PCP
PLANT & CELL PHYSIOLOGY

Special Focus Issue – Review

Singlet Oxygen Production by PSII Under Light Stress: Mechanism, Detection and the Protective role of β-Carotene

Alison Telfer*

Department of Life Sciences, Sir Ernst Chain Building, Imperial College London, London SW7 2AZ, UK
*Corresponding author. E-mail, a.telfer@imperial.ac.uk
(Received January 22, 2014; Accepted February 14, 2014)

In this review, I outline the indirect evidence for the formation of singlet oxygen ($^1O_2$) obtained from experiments with the isolated PSII reaction center complex. I also review the methods we used to measure singlet oxygen directly, including luminescence at 1,270 nm, both steady state and time resolved. Other methods we used were histidine-catalyzed molecular oxygen uptake (enabling $^1O_2$ yield measurements), and dye bleaching and difference absorption spectroscopy to identify where quenchers of $^1O_2$ can access this toxic species. We also demonstrated the protective behavior of carotenoids bound within Chl–protein complexes which bring about a substantial amount of $^1O_2$ quenching within the reaction center complex. Finally, I describe how these techniques have been used and expanded in research on photoinhibition and on the role of $^1O_2$ as a signaling molecule in instigating cellular responses to various stress factors. I also discuss the current views on the role of $^1O_2$ as a signaling molecule and the distance it might be able to travel within cells.

Keywords: Chlorophyll • Photosynthesis • Reactive oxygen species • Triplet states.

Abbreviations: Car, carotenoid; EPR, electron paramagnetic resonance; $L_{1,270}$, luminescence at 1,270 nm; LHC, light-harvesting complex; PDT, photodynamic therapy; $^1O_2$, singlet oxygen, $^3O_2$, triplet oxygen; P_{680}, primary electron donor in PSII; Pheo, primary electron acceptor in PSII; RC, reaction center; Q_A and Q_B, secondary electron acceptors in PSII; RC, reaction center; RNO, *p*-nitrosodimethylaniline; ROS, reactive oxygen species; RP, radical pair; SOSG, singlet oxygen sensor green; TM, thylakoid membranes; Yz, tyrosine electron donor to P_{680}^+.

Introduction

Singlet oxygen ($^1O_2$) is an electronically excited state of molecular oxygen which is extremely reactive (Ogilby 2010). It attacks and oxidizes proteins, lipids and nucleic acids, and consequently it is an important reactive oxygen species (ROS) in biological systems. It is less stable than triplet oxygen ($^3O_2$), and may be formed in a variety of ways; however, a common way is by electronic energy transfer from the triplet state of a photosensitized pigment or dye molecule.

$$S+h\nu \rightarrow {}^1S^* \rightarrow {}^3S^*+{}^3O_2 \rightarrow S+{}^1O_2 \quad (1)$$

where S is a sensitizer molecule, dye or pigment. During oxygenic photosynthesis (Blankenship 2014), $^1O_2$ is easily formed as Chl molecules are very good photosensitizers and the nature of the photosynthetic process means that there is always plenty of ground state, $^3O_2$, around.

Photosensitization of the triplet state of Chl leads to formation of $^1O_2$ unless Chl triplets are removed rapidly before $^1O_2$ formation can take place (Ogilby 2010). Carotenoid (Car) molecules are very effective quenchers of triplet Chl (Frank and Cogdell 1996) and also directly of $^1O_2$ (Hirayama et al. 1994) in photosynthetic systems. However, despite their effectiveness in the protection of photosynthetic organisms, high light intensities do bring about loss of photosynthetic activity in oxygenic organisms as reflected by the physiological phenomenon of photoinhibition (Prasil et al. 1992, Aro et al. 1993, Adir et al. 2003).

The phenomenon of photoinhibition has been localized mainly to the photosynthetic reaction center (RC) of PSII. High light initially causes a decrease in the rate of electron transport through PSII and preferential degradation of the Dl protein, an intrinsic subunit of the complex. Restoration of activity requires de novo protein synthesis. Molecular oxygen has been implicated in photoinhibition (Prasil et al. 1992), and damaging oxygen species, $^1O_2$ and other ROS, are likely to be the agents that activate Dl protein degradation (Barber and Andersson 1992, Fischer et al. 2013). Keren et al. (1997) have also argued that PSII can be inactivated at low light levels and that formation of the Chl triplet state in PSII and $^1O_2$ is involved. This is discussed in more detail later.

Here I will describe the history of the detection of $^1O_2$ formed by isolated photosynthetic complexes and demonstrate the protective behavior of Cars bound within Chl–protein complexes, and then relate this information to current research in photoinhibition and its function as a signaling molecule in instigating cellular responses to various stress factors.

Plant Cell Physiol. 55(7): 1216–1223 (2014) doi:10.1093/pcp/pcu040, available online at www.pcp.oxfordjournals.org
© The Author 2014. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

Photosynthetic Pigment–Protein complexes

Photosynthetic electron transport is carried out by a series of Chl–protein complexes. The antenna pigments are bound to light-harvesting pigment–protein complexes (LHCI and LHCII), which absorb light, producing the first excited singlet state of Chl, and then there are a series of energy transfer reactions, between the antenna Chl and the RCs. Here the first photochemical step occurs in which a specialized Chl molecule (P) on excitation to its excited singlet state passes on an electron to an acceptor molecule (A) to form the primary radical pair, P^+A^-. During oxygenic photosynthesis, two photochemical reactions occur in series catalyzed by two pigment–protein complexes known as PSII and PSI. In PSII, the oxidized electron donor is re-reduced by electrons extracted from water (a by-product being molecular oxygen after extraction of four electrons from two water molecules), while in PSI the reduced acceptor donates two electrons to $NADP^+$ to form NADPH.

The four pigment–protein complexes of green plants (PSI, PSII, and the antenna complexes LHCI and LHCII) all bind approximately 1 Car molecule per 4 Chl molecules and it is the Cars that normally prevent the formation of $^1O_2$. Car-deficient mutants can grow from seed but are bleached and die as soon as they see normal light (Walles 1965).

Car molecules, provided they are bound within van der Waals distance of the Chl, are extremely efficient quenchers of Chl triplets (**Fig. 1**). One of the earliest experiments demonstrating the transfer of energy from the triplet excited state of Chl to Car was the so-called 'valve reaction' of Witt (1971) in which an increase in the size of an absorbance change (due to ^{3}Car formation) was seen only once photosynthetic electron transfer was light saturated. It then continued to rise more or less linearly with the intensity of the laser flash energy.

There are two mechanisms by which Chl triplets are formed. In the antenna complexes it is by intersystem crossing:

$$\mathrm{Chl}+h\nu \rightarrow {}^1\mathrm{Chl}^* \rightarrow {}^3\mathrm{Chl}^* + {}^3O_2 \rightarrow \mathrm{Chl} + {}^1O_2 \qquad (2)$$

while in the PSII reaction centre it is by the radical pair (RP) mechanism:

$$\mathrm{Chl}+h\nu \rightarrow {}^1\mathrm{Chl}^* \rightarrow \text{ energy transfer to the RC} \rightarrow {}^1P_{680}{}^*\mathrm{Pheo} \rightarrow {}^1\left[P_{680}{}^+\mathrm{Pheo}^-\right] \rightarrow P_{680}{}^+Q_A{}^- \qquad (3)$$

$P_{680}{}^+Q_A{}^-$ recombination occurs either directly or indirectly:

$$\text{indirect} \quad P_{680}{}^+Q_A{}^- \rightarrow {}^1\left[P_{680}{}^+\mathrm{Pheo}^-\right] \rightarrow {}^3\left[P_{680}{}^+\mathrm{Pheo}^-\right] \rightarrow {}^3P_{680} + {}^3O_2 \rightarrow P + {}^1O_2 \qquad (4)$$

$$\text{direct} \quad P_{680}{}^+Q_A{}^- \rightarrow P_{680}Q_A \qquad (5)$$

where P_{680} is the primary Chl electron donor in PSII, Pheo is the primary electron acceptor in PSII, and Q_A is a plastoquinone molecule, which is the second electron acceptor in PSII. The indirect pathway leads to formation of $^1O_2$ while the direct pathway does not. In experiments where the midpoint potential of the secondary electron acceptor Q_A was made more positive (and hence decreased the likelihood of the indirect pathway), the yield of $^1O_2$ was lowered while when it was made more negative the yield was increased (Krieger-Liszkay and Rutherford 1998, Fufezan et al. 2002).

The two mechanisms of Chl triplet formation can be distinguished by their electron paramagneitc resonance (EPR) signal properties. The radical pair triplet is only formed after formation of $P_{680}{}^+\mathrm{Pheo}^-$, which gives a spin-polarized EPR triplet signal, after spin dephasing, which has a characteristic absorption (A) and emission (E) spectrum (AEEAAE) as opposed to the pattern seen in triplets formed by intersystem crossing (AEAEAE) (van Mieghem et al. 1991).

The D1D2 Reaction Center Complex of PSII and Indirect Evidence for $^1O_2$ Formation

It was the instability, in the presence of molecular oxygen, of the PSII RC complex (also known as the D1D2 complex) isolated by

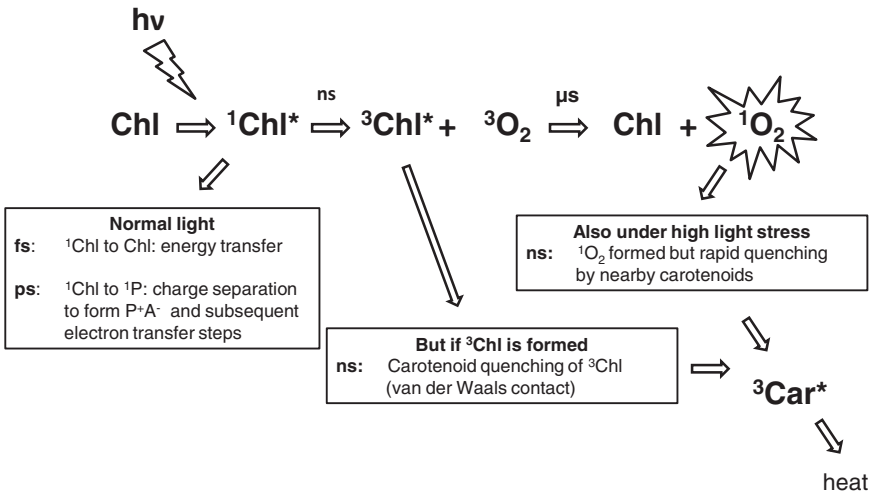

Fig. 1 Avoidance of ^{3}Chl and $^1O_2$ formation: time scales involved.

Satoh and colleagues (Nanba and Satoh 1987) which first led to the suggestion that large amounts of $^1O_2$ were being formed by this complex due to interaction of the radical pair triplet state with molecular oxygen (Barber et al. 1987, Durrant et al. 1990) (see **Fig. 2**).

The presence of molecular oxygen was found to bleach the sample and to inactivate P_{680}, the primary electron donor, it also shortened the P_{680} triplet lifetime from 1 ms (under anaerobic conditions) to 33 μs (Durrant et al. 1990). The first observation was that the complex showed a high yield of the radical pair recombination triplet state (Okamura et al. 1987), which shows the distinctive and specific absorption and emission EPR spectrum (AEEAAE), indicative of the formation of the P_{680} triplet state via the RP mechanism (see Equations 3–5). There was virtually no triplet Car formed by the D1D2 RC complex (Takahashi et al. 1987, Durrant et al. 1990).

The question arose as to why the two Cars bound to the RC were not protecting against $^1O_2$ formation by quenching the RP triplet (Telfer 2002). This was shown by De Las Rivas et al. (1993) to be because the Car is oxidized by P_{680}^+ if this highly oxidizing species is allowed to persist for any length of time, i.e. in the presence of an added artificial electron acceptor which can stabilize P_{680}^+ (Barber et al. 1987). The oxidized Car is very unstable and its absorption (420–520 nm) is rapidly irreversibly bleached (De Las Rivas et al. 1993).

$^1O_2$ Production by Photosynthetic Pigment–Protein Complexes

Conditions arise where $^1O_2$ can be formed in photosynthetic light-harvesting antenna complexes by triplet–triplet excitation transfer (intersystem crossing), as was seen by Wolff and Witt (1969) at high light intensities, when electron transport is light saturated and the Car triplet yield is increased substantially. Here Cars can prevent $^1O_2$ formation as they are bound in the antenna complexes within van der Waals contact and so quench Chl triplets which are formed on a nanosecond time scale (Schödel et al. 1998). The Car triplets then decay harmlessly, releasing heat.

It is very unlikely that Chl triplets are formed in the PSI RC under photoinhibitory conditions (Hideg and Vass 1995, Rutherford et al. 2012), but there is evidence for their formation in PSII which is related to the very high oxidizing potential of P_{680}^+, which is required for water oxidation to occur. The β-carotene in the RC has been shown to be bound well beyond van der Waals distance from the Chls of the RC cofactor cluster (**Fig. 2**; Loll et al. 2005) and so cannot be invoked to quench directly any $^3P_{680}$ which might be formed. There is strong evidence that both under high light (van Mieghem et al. 1989, Vass et al. 1992) and also under very low light $^3P_{680}$ is formed via the radical pair recombination pathway (Keren et al. 1997). The significance of this mechanism for formation of the primary donor triplet state is that P_{680}^+ must be formed first (Equations 3–5) and as it has such a high redox potential any Car bound close enough to quench a triplet would have been oxidized previously by the cationic P_{680}.

It was not only the very high sensitivity of the isolated D1D2 complex to light in the presence of molecular oxygen which suggested that $^1O_2$ was being formed at a high yield. The lifetime of the $^3P_{680}$ was lengthened dramatically under anaerobic conditions (from ~30 μs to 1 ms) and the consequent inactivation of P_{680}, loss of the red-most absorbance due to P_{680} and degradation of the D1 and D2 proteins were ascribed to the damaging effect of $^1O_2$ (Durrant et al. 1990, Barber and Archer 2001). Note that during the early 1990s it became clear that $^1O_2$

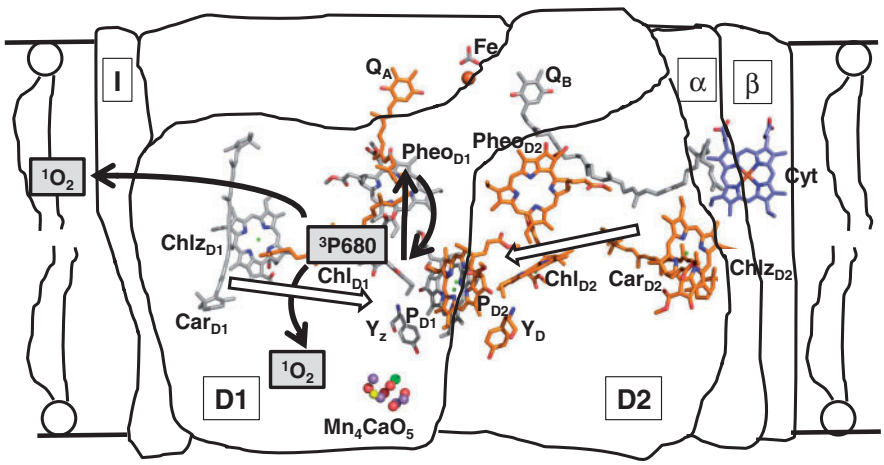

Fig. 2 Schematic diagram of the electron transfer reactions occurring in the membrane-bound PSII RC and the formation of singlet oxygen at the site of ^{3}P680. The purified complex (D1, D2, α and β subunits of Cyt b_{559} and the PsbI protein) has lost both of the secondary electron acceptors, Q_A and Q_B, the non-heme iron (Fe) and also the water-splitting Mn cluster, Mn_4CaO_5. The figure shows that if the triplet state of P_{680} is formed it will be quenched by ground state oxygen to form $^1O_2$ which can damage either the pigment–protein complex or the lipid membrane. The cofactor arrangement in the RC complex shows the distance of the two β-carotenes from the four central Chl cofactors, based on Umena et al. (2011).

formation by the isolated D1D2 complex occurs but it had only been detected by indirect methods.

Direct Detection of $^1O_2$ Formed by Isolated PSII RCs

It was in the early 1990s that we began using the techniques used by experimentalists studying photosensitization of $^1O_2$ by dyes to be used in photodynamic therapy (PDT) (Allison 2004) to look for direct evidence for $^1O_2$ formation by the D1D2 complex. The dramatic change in the lifetime of the P_{680} triplet and the fact that the Chl triplet yield was very high, about 0.3, whereas that of the Car triplet was very low (0.03) all suggested that the yield of $^1O_2$ should also be high (Durrant et al. 1990).

The first technique we used was to look for the very weak luminescence at 1,270 nm from $^1O_2$. This is so weak that it is only detected using a 77 K cooled photomultiplier (Macpherson et al. 1993). Here we showed steady-state emission of luminescence at 1,270 nm from the isolated D1D2 complex on illumination under aerobic conditions. The luminescence ($L_{1,270}$) was partially quenched by azide, a known quencher of $^1O_2$. The azide-quenchable part of the signal (30–50%) was concluded to be due to $^1O_2$ and the remaining part to infrared phosphorescence from the Chls in the PSII RC. Note that it was necessary to exchange the RCs into a D_2O medium as water itself is a very good quencher of $^1O_2$, shortening its lifetime from ~70 μs to ~3 μs and hence reducing the size of the steady-state emission signal until it was undetectable (Gorman and Rogers 1989, Wilkinson et al. 1995). As concluded by Telfer et al. (1994a), this was probably the first direct observation of $^1O_2$ luminescence sensitized by an intrinsically bound chromophore in a defined biological system as opposed to a sensitizer-doped biological material (e.g. Firey and Rogers 1998).

Complementary to the $L_{1,270}$ method, we also used a chemical trapping technique to estimate the yield of $^1O_2$ formed on illumination of the isolated D1D2 complex (Telfer et al. 1994a). The uptake of molecular oxygen due to the reaction of $^1O_2$ with histidine or imidazole was measured using an oxygen electrode, and the yield was compared with similar experiments using $^1O_2$ sensitizing dyes such as mesotetra-(4-sulfonatophenyl)porphine (TPPS) and aluminum phthalocyanine disulfonate (AlPcS) for which the $^1O_2$ yield is already known. We found that the yield of $^1O_2$ was about 0.16 whereas the yield of $^3P_{680}$ in the complex is 0.3 (Durrant et al. 1990). The lower yield of $^1O_2$ as compared with that of the Chl triplet is to be expected as some $^1O_2$ will be quenched rapidly by the protein and pigments within the RC complex before it escapes into the medium.

We also used the dye bleaching technique of Kraljic and El Mohsni (1978) to detect $^1O_2$. This technique is based on the bleaching of *p*-nitrosodimethylaniline (RNO) to the nitro form caused by the trans-annular peroxide product of the reaction of $^1O_2$ with either histidine or imidazole. We measured the bleaching of the dye due to $^1O_2$ simultaneously with the bleaching of Chl associated with the inactivation of the sample by this ROS (Telfer et al. 1994a).

All these techniques indicated that under illumination and aerobic conditions, the D1D2 complex produces a large amount of $^1O_2$. It escapes from the complex into the aqueous medium, and we conclude that it was quenched or detected there as there was no protection against bleaching of the Chl by added quenchers such as azide or histidine or by water vs. D_2O (Telfer et al. 1994a). This effect had been noted by Macpherson et al. (1993) where the bleaching of Chl was not prevented by the addition of azide, although the $L_{1,270}$ was quenched, leading us to conclude that the $^1O_2$ detected as emission at 1,270 nm is in a different environment (accessible to quenchers) from that giving rise to the beaching of Chl. In essence the $^1O_2$ is formed within the D1D2 complex on the Chl of P_{680} and then it diffuses out of the complex into the aqueous medium where not only is it accessible to water-soluble quenchers such as azide but its lifetime is lengthened by the presence of D_2O as compared with H_2O. In experiments where H_2O and D_2O buffers were compared, there was no stimulation of the inactivation of P_{680} in the latter medium compared with H_2O medium. This indicates that several rounds of buffer exchange of the complex (using Millipore concentrator tubes; see Macpherson et al. 1993), which was originally isolated in an H_2O-based medium, into a D_2O-based medium either does not exchange the water molecules within the complex or that there are no water molecules close enough to P_{680} to quench the $^1O_2$. The latest structure of the PSII core complex, which is at 1.9 Å resolution, shows that the majority of the very many water molecules in the structure are located in two layers on the surfaces of the stromal and lumenal sides (Umena et al. 2011). Of the few water molecules found in the interior of the complex, most of them serve as ligands to Chls. Note that the magnesium of the accessory Chl on D1, which is thought to be where the $^3P_{680}$ is located, is liganded by a water molecule as is that of accessory Chl_{D2}.

This evidence that the site of $^1O_2$ formation is deep within the D1D2 complex was confirmed by Telfer et al. (1994a) in RNO bleaching experiments. Absorption difference spectra show clearly that although the quencher, azide, prevents the RNO bleaching it does not stop the loss of absorption of P_{680}, which we concluded is caused by $^1O_2$ before it escapes from the complex, i.e. internal intrinsic quenching mechanisms compete very effectively with externally added water-soluble quenchers. However, the presence of D_2O in place of water increased the rate of RNO bleaching approximately 3-fold, which is consistent with the increase in the lifetime of $^1O_2$ in the external medium when it is present in place of H_2O.

Telfer et al. (1994a) also carried out a number of experiments to show that it was $^1O_2$ causing the inactivation of P_{680} and that it was not due to any other ROS. As Foote (1990) warned, 'detection of a species does not indicate its intermediacy in a process', and in PDT it has been difficult to demonstrate that it is actually $^1O_2$ causing cell death. We definitively showed that it is $^1O_2$ that brings about Chl bleaching

and inactivation of P_{680} in the isolated D1D2 complex (Telfer et al. 1994a).

Correlation of P_{680} Triplet Decay and $L_{1,270}$ Signal Rise Rates in the Isolated PSII RC

We also measured time-resolved 1,270 nm luminescence of $^1O_2$, formed on illumination of the D1D2 complex in the presence of D_2O when suspended in air-saturated medium (Macpherson et al. 1993, Telfer et al. 1994b), and later correlated the rise time of the $L_{1,270}$ signal with the decay of the $^3P_{680}$ absorption decrease at 680 nm (Telfer et al. 1999). Here we showed the similarity in the triplet decay rate and $L_{1,270}$ rise times and the dependence on the molecular oxygen concentration for the rate of quenching of the triplet and rise of the $L_{1,270}$ which indicated that $^1O_2$ is formed directly by quenching of $^3P_{680}$. Li et al. (2012) carried out similar experiments, measuring time-resolved $L_{1,270}$, on isolated PSII RCs in aqueous media and concluded that the lifetime of the $^1O_2$ would be so short (<0.5 μs) that determining the $^1O_2$ rate constants in chloroplasts suspended in aqueous medium, i.e. in vivo conditions, would be 'a tall order', i.e. they imply it would be impossible.

Role of β-Carotene in Protection against Photodamage

As shown in **Fig. 2**, the β-carotene bound to the PSII RC can act as an admittedly relatively inefficient electron donor to P_{680}^+ (De Las Rivas et al. 1993). This occurs if the lifetime of the oxidized donor is prolonged by the addition of an artificial electron acceptor, e.g. silicomolybdate and dibromothymoquinone, which are able to accept electrons directly from the pheophytin primary electron acceptor which is bound to the D1 protein of the RC complex. In addition to this role of rereduction of P_{680}^+ (if it is not reduced rapidly by the tyrosine electron donor, Y_Z, and then by electrons from water), the Car should also quench $^1O_2$, diffusing within the Chl protein complex, directly. The idea, as discussed already, is that the Car cannot be bound closely enough to quench $^3P_{680}$ directly as it would be oxidized first by P_{680}^+ which has to be formed prior to the formation of the triplet by the radical pair mechanism (Telfer 2002). Indeed the crystal structure of PSII by Loll et al. (2005) shows that the closest approach of the two β-carotenes is 13.2 Å for Car_{D2} to Chl_{D2} and 19.9 Å for Car_{D1} to Chl_{D1} (**Fig. 2**). Using the Moser and Dutton rule (1992), both distances are far too great to allow either rapid quenching of $^3P_{680}$ or rapid electron transfer directly to P_{680}^+.

The question arises as to what is the role of the Cars bound to the PSII RC aside from rereduction of any stabilized P_{680}^+. As discussed earlier, it is inevitable that some $^3P_{680}$ will be formed during turnover of the PSII RC (van Mieghem et al. 1989, Keren et al. 1997) and, because there will always be molecular oxygen around, $^1O_2$ will be formed. It was known that carotenoids can quench $^1O_2$ directly (Hirayama et al. 1994) and so we tested the proposition that this is another role for the Cars in the RC. The β-carotene level of isolated PSII RCs was lowered by extensive washing of the preparatory anion exchange column with low salt buffer before elution with high salt, and thus we prepared complexes with various levels of Car. We were then able to show an inverse correlation between the size of the $L_{1,270}$ signal and the Car level and the rate of irreversible bleaching of Chl, indicating that when the normal two Cars were present the complex was less susceptible to inactivation on illumination in the presence of molecular oxygen (Telfer et al. 1994b). The fact that when two Cars were present [i.e. as seen in the native structure, Loll et al. (2005) and see **Fig. 2**], they could not quench all of the $^1O_2$ formed is due to the fact that the $^1O_2$ formed at P_{680} can diffuse in all directions within the complex and, because of the distance of the Cars from the source of the $^1O_2$, a certain amount of damage will be done by $^1O_2$ not scavenged by them (Telfer 2002).

Conclusions

$^3P_{680}$ is inevitably formed within the PSII RC when operating in oxygenic organisms, which are also continuously evolving molecular oxygen, both at low light intensities and under high light, i.e. photoinhibitory, conditions. The P_{680} triplet thus forms $^1O_2$ as the RC Cars are unable to quench the triplet before its reaction with ground state $^3O_2$. $^1O_2$ scavenging mechanisms are in place, including the two β-carotene molecules bound to the D1 and D2 RC polypeptides and, in vivo, some of the other Cars bound near the interface between the inner antenna polypeptides CP43 and CP47 close to the D1 and the D2 polypeptides, respectively (Loll et al. 2005), may well also scavenge some $^1O_2$.

$^1O_2$ scavengers

$^1O_2$ that is not quenched by Car and hence escapes from the PSII core complex into the membrane will be quenched by tocopherol (Kruk et al. 2005) and plastoquinone (Kruk and Trebst 2008, Yadav et al. 2010), which is present in the thylakoid lipid membranes. Tocopherol has been implicated in protection against $^1O_2$ damage to the membrane lipids (Kruk et al. 2005, Krieger-Liszkay and Trebst 2006). However, this is a sacrificial reaction and depends on resynthesis, using ascorbate, to restore depleted stocks of tocopherol. Inevitably some $^1O_2$ will escape quenchers and may exit into the aqueous thylakoid lumen or stroma where it may damage proteins and nucleic acids. In the stroma, ascorbate is a good scavenger (Bisby et al. 1999) and it is usually present at high levels. It is replenished using glutathione and NADPH (Smirnoff 2000). For a discussion on scavenger effectiveness in photosynthetic systems. see Li et al. (2012).

Relevance in vivo—photoinhibition and retrograde signaling

After the initial demonstration of formation of $^1O_2$ by D1D2 complexes (Macpherson et al. 1993), $^1O_2$ formation by isolated

thylakoid membranes (TMs) after a high light treatment (photoinhibition) was demonstrated by Hideg et al. (1994), using a spin trapping technique, using the spin label 2,2,6,6,tetramethylpiperidine (TEMP) and EPR spectroscopy. These techniques have subsequently been used to demonstrate formation of $^1O_2$ in PSII-enriched particles subjected to high light conditions (Krieger et al. 1998, Fufezan et al. 2002) though use of this technique in leaves is not possible (Fischer et al. 2013).

Since our initial demonstrations, during the early 1990s, of $^1O_2$ detection in PSII RCs the techniques have been expanded greatly not only to show its involvement in photoinhibition in cells (see reviews by Krieger-Liskay et al. 2008, Fischer et al. 2013) but also it has been invoked in retrograde signaling (from the chloroplast to the nucleus) inducing cellular responses to environmental stresses including high light (op den Camp et al. 2003, Apel and colleagues; Fischer et al. 2004, Trianphylidès et al. 2009, Reinbothe et al. 2010).

Some of the new techniques employed recently, to detect $^1O_2$, are changes in fluorescence or luminescence of $^1O_2$-specific probes (such as DanePy and singlet oxygen sensor green, SOSG) along with imaging (for further information on the success and problems with these techniques, see Fischer et al. 2013). Fischer et al. (2007) used the DanePy technique to detect $^1O_2$ in the cytoplasm of *Chlamydomonas reinhardtii* cells after very high intensity light stress. The significant result they found was that treatment with herbicides, which change the redox potential of Q_A (Rutherford and Krieger-Liszkay 2001), had the expected effect on the size of the $^1O_2$ signal, indicating the PSII origin of the $^1O_2$. SOSG was also used with confocal microscopy to image $^1O_2$ formation by *Synechocystis* sp. PCC 6803 cells (Sinha et al. 2012). Additionally histidine-catalyzed molecular oxygen uptake has been demonstrated in high light-stressed *Synechocystis* sp. PCC 6803 cells (Rehman et al. 2013), and exogenously formed $^1O_2$ (Rose Bengal sensitized) has been shown to stimulate gene expression in *Arabidopsis thaliana* (see Fischer et al. 2013).

As discussed already there are so many quenching processes going on that it was thought that it was not possible for $^1O_2$ to get far enough to act as a signal to activate protein synthesis in the nucleus, and the debate rages as to whether $^1O_2$ can travel that far and induce gene expression directly or whether the $^1O_2$ detected in the cytoplasm is produced by secondary reactions. It is more likely that lipid peroxidation side products, i.e. peroxyl radicals, regenerate $^1O_2$ by the Russell mechanism (Miyamoto et al. 2007). Recent evidence for this mechanism comes from Pospíšil and colleagues in response to both heat (Chan et al. 2012) and high light (Yadav and Pospíšil 2012).

Many calculations have been carried out to try and work out the distance $^1O_2$ might be able to travel in the chloroplast and cell— and the projected distance is getting longer and longer. According to Moan (1990), it should only travel $<$70 nm before being quenched or decaying, but a more recent estimate in liposomes was $>$100 nm in 10 μs (Ehrenberg et al. 1998). However, it is likely that this would be much less in TMs as they contain, in addition to ascorbate, a lot of unsaturated lipids and proteins which would act as physical quenchers of $^1O_2$, as pointed out by Ehrenberg et al. (1998). This will also be the case in the highly dense stroma and cytoplasm. More recently, Skovsen et al. (2005) showed that dye-sensitized $^1O_2$ can travel ~270 nm in 6 μs in the aqueous region of cells. However, this was measured in rat neurons and, as already mentioned, plant cells have high concentrations of antioxidants such as ascorbate in their cytoplasm (Bisby et al. 1999) which would reduce this distance considerably.

There is also the question of how much $^1O_2$ is formed. The greater the amount, the more chance that a few molecules will travel some distance before meeting a quencher. Fischer et al. (2007) showed that it required very high light intensities to produce detectable levels of $^1O_2$ in the cytoplasm of *C. reinhardtii* cells. However, it should be noted that in leaves TMs come very close to the chloroplast envelope and some chloroplasts are very close to the nucleus, although distances will still be greater than 270 nm. There is also a question of whether $^1O_2$ originating from PSII can carry out retrograde signaling directly or whether it activates a signal transduction pathway directed to the nucleus to activate gene expression in which secondarily produced $^1O_2$ may well play a part (Laloi et al. 2007, Baruah et al. 2009). The low light stress that also results in formation of some $^1O_2$ in the PSII RC (Keren et al. 1997) probably only yields levels that are sufficient to be involved in photoinhibition and triggering of the turnover of the D1 protein, and not for retrograde signaling.

Funding

This study was supported by the Agricultural and Food Research Council (AFRC) and the Biotechnology and Biological Sciences Research Council (BBSRC) [funding to Professor Jim Barber] and the Science and Engineering Research Council (SERC) and the Engineering and Physical Sciences Research Council (EPSRC) [funding to Professor David Phillips].

Acknowledgments

I am extremely grateful to all my colleagues who were involved in the detection of singlet oxygen in the 1990s. I would also like to thank Anja Krieger-Liszkay for discussions and critical advice about the manuscript. I am especially grateful to Professor Jim Barber (Biochemistry Department) and to Professor David Phillips (Chemistry Department) in Imperial College London and members of their research groups at the time.

Disclosures

The author has no conflicts of interest to declare.

References

Adir, N., Zer, H., Shochat, S. and Ohad, I. (2003) Photoinhibition—a historical perspective. *Photosynth. Res.* 76: 343–370.

Allison, R.R., Downie, G.H., Cuenca, R., Hu, X.-H., Childs, C.J.H. and Sibata, C.H. (2004) Photosensitizers in clinical PDT. *Photodiagn. Photodyn. Ther.* 1: 27–42.

Aro, E.M., Virgin, I. and Andersson, B. (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim. Biophys. Acta* 1143: 113–134.

Barber, J. and Andersson, B. (1992) Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem. Sci.* 17: 61–66.

Barber, J. and Archer, M.D. (2001) P680, the primary electron donor of photosystem II. *J. Photochem. Photobiol. A: Chem.* 142: 97–106.

Barber, J., Chapman, D.J. and Telfer, A. (1987) Characterisation of a PS II reaction centre isolated from the chloroplasts of *Pisum sativum*. *FEBS Lett.* 220: 67–73.

Baruah, A., Simkova, K., Apel, K. and Laloi, C. (2009) Arabidopsis mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development. *Plant Mol. Biol.* 70: 547–563.

Bisby, R.H., Morgan, C.G., Hamblett, I. and Gorman, A.A. (1999) Quenching of singlet oxygen by Trolox C, ascorbate, and amino acids: effects of pH and temperature. *J. Phys. Chem. A* 103: 7454–7459.

Blankenship, R.E. (2014) Molecular Mechanisms of Photosynthesis, 2nd edn. Wiley-Blackwell, Oxford.

Chan, T., Shimizu, Y., Pospíšil, P., Nijo, N., Fujiwara, A., Taninaka, Y. et al. (2012) Quality control of photosystem II: lipid peroxidation accelerates photoinhibition under excessive illumination. *PLoS One* 7: e52100.

De Las Rivas, J., Telfer, A. and Barber, J. (1993) Two coupled β-carotene molecules protect P680 from photodamage in isolated photosystem II reaction centres. *Biochim. Biophys. Acta* 1142: 155–164.

Durrant, J.R., Giorgi, L.B., Barber, J., Klug, D.R. and Porter, G. (1990) Characterization of triplet-states in isolated photosystem-II reaction centres—oxygen quenching as a mechanism for photodamage. *Biochim. Biophys. Acta* 1017: 167–175.

Ehrenberg, B., Anderson, J.L. and Foote, C.S. (1998) Kinetics and yield of singlet oxygen photosensitized by hypericin in organic and biological media. *Photochem. Photobiol.* 68: 135–140.

Firey, P.A. and Rodgers, M.A.J. (1988) Photochemical properties of ethrythrocyte ghosts containing porphyrins. *Photochem. Photobiol.* 47: 615–619.

Fischer, B.B., Hideg, E. and Krieger-Liszkay, A. (2013) Production, detection and redox signaling of singlet oxygen in photosynthetic organisms. *Antioxidants Redox Signal.* 18: 2145–2162.

Fischer, B.B., Krieger-Liszkay, A. and Eggen, R.I.L. (2004) Photosensitizers neutral red (type I) and rose bengal (type II) cause light dependent toxicity in *Chlamydomonas reinhardtii* and induce the Gpxh gene via increased singlet oxygen formation. *Environ. Sci. Technol.* 38: 6307–6313.

Fischer, B.B., Krieger-Liszkay, A., Hideg, E., Snyrychova, I., Wiesendanger, M. and Eggen, R.I.L. (2005) Role of singlet oxygen in chloroplast retrograde signalling in *Chlamydomonas reinhardtii*. *FEBS Lett.* 581: 5555–5560.

Foote, C. S. (1990) Future Directions and Applications in Photodynamic Therapy, SPIE Institute Series Vol. IS 6, pp. 115–126. SPIE Optical Engineering Press.

Frank, H.A. and Cogdell, R.J. (1996) Carotenoids in photosynthesis. *Photochem. Photobiol.* 63: 257–264.

Fufezan, C., Rutherford, A.W. and Krieger-Liszkay, A. (2002) Singlet oxygen production in herbicide-treated photosystem II. *FEBS Lett.* 532: 407–410.

Gorman, A.A. and Rodgers, M.A.J. (1989) Singlet oxygen. *In* The Handbook of Organic Photochemistry, Vol. 2. Edited by Scaiano, J.C. pp. 229–247. CRC Press, Boca Raton, FL.

Hideg, E., Spetea, C. and Vass, I. (1994) Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. *Photosynth. Res.* 39: 191–199.

Hideg, E. and Vass, I. (1995) Singlet oxygen is not produced in photosystem I under photoinhibitory conditions. *Photochem. Photobiol.* 62: 949–952.

Hirayama, O., Nakamura, K., Hamada, S. and Kobayasi, Y. (1994) Singlet oxygen quenching ability of naturally occurring carotenoids. *Lipids* 29: 149–150.

Keren, N., Berg, A., van Kan, P.J.M., Levanon, H. and Ohad, I. (1997) Mechanism of photosystem II photoinactivation and D1 protein degradation at low light: the role of back electron flow. *Proc. Natl Acad. Sci. USA* 19: 1579–1584.

Kraljic, I. and El Mohsni, S. (1978) A new method for detection of singlet oxygen in aqueous solutions. *Photochem. Photobiol.* 28: 577–581.

Krieger, A., Rutherford, A.W., Vass, I. and Hideg, E. (1998) Relationship between activity, D1 loss and Mn binding in photoinhibition of photosystem II. *Biochemistry* 37: 16262–16269.

Krieger-Liszkay, A., Fufezan, C. and Trebst, A. (2008) Singlet oxygen production in photosystem II and related protection mechanism. *Photosynth. Res.* 98: 551–564.

Krieger-Liszkay, A. and Rutherford, A.W. (1998) Influence of herbicide binding on the redox potential of the quinone acceptors in photosystem II: relevance to photodamage and phytotoxicity. *Biochemistry* 37: 17339–17344.

Krieger-Liszkay, A. and Trebst, A. (2006) Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction centre. *J Exp. Bot.* 57: 1677–1684.

Kruk, J., Holländer-Czytko, H., Oettmeier, W. and Trebst, A. (2005) Tocopherol as a singlet oxygen scavenger in Photosystem II. *J. Plant Physiol.* 162: 749–757.

Kruk, J. and Trebst, A. (2008) Plastoquinol as a singlet oxygen scavenger in photosystem II. *J Exp. Bot.* 1777: 154–162.

Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgia, I. and Apel, K. (2007) Cross-talk between singlet oxygen and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* 104: 672–677.

Li, H., Melo, T.B., Arellano, J.B. and Naqvi, K.R. (2012) Temporal profile of the singlet oxygen emission endogenously produced by photosystem II reaction centres in an aqueous buffer. *Photosynth. Res.* 112: 75–79.

Loll, B., Kern, J., Saenger, W., Zouni, A. and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438: 1040–1044.

Macpherson, A.N., Telfer, A., Truscott, T.G. and Barber, J. (1993) Direct detection of singlet oxygen from isolated photosystem two reaction centres. *Biochim. Biophys. Acta* 1143: 301–309.

Miyamoto, S., Ronsein, G.E., Prado, F.M., Uemi, M., Correa, T.C., Toma, I.N. et al. (2007) Biological hydroperoxides and singlet molecular oxygen generation. *IUBMB Life* 59: 322–331.

Moan, J. (1990) On the diffusion length of singlet oxygen in cells and tissues. *J. Photochem. Photobiol. B: Biol.* 6: 343–347.

Moser, C.C. and Dutton, P.L. (1992) Engineering protein structure for electron transfer function in photosynthetic reaction centers. *Biochim. Biophys. Acta* 1101: 171–176.

Nanba, O. and Satoh, K. (1987) Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. *Proc. Natl Acad. Sci. USA* 84: 109–112.

Ogilby, P.R. (2010) Singlet oxygen: there is indeed something new under the sun. *Chem. Soc. Rev.* 39: 3181–3209.

Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) Evidence of the primary charge separation in theD1/D2 complex of photosystem II from spinach: Epr of the triplet state. *In* Progress in Photosynthesis Research, Vol. 1. Edited by Biggins, J. pp. 379–381, Martinus Nijhoff, Dordrecht.

op den Camp, R.G.L., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A. et al. (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *Plant Cell* 15: 2320–2332.

Prasil, O., Adir, N. and Ohad, I. (1992) Dynamics of photosystem II: mechanism of photoinhibition and recovery process. *In* Topics in Photosynthesis. The Photosystems: Structure, Function and Molecular Biology, Vol. 11. Edited by Barber, J. pp. 220–250. Elsevier, Amsterdam.

Rehman, A.U., Cser, K., Sass, L. and Vass, I. (2013) Characterization of singlet oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium *Synechocystis* PCC 6803 by histidine-mediated chemical trapping. *Biochim. Biophys. Acta* 1827: 689–698.

Reinbothe, C., Pollmann, S. and Reinbothe, S. (2010) Singlet oxygen signalling links photosynthesis to translation and plant growth. *Trends Plant Sci.* 15: 499–506.

Rutherford, A.W. and Krieger-Liszkay, A. (2001) Herbicide-induced oxidative stress in photosystem II. *Trends Biochem. Sci.* 26: 648–653.

Rutherford, A.W., Osyczka, A. and Rappaport, F. (2012) Back-reactions, short circuits, leaks and other energy wasteful reactions in biological electron transfer: redox tuning to survive life in O_2. *FEBS Lett.* 586: 603–616.

Schödel, R., Irrgang, K.D., Voigt, J. and Renger, G. (1998) Rate of carotenoid triplet formation in solubilized light-harvesting complex II (LHCII) from spinach. *Biophys J.* 75: 3143–3153.

Sinha, R.K., Komenda, J., Knoppova, J., Sedlarova, M. and Pospíšil, P. (2012) Small CAB-like proteins prevent formation of singlet oxygen in the damaged photosystem II complex of the cyanobacterium Synechocystis sp. PCC 6803. *Plant, Cell Environ.* 35: 806–818.

Skovsen, E., Snyder, J.W., Lambert, J.D.C. and Ogilby, P.R. (2005) Lifetime and diffusion of singlet oxygen in a cell. *J. Phys. Chem. B* 109: 8570–8573.

Smirnoff, N. (2000) Ascorbate biosynthesis and function in photoprotection. *Philos. Trans. R. Soc. B: Biol. Sci.* 355: 1455–1464.

Takahashi, Y., Hansson, Ö., Mathis, P. and Satoh, K. (1987) Primary radical pair in the photosystem II reaction centre. *Biochim. Biophys. Acta* 893: 49–59.

Telfer, A. (2002) What is β-carotene doing in the photosystem II reaction centre? *Philos. Trans. R. Soc. B: Biol. Sci.* 357: 1431–1440.

Telfer, A., Bishop, S.M., Phillips, D. and Barber, J. (1994a) Isolated photosynthetic reaction center of photosystem two as a sensitizer for the formation of singlet oxygen. *J. Biol. Chem.* 269: 13244–13253.

Telfer, A., Dhami, S., Bishop, S.M., Phillips, D. and Barber, J. (1994b) β-Carotene quenches singlet oxygen formed by isolated photosystem II reaction centers. *Biochemistry* 33: 14469–14474.

Telfer, A., Oldham, T.C., Phillips, D. and Barber, J. (1999) Singlet oxygen formation detected by near-infrared emission from isolated photosystem II reaction centres: direct correlation between P680 triplet decay and luminescence rise kinetics and its consequences for photoinhibition. *J. Photochem. Photobiol. B: Biol.* 48: 89–96.

Triantaphylidès, C. and Havaux, M. (2009) Singlet oxygen in plants: production, detoxification and signaling. *Trends Plant Sci.* 14: 219–228.

Umena, Y., Kawakami, K., Shen, J.-R. and Kamiya, N. (2011) Crystal structure of oxygen evolving photosystem II at a resolution of 1.9 Å. *Nature* 473: 55–61.

van Mieghem, F.J.E., Nitschke, W., Mathis, P. and Rutherford, A.W. (1989) The influence of the quinone–iron electron acceptor complex on the reaction centre photochemistry of photosystem II. *Biochim. Biophys. Acta* 977: 207–214.

van Mieghem, F.J.E., Satoh, K. and Rutherford, A.W. (1991) A chlorophyll tilted 30° relative to the membrane in the photosystem II reaction centre. *Biochim. Biophys. Acta* 1058: 379–385.

Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.M. and Andersson, B. (1992) Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced Q_A species promote chlorophyll triplet formation. *Proc. Natl Acad. Sci. USA* 89: 1408–1412.

Walles, B. (1965) Plastid structures of carotenoid-deficient mutants of sunflower (*Helianthus annus L.*) I. The white mutant. *Hereditas* 53: 247–256.

Wilkinson, F., Helman, W.P. and Ross, A.B. (1995) Rate constants for the decay and reactions of the lowest electronically excited singlet-state of molecular-oxygen in solution—an expanded and revised compilation. *J. Phys. Chem. Ref. Data* 24: 663–1021.

Witt, H.T. (1971) Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. *Q. Rev. Biophys.* 4: 365–477.

Wolff, C. and Witt, H.T. (1969) On metastable states of carotenoids in primary events of photosynthesis. Registration by repetitive ultrashort-flash photometry. *Z. Naturforsch.* 24B: 1031–1037.

Yadav, D.K., Kruk, J., Sinha, R.K. and Pospíšil, P. (2010) Singlet oxygen scavenging activity of plastoquinol in photosystem II of higher plants: electron paramagnetic resonance spin-trapping study. *Biochim. Biophys. Acta* 1797: 1807–1811.

Yadav, D.K. and Pospíšil, P. (2012) Evidence on the formation of singlet oxygen in the donor side photoinhibition of photosystem II: EPR spin-trapping study. *PLoS One* 7: e45883.