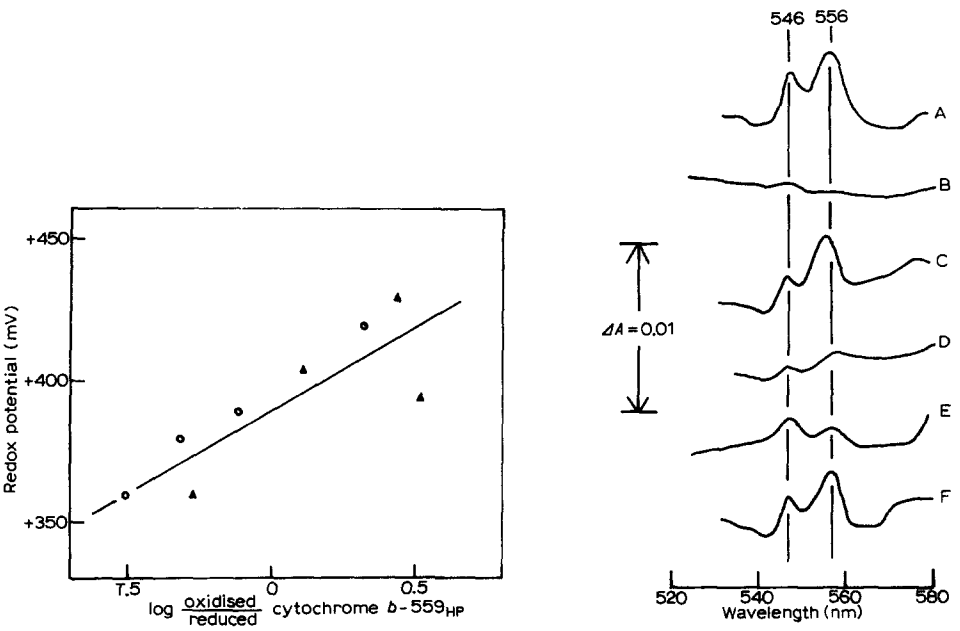

Fig. 2. Measurement of the redox potential of cytochrome b-559$_{HP}$ in freeze-dried pea chloroplasts (▲) and in extracted chloroplasts reconstituted with plastoquinone (○). Chloroplasts were resuspended at a concentration equivalent to 170 μg chlorophyll/ml in the pH-6.5 medium used for the cytochrome assays. The negative sample was oxidised with 1.25 mM potassium ferricyanide. The height above the baseline at 560 nm was measured for different mixtures of potassium ferricyanide and potassium ferrocyanide (total concentration 3–5 mM) added to the positive sample. The fully reduced cytochrome was obtained from the difference spectrum: hydroquinone reduced minus ferricyanide oxidised. For the ferricyanide couple a value $E'_0 = +430$ mV was assumed. The line was drawn with the theoretical slope of 59 mV per decade, indicating $n = 1$.

Fig. 3. Light-induced absorbance changes of orache chloroplasts at 77 °K. Chloroplasts were resuspended at a concentration of 70 μg chlorophyll/ml and reduced with hydroquinone (2.5 mM) before being frozen in the dark. The difference spectrum produced by actinic illumination of the negative sample is shown. A, freeze-dried chloroplasts; B, freeze-dried chloroplasts after extraction with heptane; C–F, extracted chloroplasts after reconstitution with the following: C, extract, D, plastoquinone; E, β-carotene; F, β-carotene and plastoquinone. For further details see Methods.

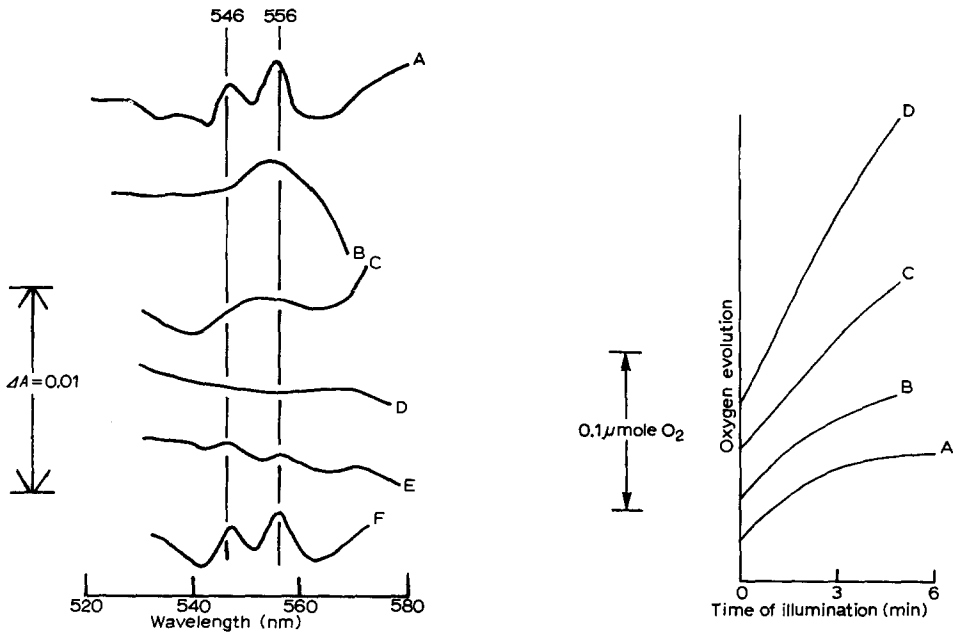

Fig. 4. Light-induced absorbance changes of orache chloroplasts at 77 °K in the presence of low concentrations of β-carotene. Chloroplasts were resuspended at a concentration of 120 μg chlorophyll/ml in the pH-6.5 medium used for the cytochrome assays and reduced with hydroquinone (2.5 mM) before being frozen in the dark. The difference produced by actinic illumination of the negative sample is shown. Freeze-dried chloroplasts were extracted with heptane and reconstituted with A, extract; B, β-carotene (100 μg/mg chlorophyll); C–F, plastoquinone (250 μg/mg chlorophyll) and the following amounts of β-carotene per mg of chlorophyll: C, none; D, 7 μg; E, 17 μg; F, 100 μg. For further details see Methods.

Fig. 5. O_2 evolution by freeze-dried pea chloroplasts extracted with heptane and reconstituted with plastoquinone (250 μg/mg chlorophyll) and the following amounts of β-carotene per mg of chlorophyll: A, none; B, 2 μg; C, 30 μg; D, 100 μg. The ordinate represents the amount of O_2 liberated in a reaction volume of 2 ml. The chloroplasts were resuspended at a concentration of 60 μg chlorophyll/ml. The electron acceptor was DCIP. Further details are given in the Methods section.

quantity of plastoquinone normally added to the chloroplasts. The results (Fig. 4) showed that the quantity of β-carotene required for a substantial extent of photooxidation of cytochrome b-559_{HP} and photoreduction of C550 was of the same order as that present in normal chloroplasts. In this experiment, which was more typical than those shown in Fig. 3, no restoration could be seen with β-carotene alone.

Similarly the rates of O_2 evolution with DCIP as electron acceptor were studied in the presence of varying amounts of β-carotene and the normal quantity of plastoquinone. Two effects of β-carotene are apparent in Fig. 5. Firstly β-carotene protected the system from a rapid photoinactivation which occurred in extracted chloroplasts to which plastoquinone alone had been added. This is in agreement with the well-known protective effect of carotenoids [20]. Secondly β-carotene stimulated the initial rate of O_2 evolution as already shown for dye reduction in Table I and in refs 5 and 6. Fig. 5 showed that both effects depended on a rather large amount of β-carotene. The best results were obtained with a quantity of β-carotene about three times greater

than that present in extracted chloroplasts. The requirement for such large quantities may reflect the difficulty of returning β-carotene to its normal position in the chloroplast simply by evaporating a heptane solution containing a suspension of the particles.

We would have been unable to demonstrate any effect dependent on a small tightly-bound fraction of plastoquinone because of the difficulty of its extraction from the chloroplasts. However, Table II and Fig. 1 show that the amount of cytochrome b-559$_{HP}$ present in extracted chloroplasts is comparable to that of the residual plastoquinone. In chloroplasts extracted for only 2 min, which contained a larger amount of residual plastoquinone (Fig. 1), there was an even larger amount of cytochrome b-559$_{HP}$, again comparable to the amount of plastoquinone. This suggests that the high-potential character of cytochrome b-559$_{HP}$ depends on the presence of the bulk plastoquinone rather than on a special fraction more or less resistant to heptane extraction.

Finally, we have investigated the dependence of the rate of O_2 evolution on light intensity in various preparations. This type of experiment enables a distinction to be made between effects on photochemical reactions and dark electron transfer processes.

The effect of freeze-drying on the light intensity curve is shown in Fig. 6. In this experiment the recovery of activity, measured at high light intensity, was about 50%; in general the recovery varied between 50 and 65%. The results at low intensities indicated that the activity of the reaction centres or the water oxidising enzyme system which is closely bound to the centres, tended to suffer even more during the process of freeze-drying. A good recovery of activity was found to depend on the presence of sugar in the medium from which the chloroplasts were dried. Although a higher concentration of sugar than that used in these experiments would improve the recovery, plastoquinone would then become more difficult to extract.

Fig. 7 shows that the stimulatory effect of β-carotene on O_2 evolution (Fig. 5) is apparent at low light intensities as well as at high intensities. Thus the stimulatory

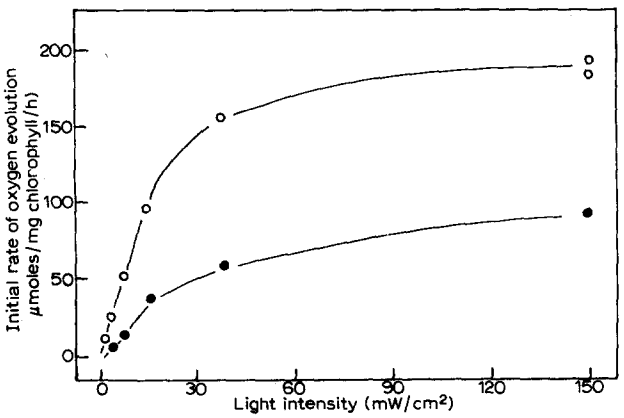

Fig. 6. Effect of light intensity on the rate of O_2 evolution by pea chloroplasts with DCIP as electron acceptor. ○, fresh chloroplasts; ●, freeze-dried chloroplasts. Further details are given in the Methods section.

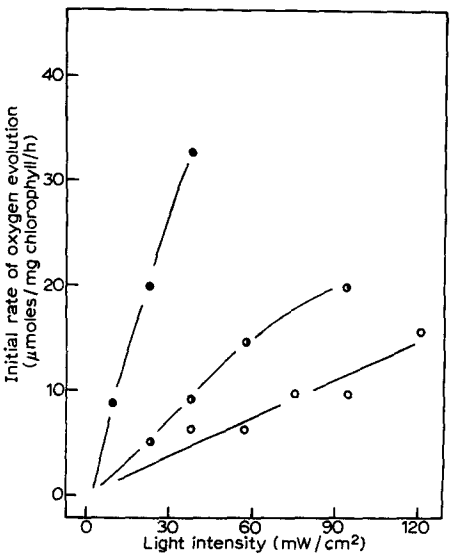

Fig. 7. Effects of plastoquinone and β-carotene on the initial rate of O_2 evolution by pea chloroplasts at low light intensities. ●, freeze-dried chloroplasts; ○, freeze-dried chloroplasts extracted with heptane and reconstituted with plastoquinone; ◑, freeze-dried chloroplasts extracted with heptane and reconstituted with both β-carotene and plastoquinone. The electron acceptor was DCIP. Further details are given in the Methods section.

effect of β-carotene is due, at least partially, to a more efficient use of light quanta by the reaction centre.

DISCUSSION

The extraction of freeze-dried chloroplasts with heptane is a reversible process that can be used to give information about the roles of plastoquinone and β-carotene in Photosystem II.

Classically, two roles have been ascribed to β-carotene in photosynthetic systems. Its role in the absorption of light to provide energy for photosynthesis need not be considered in the interpretation of the experiments reported in the present paper, for in these red light was used for all actinic illumination. The ability of β-carotene to protect the chloroplast from irreversible photochemical damage is illustrated in Fig. 5. However, in addition, the experiments of Girault et al. [5], Sofrová and Bendall [6] and Okayama and Butler [12], as well as those reported here, demonstrate that β-carotene has a more direct function in the photochemistry of Photosystem II. This is shown most clearly by the observation that β-carotene is able to stimulate the rate of O_2 evolution at low light intensities (Fig. 7). In the absence of added β-carotene (but in the presence of plastoquinone) a significant rate of O_2 evolution was still observed and thus the requirement for β-carotene does not seem to be obligatory because the residual β-carotene in the extracted chloroplasts was of the order of 0.2 molecule per Photosystem II reaction centre. β-Carotene has also been shown to be required for the photooxidation of cytochrome b-559_{HP} and the photoreduction of

C550 at low temperature [6, 12]. Both reactions depend on the activity of Photosystem II [7–11] but in the case of the cytochrome the reaction has been shown not to be an essential part of the O_2 evolution process [18]. Many of the properties of C550 are consistent with its proposed function as the primary acceptor of Photosystem II. However, our results strongly suggest that it cannot play an essential part in normal electron transport through Photosystem II because substantial rates of O_2 evolution could be observed in the absence of detectable C550 (we estimate the residual C550 to be less than 10% of normal). C550 could then be regarded either as an alternative acceptor, perhaps one which functions only under special conditions, or as a pigment which does not itself undergo oxidation or reduction but which is sensitive to the redox state of the actual acceptor.

The main role of plastoquinone, as a relatively large, semi-mobile pool of carrier between the two photosystems, is well established [21]. The type of experiment reported in the present paper raises the question of whether plastoquinone is more intimately concerned with the mechanism of Photosystem II. The most clear-cut result in the present work was that extraction with heptane caused a dramatic fall in the potential of cytochrome b-559_{HP} which could be fully reversed by the addition of plastoquinone alone. The quantity of plastoquinone required to produce this effect was of the same order as that normally present in the chloroplast, and thus the plastoquinone pool seems to influence the redox potential of the cytochrome, presumably through an effect on the conformational state of the protein. However, the reports of Okayama and Butler [12] and Sofrová and Bendall [6] suggest that after some kinds of treatment β-carotene can also have an influence on the potential of the cytochrome.

Stiehl and Witt [22] have discovered in chloroplasts a very rapid increase in absorption at 320 nm which they ascribe to the primary photochemical reaction (reduction of an electron acceptor) of Photosystem II. Their proposal that the observed difference spectrum represents the reduction of plastoquinone to its semiquinone has received some, although not unqualified, support from the recent observations of Bensasson and Land [23] on the absorption spectra of plastosemiquinone in methanol solution. Unfortunately, our extraction procedure leaves a quantity of plastoquinone in the chloroplast which is equivalent to at least one molecule per reaction centre of Photosystem II, so no direct conclusions can be drawn concerning the postulated role of plastoquinone as primary acceptor. However, the simplest interpretation of the fact that photoreduction of C550 seemed to depend on the presence of plastoquinone is that the primary acceptor is a small fraction of plastoquinone in a special environment whereas C550 is a component which depends on the presence of β-carotene and which exhibits a small shift in absorption spectrum in response to the reduction of the primary acceptor.

Okayama and Butler [12] reported some restoration of the photoreduction of C550 by the addition of β-carotene alone to extracted chloroplasts, but they provided no information on the efficiency of extraction of plastoquinone. Apart from this, and the failure of Okayama and Butler to observe O_2 evolution in the absence of β-carotene, which seems likely to be due to the greater inactivation produced by illumination under their conditions, our results are in close agreement with theirs.

From the functional point of view some important questions remain unanswered. From the molecular point of view, it has now become clear that not only do β-carotene and plastoquinone have specific functions of their own but they can also

have secondary effects through modification of certain protein components of the membrane. In the case of plastoquinone this has become evident from its effect on cytochrome b-559_{HP}; in the case of β-carotene the most plausible explanation of its effect on O_2 evolution would seem to be in terms of an influence on the conformational state of certain components, as yet undetermined, of Photosystem II.

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