Biochimica et Biophysica Acta, 1060 (1991) 106–114

ADONIS 000527289100227C

BBABIO 43477

β-Carotene within the isolated Photosystem II reaction centre: photooxidation and irreversible bleaching of this chromophore by oxidised P680

A. Telfer, J. De Las Rivas and J. Barber

AFRC Photosynthesis Research Group, Department of Biochemistry, Wolfson Laboratories, Imperial College of Science, Technology and Medicine, London (U.K.)

(Received 18 March 1991)

Key words: Photosynthesis; Photosystem II; Reaction center; Photoinhibition; β-Carotene; Primary donor; P680

Illumination of isolated Photosystem II reaction centres in the presence of the electron acceptors, silicomolybdate (SiMo) or 3,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), leads to selective photooxidation and irreversible photobleaching of β-carotene. No such effect is observed in the absence of the electron acceptors and it is dependent on the ability of the reaction centres to carry out charge separation. Flash absorption studies indicate that prior to the irreversible photobleaching, β-carotene is photooxidised by electron transfer to $P680^+$. The rate of photobleaching of β-carotene is faster when SiMo is used as the acceptor and occurs both in the presence and absence of oxygen. However, with DBMIB present photobleaching is more clearly observed when oxygen is present. It is argued that when oxygen is absent, photoreduced DBMIB can rapidly rereduce $P680^+$ by an electron transfer cycle involving cytochrome *b*-559, while in aerobic conditions the cycle is partially inhibited by oxygen acting as an electron acceptor. When Mn(II) is added as an electron donor to $P680^+$, no photobleaching of β-carotene occurs. The kinetics of photobleaching shows two phases, with 50% loss of the total β-carotene pool occurring rapidly. Coupled with the loss of β-carotene is a photobleaching of accessory chlorophyll which absorbs at 670 nm. Therefore our results indicate that, when the Photosystem II reaction centre is photoactivated under conditions in which $P680^+$ can photoaccumulate, there is a secondary oxidation of β-carotene and accessory chlorophyll which leads to irreversible photobleaching. No such photobleaching occurs if $P680^+$ is rapidly reduced by an exogenous electron donor or by a quinone dependent cyclic flow of electrons around PSII. We discuss the physiological role of β-carotene oxidation and cyclic electron transport in the function of PSII in vivo.

Introduction

The isolated Photosystem II (PSII) reaction centre consisting of the D1 and D2 polypeptides, the cytochrome *b*-559 α and β subunits and the I protein [1–4] is not only proving to be an excellent experimental system for studying primary and secondary electron transfer processes [5–10] but also for investigating the susceptibility of this reaction centre to photodamage [11,12]. These studies may have important implications for understanding the molecular processes which underlie the vulnerability of the PSII reaction centre to damage within intact photosynthetic organisms as expressed by the physiological stress condition known as photoinhibition [13,14].

In previous communications [11,12] we, and also others [15,16], have emphasised and studied aspects of the photodamaging processes which occur when the isolated PSII reaction centre is exposed to strong light. In particular, when molecular oxygen is present, the illumination of the isolated D1/D2/cytochrome *b*-559 complex causes a selective photobleaching of chlorophyll which absorbs at about 680 nm and which almost

Abbreviations: Car, carotenoid; Chl, chlorophyll; Cyt, cytochrome; D1 and D2 polypeptides, products of the *psb*A and *psb*D genes respectively; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FeCy, ferricyanide; HPLC, high performance liquid chromatography; P680, primary electron donor to PSII; Pheo, pheophytin; PSII, Photosystem II; Q_A and Q_B, secondary quinone electron acceptors in PSII; SiMo, silicomolybdate ($SiMo_{12}O_{40}^{4-}$); Y_Z and Y_D, redox active tyrosines in PSII.

Correspondence: A. Telfer, AFRC Photosynthesis Research Group, Department of Biochemistry, Wolfson Laboratories, Imperial College of Science, Technology and Medicine, London SW7 2AY, U.K.

certainly constitutes the primary electron donor P680 [12]. In agreement with this conclusion is the finding that the loss of this chlorophyll is paralleled by a loss of photochemical activity measured by electron transport capacity [11] and by inhibition of radical pair formation [9,17]. When oxygen is removed the reaction centre becomes more resistant to photodamage and the lifetime of the P680 triplet state lengthens from 33 μs to 1 ms [8,10]. These results indicate that under aerobic conditions the selective photobleaching of the P680 chlorophylls is probably due to singlet oxygen formation generated by the P680 triplet state [10]. Partial protection against photodamage can also be achieved by adding an electron acceptor such as silicomolybdate (SiMo). This reduces the yield of the P680 triplet by competing with the recombination reaction which generates the triplet state [12,18,19]. In this case the effect of prolonged illumination is to cause, initially, a preferential irreversible bleaching of chlorophylls which absorb maximally at 670 nm [12]. This bleaching also occurs under anaerobic conditions and seems to be due to the oxidation of accessory chlorophyll(s) within the PSII reaction centre by $P680^+$, which is photoaccumulated in the presence of SiMo [2,19,20].

Recently it has become clear that, despite the close homology between the reaction centres of purple photosynthetic bacteria and PSII, there are significant differences in the chromophore content [21–23]. If PSII reaction centres are isolated under stabilising conditions, there are apparently two β-carotene and five to six chlorophyll molecules per two pheophytins, i.e. per reaction centre. Recently spectroscopic studies of the chromophores of isolated PSII reaction centres have shown that the two β-carotenes are in the all *trans* conformation in the native purified complex [24] and that they are sufficiently close to allow excitonic interaction [25]. In this paper we have extended our studies of photodamage within the isolated PSII reaction centre by investigating the susceptibility of the carotenoids and have detected photooxidation and irreversible photobleaching of β-carotene as well as accessory chlorophyll. It has been argued for some time that carotenoids have two important roles in photosynthesis: to act as accessory light harvesting pigments in the antenna system and to protect against photodynamic damage [26,27]. We therefore discuss our results in terms of the ability of β-carotene to protect the PSII reaction centre against photodamage.

Materials and Methods

Reaction centres of PSII were isolated from pea thylakoids using the method previously described [28] in which the second column chromatography step was conducted in the presence of 2 mM dodecyl maltoside and 50 mM Tris-HCl (pH 7.2). All measurements were carried out in 50 mM Tris-HCl buffer (pH 8.0) plus 2 mM dodecyl maltoside.

Absorption spectra were measured in an SLM Instruments DW2000 dual wavelength spectrophotometer equipped for side illumination and set with a bandwidth of 2 nm. Light induced absorbance changes were excited with red light (2 mm Schott RG665 cut off filter and a KG1 heat filter). The photomultiplier was protected from stray light with a 4 mm Schott BG18 filter.

Photodamage of PSII reaction centres with and without electron acceptors, was induced by red (RG665 filter) or white light in the temperature controlled cuvette holder, which was maintained at 10°C. Reaction conditions and chlorophyll concentrations were as described in the figure legends. Preinactivation of PSII reaction centres, for the experiment of Fig. 6, was carried out as follows. PSII reaction centres equivalent to 22 μg ml^{-1} chlorophyll were illuminated for various times with white light (approx. 5000 μmol photons m^{-2} s^{-1}) in the absence of electron acceptor. Samples were withdrawn at fixed intervals and stored on ice in the dark. They were then diluted 10-fold with the same buffer and their photodamage difference spectra were measured (light treated minus dark control). 100 μM SiMo was then added and their ability to show a light-induced irreversible decrease in absorbance at 485 nm was measured using 500 μmol photons m^{-2} s^{-1}, red light.

High performance liquid chromatography (HPLC) analysis of the pigments was carried out on a reverse phase Spherisorb S5ODS1 column (5 μm, 250 × 4.6 cm I.D.) using the mobile phase described by De Las Rivas et al. [29]. The pigments were extracted from the reaction centres dissolved in buffers by addition of 9 vol. of 100% HPLC grade acetone and after mild agitation they were filtered through a 0.2 μm fluoropolymer filter (ACRO™ LC13). 20 μl aliquots (equivalent to 100 ng chlorophyll in the original dark control samples) were injected for analysis and peak detection was measured with a UV-VIS Kontron spectrophotometer, at 450 nm for β-carotene and 663 nm for chlorophyll *a* and pheophytin *a*.

Flash induced transient absorption changes were measured at 4°C according to Durrant et al. [10] using a nitrogen laser giving subnanosecond flashes at 337 nm to excite the samples. The measuring beam, from a xenon source, was passed through a cut-off filter (transmitting above 615 nm) in addition to the monochrometer. Signals are the average of 64 flashes, measured with a 3 μs time constant and 4 nm bandwidth.

Results

Fig. 1 shows the effect of illuminating isolated PSII reaction centres with red light on their absorption at

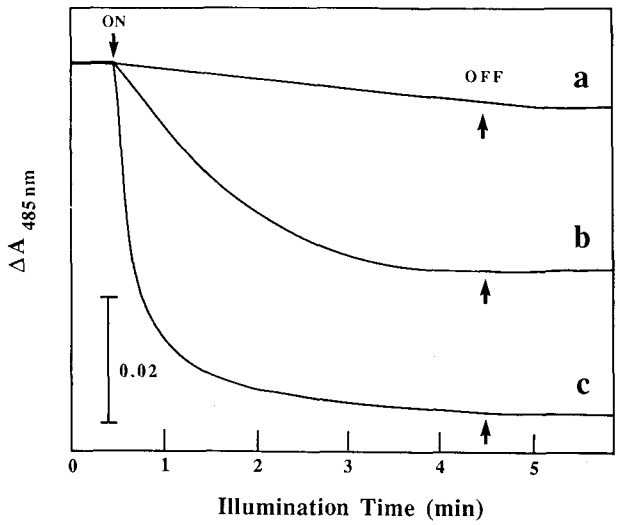

Fig. 1. Irreversible photobleaching of absorption at 485 nm in isolated PSII reaction centres requires the presence of an electron acceptor to stabilise $P680^+$. Reaction centres equivalent to 2.2 μg ml^{-1} chlorophyll were suspended in 50 mM Tris-HCl buffer (pH 8.0) and 2 mM dodecyl maltoside. (a) no additions, (b) plus 100 μM DBMIB, (c) plus 100 μM SiMo. Illumination was with 600 μmol photons m^{-2} s^{-1} of red light (> 665 nm).

485 nm. There is a decrease in absorbance but only when an electron acceptor is present (traces c and d). The decrease is essentially irreversible. With 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) present there are two kinetic phases to the bleaching, an initial fast component followed by a slower decrease (confirmed by semilog plots, not shown). In the presence of SiMo the rate of bleaching is much faster but the decrease is still biphasic.

In order to identify the absorbing species bleached in the experiment of Fig. 1 we measured the absorption spectra of PSII reaction centres (Fig. 2a and c) illuminated with white light for 0, 1 and 4 min, with and without DBMIB. In the absence of electron acceptor the major bleaching occurs in the blue and red part of the spectrum which is characteristic of chlorophyll absorption (Fig. 2a). The presence of DBMIB has two effects (Fig. 2c). It reduces the rate of chlorophyll bleaching but stimulates a dramatic decrease in absorbance around 485 nm which suggests a loss of β-carotene, the carotenoid present in this preparation [1,21]. The absorption difference spectra (light treated-minus-dark control, which we will refer to as the photodamage absorption difference spectra) are shown in Fig. 2b and d. The bleaching at 435 nm and 679 nm (which is smaller when DBMIB is present) is presumably due mainly to loss of P680, while the bleach at 486 nm, with shoulders at approximately 460 and 510 nm (seen in the presence of DBMIB, Fig. 2c and d), is presumably due to loss of β-carotene.

A very similar bleaching is seen when using SiMo as acceptor, except that as found previously, there is an initial preferential bleaching at 670 nm compared to 680 nm (Fig. 3) which is not seen with DBMIB (Fig. 2d). The photodamage absorption difference spectra with the two acceptors are otherwise very similar except that again the bleaching in the 486 nm region is faster with SiMo despite the fact that in this experi-

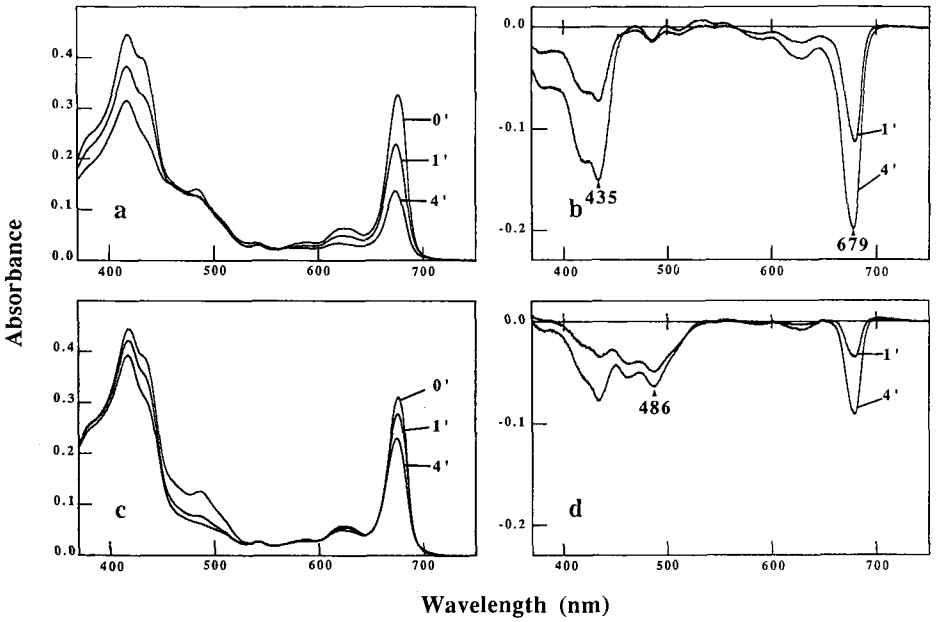

Fig. 2. Absorption characteristics of PSII reaction centres after photobleaching in the presence (c, d) and absence (a, b) of 100 μM DBMIB. Reaction centres equivalent to 5 μg ml^{-1} chlorophyll were suspended in buffer as in Fig. 1. Photobleaching was induced by 5000 μmol photons m^{-2} s^{-1} white light. (a) Absorption spectra after 0, 1 and 4 min illumination. (b) Absorption difference spectra after 1 and 4 min illumination (light treated minus dark control spectra).

ment photodamage was brought about by a lower intensity of red light as compared to the white light used in the experiment of Fig. 2. The rate of photobleaching was both light intensity and chlorophyll concentration dependent. In Fig. 3 the baseline is somewhat shifted over the wavelength range examined because of colour changes occurring as the result of the reduction of SiMo.

The data presented here show that, when an electron acceptor is present, illumination of PSII reaction centres leads to the photobleaching of a substantial amount of the β-carotene present in this preparation. The most likely mechanism is that stabilisation of $P680^+$ by electron acceptors brings about the oxidation of carotenoid in addition to the accessory chlorophyll, Chl670, reported previously [12], and this subsequently leads to irreversible bleaching of β-carotene as well as the chlorophyll. The bleaching (seen in Fig. 2, for example) could not be reversed by addition of reducing agents; neither ascorbate nor dithionite were effective. A number of artificial electron donors (e.g. $MnCl_2$), however, will rereduce $P680^+$ and support net electron transport to SiMo [2,11,15]. We therefore examined the effect of the presence of $MnCl_2$ during the exposure of the reaction centre to illumination with SiMo as the acceptor. As can be seen in Fig. 3 (dotted line) the donor provides very substantial protection against photooxidation and destruction of both β-carotene and the chlorophylls of the reaction centre. Again the baseline change can be attributed to net reduction of SiMo. This experiment suggests that β-carotene and Chl670 are oxidised by $P680^+$ but that $MnCl_2$ is a more

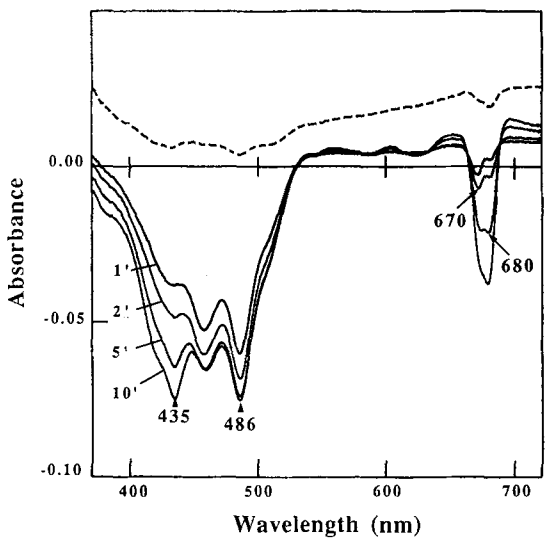

Fig. 3. Difference spectra of irreversible changes (light treated minus dark control) in absorbance of PSII reaction centres illuminated in the presence of 100 μM SiMo. Illumination was with red light, 600 μmoles photons m^{-2} s^{-1} and chlorophyll concentration was 4 μg ml^{-1}. (Solid line) no additions, after 1, 2, 5 and 10 min illumination; (dotted line) plus 1 mM $MnCl_2$ after 1 min illumination.

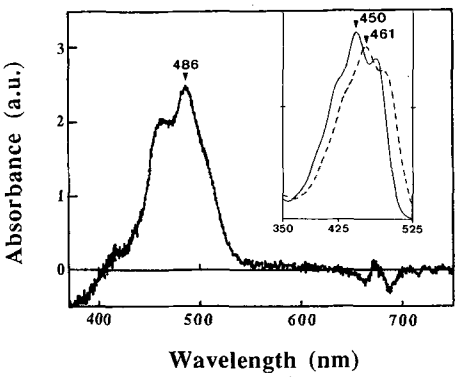

Fig 4. 'In situ' spectrum of β-carotene bleached in isolated PSII reaction centres plus 100 μM DBMIB. Inset: the spectrum of β-carotene extracted in *n*-hexane (solid line) and chloroform (dotted line). The 'in situ" spectrum is the difference between the 4 min spectrum of Fig. 2d (normalised at 679 nm to the 1 min spectrum) and the 1 min spectrum.

efficient electron donor than either of these chromophores thus rereducing $P680^+$ sufficiently rapidly to provide almost complete protection against photodamage.

As carotenoid bleaching is much more rapid than the chlorophyll loss it is possible to obtain the spectrum of the carotenoid 'in situ' (Fig. 4). Assuming that there is no contribution of carotenoid to the absorbance at 679 nm, the 4 min difference spectrum of Fig. 2d (plus DBMIB) was normalised at 679 nm to the 1 min data. The 1 min data was then subtracted from the 4 min normalised spectrum to give the 'in situ' spectrum of β-carotene. Fig. 4 compares the spectrum obtained with the spectrum of β-carotene extracted from whole leaves in *n*-hexane and chloroform. Presumably because of its association with protein, the spectrum is red-shifted, narrowed and the peak ratios are different in comparison to the spectrum in organic solvent. The 'in situ' spectrum shows a small amount of interference from a chlorophyll bandshift, seen in the 670 to 680 nm region, which also causes some distortion of the 'in situ' spectrum of β-carotene to the blue of its absorption peaks.

So far we have equated the light induced absorbance decrease at 485 nm (Fig. 1) with loss of β-carotene. In Fig. 5 we show the actual loss of β-carotene, chlorophyll *a* and pheophytin *a* induced by illumination in the presence of DBMIB, detected by high performance liquid chromatography (HPLC) of the extracted pigments. Fig. 5 shows that approx. 50% of the β-carotene is lost rapidly during the first approx. 7 min illumination. There was a linear relationship between the kinetics of loss of β-carotene measured by HPLC and the loss of absorbance at 485 nm (data not shown). However, a 50% loss of β-carotene was equivalent to a 35% decrease in absorbance at 485 nm indicating that there is some other chromophore in addition to β-carotene which absorbs at this wave-

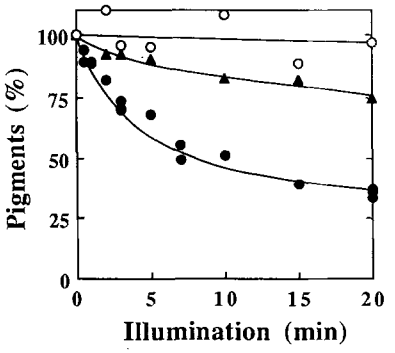

Fig. 5. The effect of illumination of PSII reaction centres in the presence of 100 μM DBMIB on β-carotene (•), chlorophyll *a* (▲) and pheophytin *a* (○) levels. Reaction centres equivalent to 50 μg ml^{-1} chlorophyll were illuminated with 600 μmol photons m^{-2} s^{-1} red light and 100 μl samples were withdrawn at fixed times for analysis by HPLC as described in the Materials and Methods. All results were expressed as the % of the initial dark control level.

length but which is not bleached under these conditions. The rate of bleaching was relatively slow because of the high chlorophyll concentration used in this experiment. Although pheophytin *a* was not bleached, chlorophyll *a* was destroyed slowly and essentially monophasically. We consistently found, both with DBMIB and SiMo as electron acceptor, that there was a rapid loss of 50% of the β-carotene pool followed by a slower bleaching of the remainder.

If carotenoid bleaching is the result of carotenoid oxidation by P680$^+$, it should occur only in PSII reaction centres which are able to carry out light-induced charge separation. Inactivation of charge separation can be brought about by illumination of reaction centres in the absence of electron acceptor, under aerobic conditions [11,15]. In the experiment of Fig. 6, reaction centres were illuminated first in the absence of electron acceptor. This was carried out at a relatively high concentration of chlorophyll (22 μg ml^{-1}). After preillumination, aliquots were removed and diluted 10-fold for measurement of their absorption spectrum (to monitor loss of P680). The photodamage difference spectra obtained (Fig. 6a) were essentially the same as those of Fig. 2b. SiMo was then added to these samples and their ability to show bleaching at 485 nm was measured (Fig. 6b). Bleaching of carotenoid was essentially totally inhibited in samples which had been preilluminated for approx. 4 min or longer. This correlated well with the rapid rate of loss of absorbance at 680 nm seen during preillumination which relates directly to loss of charge separation capability (not shown). This shows that β-carotene bleaching occurs only in photochemically active reaction centres.

We have suggested that the mechanism leading to irreversible bleaching of the carotenoid may be via its oxidation. The Car$^+$ radical absorbs in the infrared, with a broad peak at about 980 nm [30,31]. Fig. 7 shows flash induced transient absorption changes of PSII reaction centres plus an electron acceptor (DBMIB). The changes at 950 nm are due mainly to Car$^+$, while those at 820 nm are attributable to P680$^+$. On this time scale no absorption changes were seen at either 820 nm or 950 nm in the absence of electron acceptor. The decay of P680$^+$ is multiphasic but includes an intermediate component that correlates with the slow rise in the 950 nm signal (τ approx. 0.8 ms). The two signals

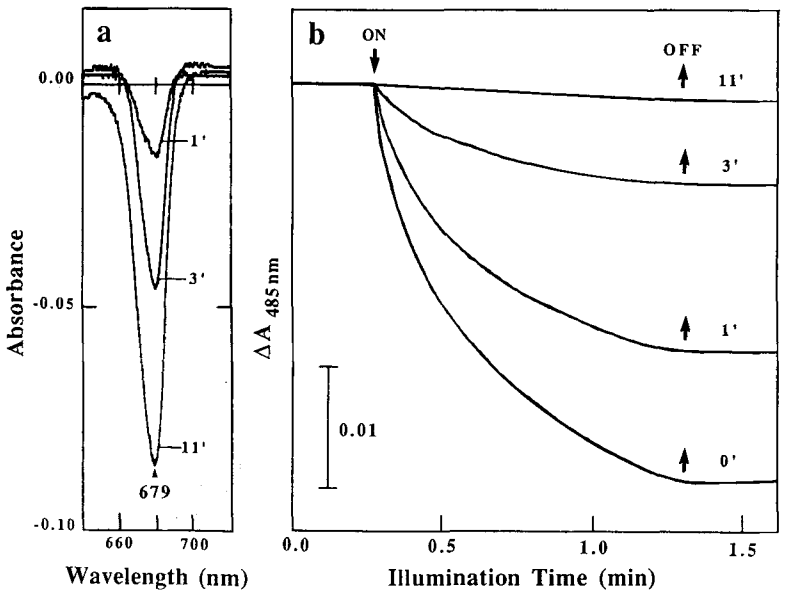

Fig. 6. Preinactivation of P680 by illumination of PSII reaction centres in the absence of an added electron acceptor (a) results in the inability of these reaction centres to show the light induced electron acceptor-dependent loss of absorbance at 485 nm, due to bleaching of β-carotene (b). Preinactivation and measurement of the photodamage difference spectra (a) after 1, 3 and 11 min preillumination and SiMo-dependent bleaching at 485 nm (b) was carried out as described in the Materials and Methods.

subsequently decay on the ms time scale (τ approx. 15 ms in both cases). Both in the presence of ferricyanide with DBMIB or with SiMo alone there is an increase in the stability of $P680^+$ and also of the Car^+ signal as is shown by an increase in the lifetime of the long decay components (data not shown). The designation of the 950 nm signal to Car^+ was confirmed by its infrared spectrum (Kleinherenbrink, F.A.M. and van Gorkom, H.J., personal communication), which peaked at 980 nm as originally shown in PSII enriched preparations by Schenck et al. [31].

The flash yield of Car^+ is low i.e., 1 Car^+ per approx. 30 long-lived $P680^+$ ($\tau \geqslant 0.8$ ms), using extinction coefficients of 94000 M^{-1} cm^{-1} for Car^+ at 950 nm [31] and 7000 M^{-1} cm^{-1} for $P680^+$ at 820 nm [6]. It should be borne in mind that in these experiments as DBMIB was the acceptor with oxygen present that this is a relatively protected situation as compared to the presence of SiMo. The immediate increase in the 950 nm signal due to the flash was also seen by Schenck et al. [31], although it was smaller in proportion to the Car^+ signal. This immediate increase could be due to $P680^+$, which may absorb significantly at this wavelength. We know the relative yield of $P680^+$ is high compared to Car^+ but assume that the extinction coefficient is relatively low so it is not surprising that a greater than 20-times yield of $P680^+$ may give a significant 950 nm signal relative to that of Car^+. We cannot however rule out that there is some rapid donation by β-carotene, which would imply that the isolated PSII reaction centres are heterogeneous, at least in their electron donation reactions.

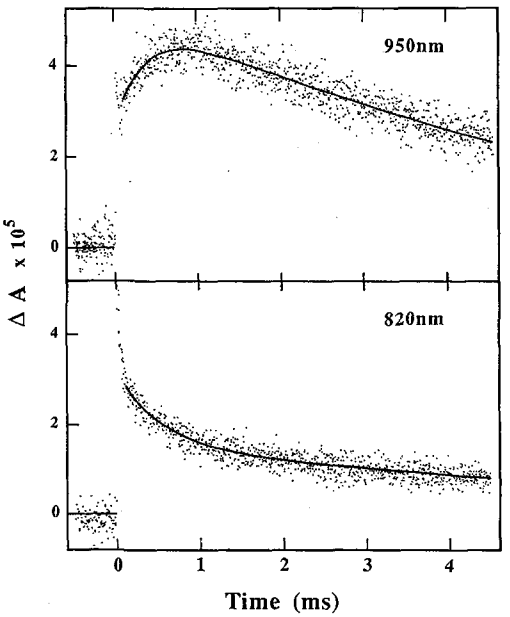

Fig. 7. Flash induced transient absorption changes at 950 nm and 820 nm in PSII reaction centres (chlorophyll 5 μg ml^{-1}) plus 100 μM DBMIB. The solid line is a two exponential fit for the intermediate and slow decay components of the signals. All measurements were at 4 °C.

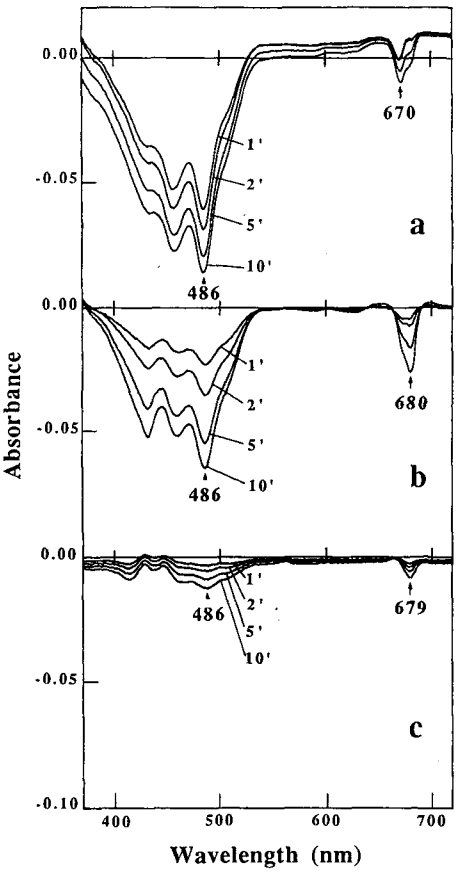

Fig. 8. The effect of anaerobic conditions on the photodamage difference spectra of PSII reaction centres induced by illumination for various times with 600 μmol photons m^{-2} s^{-1} red light. Conditions were as in Fig. 1 except that the chlorophyll concentration was 4 μg ml^{-1}. Oxygen was removed by incubation for 5 min with 0.1 mg ml^{-1} each of glucose oxidase and catalase plus 5 mM glucose prior to measurement. (a) plus 100 μM SiMo, (b) plus 200 μM ferricyanide, (c) plus 100 μM DBMIB.

Fig. 8 shows the effect of anaerobic conditions on the photodamage difference spectra of PSII reaction centres in the presence of different electron acceptors. Absence of oxygen does not prevent the SiMo-dependent bleaching of β-carotene (Fig. 8a), although chlorophyll absorbing at 680 nm is somewhat protected compared to the effect seen under aerobic conditions (compare with Fig. 3). The presence of ferricyanide under anaerobic conditions also allows a pronounced bleaching of β-carotene (Fig. 8b), though at a considerably slower rate, and preferential bleaching at 680 nm as compared to 670 nm. However, when DBMIB is the electron acceptor (Fig. 8c) very little bleaching of either chlorophyll or β-carotene is seen. As bleaching of β-carotene is apparently an oxygen independent process (Fig. 8a and b), the results with DBMIB suggest that under anaerobic conditions there is no oxidation of secondary electron donors because $P680^+$ is rapidly

rereduced via a DBMIB-catalysed cycle of electrons. Indeed rereduction is apparently so rapid that P680 itself is substantially protected from irreversible bleaching.

Discussion

It has been shown previously that the back reaction between $P680^+$ and $Pheo^-$ in the isolated PSII reaction centre is prevented by the addition of the artificial electron acceptors, SiMo and DBMIB [2,8,19,32,33]. In this paper we have shown that, under these conditions, β-carotene as well as the accessory chlorophyll, Chl670, can be photooxidised by $P680^+$. Using transient spectroscopy we found a flash induced absorption increase at 950 nm (Fig. 7) characteristic of Car^+, the kinetics of which correlated with a component in the rereduction of $P680^+$. The oxidised state of β-carotene appears to be unstable and is rapidly bleached, irreversibly. We found that with prolonged illumination both molecules of β-carotene present in the reaction centre could be photobleached.

Previously it has been suggested that the possible function of carotenoids in photosynthesis, in addition to their role in energy transfer, is to protect against singlet oxygen damage [26,27]. This is achieved by direct interaction with chlorophyll triplets to form the carotenoid triplet or by bringing about a rapid disposal of singlet oxygen before it causes any damage as follows:

Singlet oxygen production

$$^3Chl + {}^3O_2 \rightarrow {}^1Chl + {}^1O_2$$

Carotenoid action

$$^3Chl + {}^1Car \rightarrow {}^1Chl + {}^3Car \quad (1)$$

$$^1O_2 + {}^1Car \rightarrow {}^3O_2 + {}^3Car \quad (2)$$

$$^1O_2 + {}^1Car \rightarrow Car^+ \quad (3)$$

These mechanisms do not, however, explain our results. In the absence of added electron acceptors the yield of carotenoid triplet is low in these preparations (approx. 5–10%, Refs. 6 and 10) so intersystem crossing (mechanism 1) cannot play an important part in the action of β-carotene. Carotenoid bleaching only occurs if $P680^+$ has been stabilised by the presence of an electron acceptor (e.g., Fig. 1) and also occurs under anaerobic conditions (Fig. 8). It therefore cannot involve quenching of singlet oxygen by either mechanism 2 or 3. We therefore propose that the main mechanism of carotenoid action in the isolated reaction centre is its direct oxidation by $P680^+$ followed by irreversible bleaching.

$$P680^+ + Car \rightarrow P680 + Car^+ \quad (4)$$

The effect of the electron donor, Mn(II), in preventing photodamage to carotenoid and chlorophyll (Fig. 3) and the absence of photobleaching if primary charge separation is inhibited (Fig. 6) confirms that the loss of these pigments occurs only after they have been oxidised by $P680^+$.

The evidence presented in this paper does not indicate whether the tyrosines, Y_Z and Y_D, present on the D1 and D2 polypeptides, respectively, are active in electron donation to $P680^+$, and more particularly, whether in this preparation Mn^{2+} donates via Y_{Z^+} as reported by Hoganson et al. [34] or directly to $P680^+$. Petersen et al. [35] show in a CP47/D1/D2/Cyt b559 complex which has no active bound Q_A that the spin polarised triplet cannot be formed. This is because the tyrosine donor is active and there is accumulation of the state $Y_{Z^+}Pheo^-$ (or $Y_{Z^+}Pheo$ if ferricyanide is present). However the ability of the isolated PSII reaction centre (D1/D2/Cyt b559) to form the spin polarised triplet and the fact that the EPR signal II, due to Y_{Z^+} and/or Y_{D^+}, can only be seen in 10–20% of reaction centres when they are frozen under illumination with an electron acceptor [18] suggests that Y_Z is either inactive or its rate of donation of electrons is decreased to such an extent that it cannot compete with radical pair recombination. It therefore is possible that, in the experiment of Fig. 3, Mn^{2+} donates directly to $P680^+$. It is also possible, however, that the behaviour of Y_Z is such that it can catalyse enough electron transfer from Mn^{2+} to compete with other secondary donors and afford protection against photodamage but not sufficiently to compete with the back-reaction in the absence of added electron acceptor. Two reports on transient absorption experiments have attributed a 5–10 μs component of the rereduction of $P680^+$ to donation from Y_Z [8,18]. It is therefore possible that the fast phase in the rereduction of $P680^+$ ($\Delta A820$) seen in Fig. 7 is due to donation by Y_Z.

In this paper we have shown that quantitative determination of the loss of β-carotene indicates that the whole pool, assumed to be two β-carotenes per reaction centre (i.e., per two pheophytins, see Ref. 21), can be irreversibly bleached. Despite a variation in the rate of loss, which is electron acceptor dependent (Figs. 1 and 8), the loss is always biphasic. This may be because one of the two β-carotenes in each reaction centre complex is more easily oxidised than the other or because there is a heterogeneous population of reaction centres. However, as we consistently found that 50% of the β-carotene pool is bleached more rapidly than the remainder the former explanation is the more likely. The loss of chlorophyll is apparently less dramatic than that of β-carotene. Fig. 5, for example, shows a 15–20% loss of chlorophyll over the time it takes to bleach 50% of the β-carotene pool. Assuming there are five to six chlorophylls per reaction centre

[21] this means that in this experiment at least one chlorophyll molecule was bleached in the time it took to bleach the first β-carotene. The lack of bleaching of pheophytin indicates that the β-carotene and chlorophyll bleaching is a consequence of photooxidation. The correlation between loss of β-carotene detected by HPLC and by the decrease in absorbance at 485 nm shows that at least 30% of the absorbance at 485 nm is not due to β-carotene. No other peaks were observed at 485 nm in the HPLC analysis, thus the absorbance at 485 nm not due to β-carotene in the spectrum of the isolated PSII reaction centre, must be due to the red shift in absorbance of another pigment bound to protein, presumably pheophytin. We found no evidence for conversion of the all-*trans* form of β-carotene to the *cis* form or for any breakdown products absorbing in the visible region of the spectrum. We have detected the appearance of some new peaks in the ultraviolet region (280 nm), when reaction centres are photodamaged, which are presumably colourless isoprenoid products of the bleached pigments but we have not identified them further.

Because of the selective nature of the initial bleaching at 485 nm, seen in the presence of an electron acceptor, we have been able to obtain a spectrum of β-carotene in the reaction centre, i.e., in situ on the protein complex. The spectrum of Fig. 4 shows the expected bathochromic effect (red shift) compared to the spectra of β-carotene in organic solvents (Fig. 4, inset). We did not observe any significant change in the in situ spectrum during the time course of photobleaching. The absorption spectra of PSII reaction centres has been examined in detail by several groups [16,25,36–38]. CD measurements seem to indicate that, provided PSII reaction centres are stabilised in a suitable detergent, there is excitonic interaction of two β-carotene molecules [37,38] and low temperature LD shows these molecules have different orientations [25,36]. Apparently the accuracy of our in situ spectrum is not sufficient to detect spectral changes due to any decoupling of the excitonic interactions. He [38] found, however, that the loss of excitonic interaction as measured by CD (induced by illumination of PSII reaction centres in the presence of an electron acceptor) is correlated to the rate of loss of the rapidly bleached β-carotene. We therefore conclude that in our experiments there is rapid oxidation and bleaching of one of a pair of excitonically coupled β-carotene molecules followed by a slower oxidation and bleaching of the second. This is similar to the assymetric oxidation of the electron donors to $P680^+$, Y_Z as compared to Y_D [39–41], which show a two-fold symmetry for binding to the D1 and D2 polypeptides, respectively. It is therefore possible that the differential oxidation rates of the two molecules of β-carotene may be due to binding of one molecule to the D1 protein and the other to the D2 protein though the excitonic interaction data indicate that they must be rather close to each other.

We found that the relative amount of irreversible bleaching of β-carotene, Chl670 and P680 (Figs. 2, 3, and 8) was dependent on the type of electron acceptor used. Certain acceptors catalyse non-cyclic electron transfer which results in the accumulation of oxidised donors and their consequent bleaching. The greater the efficiency of the acceptor to bring about non-cyclic electron transfer the greater is the bleaching of the secondary electron donors relative to P680, i.e., SiMo allows photoaccumulation of $P680^+$ under steady state illumination (e.g. Refs. 2 and 12), while ferricyanide, though being capable of accepting electrons [18], does not allow photoaccumulation of $P680^+$ in the steady state (Telfer, A., unpublished data). This difference is reflected in the rate and selectivity of the photobleaching as shown in Fig. 8.

DBMIB behaves rather differently. It catalyses photoreduction of cytochrome *b*-559 [11,42] and allows a cyclic flow of electrons back to P680 [8,32]. Other quinones also catalyse cytochrome *b*-559 photoreduction when reconstituted with the reaction centre [11,42]. Thompson and Brudvig [43] have argued that a quinone dependent cycle, involving cytochrome *b*-559, occurs in vivo. Our work shows almost no photobleaching of pigments in the isolated reaction centre when DBMIB is present under anaerobic conditions (Fig. 8c). This indicates that the rereduction of $P680^+$ via cytochrome *b*-559 is not only sufficiently rapid to prevent oxidation of the secondary donors, β-carotene and Chl670, but also shortens the lifetime of $P680^+$ to such an extent that it is not itself photobleached by steady state illumination. Significant protection against photobleaching is also provided by the presence of a good electron donor to stimulate non-cyclic electron flow e.g. Mn(II) with SiMo (see Fig. 3). The bleaching observed with DBMIB in the presence of oxygen (Fig. 2d) indicates that oxygen acts as an electron acceptor hence competing with cyclic electron flow via cytochrome *b*-559 and increasing the lifetime of $P680^+$. This allows the oxidation of β-carotene to occur.

The results presented here suggest that the major role of β-carotene in the PSII reaction centre is to protect against photodynamic damage to P680. P680 is probably also protected against photodamage by a cycling of electrons around PSII via cytochrome *b*-559 and a monomeric chlorophyll as discussed by Thompson and Brudvig [43]. We therefore suggest that the photooxidation of β-carotene and cyclic electron transfer both play an important role in the protection of P680 in vivo. These two mechanisms probably prevent photoinactivation of PSII which is the basis for the physiological phenomenon of photoinhibition. It is also possible that it is the irreversible bleaching of one or

more of the chromophores of the PSII reaction centre which triggers the steps leading to breakdown of the D1 protein, as discussed by Shipton and Barber [44].

Acknowledgements

We thank the Agricultural and Food Research Council for financial support. J. De Las Rivas is the recipient of a FEBS Research Fellowship. We are very grateful to Dr. David Klug, Dr. Chris Barnett and Ms. Soley Özer for assistance with measuring and analysing the transient absorption data. We also would like to thank Dr. James Durrant and Ms. Catherine Shipton for helpful discussions and Mr. Niall Walsh and Ms. Caroline Woollin for valuable technical back-up. We are also indebted to Mr. Frank Kleinherenbrink, Dept. of Biophysics, University of Leiden, for measuring the spectrum of the infrared transient absorption changes.

References

1 Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
2 Barber, J., Chapman, D.J. and Telfer, A. (1987) FEBS Lett. 220, 67–73.
3 Ikeuchi, M. and Inoue, Y. (1988) FEBS Lett. 241, 99–104.
4 Webber, A.N., Packman, L., Chapman, D.J., Barber, J. and Gray, J.C. (1989) FEBS Lett. 24, 259–262.
5 Danielius, R.V., Satoh, K., Van Kan, P.J.M., Plijter, J.J., Nuijs, A.M. and Van Gorkom, H.J. (1987) FEBS Lett. 213, 241–244.
6 Takahashi, Y., Hansson, O., Mathis, P. and Satoh, K. (1987) Biochim. Biophys. Acta 893, 49–59.
7 Wasielewski, M.R., Johnson, D.G., Seibert, M. and Govindjee (1989) Proc. Natl. Acad. Sci. USA 86, 524–528.
8 Mathis, P., Satoh, K. and Hansson, O. (1989) FEBS Lett. 251, 241–244.
9 Booth, P.J., Crystall, B., Barber, J., Klug, D.R. and Porter, G. (1990) Biochim. Biophys. Acta 1016, 141–152.
10 Durrant, J.R., Giorgi, L.B., Barber, J., Klug, D.R. and Porter, G. (1990) Biochim. Biophys. Acta 1017, 167–175.
11 Chapman, D.J., Gounaris, K., and Barber, J. (1989) Photosynthetica 23, 411–426.
12 Telfer, A., He, W.-Z. and Barber, J. (1990) Biochim. Biophys. Acta 1017, 143–151.
13 Powles, S.B. (1984) Annu. Rev. Plant Physiol. 35, 15–44.
14 Kyle, D.J. (1987) in Topics in Photosynthesis, Vol. 9, Photoinhibition (Kyle, D.J., Osmond, C.B. and Arntzen, C.J. eds.), pp. 197–226, Elsevier, Amsterdam.
15 McTavish, H., Picorel, R. and Seibert, M. (1989) Plant Physiol. 35, 15–44.
16 Braun, P., Greenberg, B.M. and Scherz, A. (1990) Biochemistry 29, 10376–10387.
17 Crystall, B., Booth, P.J., Klug, D.R., Barber, J. and Porter, G. (1989) FEBS Lett. 249, 75–78.
18 Nugent, J.H.A., Telfer, A., Demetriou, C. and Barber, J. (1989) FEBS Lett. 255, 53–58.
19 Takahashi, Y., Satoh, K. and Itoh, S. (1989) FEBS Lett. 255, 133–138.
20 Telfer, A. and Barber, J. (1989) FEBS Lett. 246, 223–228.
21 Gounaris, K., Chapman, D.J., Booth, P.J., Giorgi, L.B., Klug, D.R., Porter, G. and Barber, J. (1990) FEBS Lett. 265, 88–92.
22 Kobayashi, M., Maeda, H., Watanabe, T., Nakane, H. and Satoh, K. (1990) FEBS Lett. 260, 138–140.
23 Dekker, J.P., Bowlby, N.R. and Yocum, C.F. (1989) FEBS Lett. 254, 150–154.
24 Ghanotakis, D.F., De Paula, J.C., Demetriou, D.M., Bowlby, N.R., Petersen, J., Babcock, G.T. and Yocum, C.F. (1989) Biochim. Biophys. Acta 974, 44–53.
25 Newell, W.R., Van Amerongen, H., Barber, J. and Van Grondelle, R. (1991) Biochim. Biophys. Acta 1057, 232–238.
26 Witt, H.T. (1971) Q. Rev. Biophys. 4, 365–477.
27 Cogdell, R.J. and Frank, H.A. (1987) Biochim. Biophys. Acta 895, 63–79.
28 Chapman, D.J., Gounaris, K. and Barber, J. (1988) Biochim. Biophys. Acta 933, 423–431.
29 De Las Rivas, J., Abadia, A. and Abadia, J. (1989) Plant Physiol. 91, 190–192.
30 Velthuys, B.R. (1981) FEBS Lett. 126, 272–276.
31 Schenck, C.C., Diner, B.A., Mathis, P. and Satoh, K. (1982) Biochim. Biophys. Acta 680, 216–227.
32 Satoh, K., Hansson, O. and Mathis, P. (1990) Biochim. Biophys. Acta 1016, 121–126.
33 Telfer, A., Durrant, J.R. and Barber, J. (1990) Biochim. Biophys Acta, 1018, 168–172.
34 Hoganson, C.W., Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1989) Photosynthesis Res. 22, 285–293.
35 Petersen, J., Dekker, J.P., Bowlby, N.R., Ghanotakis, D.F., Yocum, C.F. and Babcock, G.T. (1990) Biochemistry 29, 3226–3231.
36 Van Dorssen, R.J., Breton, J., Plijter, J.J., Satoh, K., Van Gorkom, H.J. and Amesz, J. (1987) Biochim. Biophys. Acta 893, 267–274.
37 Tetenkin, V.L., Gulyaev, B.A., Seibert, M. and Rubin, A.B. (1989) FEBS Lett. 250, 459–463.
38 He, W.-Z. (1991) Ph.D. Thesis, Imperial College, University of London.
39 Debus, R.J., Barry, B.A., Babcock, G.T. and McIntosh, L. (1988) Proc. Natl. Acad. Sci. USA 85, 427–430.
40 Debus, R.J., Barry, B.A., Sithole, I., Babcock, G.T. and McIntosh, L. (1988) Biochemistry 27, 9071–9074.
41 Vermaas, W.F.J., Rutherford, A.W. and Hansson, O. (1988) Proc. Natl. Acad. Sci. USA 85, 8477–8481.
42 Gounaris, K., Chapman, D.J. and Barber, J. (1988) FEBS Lett. 240, 143–147.
43 Thompson, L.K. and Brudvig, G.W. (1988) Biochemistry 27, 6653–6658.
44 Shipton, C.A. and Barber, J. (1991) Proc. Natl. Acad. Sci. USA, in press.