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Characterisation of triplet states in isolated Photosystem II reaction centres: oxygen quenching as a mechanism for photodamage

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Transient absorption spectroscopy has been used to study the isolated D1/D2/cytochrome *b*-559 reaction centre complex at 4°C. The D1/D2 reaction centre is observed to have an increased susceptibility to photodamage under aerobic conditions. This is attributed to oxygen quenching of a P680 triplet state, which results in the formation of highly oxidising singlet oxygen. This P680 triplet state is observed to have a lifetime of (1.0 ± 0.1) ms under anaerobic conditions, shortening to (33 ± 3) μs in the presence of oxygen. This state, which has a quantum yield of approx. 30%, is identified as residing upon the primary electron donor P680 by the transient bleaching of its reddest absorption band, which peaks at (680.5 ± 0.5) nm. The shape of the P680 triplet-minus-singlet absorption difference spectrum, and particularly the (12 ± 1) nm bandwidth of the red absorption band bleach, indicate that P680 is probably a pair of excitonically coupled chlorophyll molecules, with the P680 triplet state being localised upon one of these chlorophyll molecules. The red absorption band bleached by P680 triplet formation has a peak extinction coefficient of 133 000 $M^{-1} \cdot cm^{-1}$ and an oscillator strength 1.1-times larger than that of the Q_y-band of a chlorophyll *a* monomer in ether. It is shown that this P680 triplet state is formed primarily by charge recombination from the primary radical pair state at 4°C. A 3% quantum yield of a carotenoid triple state characterised by an absorption peak at 526 nm is also observed. The observed P680 triplet does not appear to be quenched by this carotenoid.

Introduction

In vivo, Photosystem II (PS II) is involved in the oxidation of water which results in the liberation of oxygen, the mechanism of which is not fully understood. Over the last few years, studies of PS II have been greatly advanced by the isolation of the D1/D2-cytochrome *b*-559 reaction centre complex [1,2]. In particular, the availability of a reaction centre without an associated antenna complex has greatly simplified spectroscopic studies.

This D1/D2 reaction centre preparation consists of the D1 and D2 polypeptides together with α and β subunits of cytochrome *b*-559 and the 4.8 kDa product of the *psbI* gene [1–5]. It has been reported to bind chlorophyll *a*, pheophytin *a* and β-carotene in a molar ratio of 4:2:1 [6], although recent studies have suggested slightly higher chlorophyll stoichiometries of 6 per 2 pheophytin [7] or 8–10 per 2 cytochrome *b*-559 [8]. It does not, however, contain the secondary electron acceptors Q_A and Q_B and therefore, in the absence of added artificial electron donors and acceptors, its light-induced electron transfer properties are restricted to the formation of the radical pair $P680^+$pheophytin$^-$ and charge recombination pathways from this state [9,10]. The radical pair has a lifetime of 37 ns [9,11], decaying primarily to the ground state, but also giving a high yield of a chlorophyll triplet with a spin polarisation indicative of charge recombination, detected by EPR spectroscopy at liquid helium temperatures [12,13]. Flash absorption spectroscopy has also been used to detect the chlorophyll triplet in the isolated reaction centre [10]. At 276 K the $t_{1/2}$ lifetime of this triplet state is 30 μs, lengthening to 0.9 ms at 5 K. In the former case the triplet yield is about 23% increasing to 80% at 10 K.

Abbreviations: P680, primary electron donor in Photosystem II; PS II, Photosystem II; fwhm, full width half maximum; ADMR, absorption detected magnetic resonance; EPR, electron paramagnetic resonance.

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Recently it has been observed that the lifetime of this chlorophyll triplet is strongly dependent upon the presence of oxygen, removal of oxygen from the solution results in a lengthening of the $t_{1/2}$ lifetime of this state to 600–800 μs at room temperature [14]. Takahashi et al. [10] also observe in the D1/D2 reaction centre preparation a carotenoid triple characterised at 50 K by an absorption maximum at 545 nm. This triplet state, at 276 K, has a $t_{1/2}$ rise time of 12 ns and decay time of 5 μs, with a quantum yield of 3%.

A chlorophyll triplet state, assigned to P680, has also been studied in larger PS II core particles at 1.2 K using the technique of absorbance-detected EPR in zero magnetic field (ADMR) [15]. A low-temperature triplet-minus-singlet absorbance difference spectrum was obtained for the P680 triplet state, characterised by a strong bleaching centred at 682 nm and an absorption increase at 665 nm. This difference spectrum was shown to be similar to the triplet-minus-singlet spectrum observed both for chlorophyll *a* dimers in methylcyclohexane and also for the primary donor of Photosystem I (P700), but was different from that observed for monomeric chlorophyll *a* in ethanol. It was suggested [15] that this was evidence for the dimeric nature of P680, with the P680 triplet state being localised (at least at liquid helium temperatures) on one of the two chlorophyll *a* molecules of the dimer. The absorption increase at 665 nm was attributed to the appearance of a monomeric ground state absorption band caused by the breaking of the excitonic interaction due to triplet formation.

Early experiments with the isolated PS II reaction centre revealed its instability to prolonged illumination [2]. Recently it has been reported that the removal of oxygen from a suspension of the isolated PS II reaction centre significantly increased its resistance to photodamage [11,16,17]. The use of anaerobic conditions allowed the observation of a high yield of a 37 ns fluorescence component attributed to charge recombination from the radical pair state [11].

In this paper we report on flash absorption experiments designed to explore the photochemical properties of the anaerobically stabilised isolated PS II reaction centre at 4° C, with particular focus on the triplet states.

Materials and Methods

Photosystem II reaction centre complexes were prepared from pea chloroplasts as described by Chapman et al. [18], with the following variations. Exchange to 2 mM lauryl β-maltoside was carried out after the first column purification in Triton X-100. Elution from the column was achieved using an NaCl gradient of 2 mM · ml^{-1}. The preparation was then stored at −196° C. For experiments, all samples were resuspended in a buffer of 50 mM Tris-HCl (pH 8 at room temperature) containing 2 mM maltoside to give a final chlorophyll concentration of 10 μg · ml^{-1}. The use of maltoside rather than Triton X-100 helps to stabilise the reaction centre complex [18]. Anaerobic conditions were achieved by adding 5 mM glucose, 0.1 mg · ml^{-1} glucose oxidase and 0.05 mg · ml^{-1} catalase to 2 ml of the sample in a 1 cm pathlength stoppered cuvette. The addition of these reagents reduces the concentration of oxygen in the solution, after 5 min at 4° C, by a factor of more than 100, as measured by an oxygen electrode (Hansatech King's Lynn, Norfolk).

The state of the isolated PS II reaction centres was monitored during all experiments by noting the position of the reddest absorption band maximum. If degradation occurs there is a blue shift in this maximum from its initial value of 675.5–676 nm [11,19,20]. The red absorption band of the reaction centre preparation peaked at (675.5 ± 0.5) nm throughout the experiments described below, unless otherwise stated. Samples were maintained at 4° C during all measurements and had an optical absorbance at 676 nm of approx. 0.8.

The proportion of chlorophyll uncoupled from the process of charge transfer in this preparation was estimated as follows. Chlorophyll *a* in ether was measured to be 8.5-times more fluorescent than the isolated D1/D2 reaction centre preparation. It has been shown that there are two main contributors to the fluorescence of this preparation, uncoupled chlorophyll and radical pair charge recombination, both having approximately the same fluorescence yield [11,21]. Allowing for the contribution of uncoupled chlorophyll, the intact D1/D2 reaction centres themselves can be calculated to be 0.06 as fluorescent as chlorophyll *a* in ether. A comparison of the fluorescence yield of the uncoupled chlorophyll to that of the chlorophyll *a* in ether shows that only (6 ± 1)% of the total chlorophyll present in our samples is chlorophyll uncoupled from the active D1/D2 reaction centres. This also indicates that, given the 32% fluorescence quantum yield of chlorophyll *a* in ether [22], the isolated D1/D2 reaction centre has a fluorescence yield of approx. 2%.

Transient absorption data were measured with a flash photolysis system comprising a PRA LN1000 nitrogen laser excitation source, a tungsten lamp monitoring source, a silicon photodiode detector with a 4700 Ω load resistor, a two stage 400-times amplifier with a variable d.c. input voltage offset and a Gould 4071 digital storage oscilloscope. The excitation wavelength was 337 nm and the detection wavelength was selected between 500 and 900 nm using a monochromator placed after the sample. The detection bandwidth was variable from 2 nm to 20 nm. Photodamage due to the monitoring light was reduced by the use of a broad bandwidth monochromator placed between the sample and the tungsten lamp. The duration of the excitation pulse was 800 ps and this was passed down a liquid-filled light-

guide to the sample with an exit pulse energy of 0.45 mJ and a repetition rate of 3 Hz. The response time of the system was 150 ns, limited by an electronic filter. However, times less than approx. 2 μs could not be observed due to a saturating fluorescence burst. After this saturating period any fluorescence artifacts were subtracted from the signal. All transients were averaged for 50–250 flashes, depending upon the size of the signal under study. Steady-state absorption measurements were made using a Perkin-Elmer 554 spectrophotometer.

Results

Fig. 1 shows a plot of the effect of preillumination with white light upon the position of the maximum of the reddest absorption band of the maltoside-resuspended D1/D2 reaction centre under both aerobic and anaerobic conditions. This clearly shows that the vulnerability of the reaction centre to photodamage is significantly reduced if oxygen is removed from the suspension. This result is in agreement with other studies [11,16,17]. A recent study [23] under similar experimental conditions has shown that the blue shift observed in Fig. 1 is caused by bleaching of a spectral species with an absorption maximum at approx. 680 nm, bandwidth 12 nm and assigned to P680. This bleaching is accompanied by a reduction in circular dichroic activity of the sample [17].

Fig. 2 shows flash induced absorption transients observed at 740 nm in the PS II reaction centre preparation. These have monoexponential lifetimes of (33 ± 3) μs under aerobic conditions, lengthening to (1.0 ± 0.1) ms when oxygen is removed from the sample. This lifetime change is reversible upon storage of the sample overnight at −10 °C, as the glucose necessary for oxygen removal is consumed; subsequent addition of more glucose lengthens the lifetime again to 1 ms. The initial absorbance changes (determined by plotting the decays on a log/linear scale and extrapolating back to zero time) are similar under both aerobic and anaerobic conditions. A similar lengthening of this lifetime has recently been observed upon the removal of oxygen by degassing or by the addition of dithionite [14]. These long-lived transients were attributed to a chlorophyll triplet state, probably residing upon P680 [14]. We use the following data to support this assignment and indicate that the P680 triplet is formed by charge recombination from the primary radical-pair state, a conclusion also reached by low-temperature EPR studies of the D1/D2 reaction centres [12,13].

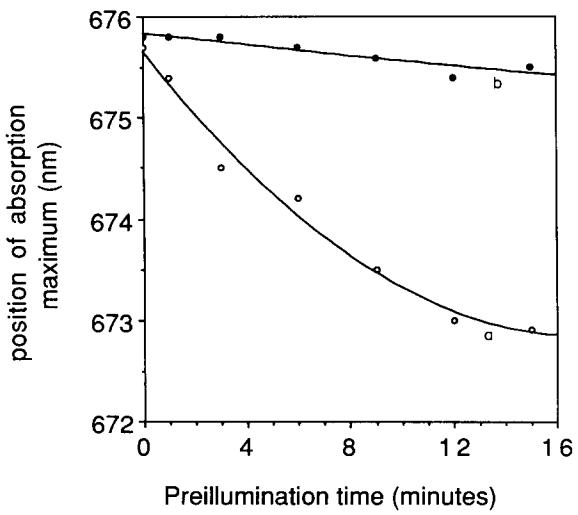

Fig. 1. A plot of the position of the red absorption maximum of the D1/D2 reaction centre preparation as a function of white light preillumination time under (a) aerobic (○) and (b) anaerobic (●) conditions. Light preillumination was carried out progressively upon the same sample, all points are the average of two experiments. Illumination intensity approx. 800 $\mu E \cdot m^{-2} \cdot s^{-1}$. The sample had a chlorophyll concentration of 10 $\mu g \cdot ml^{-1}$ suspended at 4 °C in 50 mM Tris-HCl (pH 8 at room temperature), 5 mM NaCl and 2 mM lauryl β-maltoside. In (b) 5 mM glucose, 0.1 $mg \cdot ml^{-1}$ glucose oxidase and 0.05 $mg \cdot ml^{-1}$ catalase were added.

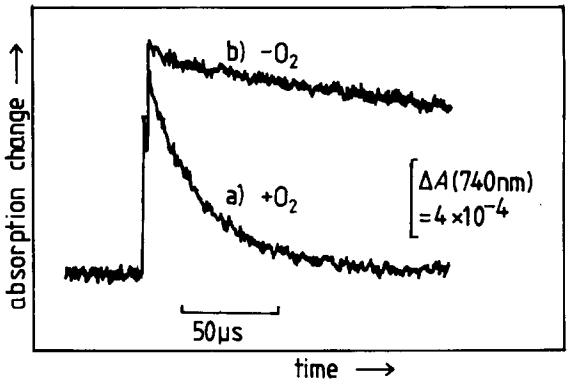

Fig. 2. Flash induced absorption changes observed in D1/D2 reaction centres at 740 nm under (a) aerobic and (b) anaerobic conditions. Sample conditions as in Figs. 1a and 1b, respectively. Averages of approx. 50 flashes.

The transient absorption difference spectrum of the 1 ms transient observed under anaerobic conditions is shown in Fig. 3a. This spectrum, taken at 4 °C, is similar to spectra observed at low temperatures in both D1/D2 reaction centres suspended in Triton X-100 [10] and larger PS II core particles [15]. These low-temperature spectra have been attributed to the P680 triplet state [10,15]. The spectral features are narrower in the spectra in Refs. 10 and 15 due to the lower temperatures used. The peak observed at 526 nm in the 1 ms spectrum shown in Fig. 3a was also observed, but less resolved, by Takahashi et al. [10]. However, it was not observed by Den Blanken et al. [15] (using a different experimental technique and larger particles, see also Introduction), nor was it observed in studies of the triplet state of chlorophyll *a* in vitro [26]. The significance of this feature is not clear. A careful analysis of the transient absorption bleach in the 660 to 690 nm region is shown in Fig. 4, where the spectral resolution

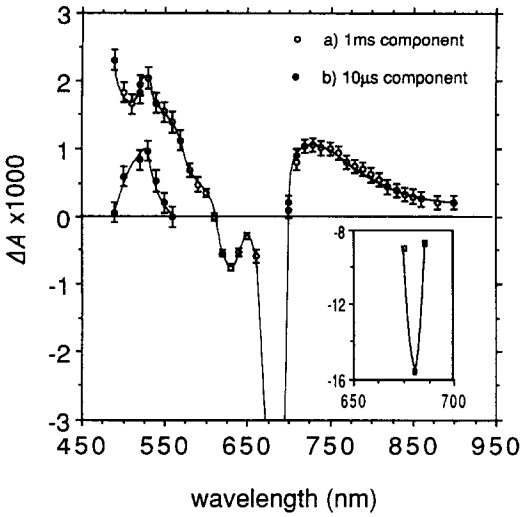

Fig. 3. Transient absorption difference spectra, measured under anaerobic conditions with a 4 nm resolution, of (a) the 1 ms kinetic ascribed to P680 triplet (○) and (b) the 10 μs kinetic ascribed to the carotenoid triplet (●). ΔA values are extrapolated back, when necessary, to zero time. ΔA values shown in the inset around 680 nm are taken from Fig. 4. Sample conditions as in Fig. 1b (anaerobic).

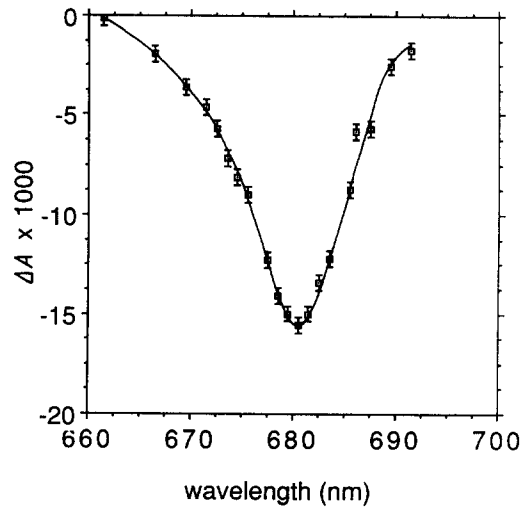

Fig. 4. Transient absorption difference spectrum of the 1 ms kinetic shown in Fig. 2a, measured with a 2 nm resolution. Sample conditions as in Fig. 1b (anaerobic).

is 2 nm. This bleach has a peak at (680.5 ± 0.5) with a bandwidth (fwhm) of (12 ± 1) nm.

Fig. 5a shows the loss of relative P680 triplet yield, measured under aerobic conditions, plotted against total exposure to the 337 nm excitation of the nitrogen laser. The relative P680 triplet yield was measured by extrapolating the triplet decay curve to $t = 0$. The loss of P680 triplet yield is accompanied by a blue shift in the red absorption band maximum (Fig. 5b). The P680 triplet lifetime shortened from initially 34 μs to a final value 30 μs (after 16000 flashes). Under anaerobic conditions, there is a significantly slower blue shift in the red absorption maximum caused by the laser excitation (data not shown). The increased stability of the sample resulting from the removal of oxygen is not as marked under ultraviolet laser illumination as that observed under white light illumination (Fig. 1). Care was taken to ensure that the photodamaging effect of the laser excitation did not significantly affect the results presented in this paper, achieved by monitoring the peak position of the reddest absorption band (as discussed in Materials and Methods).

The observed transient absorption decay in these PS II reaction centre particles is biexponential at 526 nm. This is most clear under anaerobic conditions, where the 1 ms P680 triplet decay is preceded by a microsecond component (Fig. 6). The transient absorption spectrum of this microsecond component is shown in Fig. 3b. It has a peak at (526 ± 4) nm and is characteristic of

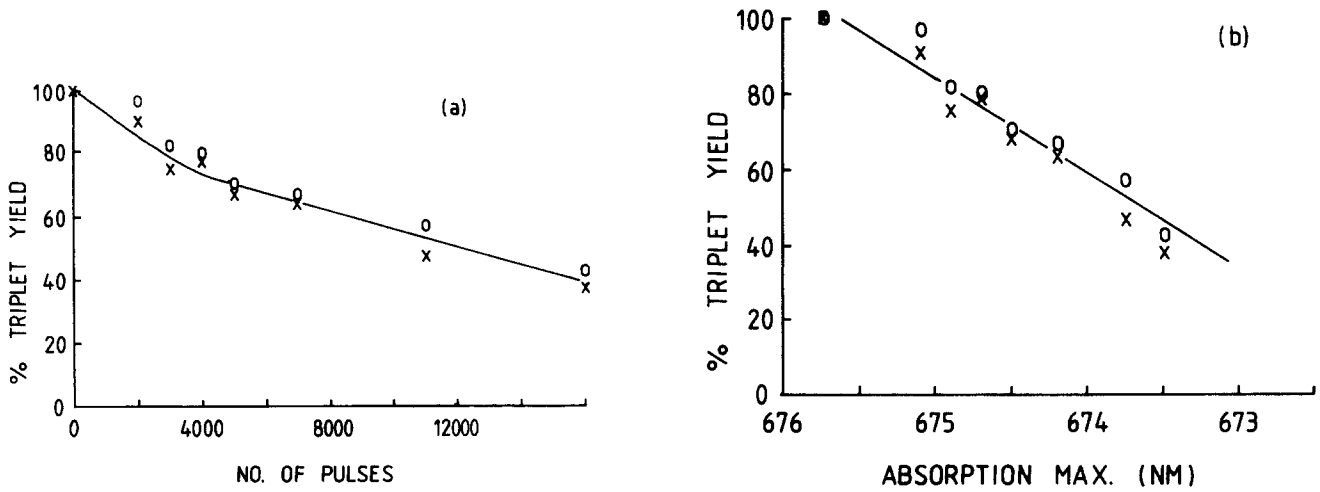

Fig. 5. (a) A plot of the relative triplet yield in PS II reaction centres against exposure to laser illumination, at different wavelengths: ×, 680 nm; ○, 740 nm. The laser pulse energy was 0.45 mJ at 337 nm. The total exposure is in terms of total number of 337 nm pulses absorbed by the sample. (b) Correlation in relative triplet yield with the blue shift in the Q_y-band absorption maximum. Sample conditions as in Fig. 1a (aerobic).

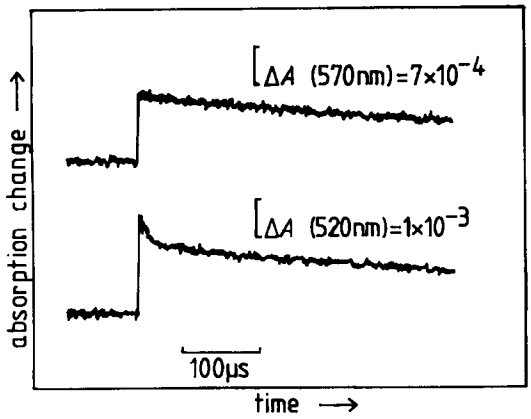

Fig. 6. Flash induced absorption changes observed in PS II reaction centres at 570 nm and 520 nm. Sample conditions as in Fig. 1b (anaerobic). Averages of approx. 100 flashes.

the spectrum for the carotenoid triplet state. This carotenoid triplet state has a lifetime of (10 ± 1) μs under anaerobic conditions, which shortens to (4 ± 1) μs under aerobic conditions (data not shown). The yield of this state is not significantly reduced by photodamage induced by ultraviolet laser illumination under aerobic conditions.

Triplet yields start to become non-linear versus excitation energy at high pump intensities due to multiple excitations of individual reaction centres. The triplet quantum yields given below are calculated for low pump intensities.

Quantum yields are defined as the yield of triplet state per excited reaction centre. The concentration of excited reaction centres is determined from the absorbance of the sample at 337 nm and the energy of the excitation pulse illuminating 1 cm^3 of the sample. The concentration of triplet state formed is determined from the magnitude of the flash induced change in absorbance, extrapolated to $t = 0$. Extinction coefficients of 3800 $M^{-1} \cdot cm^{-1}$ at 820 nm and 15000 $M^{-1} \cdot cm^{-1}$ at 520 nm are used for the P680 triplet-minus-singlet absorption change [25]. An extinction coefficient of 100000 $M^{-1} \cdot cm^{-1}$ at 526 nm is used for the carotenoid triplet-minus-singlet absorption change [26]. From these calculations, the P680 triplet quantum yield, at both 820 nm and 520 nm, is estimated to be approx. (30 ± 6)%, under both aerobic and anaerobic conditions, and the carotenoid triplet quantum yield is estimated to be 2–3%. The calculation of the quantum yields is independent of the number of chlorophylls per reaction centre.

Discussion

Oxygen quenching of P680 triplet as a mechanism of photodamage

It has previously been observed that carotenoidless reaction centres isolated from purple bacteria undergo photodamage when exposed to prolonged illumination under aerobic conditions [27]. For example, Boucher et al. [28] observed the irreversible bleaching of the reaction centre bacteriochlorophylls under exposure to intense infrared light in the presence of oxygen, and used reporter molecules to determine that this bleaching was due to the formation of singlet oxygen. Singlet oxygen is generated by oxygen quenching of the bacteriochlorophyll triplet state [27] and is an extremely reactive species which is known to damage biological tissue [29]. Therefore, it seems likely that the photodamage of the isolated D1/D2 reaction centre by prolonged illumination under aerobic conditions observed in Fig. 1 is also due largely to the formation of singlet oxygen, generated by the reaction:

$$^3P680 + {}^3O_2 \rightarrow P680 + {}^1O_2 \quad (1)$$

where $^3O_2$ is the ground state of oxygen and $^1O_2$ is a singlet state. That this mechanism is active is confirmed by the reduction in the lifetime of the P680 triplet state observed in this D1/D2 reaction centre in the presence of oxygen (Fig. 2). It has yet to be established whether this mechanism is a cause of the photodamage of PS II in vivo which brings about photoinhibition and the necessity for the rapid turnover of the D1 polypeptide [30]. The role of oxygen in photoinhibition has been the subject of much debate [29], although it has been observed that much larger oxygen-evolving PS II core particles are also more susceptible to certain types of photoinhibition in the presence of oxygen [31].

The rates of oxygen quenching of both the P680 and carotenoid triplet states are approx. 10-times less than those seen in nonviscous solvents for chlorophyll *a* and β-carotene [26]. This indicates that either the local oxygen concentration (inside or near the protein), or the rate of oxygen diffusion, is lower within the reaction centre than in the aqueous medium.

The P680 triplet state

There are two possible mechanisms of P680 triplet formation in the D1/D2 reaction centre: (i) primary charge separation to the singlet radical-pair state, intersystem crossing from the singlet to the triplet radical-pair state and subsequent charge recombination from the triplet radical-pair state (for review see Ref. 32) and (ii) direct intersystem crossing from the P680 singlet excited state. Our results indicate that the P680 triplet is predominantly formed by the charge recombination mechanism at 4 °C. A similar conclusion was also reached from EPR studies of the reaction centre conducted at low temperatures [12,13]. The reasons for our assignment are as follows. Firstly, increased photodamage of the reaction centre under aerobic conditions, as judged by the blue shifting of the reddest absorption peak, shows a correlation between the loss of triplet

yield (Fig. 5b) and the loss of the 37 ns fluorescence decay component attributed to charge recombination from the singlet radical-pair state [11]. Secondly, if the P680 triplet was predominantly formed directly by intersystem crossing from the P680 singlet excited state, then it is estimated (see below) that its quantum yield would be only about 4% at 4°C and not 30% as we observe; moreover the triplet yield would increase during photodamage. The 4% value is estimated from the fact that for chlorophyll *a* in organic solution the triplet yield is about twice the fluorescence yield [22]. Since the fluorescence quantum yield for the isolated PS II reaction centre is only 2% (see Materials and Methods), it follows that the P680 triplet yield, formed by direct intersystem crossing, should be only of the order of 4%, the remaining 26% being formed by charge recombination from the radical-pair state. During photodamage induced by white light illumination, there is an increase in the fluorescence quantum yield of the D1/D2 reaction centre preparation [21], and it is therefore likely that the yield of chlorophyll triplet formed by direct intersystem crossing will also increase during photodamage. The fact that we observe a decrease in triplet yield during photodamage under aerobic conditions confirms that the amount of triplet formation due to this direct intersystem crossing mechanism is relatively low for intact D1/D2 reaction centres.

The transient triplet-minus-singlet absorption difference spectrum shown in Fig. 4 supports the suggestion that the observed chlorophyll triplet state resides upon P680. If this is so, then the spectrum in Fig. 4 will contain negative contributions from the bleaching of any P680 absorption, and positive contributions from any triplet state absorption. The latter contribution is relatively constant and small over the wavelength range shown [24]. The spectrum in Fig. 4 can not be caused by formation of a pheophytin triplet state as this would be inconsistent with the position of the sidebands observed at 630 nm and 590 nm in Fig. 3. Therefore, it must be due to formation of a triplet state residing upon a chlorophyll species with its red absorption band maximum at 680.5 nm. The question as to whether other chlorophylls, in addition to the primary donor, may also have their absorption maximum near 680 nm depends upon the number of chlorophylls per P680, a subject of some contention currently [7,8]. If there are only four chlorophyll molecules per reaction centre, all the accessory chlorophylls must have absorption maxima several nanometers to the blue of P680 and the pheophytins, in order to shift the mean peak position over to 676 nm. However with six or more chlorophylls per reaction centre, one of the accessory chlorophyll molecules could have an absorption maximum at 680 nm and still allow the D1/D2 spectrum to peak at 676 nm. Therefore, the observed absorption bleach maximum at 680.5 nm is not, by itself, clear evidence for the triplet residing upon P680. Instead, we base our assignment of the observed chlorophyll triplet state as the P680 triplet state upon the following discussion, which suggests that this triplet probably resides upon a chlorophyll species exhibiting significant excitonic coupling in its singlet state.

We believe that our spectra (Figs. 3a and 4), whilst not conclusive, are at least consistent with P680 being a special pair of chlorophyll molecules whose singlet states exhibit significant excitonic coupling. Firstly, a triplet-minus-singlet absorption difference spectrum similar to that shown in Fig. 3a has been observed by Den Blanken et al. [15] in PS II core particles at 1.2 K. They attributed their spectrum to the formation of the P680 triplet state, and suggested that the shape of this spectrum between 630 nm and 670 nm was evidence for P680 being a pair of excitonically coupled chlorophyll molecules with the triplet state localised upon one of these molecules (as discussed in the Introduction above). The spectrum we have obtained has a similar shape over this spectral range, although the features observed are broader and less well resolved, presumably due to the higher temperature used. Our spectrum is significantly different from the room-temperature difference spectrum of the triplet state of monomeric chlorophyll *a*, observed in a variety of solvents in vitro [33].

Secondly, it should be noted that the spectrum shown in Fig. 4 around 680 nm is narrow, with a bandwidth of only 12 nm. Whilst it is true that the width of an absorption line is a complicated function of a manifold of vibrational states as well as the effect of any exciton coupling, this 12 nm bandwidth is significantly narrower than that observed for the Q_y-absorption band of chlorophyll *a* monomers in organic solvents (for example, the bandwidth is 17 nm in ether). It has not been demonstrated that a protein environment can cause this degree of narrowing for monomeric chlorophyll species at room temperature, whilst it is known that exciton coupling can narrow absorption bands [34]. Indeed it is unlikely that a protein, composed as it is of many polar groups, would interact less, and therefore give less broadening, than solvents such as ether. It is therefore possible that this narrow bandwidth is caused by the process of exciton narrowing [34], in agreement with the suggestion that P680 is a special pair of chlorophyll molecules which show some degree of singlet state exciton coupling. This does not rule out the possible contribution of other chromophores to the band shape, as the transitions of the other chromophores are likely to mix in to some extent via additional exciton coupling. The presence of exciton coupling between the chromophores in this D1/D2 reaction centre is also indicated by the circular dichroism spectrum of the reaction centre [17,35].

The extinction coefficient at the peak of the reddest absorption band of P680 can be calculated as 133000 $M^{-1} \cdot cm^{-1}$, by normalising the absorbance change at

680.5 nm (Fig. 4) against the extinction coefficients at 820 nm and 520 nm for the P680 triplet (see above). From this, the oscillator strength of this red absorption band can be estimated to be 1.1 that of a chlorophyll *a* monomer in ether. Values used for the chlorophyll *a* Q_y-band in this calculation were an absorption bandwidth of 17 nm and peak extinction coefficient of 86000 $M^{-1} \cdot cm^{-1}$ [36]. That this band should show an oscillator strength similar to that of a chlorophyll monomer may simply be because P680 is a monomeric chlorophyll species, although we believe this possibility unlikely for the reasons discussed above. If P680 is a special pair of chlorophyll molecules, it is possible that the rest of the oscillator strength of the special pair lies in some other band to the blue of 680 nm, and that the bleach of this band may not be apparent because the triplet state of P680 is highly localised. The bleaching of the bluer band will not be apparent due to the increase of monomeric chlorophyll absorption as the original exciton interaction is broken by triplet formation. The localisation of the P680 triplet state upon one molecule of a special pair is consistent with EPR studies of PS II particles, as reviewed by Rutherford [37] and discussed below. Alternatively, effects such as hypochromism could genuinely be producing a reduced oscillator strength for the total special pair Q-band transition.

The natural radiative lifetime of an excited singlet state is inversely proportional to the area under the curve (and therefore oscillator strength) for the corresponding absorption band. The proportionality factor is given by the Strickler-Berg equation [38]. Using this equation the natural radiative lifetime of chlorophyll *a* in ether can be calculated to be 19 ns. The natural radiative lifetime of the singlet excited state of P680 can now be estimated, using this value of 19 ns and the ratio of the oscillator strengths determined above. If it is assumed that the P680 triplet is either delocalised over both molecules of the special pair or that P680 is a monomeric chlorophyll species, the natural radiative lifetime of P680 is about 17 ns. If, however, as we feel is more consistent with the data discussed above, the P680 triplet state is localised upon one molecule of a special pair, there is probably additional oscillator strength in an excitonic band just to the blue of 680 nm whose bleaching will not be apparent in Figs. 3 and 4 (as discussed above), and the natural radiative lifetime may be upto a factor of 2 shorter.

Comparison with the reaction centres of purple bacteria

The 1 ms lifetime of the P680 triplet state observed under anaerobic conditions is much longer than the lifetime of the primary donor triplet state (^3P) observed in purple bacterial reaction centres, which is, for example, only 50 μs in the case of *Rhodopseudomonas sphaeroides* R-26 [39]. More importantly, whereas the P680 triplet lifetime is similar to the lifetime of the chlorophyll *a* triplet observed in vitro in the absence of any rapid quenching mechanisms (given as, for example, 1.5 ms in toluene [40] and 0.7 ms in both cyclohexanol and sodium dodecyl sulphate micelles [25]), the 50 μs lifetime of ^3P in purple bacteria is significantly shorter than the 300–500 μs lifetime of the bacteriochlorophyll *a* triplet state in vitro [26]. This difference indicates that the P680 triplet state does not decay by thermal repopulation of the radical-pair state, which is a prominent decay mechanism observed in bacterial reaction centres [39].

It is also interesting that the bandwidth of the red absorption band of the bacteriochlorophyll special pair in purple bacteria (for example, P870 in *Rhodopseudomonas sphaeroides*) is significantly broader (for example, Ref. 40) than the bandwidth observed here for PS II, suggesting that the nature of the special pair is different in these two systems. The broad nature of the P870 band has been the subject of much debate. Recently, it has been suggested that this band is vibrationally broadened, with a large inhomogeneous linewidth caused by strong electron-phonon coupling [41,42]. This vibrational broadening appears to be weaker for the red absorption band of P680, allowing observation of the exciton narrowing discussed above.

Previous EPR studies of a spin-polarised chlorophyll triplet state, induced by continuous illumination of PS II cores at liquid helium temperatures, have been reviewed by Rutherford [37]. These indicated that this triplet state resided upon a single chlorophyll molecule whose plane was oriented parallel to the membrane plane. It was not clear whether this single chlorophyll molecule was an accessory chlorophyll molecule or a constituent of P680 itself. Our results indicate that, when flashes of light are used and the sample is at 4 °C, then the PS II reaction centre triplet state resides upon the chlorophyll special pair, P680. The ADMR study of the chlorophyll triplet observed in PS II core particles at 1.2 K [15] indicates that the triplet remains on P680 at liquid helium temperatures. If the triplet state does indeed remain on P680 under the conditions used for the EPR measurements, this would indicate that the special pair of PS II is oriented parallel to the membrane and not perpendicular as expected for a special pair similar to that in the reaction centre of purple bacteria [43]. This is a further indication that the nature of the special pair may be rather different in PS II compared to purple bacteria.

The carotenoid triplet state

Like Takahashi et al. [10], we were able to detect a low yield of carotenoid triplet, the lifetime of which increased with the removal of oxygen. Our transient absorption peak at 526 nm (Fig. 3b), however, is at a wavelength shorter than the 545 nm peak reported by Takahashi et al. at 50 K [10], but similar to that

previously observed for PS II core complexes at room temperature [44]. As the yield of intersystem crossing from the carotenoid singlet excited state to the triplet state is less than 10^{-4} [26], the carotenoid triplet state observed in this D1/D2 reaction centre preparation must be formed by energy transfer from another chromophore's triplet state. It is possible that this carotenoid triplet is formed by fast (submicrosecond) energy transfer from the P680 triplet state in a subpopulation of reaction centres in which this mechanism is active, a suggestion made by Takahashi et al. [10]. Alternatively energy transfer to the carotenoid triplet from the triplet state of the 6% of chlorophylls uncoupled from the process of charge separation in this preparation (see Materials and Methods) is sufficient to give rise to the carotenoid triplet yield observed. The triplet yield of uncoupled chlorophyll is approx. 66% [22], giving an apparent yield of 4% for the uncoupled chlorophyll triplet state in this preparation, a little more than the observed 2–3% carotenoid triplet yield. Therefore the carotenoid triplet state observed in this preparation may be formed via a small subpopulation of contaminating uncoupled chlorophyll molecules, independent of the reaction centres themselves.

The inability of the carotenoids to quench the P680 triplet state in the majority of reaction centres is surprising given that this preparation is thought to have one carotenoid per reaction centre. This inactivity may be a consequence of the isolation procedure for these D1/D2 reaction centres. It is also clear from the susceptibility of the D1/D2 reaction centre to photodamage under aerobic conditions that the protection from photodamage attributed to carotenoids in other photosynthetic systems (for review see Ref. 27) does not function in this D1/D2 reaction centre preparation.

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Two studies [7,45] have recently suggested that the reaction centres used in this preparation may contain six chlorophyll *a* molecules and two β-carotene.