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What is β-carotene doing in the photosystem II reaction centre?

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During photosynthesis carotenoids normally serve as antenna pigments, transferring singlet excitation energy to chlorophyll, and preventing singlet oxygen production from chlorophyll triplet states, by rapid spin exchange and decay of the carotenoid triplet to the ground state. The presence of two β-carotene molecules in the photosystem II reaction centre (RC) now seems well established, but they do not quench the triplet state of the primary electron-donor chlorophylls, which are known as P_{680}. The β-carotenes cannot be close enough to P_{680} for triplet quenching because that would also allow extremely fast electron transfer from β-carotene to P_{680}^{+}, preventing the oxidation of water. Their transfer of excitation energy to chlorophyll, though not very efficient, indicates close proximity to the chlorophylls ligated by histidine 118 towards the periphery of the two main RC polypeptides. The primary function of the β-carotenes is probably the quenching of singlet oxygen produced after charge recombination to the triplet state of P_{680}. Only when electron donation from water is disturbed does β-carotene become oxidized. One β-carotene can mediate cyclic electron transfer via cytochrome *b*559. The other is probably destroyed upon oxidation, which might trigger a breakdown of the polypeptide that binds the cofactors that carry out charge separation.

Keywords: β-carotene; photosystem II; triplet state; singlet oxygen; photoinhibition

1. INTRODUCTION

Carotenoids increase the efficiency of photosynthesis by absorbing blue-green light and transferring this energy to chlorophyll. They are bound to 'antenna protein complexes' that channel the energy to photochemical pigment binding 'RCs' where the first energy storing electron-transfer events take place. It appears to be for a different reason, however, that carotenoids are essential components of the photosynthetic apparatus. Wherever there is chlorophyll there are also carotenoids close by (Cogdell & Frank 1987; Frank & Cogdell 1993; Frank *et al.* 1999). Their importance is demonstrated by the fact that when mutant purple bacteria lacking carotenoids are put under severe light stress they produce revertants that are able to synthesize carotenoids (Ouchane *et al.* 1997). This is because carotenoids are required in order to quench chlorophyll triplets and prevent their quenching by oxygen. The latter process produces singlet molecular oxygen ($^1O_2$) that is a highly reactive and extremely toxic species that causes oxidative damage: bleaching pigments and bringing about protein inactivation and lipid peroxidation (Shigenaga *et al.* 1994). At high light intensities, once the rate of photosynthetic electron transport reaches a maximum, there is a gradual intensity-dependent increase in the yield of the carotenoid triplets. This was called the valve reaction by Witt (1971) and reflects the fact that, once the photochemical reactions are saturated, the yield of chlorophyll triplets increases and hence more quenching by carotenoid occurs.

A series of transmembrane protein complexes embedded in the inner chloroplast membranes act as an electron transport chain providing reducing power for carbon dioxide fixation. PSII is a complex that catalyses electron transfer from water to plastoquinone, a diffusible molecule that connects PSII to the next component in the chain. The PSII complex consists of the photochemical RC, the site of primary charge separation in the PSII complex, surrounded by light harvesting (or antenna) complexes. These are the core antenna CP complexes (CP47, CP43), the peripheral antenna consisting of an inner part (minor CP proteins) and the main outer LHCs (LHCII). The RC and core antenna CP47 and CP43 complexes usually bind only chlorophyll *a* and β-carotene while the minor CP proteins and the LHCII family of complexes bind chlorophyll *a*, chlorophyll *b* and xanthophylls (oxygenated derivatives of carotenoid hydrocarbons) (see Simpson & Knoetzel 1996).

High light intensities that saturate the electron-transfer reactions lead to photoinhibition, a loss of photosynthetic activity, *in vivo*. The PSII RC is known to be the target of photoinhibition (Barber & Andersson 1992; Aro *et al.* 1993; Long *et al.* 1994). The result of photoinhibitory light treatment is that a particular polypeptide in the RC of PSII, the D1 protein, is subject to photo-oxidation. This necessitates a complex cycle where the D1 protein is continually broken down and replaced far more rapidly than any other chloroplast protein (Aro *et al.* 1993). In fact the D1 protein is being continuously turned over at all light intensities but the net decrease in photosynthesis

One contribution of 21 to a Discussion Meeting Issue 'Photosystem II: molecular structure and function'.

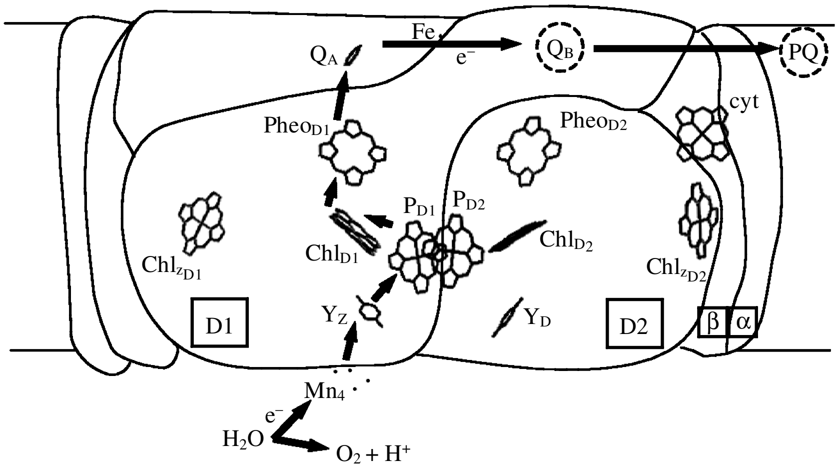

Figure 1. Model of the PSII complex in the thylakoid membrane showing the electron-transfer reactions involved in its water–plastoquinone oxidoreductase activity and the cofactors seen in the X-ray structure of the PSII complex, after Zouni *et al.* (2001). Abbreviation: PQ, plastoquinone; see glossary.

is seen only when the repair mechanism cannot keep up with the rate of breakdown of D1. The PSII RC binds two molecules of β-carotene, but they apparently cannot prevent the photo-oxidative damage leading to D1 turnover. The question addressed here is: what is this β-carotene doing in the PSII RC?

2. THE PHOTOSYSTEM II REACTION CENTRE

When antenna chlorophylls of PSII absorb light, the excitation energy is transferred by resonance transfer to the primary chlorophyll electron donor, special chlorophyll *a* molecule(s) known as P_{680}, in the RC. Primary charge separation occurs from the first excited singlet state of the donor, $P_{680}{}^{*}$, which gives up an electron to reduce an acceptor molecule, Pheo, forming a primary radical pair, $P^{+}_{680}Pheo^{-}$. The oxidized donor, P^{+}_{680}, is then reduced by electrons from water via a tyrosine molecule bound to the D1 protein (Y_Z). Water is oxidized to molecular oxygen, electrons and protons, in a reaction catalysed by a cluster of four Mn atoms. The $Pheo^{-}$ bound to the D1 protein passes its electron to two bound plastoquinone acceptors, Q_A and Q_B. Q_A accepts one electron to form the semi-quinone state and this electron is passed rapidly onto Q_B. On the next charge separation Q_B accepts a second electron and two protons. The fully reduced molecule leaves its binding site and moves into the lipid phase of the membrane allowing a new oxidized plastoquinone molecule to be bound to the binding site on the D1 polypeptide.

Figure 1 shows a schematic model of the structure and electron-transfer reactions of the PSII RC complex. The figure shows the organization of the polypeptides, in particular D1, D2 and cyt *b*559, the electron-transfer reactions from water to plastoquinone and also all the cofactors (or headgroups of cofactors) bound to the complex. The cofactor organization is adapted from the recent X-ray structure of a larger complex, the oxygen-evolving PSII core complex, isolated from the cyanobacterium *Synechococcus elongatus* (Zouni *et al.* 2001). The current resolution of the core complex is 3.8 Å (Zouni *et al.* 2001). This structure therefore gives an indication of the position but not the full orientation of the six chlorophylls, two Pheo and one quinone (Q_B is missing from the isolated core complex) bound to the D1 and D2 proteins and the haem bound to the α- and β-polypeptides of cyt *b*559.

The cofactors are bound in a twofold symmetry homologous to that seen in the pBRC (Michel & Deisenhofer 1988). Electron transfer occurs along an active electron-transfer branch (mainly associated with D1) as it does in the pBRC. Like the L and M subunits in the pBRC, the two main polypeptides, D1 and D2, are entwined so that, although most of the active branch cofactors are liganded to D1, Q_A is mainly associated with D2 (figure 1). The primary electron donor in pBRC is composed of two closely aligned (dimeric) bacteriochlorophylls, orientated perpendicular to the membrane plane with two accessory bacteriochlorophylls at a 30° angle and two bacteriopheophytins, one active in electron transport and one inactive. Figure 1 shows that in the PSII RC the four central chlorophylls (P_{680}) are similarly orientated but evenly spaced (P_{D1}, P_{D2}, Chl_{D1} and Chl_{D2}) i.e. there is no dimer. The X-ray structure confirms and extends the previous electron crystallographic data on the RC–CP47 complex (Rhee *et al.* 1998) in particular emphasizing the even spacing of the four chlorophylls of the primary electron donor.

There are other significant differences between the PSII RC and the pBRC. The PSII RC binds two extra, peripherally located chlorophyll molecules, $Chl_{z_{D1}}$ and $Chl_{z_{D2}}$, that are liganded at homologous positions on D1 and D2 to His118 (His117 on D2 in cyanobacteria and the green alga *Chlamydomonas reinhardtii*) (see Stewart *et al.* 1998; Ruffle *et al.* 2001). This binding position was confirmed by the X-ray structure of Zouni *et al.* (2001), as shown in figure 1. In addition, there is the presence of the membrane-intrinsic cyt *b*559, that is bound closely to the RC on the D2 side with the haem closer to Q_B than to Q_A. The X-ray structure of the PSII core complex has resolved the position of nearly all the cofactors in the RC: the pigments, Q_A, the non-haem Fe atom and the four Mn atoms of the water-oxidizing complex. In addition, the PSII RC binds two, all-*trans* β-carotene molecules whereas the pBRC binds only one 15-*cis* carotenoid (see § 3). The X-ray structure of the purple photosynthetic bacterium *Rhodobacter sphaeroides*' RC shows that the carotenoid is closely associated with the accessory bacteriochlorophyll on the L polypeptide i.e. inactive branch (Arnoux *et al.* 1989). However, none of the β-carotenes bound to either the PSII RC or the CP47 and CP43 antenna proteins have been seen at the current resolution of the PSII core structure (Zouni *et al.* 2001).

3. THERE ARE TWO DIFFERENT β-CAROTENES IN THE ISOLATED PHOTOSYSTEM II REACTION CENTRE

The PSII RC can be isolated from larger PSII preparations as a complex composed of the D1 and D2 polypeptides and a few other small transmembrane polypeptides including the α- and β-subunits of cyt *b*559 (Nanba & Satoh 1987). *In vivo* these polypeptides plus some extrinsic proteins bind and stabilize the electron transport cofactors of PSII. The isolation procedure removes the quinone acceptors, Q_A and Q_B, the non-haem Fe and the Mn and polypeptides that catalyse and stabilize water oxidation, respectively (see Satoh 1996). It also inactivates the tyrosine electron donors to P_{680}, Y_Z in D1 and the analogous slow electron donor, Y_D, in the D2 protein. However, the isolated PSII RC retains six chlorophyll, two pheophytin and two β-carotene molecules and cyt *b*559 (Kobayashi *et al.* 1990; Gounaris *et al.* 1990; Satoh 1996). The two RC β-carotenes are clearly distinguished by their spectroscopic properties; Car_{507} absorbs at 507, 473 and 443 nm whereas Car_{489} absorbs at 489, 458 and 429 nm (Van Dorssen *et al.* 1987*a*; Breton *et al.* 1988; Kwa *et al.* 1992; Tomo *et al.* 1997).

Prolonged washing with aqueous-based buffers containing detergent during the isolation procedure tends to remove some β-carotene (see De Las Rivas *et al.* 1993), which is one reason the pigment stoichiometry of PSII RC has been hotly debated in the past (Kobayashi *et al.* 1990; Gounaris *et al.* 1990; Eijckelhoff & Dekker 1995). However, although the β-carotene to chlorophyll ratio decreases, there is no change in the relative amplitudes of the red-most absorption bands of the two carotenoids (Eijckelhoff & Dekker (1995) at room temperature; Kwa *et al.* (1992) at 77 K). This non-selective extraction must therefore result in a heterogeneous mixture composed of 0-Car, 1-Car_{507}, 1-Car_{489} and 2-Car containing RC complexes.

Preparation of PSII RC with two β-carotenes per centre is easier from pea, *Pisum sativum*, and spinach, *Spinacea oleracea* (A. Telfer, personal observations). It seems to be more difficult to wash off the CP47 and CP43 antenna complexes, when using spinach as source material, and so great care must be taken if the RCs are not to become depleted of β-carotene. A simple way to roughly estimate the average number of β-carotenes in any PSII RC preparation is to measure the A_{417}/A_{484} ratio at room temperature. Analysis of published pigment level determinations shows that ratios greater than *ca.* 3.8 indicate significant loss of β-carotene (see table 1; Telfer *et al.* 1994*b*). In fact, many published spectra, particularly preparations from spinach, show an average of less than two β-carotenes per centre, i.e. the preparations must be heterogeneous (e.g. Nanba & Satoh 1987; Ghanotakis *et al.* 1989; Kwa *et al.* 1992). It has been reported that one of the β-carotene molecules (Car_{489}) can be removed preferentially from the isolated RC2, but to do this it is necessary to use organic solvents (Tomo *et al.* 1997).

Table 1. Ratio of absorbance at 417 and 484 nm, measured at room temperature, giving an indication of the average number of β-carotene molecules per RC (two pheophytin molecules) and hence the heterogeneity of various PSII RC preparations reported in the literature.

source material	wash time	A_{417}/A_{484}	Chl : Car : Pheo
pea[a]	short	3.1	6.4 : 1.8 : 2
	long	5.6	6.4 : 1.0 : 2
pea[b]	medium	4.7	6.6 : 1.6 : 2
	long	5.5	6.4 : 1.2 : 2
spinach[c]	short	3.8	6.4 : 1.6 : 2
	long	5.0	6.3 : 1.3 : 2
spinach[d]	short	3.5	6.2 : 2.2 : 2

[a] De Las Rivas *et al.* (1993).
[b] Telfer *et al.* (1994*b*).
[c] Eijckelhoff & Dekker (1995).
[d] Tomo *et al.* (1997).

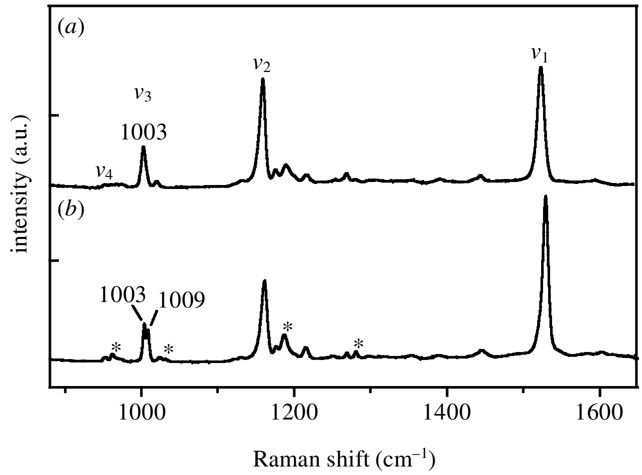

Figure 2. Resonance Raman spectra of neutral β-carotene in isolated PSII RCs at 77 K: excitation at (*a*) 514.5 nm compared with (*b*) 488.0 nm. Adapted from Telfer *et al.* (2003). Asterisks denote the modes associated only with Car_{489}.

The absorption transitions of the two β-carotenes are clearly seen in low-temperature LD spectroscopy and the positive and negative transitions have been interpreted as indicating that Car_{507} is more parallel to the membrane plane while Car_{489} is more perpendicular (Van Dorssen *et al.* 1987*a*; Breton *et al.* 1988; Kwa *et al.* 1992; Tomo *et al.* 1997). The LD and absorption data have also been interpreted as indicating excitonic interaction between the two β-carotenes (Newell *et al.* 1991; Tetenkin *et al.* 1989; De Las Rivas *et al.* 1993). However, it is increasingly clear that this is not the case and that there are two spectroscopically distinct β-carotenes in the RC (see Germano *et al.* 2001).

Recently the two β-carotenes have also been distinguished in resonance Raman spectra excited in the main absorption band of the β-carotenes (figure 2). Excitation with 514.5 nm light preferentially excites Car_{507} whereas 488.0 nm excites the two β-carotenes approximately equally. Figure 2 shows that various specific modes can be attributed to one or other of the two β-carotenes. Two of the main four modes attributed to carotenoid (see Robert 1999), known as ν_1 and ν_3, are shifted in the spectrum of one with respect to the other and there are certain other small bands specifically related to one or other of the β-carotenes (see figure 2). An earlier study on isolated PSII RCs, using excitation at 488.0 nm only, failed to resolve the two ν_3 bands (figure 2: 1003 cm^{-1} due to Car_{507} and 1009 cm^{-1} due to Car_{489}) but otherwise yielded essentially the same modes seen here (Ghanotakis *et al.* 1989).

The single carotenoid molecule present in the pBRC is in the 15-*cis* conformation (Arnoux *et al.* 1989). Initially, resonance Raman data indicated that the β-carotenes of the PSII RC are all *trans* (Fujiwara *et al.* 1987; Ghanotakis *et al.* 1989). Later, using HPLC, Bialek-Bylka *et al.* (1995) showed that β-carotenes have a 15-*cis* structure. However, in another HPLC, study, Yruela *et al.* (1998) found that, although initially only the all-*trans* structure was detectable, a *cis* component appeared with time. They suggest therefore that the *cis* form is not the *in vivo* structure. This is also confirmed by the data of figure 2, as there is no specific band in the 1245 cm^{-1} region, which would indicate the presence of the 15-*cis* structure of β-carotene (Koyama *et al.* 1983). This structural difference emphasizes the fact that the β-carotenes of the PSII RC have very different roles to the single carotenoid of the pBRC.

4. NO CHLOROPHYLL TRIPLET QUENCHING BY β-CAROTENE

In spite of the presence of β-carotene in the isolated PSII RC, the yield of ^{3}Car on illumination was low (less than 3%) whereas the triplet yield of the primary electron-donor chlorophyll, $^3P_{680}$, was high (30%) (Takahashi *et al.* 1987; Durrant *et al.* 1990). As discussed in § 3 secondary electron transport is inhibited in the isolated PSII RC complex. Consequently on illumination it is only capable of forming the radical pair, $P_{680}^{+}Pheo^{-}$, which then undergoes rapid charge recombination with a 30% yield of the triplet state of P_{680}. That this triplet is formed after charge separation has occurred, i.e. by the radical-pair mechanism, is shown by the characteristic AEEAAE polarization of the EPR signal of the triplet (Okamura *et al.* 1987).

In contrast to the carotenoid in the pBRC, which efficiently quenches the corresponding bacteriochlorophyll triplet state (see Frank & Cogdell 1993), the PSII RC-bound β-carotene is apparently unable to quench chlorophyll triplets and hence there should be no protection against $^1O_2$ formation by $^3P_{680}$. The fact that the presence of oxygen during illumination shortens the $^3P_{680}$ lifetime from 1 ms to *ca.* 30 μs, causes irreversible bleaching of chlorophyll and decreases the stability of the isolated complex, was considered to be indirect evidence for $^1O_2$ formation (Barber *et al.* 1987; McTavish *et al.* 1989; Durrant *et al.* 1990). Subsequently, $^1O_2$ formation on illumination of isolated PSII RCs was detected directly by its luminescence at 1270 nm (Macpherson *et al.* 1993; Telfer *et al.* 1994*a*).

Takahashi *et al.* (1987) found that, although the isolated RC had a low yield of ^{3}Car, a core preparation (RC–CP47–CP43 complex) showed not only a high yield of ^{3}Car but also a high yield of $^3P_{680}$. These results show that the inability of the isolated PSII RC to form ^{3}Car is not simply because the normal quenching ability of the β-carotenes was disturbed by the isolation technique. The most probable explanation is that chlorophyll triplets were quenched by the β-carotenes in the core antenna proteins, CP47 and CP43, while in the RC the long-lived $^3P_{680}$ was detected because the β-carotenes in the RC were unable to quench it.

The spin exchange required for transfer of the triplet state from ^{3}Chl to carotenoid can only occur if the electron orbitals have some overlap. The edge-to-edge distance between the two molecules must be less than the van der Waals' distance (3.6 Å). Indeed, in all the main photosynthetic pigment protein complex crystallographic structures known to date the carotenoids are bound a few angstroms from (bacterio)chlorophyll headgroups (e.g. Arnoux *et al.* 1989; Kühlbrandt *et al.* 1994; McDermott *et al.* 1995; Jordan *et al.* 2001).

The reason why β-carotene does not quench $^3P_{680}$ is probably the extremely high oxidizing power PSII has to generate in order to oxidize water, as discussed by Van Gorkom & Schelvis (1993). The midpoint potential of P_{680}^{+}/P_{680} must be higher than the *ca.* 1.0 V of β-carotene (Edge *et al.* 2000) and at the van der Waals' distance electron transfer between them would be exceedingly fast and would prevent electron transfer from Y_Z to P_{680}^{+}. This problem is unique for PSII; the redox potentials of the primary electron donors in other photosystems are less oxidizing than the other pigments in the complexes and consequently the cationic forms are not dangerous (Thompson & Brudvig 1988).

5. IMPORTANT ROLE OF β-CAROTENE AS A SINGLET OXYGEN SCAVENGER

It is clear that β-carotene cannot quench chlorophyll triplets in PSII and hence prevent them from forming $^1O_2$. It is thus possible that a second line of defence comes in and that they scavenge $^1O_2$ directly. Quenching of $^1O_2$ by carotenoids is known to occur in a diffusion-controlled reaction in solution, forming ^{3}Car, which then decays harmlessly (Krinsky 1968) and was postulated to play a part in photosynthesis (Cogdell & Frank 1987; Frank & Cogdell 1993). The yield of $^1O_2$ formation (detected by its luminescence at 1270 nm) was subsequently found to be inversely proportional to the average level of β-carotene in PSII RC preparations as was the rate of irreversible chlorophyll bleaching (Telfer *et al.* 1994*b*). This showed that the β-carotenes present in the RC do indeed scavenge $^1O_2$ and provide significant protection against oxidative damage (see Van Gorkom & Schelvis 1993). However, the protection can only be partial because the β-carotenes are bound some distance away from the source of the $^1O_2$. Consequently, the ability of the β-carotenes to scavenge $^1O_2$ is always in competition with other target molecules that may become oxidized and hence it is not surprising that $^1O_2$ severely damages isolated RC complexes (Barber *et al.* 1987) and PSII core complexes (Mishra *et al.* 1994) subjected to high light intensities.

6. POSSIBLE ROLE OF THE D2-SIDE β-CAROTENE IN A PHOTOPROTECTIVE ELECTRON-TRANSFER CYCLE

Recombination of the radical pair ($P_{680}^{+}Pheo^{-}$) is prevented if an electron acceptor is added to PSII RC (to replace Q_A) and thus on illumination P_{680}^{+} can be accumulated (Barber *et al.* 1987; Takahashi *et al.* 1989; Telfer & Barber 1989). Further examination of the oxidation reactions showed that, in fact, initially β-carotene is oxidized by P_{680}^{+} but that the carotenoid cation is unstable and is irreversibly bleached in an oxygen-independent reaction. Only once all the β-carotene is inactivated can stable accumulation of P_{680}^{+} be observed (Telfer & Barber 1989; De Las Rivas *et al.* 1993). In flash-induced absorption experiments on PSII RC β-carotene oxidation was detected by its reversible absorption increase due to absorption by the radical cation, a very broad signal peaking at *ca.* 1000 nm (Telfer *et al.* 1991). Oxidation of the β-carotene by P_{680}^{+} occurs relatively slowly ($\tau = ca.$ 1 ms) and using the rule of Moser & Dutton (1992), it was calculated that the β-carotene must be at a distance of 18–20 Å from the nearest oxidized chlorophyll (Telfer & Barber 1995). In experiments on PSII-enriched preparations where carotenoid oxidation could be observed, it was presumably the β-carotene within the PSII RC that was oxidized (Schenck *et al.* 1982; Hillmann & Schlodder 1995).

In larger PSII preparations than the RC, if the normal electron-donation pathway from water via Y_Z (figure 1) is inhibited, the lifetime of P_{680}^{+} is extended and hence it oxidizes other components of the RC. A cyclic flow of electrons via Chl_Z and cyt *b*559, which *in vivo* is in its high potential form and hence is reduced at the ambient redox potential, may occur under these conditions. This was suggested to be a mechanism for protection against photoinhibition (Thompson & Brudvig 1988). However, recent papers have indicated that a β-carotene molecule, which has a radical with a narrow EPR spectrum very similar to that of Chl_Z, is the first electron donor to be oxidized by P_{680}^{+}. This β-carotene participates in a cycle around PSII via cyt *b*559 (Hanley *et al.* 1999; Faller *et al.* 2001*a*). Recent papers discuss whether Chl_Z is an intermediate between Car and cyt *b*559 or on a branched path (Faller *et al.* 2001*b*; Tracewell *et al.* 2001*a*,*b*). It was assumed that Chl_Z is one of the so-called peripheral chlorophylls bound near the edge of the complex ($Chl_{z_{D1}}$ and $Chl_{z_{D2}}$ in figure 1). Although a mutational study indicated that Chl_Z is on the D1 polypeptide (Stewart *et al.* 1998) recent spectroscopic distance measurements and mutational work locate it to D2 (Shigemori *et al.* 1998; Ruffle *et al.* 2001; Wang *et al.* 2002). The latter is more consistent with the model from X-crystallographic studies as $Chl_{z_{D2}}$ is nearer cyt *b*559 than $Chl_{z_{D1}}$. It should be noted that this terminology for the peripheral chlorophylls is defined from their position in the X-ray structure (Zouni *et al.* 2001), whereas Chl_Z is a functional definition.

The distance from $Chl_{z_{D2}}$ to the nearest chlorophylls (centre-to-centre, 24.6 Å) of the inner four, that constitute P_{680}, is too far for direct electron transport to be significant and thus it has been suggested that Car acts as an intermediate between P_{680} and both cyt *b*559 and Chl_Z. However, the X-ray structure shows that Chl_Z cannot be an intermediate in a PSII cyclic electron-transport pathway as it is too far from cyt *b*559 (centre-to-centre, 27.0 Å). The long (more than 25 Å) structure of all *trans* β-carotene means that it may be close enough to be very rapidly reduced by the cytochrome and yet still extend through the membrane to within 18–20 Å of P_{680}; acting as an 'molecular wire' (Gruszecki *et al.* 1995; Hanley *et al.* 1999). The cycling of electrons around PSII, via cyt *b*559, and the possible location of the two Cars are illustrated in figure 3 (Car_{D1} is discussed in § 7). A slight twist in Car_{D2} has been added to allow close contact with both cyt *b*559 and $Chl_{z_{D2}}$ and yet for it also to come close enough to allow electron transfer to P_{680}^{+} on a millisecond time-scale.

Resonance Raman spectra of PSII RCs (figure 2) show that the ν_4 to ν_3 band-size ratio for Car_{489} is somewhat higher than that for Car_{507}. This indicates that Car_{489} could indeed have a slightly more twisted or strained conformation than Car_{507} (Pascal *et al.* 1998) and could explain the other differences between the spectra (ν_1 and ν_3 frequency shift, etc.). These results indicate Car_{489} is bound to D2, which is in contradiction to the conclusion of Tomo *et al.* (1997). However, it is clear that more conclusive evidence is required before identification of the two β-carotenes is possible.

Evidence indicates that *in vivo* if the electron transport from water to P_{680}^{+} is limiting then electrons can be cycled around PSII via $Q_B \rightarrow$ cyt *b*559HP (i.e. in its high potential form) $\rightarrow$ Car $\rightarrow P_{680}^{+}$. If the electron supply from the cytochrome is limiting, because it is already oxidized due to a high to low potential shift for example (see Barber & Rivas 1993), the Car will oxidize Chl_Z, that in turn can quench fluorescence and reduce the photochemical pressure on PSII, as proposed by Barber & Rivas (1993) and Stewart *et al.* (1998).

7. OXIDATION OF THE D1-SIDE β-CAROTENE

There are two β-carotenes and two peripheral chlorophylls present in the RC. In the isolated PSII RC on illumination in the presence of an electron acceptor both of the β-carotenes are oxidized and irreversibly bleached at room temperature (De Las Rivas *et al.* 1993). Recently, it has been indicated that, in PSII preparations, both peripheral chlorophylls can also be oxidized but to differing extents in different organisms (Tracewell *et al.* 2001*a*). This has led to the proposal of models in which the two β-carotenes are located symmetrically between the two peripheral chlorophylls and there are thus two ways in which P_{680}^{+} can be reduced if donation of electrons from water is inhibited (Tomo *et al.* 1997; Tracewell *et al.* 2001*a*).

These proposals ignore the marked asymmetry of the RC implied by the different orientations of the two β-carotenes and by the absence of a homologue of cyt *b*559 to reduce Car and Chl cations on the D1 side. Figure 3 attempts to address the orientation problem, showing a possible more parallel position for Car_{D1}. Experiments on the isolated PSII RC indicate that the Car cation state is very unstable (De Las Rivas *et al.* 1993). Also, in a series of flash-induced cycles of Car oxidation–reduction with PSII RCs supplied with an electron acceptor, there was a gradual loss of the absorption change due to Car^{+} (ΔA_{980})

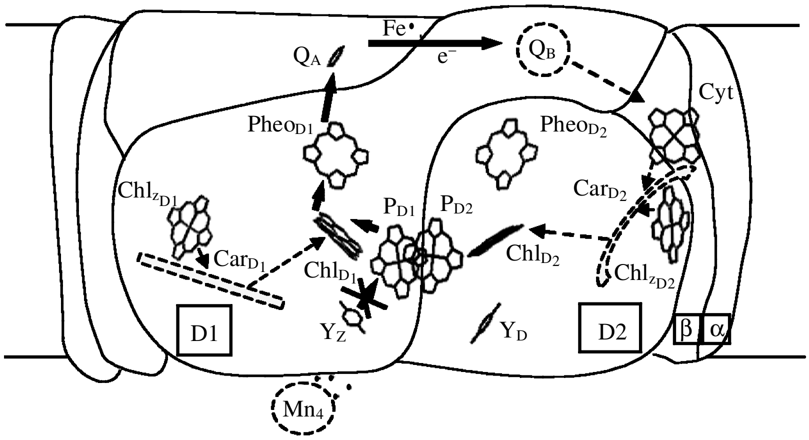

Figure 3. Model of the PSII complex in the thylakoid membrane showing cyclic electron flow around PSII when electron transfer from Y_Z is inhibited. Positions for the two Car molecules, that are unresolved in the X-ray structure (Zouni *et al.* 2001) are postulated.

within relatively few turnovers (E. Schlodder & A. Telfer, personal observations). This is presumably due to the lack of electrons to reduce either of the Car cations as isolation of the complex converts cyt *b*559 to its low potential form and it is therefore oxidized at ambient redox potential. Thus, it seems that any electron-transfer reactions involving Car^+ formation require it to be rapidly re-reduced if the pathway is to have a viable photoprotective function.

If, in contrast to the D2 side, no cyclic electron-transfer path exists on the D1 side, oxidation of Car on that side would seem to be undesirable. Perhaps it is in fact much slower than Car oxidation on the D2 side. Hanley *et al.* (1999) found that no Car^+ could be trapped at 20 K if the cytochrome was pre-reduced implying that Car^+_{D2} is not normally accumulated. However, both of the β-carotenes cations can be accumulated in isolated PSII RCs and have recently been shown to have distinctly different resonance Raman spectra (Telfer *et al.* 2003). This should prove a useful tool in distinguishing either which β-carotene is oxidized (or whether both β-carotenes are oxidized) in larger, more intact PSII complexes. Owing to the lability of the carotenoid cation state (see the discussion in Edge *et al.* 1997), Telfer & Barber (1995) proposed that β-carotene is a 'sacrificial' electron donor *in vivo* if donation of electrons from water is inhibited and P^+_{680} is in danger of being accumulated. However, even if Car oxidation on the D1 side occurs only under high light stress when all other electron sources are exhausted, the use of a sacrifice that delays the photodestruction of the RC by one or two charge separations is not obvious.

8. STRUCTURAL ROLE OF THE β-CAROTENES AND POSSIBLE MARKER FOR PROTEASE ATTACK?

Carotenoids are well known to have an important structural role in the assembly of photosynthetic protein complexes (e.g. Lang & Hunter 1994). Indeed β-carotene is essential for PSII, as shown by the fact that the D1 protein and hence PSII activity are lost if carotenoid synthesis is inhibited (Trebst & Depka 1997). Apparently, the presence of β-carotene is required for the stable assembly of the PSII complex. Trebst & Depka (1997) suggested that bleaching of β-carotene, which occurs under high light, destabilizes the structure. It is more probable that a low quantum yield oxidation of β-carotene leads to its loss (see Edge *et al.* 1997) and that it is not bleached by $^1O_2$, as they suggested (Trebst & Depka 1997). This indeed seems to be the case as the β-carotene in isolated PSII RCs is far more rapidly inactivated if the Car cation is accumulated than when large quantities of $^1O_2$ are produced on illumination in the absence of an electron acceptor (Telfer *et al.* 1991).

If the β-carotene associated with the D2 polypeptide (Car_{D2}) catalyses an efficient cycle of electrons around PSII via cyt *b*559, oxidation of the other (Car_{D1}) may perhaps be viewed as a last ditch approach when the electron supply from water to P^+_{680} is limiting. The lack of an efficient electron donor to Car_{D1} would mean that it is rapidly destroyed. Loss of this β-carotene might then be the trigger for D1 degradation occurring *in vivo* under 'donor-side' photoinhibitory conditions (Barber & Andersson 1992). This could explain why D1 is so much more labile than D2 and offers a mechanism by which PSII is inactivated without any of its other proteins being too drastically affected.

9. LIGHT-HARVESTING ROLE OF THE β-CAROTENES

The β-carotene in the PSII RC has clearly not been selected for light-harvesting efficiency: fluorescence excitation spectra indicate efficiencies in the 20–30% range for singlet–singlet excitation transfer to chlorophyll (Van Dorssen *et al.* 1987*a*; Kwa *et al.* 1992). In some, but not all, bacterial RCs and in LHC complexes efficiencies close to 100% have been observed (see Frank & Cogdell 1993). The long-distance Förster mechanism of singlet–singlet excitation transfer does not work for Car to Chl transfer and close contact between the two molecules is required. However, relatively low efficiencies, 35–40%, are also observed in the PSII core antenna complexes (Van Dorssen *et al.* 1987*a*,*b*; Van Leeuwen 1993) where the β-carotene must be within the van der Waals' distance of chlorophyll because there is Car triplet production (Takahashi *et al.* 1987). PSII-enriched membranes have a far greater efficiency of energy transfer but this was attributed to the outer xanthophyll-containing antenna complexes (Van Dorssen *et al.* 1987*b*). β-carotene is, however, twice as effective in scavenging $^1O_2$ as lutein for example, which is the main carotenoid in the outer antenna complexes (see Edge *et al.* 1997). The presence of β-carotene in and near the PSII RC may therefore have been selected for its $^1O_2$ scavenging ability, in spite of its rather poor light-harvesting efficiency.

The more interesting question, therefore, is how the β-carotene in the PSII RC can transfer singlet excitation energy to chlorophyll nearly as efficiently as in the core antenna, where β-carotene must be within the van der Waals' distance from chlorophyll. It cannot transfer energy to one of the four central chlorophylls (figure 3) because it is not rapidly oxidized by P^+_{680}. It has been indicated that the Car transfers energy to pheophytin (Mimuro *et al.* 1995), however, the lack of any effect when the pheophytins are exchanged with a pheophytin homologue makes this most unlikely (Germano *et al.* 2001). Therefore the Cars are more likely to be close to the peripheral His118 chlorophylls. The energy absorbed by the β-carotenes is hence not wasted and this may well be the main advantage for the presence of the peripheral chlorophylls in the RC complex.

10. TENTATIVE EVALUATION

The overall picture emerging from the considerations given in this paper is as follows.

(i) Quenching of $^3P_{680}$ by β-carotene is not possible because they would have to be so close to P_{680} that their oxidation by P^+_{680} would be much faster than the oxidation of Y_Z.

(ii) Consequently, $^1O_2$ will be formed whenever charge recombination to $^3P_{680}$ takes place and scavenging of this $^1O_2$ is a primary function of the β-carotene molecules in the PSII RC.

(iii) Spectroscopic measurements indicate that the two β-carotene molecules in the PSII RC are bound in rather different environments: one to the D1 protein and one to D2.

(iv) When electron transfer at the donor side fails to reduce P^+_{680} rapidly, the β-carotene on the D2 side may mediate a photoprotective electron-transfer cycle via cyt *b*559 but there is no evidence for that on the D1 side.

(v) The role of $Chl_{z_{D2}}$ in such a cycle is of doubtful significance because it is too far from both P_{680} and cyt *b*559. Its oxidation by Car^+ may merely be the consequence of its close association with Car, which is required to salvage some of the excitation energy absorbed by Car.

(vi) When all else fails, oxidation of the peripheral chlorophylls by Car^+ may help to alleviate excitation pressure by quenching of fluorescence by the chlorophyll cation. Car^+ itself is too unstable to accumulate.

(vii) If the destruction of Car^+ indeed destabilizes the RC protein, the one on the D1 side might serve as an important signalling function for protease attack, selectively destroying the D1 protein to prevent further charge separations.

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Discussion

G. W. Brudvig (*Department of Chemistry, Yale University, New Haven, CT, USA*). Because P^{+}_{680} appears to be on Chl_{D1}, do you think that the D1-side carotenoid may be preferentially oxidized over the D2-side carotenoid?

A. Telfer. Experiments with larger preparations than the PSII RC suggest that the D2-side carotenoid is preferentially oxidized (see comment by A. W. Rutherford, below). Therefore, it is likely that the D1-side carotenoid is not orientated as favourably for oxidation by P^{+}_{680} as the other, despite the fact that P^{+}_{680} appears to be located mainly on Chl_{D1}.

T. A. Moore (*Department of Chemistry and Biochemistry and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ, USA*). Regarding the singlet oxygen emission that you measured at 1270 nm, it looks very long lived. Why is this?

A. Telfer. Singlet oxygen emission was measured in deuterated medium because the lifetime is *ca.* 70 μs in D_2O, whereas it is less than 4 μs in water. The latter is faster than the reported rate of quenching of the P_{680} triplet and therefore single oxygen emission from PSII RCs cannot be detected in aqueous medium (Telfer *et al.* 1994*b*).

S. Styring (*Department of Biochemistry, Lund University, Lund, Sweden*). I have two questions. First, why do you place a carotenoid on both the D1 and D2 sides of the reaction centre? Second, you say that the two carotenoids are not coupled; what evidence is there for this?

A. Telfer. There is evidence that both peripheral chlorophylls can be oxidized and that, in each case, they are too far from the chlorophylls of P_{680} to be oxidized without an intermediary electron carrier. It is therefore suggested that the two carotenoids carry out this function. Pheophytin exchange experiments, by Germano *et al.* (2001), indicate that there is no excitonic coupling of the two carotenoids. See Germano *et al.* (2001) for a discussion of this subject.

A. W. Rutherford (*Service de Bioénergétique, Saclay, France*). I wish to make a comment. With regard to the two side branches of electron transfer to P_{680}, the cyt *b*559 always wins the electron-transfer competition. Peter Faller in my group, in a recent paper (Faller *et al.* 2001*b*) showed that cyt *b*559 is oxidized with no competition from the Chl_Z on the D1 side. This means that the D2 side Car outcompetes donation from the D1 side.

A. Telfer. I agree that it seems to be the case in larger PSII particles and presumably *in vivo* too. In isolated PSII RCs with an added external electron acceptor, both carotenoids can be oxidized to an approximately equal extent.

P. Fromme (*Max-Volmer-Laboratorium für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Berlin, Germany*). When you prepare your core reaction centres with 0, 1 and 2 carotenoids, can you selectively remove only one of the two carotenoids?

A. Telfer. No, because I used aqueous-based buffer and detergent, which removes the carotenoids non-selectively. However, Tomo *et al.* (1997) were able to remove one (Car_{507}) or both carotenoids using ether containing different amounts of water.

E.-M. Aro (*Department of Biology, Plant Physiology and Molecular Biology, University of Turku, Turku, Finland*). I have a question and a comment. What is the quantum yield for the loss of the β-carotene on the D1 side? The loss of β-carotene in relation to PSII inactivation and damage to the D1 protein would fit very well with the studies of A. Trebst and colleagues (*Biochemie der Pflanzen, Ruhr Universität, Bochum, Germany*), showing that carotenoid biosynthesis is a prerequisite for recovery from photoinhibition via D1 replacement in PSII. Do you have any comment on this?

A. Telfer. No, but I assume that it must be low for, as discussed above, Car_{D1} is not the carotenoid, which is preferentially oxidized *in vivo*. Normally, under conditions leading to an increase in the lifetime of P_{680}^{+}, Car_{D2} would be oxidized and re-reduced via a cycle involving cyt *b*559. However, I suggest that a low quantum yield oxidation of Car_{D1} may also occur. The lack of a ready electron supply for re-reduction of the cation would lead to breakdown of the β-carotene molecule and hence destabilization of D1. This is consistent with the studies of Trebst and colleagues.

A. W. Rutherford. What is known about the chemistry following the oxidization and degradation of the β-carotene?

A. Telfer. I do not know what the breakdown products are, but they do not absorb in the visible region. This is something that still needs to be investigated.

GLOSSARY

Car: carotene
Chl: chlorophyll
CP: chlorophyll protein
cyt *b*559: cytochrome *b*559
EPR: electron paramagnetic resonance
HPLC: high-performance liquid chromatography
LD: linear dichroic
LHC: light-harvesting complex
Pheo *a*: pheophytin *a*
PSII: photosystem II
pBRC: purple bacterial reaction centre
RC: reaction centre