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

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In this review, I outline the indirect evidence for the formation of singlet oxygen (1O2) 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 1O2 yield measurements), and dye bleaching and difference absorption spectroscopy to identify where quenchers of 1O2 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 1O2 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 1O2 as a signaling molecule in instigating cellular responses to various stress factors. I also discuss the current views on the role of 1O2 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; L1,270, luminescence at 1,270 nm; LHC, light-harvesting complex; PDT, photodynamic therapy; 1O2, singlet oxygen; 3O2, triplet oxygen; P680, primary electron donor in PSII; Pheo, primary electron acceptor in PSII; RC, reaction center; QA and QB, secondary electron acceptors in PSII; RNO, p-nitrosodimethylaniline; ROS, reactive oxygen species; RP, radical pair; SOSG, singlet oxygen sensor green; TM, thylakoid membranes; Yz, tyrosine electron donor to P680+.

Introduction

Singlet oxygen (1O2) 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 (3O2), 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.

\[
S+hv \rightarrow 1S^* \rightarrow 1S^*+1O_2 \rightarrow S+3O_2
\]

where S is a sensitizer molecule, dye or pigment. During oxygenic photosynthesis (Blankenship 2014), 1O2 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, 3O2, around.

Photosensitization of the triplet state of Chl leads to formation of 1O2 unless Chl triplets are removed rapidly before 1O2 formation can take place (Ogilby 2010). Carotenoid (Car) molecules are very effective quenchers of triplet Chl (Frank and Cogdell 1996) and also directly of 1O2 (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, 1O2 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 1O2 is involved. This is discussed in more detail later.

Here I will describe the history of the detection of 1O2 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.
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 \( 3\text{Car} \) 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:

\[
\text{Chl}+hv \rightarrow ^1\text{Chl}^* \rightarrow ^3\text{Chl}^*+^3O_2 \rightarrow \text{Chl}+^1O_2
\]  

(2)

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

\[
\text{Chl}+hv \rightarrow ^1\text{Chl}^* \rightarrow \text{energy transfer to the RC} \\
\rightarrow ^1\text{P}_{680}^+\text{Pheo} \rightarrow ^1\left[\text{P}_{680}^+\text{Pheo}^-\right] \rightarrow \text{P}_{680}^+Q_A^- \]  

(3)

\[
P_{680}^+Q_A^- \text{ recombination occurs either directly or indirectly:}
\]

\[
\text{indirect} \quad P_{680}^+Q_A^- \rightarrow ^1\left[\text{P}_{680}^+\text{Pheo}^-\right] \rightarrow ^1\left[\text{P}_{680}^+\text{Pheo}^+\right] \\
\rightarrow ^3\text{P}_{680}+^1O_2 \rightarrow ^3\text{P}_{680}+^1O_2
\]  

(4)

\[
\text{direct} \quad P_{680}^+Q_A^- \rightarrow P_{680}Q_A \]  

(5)

where \( \text{P}_{680} \) 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 paramagnetic resonance (EPR) signal properties. The radical pair triplet is only formed after 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).

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Satoh and colleagues (Nanba and Satoh 1987) which first led to the suggestion that large amounts of $^{1}O_2$ 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 $\mu$s (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 $^{1}O_2$ 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).

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

Conditions arise where $^{1}O_2$ 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 $^{1}O_2$ 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 $\beta$-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 $^{3}P_{680}$ 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 $^{3}P_{680}$ 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 $^{1}O_2$ being formed at a high yield. The lifetime of the $^{3}P_{680}$ was lengthened dramatically under anaerobic conditions (from $\sim$30 $\mu$s 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 $^{1}O_2$ (Durrant et al. 1990, Barber and Archer 2001). Note that during the early 1990s it became clear that $^{1}O_2$...
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$_2$O medium as water itself is a very good quencher of $^1O_2$ shorten its lifetime from $\sim 70$ µs to $\sim 3$ 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 yield of $^1O_2$ is already known. We found that the yield of $^1O_2$ was about 0.16 whereas the yield of $P_{680}$ 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 $\rho$-nitrosodimethylaniline (RNO) to the nitro form caused by the trans-annelar 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$_2$O (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 bleaching 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$_2$O as compared with H$_2$O. In experiments where H$_2$O and D$_2$O buffers were compared, there was no stimulation of the inactivation of $P_{680}$ in the latter medium compared with H$_2$O 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$_2$O-based medium, into a D$_2$O-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 luminal 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 D$_1$, which is thought to be where the $^3P_{680}$ is located, is liganded by a water molecule as is that of accessory Chl D$_2$.

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$_2$O 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$_2$O.

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 P680 in the isolated D1D2 complex (Telfer et al. 1994a).

Correlation of P680 Triplet Decay and L1,270 Signal Rise Rates in the Isolated PSII RC

We also measured time-resolved 1.270 nm luminescence of 1O2, formed on illumination of the D1D2 complex in the presence of D2O when suspended in air-saturated medium (Macpherson et al. 1993, Telfer et al. 1994b), and later correlated the rise time of the L1,270 signal with the decay of the 3P680 absorption decrease at 680 nm (Telfer et al. 1999). Here we showed the similarity in the triplet decay rate and L1,270 rise times and the dependence on the molecular oxygen concentration for the rate of quenching of the triplet and rise of the L1,270 which indicated that 1O2 is formed directly by quenching of 3P680 (Li et al. 2012) carried out similar experiments, measuring time-resolved L1,270 on isolated PSII RCs in aqueous media and concluded that the lifetime of the 1O2 would be so short (<0.5 μs) that determining the 1O2 rise constant 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 P680+ (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. siliconomylolate 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 P680+ (if it is not reduced rapidly by the tyrosine electron donor, Ye, and then by electrons from water), the Car should also quench 1O2, diffusing within the Chl protein complex, directly. The idea, as discussed already, is that the Car cannot be bound closely enough to quench 3P680 directly as it would be oxidized first by P680+ 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 CarD2 to ChlD2 and 19.9 Å for CarD1 to ChlD1 (Fig. 2). Using the Moser and Dutton rule (1992), both distances are far too great to allow either rapid quenching of 3P680 or rapid electron transfer directly to P680+.

The question arises as to what is the role of the Cars bound to the PSII RC aside from rereduction of any stabilized P680+. As discussed earlier, it is inevitable that some 3P680 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, 1O2 will be formed. It was known that carotenoids can quench 1O2 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 L1,270 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 1O2 formed is due to the fact that the 1O2 formed at P680 can diffuse in all directions within the complex and, because of the distance of the Cars from the source of the 1O2, a certain amount of damage will be done by 1O2 not scavenged by them (Telfer 2002).

Conclusions

3P680 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 P680 triplet thus forms 1O2 as the RC Cars are unable to quench the triplet before its reaction with ground state 1O2. 1O2 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 1O2.

1O2 scavengers

1O2 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 1O2 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 1O2 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 1O2 by D1D2 complexes (Macpherson et al. 1993), 1O2 formation by isolated
Singlet oxygen production by PSII under light stress

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