Biochimica et Biophysica Acta, 1143 (1993) 301–309

BBABIO 43852

Direct detection of singlet oxygen from isolated Photosystem II reaction centres

Alisdair N. Macpherson [a,1], Alison Telfer [b], James Barber [b] and T. George Truscott [a]

[a] *Department of Chemistry, Keele University, Keele (UK) and* [b] *AFRC Photosynthesis Research Group, Wolfson Laboratories, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London (UK)*

(Received 27 August 1992)
(Revised manuscript received 13 January 1993)

Key words Photosystem II, Reaction center, Photosynthesis, Singlet oxygen, Triplet state, P680, Luminescence

Both steady-state and time-resolved luminescence measurements at 1270 nm indicate that, when illuminated, isolated reaction centres of Photosystem II can generate singlet oxygen, O_2 ($^1\Delta_g$) The oxygen dependent component of the luminescence signal, which was measured in a D_2O buffer, was quenched by either azide or H_2O Singlet oxygen was detected both when primary charge separation was functioning and after it had been inactivated, suggesting that $^1O_2$ can be generated from triplet states formed by radical pair recombination (P680 chlorophyll triplet) and by intersystem crossing (accessory chlorophylls) Neither azide nor H_2O was found to protect against light-induced oxygen dependent irreversible bleaching of chlorophyll and pheophytin of reaction centres We suggest that the pool of singlet oxygen detected by steady-state luminescence at 1270 nm and quenched by azide and water is located in the bulk medium rather than in the protein matrix of the reaction centre, where the photodamage to pigments occurs, and the singlet oxygen is initially generated

Introduction

The isolated Photosystem II (PS II) reaction centre, composed of the D1 and D2 proteins, the apoproteins of cytochrome *b*-559 and the PsbI protein does not retain the secondary quinone electron acceptors, Q_A and Q_B, which normally stabilise charge separation [1,2] between the donor chlorophylls, known as P680, and the acceptor, pheophytin molecule (Pheo) Consequently, when formed, the primary radical pair, $P680^+$ $Pheo^-$ [3], recombines resulting in emission of fluorescence [4,5] and the formation of a P680 triplet state [6,7] In the latter case the triplet is spin polarised and has a yield of about 30% at 297 K [7–9] The lifetime of this triplet, which can be observed as an absorption increase at 740 nm, is about 1 ms under anaerobic conditions [8,9] However, under aerobic conditions the triplet lifetime shortens to 33 μs [7–9] and it has been assumed [9] that this efficient quenching of the triplet state involves the conversion of molecular oxygen to its first excited singlet state, $O_2(^1\Delta_g)$

$$^3P680^* + O_2(^3\Sigma_g^-) \rightarrow P680 + O_2^*(^1\Delta_g)$$

Under conditions when this reaction may occur it has been shown that there is irreversible bleaching of chromophores contained within the protein complex [10–12] The primary damage is to the chlorophylls of P680 but further exposure to illumination results in other bound chromophores being bleached and the proteins, D1 and D2, degraded [13,14] From these observations it has been suggested that singlet oxygen is the toxic species giving rise to the damage [15] In agreement with the singlet oxygen hypothesis it has been shown that under anaerobic conditions no such degradation occurs [11,12,14]

The isolated reaction centre contains β-carotene which would be expected to quench chlorophyll triplets or singlet oxygen directly by conversion to its triplet state [16] Therefore, it appears that in isolated PS II reaction centres the carotenoids are not protecting against photoinduced damage via the triplet state

Correspondence to A Telfer, AFRC Photosynthesis Research Group, Wolfson Laboratories, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, SW7 2AY, UK

[1] Present address Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287-1604, USA

Abbreviations Chl, chlorophyll, D1 and D2, products of the *psbA* and *psbD* genes, respectively, DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, $^1O_2$, singlet oxygen, $O_2(^1\Delta_g)$, P680, primary electron donor to PS II, Pheo, pheophytin, PS II, Photosystem II, PsbI protein, product of the *psbI* gene, Q_A and Q_B, secondary electron acceptors in PS II

mechanism a conclusion supported by the finding that the yield of the carotenoid triplet state in isolated PS II reaction centres is low, not exceeding 3% [7,9]

The vulnerability of the isolated PS II reaction centre to photoinduced damage is of interest for physiological reasons because one of the most important components of the PS II reaction centre, the D1 protein, is rapidly turned over in vivo and this seems to underlie the phenomenon of photoinhibition [17,18] Indeed, singlet oxygen has been invoked as a possible trigger during acceptor side photoinhibition, as discussed by Barber and Andersson [19]

The concept that singlet oxygen is a toxic species for photosynthesis, and PS II in particular, has previously relied only on the protective effect of removing oxygen [11,12,20], or circumstantial evidence, such as the presence of carotenoids [21,22] In this paper we report the direct detection of singlet oxygen, generated by isolated PS II reaction centres This toxic species was observed by measuring an oxygen-dependent luminescence signal at 1270 nm Although singlet oxygen is known to be highly reactive and is believed to be the active species responsible for effecting tumour destruction in photodynamic therapy [23], it has proved difficult to detect it directly in biological samples Singlet oxygen luminescence has been detected from stimulated intact human eosinophils [24] and recently from suspensions of dye-stained cells [25,26] However, the observed emission lifetimes suggest that the $^1O_2$ is predominantly detected from the bulk medium, and there is the possibility that it is generated by sensitiser which is not bound or is only 'loosely' bound to the cell membrane By contrast, in the experiments reported here, singlet oxygen is shown to be formed within the PS II reaction centre by natural photosensitisers, located in the protein matrix of the complex

Materials and Methods

Reaction centres of PS II were isolated from pea (*Pisum sativum*) using the method described previously [27] except that the first column chromatographic step was conducted in 50 mM Tris-HCl (pH 7 2), 0 2% Triton X-100 and 1 mM dodecyl maltoside and the second column chromatographic step in 50 mM Tris-HCl (pH 7 2) and 2 mM dodecyl maltoside The reaction centres were then exchanged into 'D_2O buffer' (50 mM Tris-HCl (pH 8 0) plus 2 mM dodecyl maltoside made up in D_2O from BDH Spectrosol) as follows reaction centre samples (4 × 0 4 ml samples equivalent to about 400 μg chlorophyll in total) were centrifuged at 6500 rpm in Ultrafree-MC Millipore concentrating tubes, with low protein binding PLGC ultrafiltration membranes, at 4°C for approx 20 min The eluate was discarded and 0 25 ml of 'D_2O buffer' was added to the concentrate in each tube and they were centrifuged

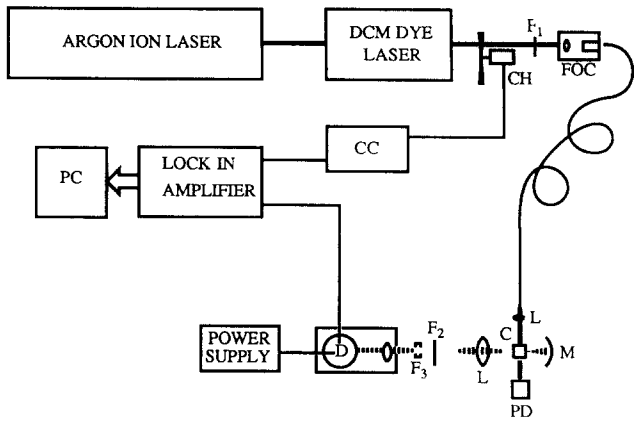

Fig 1 Scheme for the steady-state luminescence spectrometer CH optical chopper, F_1, neutral density filters, FOC, fibre optic coupler L, lens, CC, chopper controller, M mirror, C cuvette F_2 RG1000 long pass filter, F_3, 1270 nm interference filter, D, ultrasensitive germanium detector with integral preamplifier (77 K)

again This was repeated twice more before combining all the concentrated samples together in the 'D_2O buffer' to a final chlorophyll concentration of 290 μg ml^{-1} This stock solution was stored at −80°C

Anaerobic conditions were achieved by bubbling with nitrogen or by addition of 5 mM glucose, 0 1 mg ml^{-1} glucose oxidase (Sigma G2133, 100 units mg^{-1}) and 0 1 mg ml^{-1} catalase (Sigma C10, 1000 units mg^{-1}) Silicomolybdate was obtained from Pfaltz and Bauer, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was a gift from Dr W Oettmeier and chloroaluminium phthalocyanine tetrasulphonate was obtained from Porphyrin Products

Absorption spectra were measured in either an SLM Aminco DW2000 (at 10°C) or a Perkin Elmer Lambda 2 UV/VIS spectrophotometer (at room temperature) Steady-state fluorescence emission spectra were measured with a Perkin Elmer LS50 spectrofluorimeter (at room temperature) Transient absorption changes at 740 nm (4 nm bandpass) were averaged over 32 shots, using a subnanosecond nitrogen laser to excite the samples (at 4°C), as described previously [8]

A new steady-state instrument was used to detect the $^1O_2$ phosphorescence at 1270 nm (Fig 1) An argon laser (Cambridge Lasers CL5), operated in constant light mode, was used to pump a DCM dye laser (Spectra-Physics 375B) with a standard dielectric wedge A micrometer was fitted to the tuning mechanism and it was calibrated by viewing the emission through a monochromator calibrated at 632 8 nm The laser emission was modulated at 119 Hz using an optical chopper (Scitec Instruments Ltd model 300) and focused into an optical fibre (125 μm core diameter) A fast light guide lens head was used to collect the fibre output so that the beam filled the 10 mm aperture of the cuvette holder Emission from the sample (maintained at room temperature) was collected by a

lens assembly and a concave mirror, placed behind the cuvette, and was filtered with a 1000 nm long-wavelength pass filter (Schott RG1000) and a 1270 nm interference filter (FWHM 28 nm, Speirs Robertson) The interference filter was used in place of a high intensity computer-controlled $f/7$ monochromator with a 1 μm blazed grating (Bausch and Lomb 33-86-03) to allow a much higher signal throughput at 1270 nm The emission was imaged onto the 0 25 cm^2 active area of an ultrasensitive liquid-nitrogen-cooled germanium detector (North Coast Optical Systems model EO-817L, NEP (1500 nm, 100 Hz) = 8 9 10^{-16} $WHz^{-1/2}$) operated at a bias of -250 V (model 823 supply) The output from the integral preamplifier was fed to a lock-in amplifier (Scitec Instruments Ltd, model 500MC) interfaced to a PC The 1270 nm luminescence signals were obtained by manually fitting a line through plots of the lock-in output voltage against time and correcting for the number of photons absorbed ($1-10^{-A}$) at the excitation wavelength, at the start and end of each irradiation period

Time-resolved near-infrared luminescence was detected as previously described [28] The reaction centres were excited at 532 nm ($A \approx 0\,2$, < 50 mJ) by a frequency-doubled NdYAG laser (Spectron lasers SL2Q + SL3A, 16 ns pulse) operated at 1 Hz Emission at $\lambda > 1050$ nm was detected at 90° to the 10 mm sample cuvette by a germanium photodiode, amplified and then signal averaged (64 shots) on a digitising oscilloscope (Tektronix 2432A) before transferring to a PC for analysis and storage Measurements were carried out at room temperature

The visible absorption and fluorescence emission spectra were recorded before and after each irradiation The power or energy of the lasers were measured with a Gentec PSV-103 head and TPM meter and the power at the fibre output was monitored with a 1 cm^2 silicon photodiode

Results

Photodamage of PS II reaction centres in the presence of oxygen

We have shown previously that photodamage of isolated PS II reaction centres under aerobic conditions results in an irreversible bleaching of the bound chromophores Initially the damage occurs to a species absorbing maximally at 680 nm [10,11] We have further examined this phenomenon and our findings are shown in Fig 2 This figure shows the absorption difference spectrum (irreversible changes, light-treated minus initial dark control) of PS II reaction centres, as a function of illumination time with red light With a short time of illumination the initial bleaching occurs maximally at 681 nm but with increasing time there is a blue shift to 679 nm The bleaching is consistent with

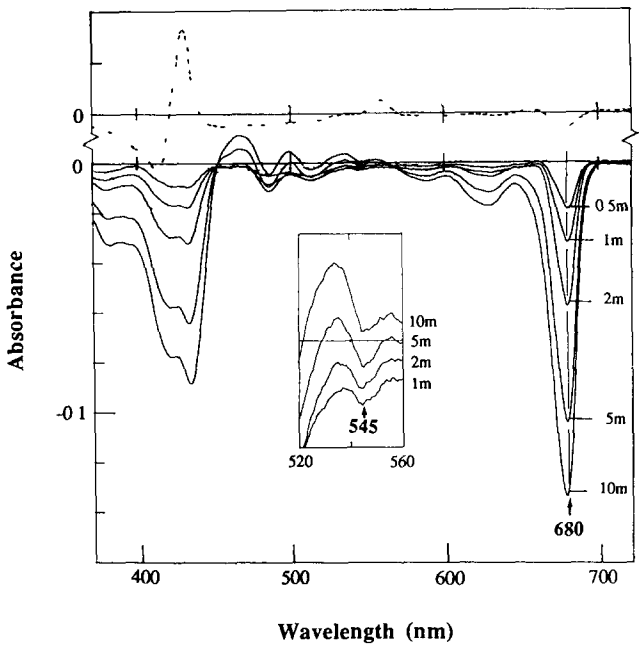

Fig 2 Absorption difference spectra of PS II reaction centres illuminated with red light (RG665 Schott glass cut off filter, 600 μE m^{-2} s^{-1}) for various times Spectra were measured in the dark after specified light treatments and the control dark spectrum was subtracted PS II reaction centres equivalent to 4 μg ml^{-1} chlorophyll were suspended in 50 mM Tris-HCl buffer (pH 8 0) and 2 mM dodecyl maltoside under aerobic conditions (———) and anaerobic conditions (- - - - -), light treated for 2 min Anaerobiosis was achieved by preincubation for 5 min with 5 mM glucose and 0 1 mg ml^{-1} each of catalase and glucose oxidase

the loss of chlorophyll(s) and of some pheophytin The inset of Fig 2 confirms the loss of pheophytin, as there is a small but definite bleach at 545 nm which is due to the loss of the Q_x absorbance band of this pigment The absorption changes in the 470–510 nm region are probably due to a bandshift of the β-carotene absorbance In the long wavelength region, Fig 2 shows a gradual broadening of the absorption difference band due to increased relative bleaching to the blue side of the maximum signal We conclude that there is an initial loss of P680 chlorophyll and that with increased illumination time there is also an irreversible bleaching of accessory chlorophyll, which absorbs more to the blue than P680, and pheophytin As shown previously we found that absence of oxygen almost completely protects against this irreversible bleaching [11] and only a stable reduction of cytochrome *b*-559 can be observed It is this photoinduced irreversible bleaching of chromophores occurring only under aerobic conditions which has been attributed to the toxic effect of singlet oxygen [11,12,15]

Activity of PS II reaction centre preparations exchanged into D_2O

The lifetime of singlet oxygen in D_2O ($\tau_\Delta = 68$ μs [29]) is approx 20-times longer than in H_2O [25] and

hence D_2O is commonly used in both direct luminescence measurements and in indirect chemical bleaching experiments as a means of detecting the formation of singlet oxygen For this reason, PS II reaction centres were exchanged into 'D_2O buffer' by a series of concentrating steps, using the ultrafiltration technique described in Materials and Methods After the final, approx 50-fold dilution into 'D_2O buffer', we estimate the H_2O content of the suspension buffer to be less than 0 1%

The absorption spectrum of PS II reaction centres in D_2O had a Q_y peak slightly blue shifted to 674 8 nm from the normal 675 5 nm value We presume this was due to the handling procedures required for the exchange As a blue shift of the red peak absorbance maximum is known to accompany loss of P680 and hence charge separation activity [5,9,30], we checked the activity of the reaction centres after exchange into D_2O This was measured by the ability of the reaction centres to form the triplet of P680 Triplet formation in reaction centres which have been exchanged into 'D_2O buffer' was measured by the flash-induced absorption change at 740 nm (see Fig 3) Under anaerobic conditions (Fig 3, trace a) the absorption decay shows a single exponential component with a lifetime (τ) of 1 0 ms which is shortened to 33 μs when oxygen is present (Fig 3, trace b) These triplet lifetimes are the same as those reported previously for PS II reaction centres in 'H_2O buffer' [9] There was, however, a slight decrease in the yield of triplet (by approx 15%) as compared to non D_2O-exchanged reaction centres (data not shown), which is consistent with a small decrease in activity as represented by the minor blue shift in the red peak absorption maximum [5,9]

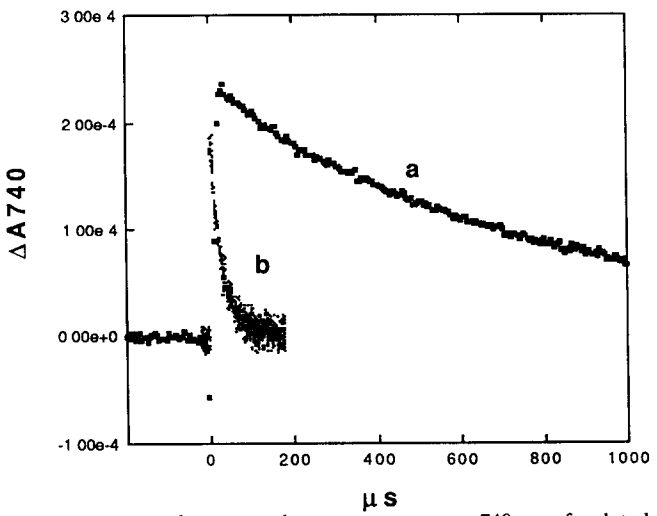

Fig 3 Transient absorption absorption changes at 740 nm of isolated PS II reaction centres in 'D_2O buffer' plus (b) and minus (a) oxygen Chlorophyll concentration was 8 μg ml^{-1} Anaerobiosis was achieved as in Fig 2

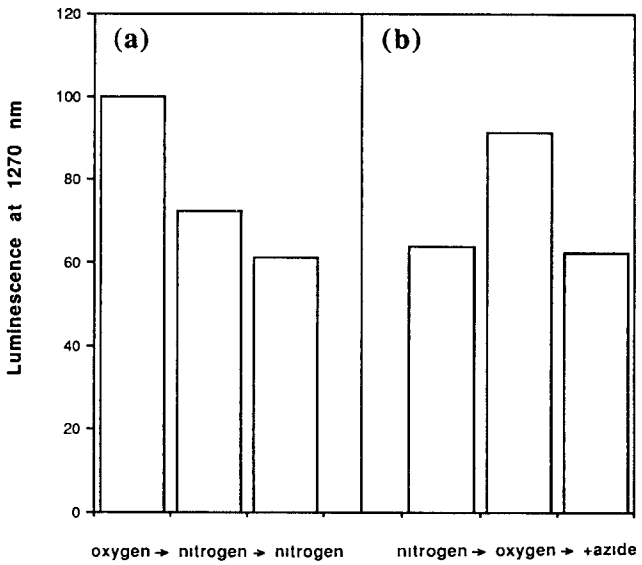

Fig 4 Corrected 1270 nm luminescence signals, L_{1270} (μV), from isolated PS II reaction centres bubbled initially with oxygen (a) and nitrogen (b) The samples were subjected in total to approx 4 min of illumination at 55 mW cm^{-2} average laser excitation at 682 nm After the initial measurements, the gases were exchanged by bubbling for a further 30 min, as indicated, and the new L_{1270} level was measured Finally in (b) the effect of addition of approx 3 mg of sodium azide was measured and compared with the sample in (b), which had been excited with the laser for the same length of time Chlorophyll concentration was 6 μg ml^{-1} L_{1270} signals were corrected for the number of photons absorbed as described in the Materials and Methods The initial, uncorrected L_{1270} levels were 925 μV plus oxygen (a) and 660 μV plus nitrogen (b), with typical noise levels of $\pm$100 μV

Detection of singlet oxygen phosphorescence

Having established that the exchange into D_2O has little effect on the activity of PS II reaction centres, we attempted to detect $^1O_2$ directly by its near-infrared luminescence, which has an emission peak at 1270 nm Because the quantum yield of $^1O_2$ phosphorescence is very low (in D_2O, ϕ_p is $\approx 2\ 10^{-5}\ \phi_\Delta$ [31]) it can be difficult to distinguish it from the long wavelength tail of the fluorescence of the singlet oxygen sensitiser With the steady-state emission apparatus used for the experiments reported here we were unable to resolve the spectrum of $^1O_2$ phosphorescence from the overlapping chlorophyll fluorescence tail We therefore carried out our experiments using a 1270 nm interference filter (fwhm = 28 nm) instead of a monochromator This substantially increased the signal to noise ratio

The luminescence detected at 1270 nm is then the sum of the chlorophyll fluorescence and the $^1O_2$ phosphorescence By determining the difference in the 1270 nm luminescence signal in the presence and absence of oxygen (or by addition of a $^1O_2$ quencher), the intensity of $^1O_2$ phosphorescence may be estimated Fig 4 shows an experiment designed to detect $^1O_2$ by this method In Fig 4a the total luminescence signal is shown for PS II reaction centres gently bubbled with

oxygen After oxygen was exchanged for nitrogen the luminescence signal decreased On the other hand, in Fig 4b when reaction centres were initially bubbled with nitrogen the reverse was seen The luminescence signal was originally low and was increased by introduction of oxygen Finally, addition of azide, a $^1O_2$ quencher, lowered the signal to the level observed with nitrogen present Addition of an enzymic trap for oxygen was equally as effective at lowering the luminescence yield as removal of oxygen by bubbling with nitrogen (data not shown)

The measurements of the $^1O_2$ phosphorescence signals required 682 nm laser excitation for at least one minute, at an intensity which caused changes in the absorption and emission spectra in the visible region of the reaction centre samples, when oxygen was present For this reason the absorption and fluorescence spectra were measured before and after each measurement and the luminescence values of Fig 4 have been corrected for decreases in absorption at the excitation wavelength

We found that, although there was a difference in the yield of the chlorophyll fluorescence (600 to 850 nm) between aerobically and anaerobically light-treated samples ($\phi_f^{O_2}/\phi_f^{N_2} = 1\,09$), this increase was much smaller than the difference in the corrected 1270 nm luminescence signals ($L_{O_2}/L_{N_2} = 1\,56$) We also found that the 1270 nm luminescence signal from reaction centres in buffer diluted into H_2O (instead of D_2O) did not increase in the presence of oxygen and the subsequent addition of azide had no effect These two observations indicate that the higher level of 1270 nm luminescence from reaction centres in oxygen-bubbled D_2O buffer (Fig 4) cannot be ascribed to an oxygen-dependent increase in the tail of chlorophyll fluorescence, but must be due to an additional luminescent species which is quenched by H_2O or azide, i e , singlet oxygen

Time dependence of singlet oxygen phosphorescence

In Fig 5 we show the dependence of 675 nm absorption, chlorophyll fluorescence emission and 1270 nm luminescence of PS II reaction centres under aerobic conditions, as a function of time of illumination with the luminescence excitation beam Fig 5a shows that the intensity of the laser induces a rapid rate of bleaching of chlorophyll and an increase in fluorescence yield Surprisingly, the presence of azide had no effect on either the rate of irreversible bleaching of chlorophyll or the increase in fluorescence (Fig 5a) However, as shown in Fig 5b the 1270 nm luminescence signal was decreased by azide which is in agreement with its role as a quencher of singlet oxygen and with the data of Fig 4 This result seems to indicate that the singlet oxygen which is detected as emission at 1270 nm is in a different environment (accessible to

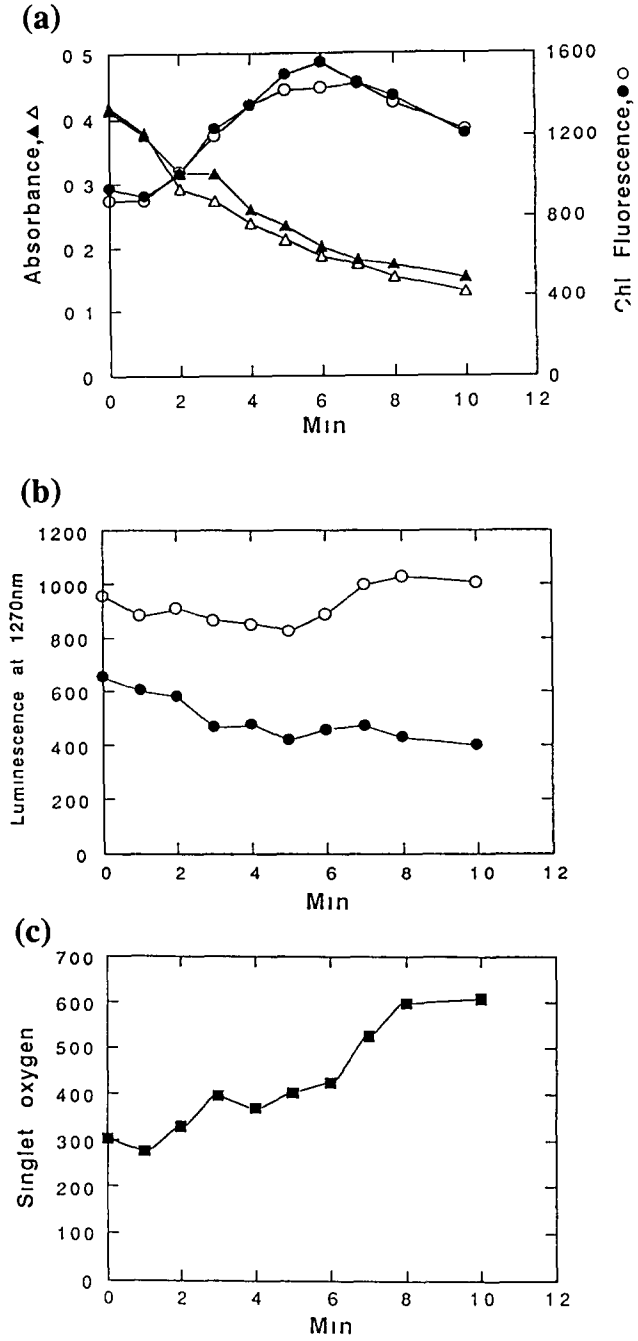

Fig 5 (a) Changes in absorption at 675 nm (triangles) and chlorophyll fluorescence yield, 600 to 850 nm (circles) (b) Corrected 1270 nm luminescence signals (c) The calculated 1270 nm phosphorescence intensity (chloride minus azide data from b) from PS II reaction centres illuminated in the presence of 5 mM sodium chloride (open symbols) or 5 mM sodium azide (closed symbols) under air saturated conditions Excitation was 6 mW cm^{-2} at 675 nm Chlorophyll concentration 6 $\mu g\ ml^{-1}$

singlet oxygen quenchers) to that giving rise to the bleaching of chlorophyll Moreover, the data in Figs 5a and b suggest that singlet oxygen continues to be generated, despite the obvious loss of the P680 chlorophyll and therefore presumably of the primary photo-

chemistry giving rise to charge separation and recombination [5,9] In fact, the yield of singlet oxygen, determined as the difference between the luminescence signals plus and minus azide (Fig 5c) indicates an increase in the level of this species with time of illumination The level of luminescence then seemed to remain relatively constant for a further 15 min illumination period Addition of azide to the 'azide-free sample' after this time caused an immediate drop in the luminescence level to that observed with azide treated reaction centres (data not shown)

We found that the longer the period of excitation with the laser the lower the final L_{1270} level Some of this light dependent decrease was reversible in the dark and we suggest that it may be due to a local depletion in the oxygen concentration at the site of singlet oxygen production, i e , the consumption rate must be faster than the rate at which oxygen can diffuse in from the medium (Macpherson et al , unpublished data)

As azide was unable to protect against damage to chromophores within the PS II reaction centre, we examined the effect of H_2O which, as stated earlier, is also an efficient quencher of $^1O_2$ We compared the rate of irreversible bleaching of chlorophyll at 680 nm in PS II reaction centres suspended in either H_2O or D_2O buffer and found that they were very similar to each other (data not shown) If H_2O was able to quench $^1O_2$ within the protein matrix of the reaction centre we would have expected to have seen a decrease in the rate of chlorophyll degradation in this buffer as compared to D_2O buffer

In the above experiments, when azide was added, the decrease in the 1270 nm emission could be attributed fully to the quenching of the $^1O_2$ signal, since this compound was found to have no significant affect on the yield of chlorophyll fluorescence In other experiments we added the electron acceptors, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) and silicomolybdate, which suppress charge recombination, P680 triplet formation and hence should prevent or reduce the rate of $^1O_2$ formation [2,8,10,11] However, large changes in the chlorophyll fluorescence yield made interpretation of these results difficult

Time-resolved measurements of $^1O_2$ phosphorescence

An alternative method to separate $^1O_2$ phosphorescence from the chlorophyll fluorescence tail at 1270 nm is by conducting time-resolved measurements The longest lifetime of the fluorescence from PS II reaction centres is approx 30 ns [5], whereas singlet oxygen phosphorescence in D_2O has a lifetime in the μs timescale [29] Fig 6 shows the time-resolved emission, in the near infra red, of PS II reaction centres bubbled with oxygen minus the signal seen in the presence of azide This figure shows that the decay of the azide-sensitive component has a lifetime of about 26 μs and we attribute this signal to $^1O_2$ phosphorescence

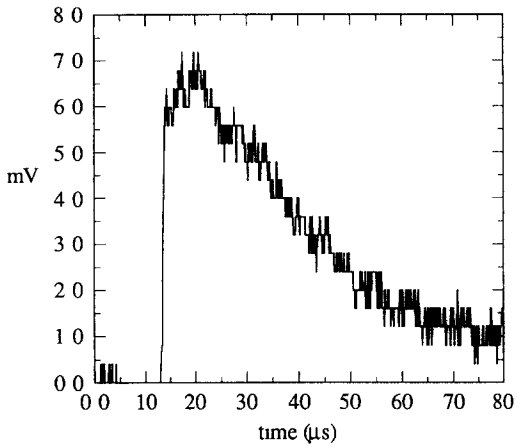

Fig 6 Azide-sensitive time-resolved singlet oxygen luminescence decay from isolated PS II reaction centres incubated in oxygen-bubbled 'D_2O buffer' at 30 μg ml^{-1} chlorophyll Luminescence, at wavelengths > 1050 nm, was measured in the presence and absence of 3 mg of sodium azide and the plus azide signal was subtracted from the minus azide signal

Discussion

Under aerobic conditions and in the absence of electron acceptors, the chlorophyll molecules within the isolated PS II reaction centre are susceptible to photodegradation (Fig 2) Initially chlorophylls which absorb maximally at 680 nm are the most vulnerable It has been argued previously that these chlorophylls function as the primary electron donor P680 and that their degradation is due to singlet oxygen production via the ^{3}P680 state [10,11,13,15] Further illumination of the isolated complex causes irreversible bleaching of the accessory chlorophylls which absorb at about 670 nm This bleaching may also be due to singlet oxygen produced directly from chlorophyll triplet states formed by intersystem crossing rather than by radical pair recombination as for ^{3}P680 Since the fraction of chlorophyll triplet states quenched by O_2 leading to $^1O_2$ formation, $S_\Delta = \phi_\Delta/\phi_T$, is expected to be high ($S_\Delta = 0\,92$ for chlorophyll *b* in C_6D_6 [32]) then the $^1O_2$ yield should be around 20 to 30% at room temperature as judged by the lifetime change of the chlorophyll triplet state between anaerobically and aerobically treated PS II reaction centres [8,9] However, until the work reported here, the formation of $^1O_2$ had not been detected directly in isolated PS II reaction centres, a situation which is also common for other biological systems which are believed to generate this reactive species

Normally, the existence of $^1O_2$ is inferred from the protective effect of removing O_2 from the experimen-

tal system or by adding agents, such as azide, as a $^1O_2$ quencher However, the most reliable method of detecting $^1O_2$ is by direct measurement of its near infrared phosphorescence at 1270 nm When measuring this 1270 nm phosphorescence from $^1O_2$ in aqueous solutions, water is usually exchanged for D_2O, as the non-radiative solvent quenching rate, k_{nr}, and hence the rate of decay of $^1O_2$, $k_\Delta = {}^1/t_\Delta = k_r + k_{nr} + k_q[Q]$, is decreased The phosphorescence yield, $\phi_p = k_r \tau_\Delta \phi_\Delta$, is extremely small in water ($\approx 6\ 10^{-7}\ \phi_\Delta$), due to the low radiative rate ($k_r \approx 0\,18$ in H_2O and $0\,3$ in D_2O [31]), but it will be increased 20 to 30-fold in D_2O, if the quenching term $k_q[Q]$ is not large The weak phosphorescence from any $^1O_2$ generated in the PS II reaction centres in aqueous solution has, therefore, to be distinguished from the long wavelength tail of the overlapping chlorophyll fluorescence ($\phi_f = 0\,04$ [5]), which peaks at 683 nm When time-resolved luminescence detection is employed, the microsecond decay of $^1O_2$ can be separated from the nanosecond chlorophyll fluorescence decay Using the steady-state method $^1O_2$ emission can be characterised spectrally, by observation of a peak at 1270 nm, but the fluorescence tail may distort or even mask the $^1O_2$ emission in aqueous solutions We have attempted to detect $^1O_2$ production in illuminated PS II reaction centres using both of these methods

We have demonstrated (Figs 4 and 5) a decrease in the total luminescence at 1270 nm of PS II reaction centres on removal of oxygen (by exchange with nitrogen or addition of an enzymic oxygen trap) or on addition of $^1O_2$ quenchers (azide or H_2O) This difference in luminescence is not due to a decrease in the intensity of the chlorophyll fluorescence tail, as the latter is unaffected by the presence and absence of azide (Fig 5) We therefore conclude that we have directly demonstrated formation of singlet oxygen, photosensitised by the chromophores of the PS II reaction centre complex This was confirmed by time-resolved measurements We detected a luminescence decay component in the μs time scale which was again dependent on the presence of oxygen (data not shown) and was sensitive to the presence of azide.

The results presented in Fig 5 also show that, even after bleaching of more than 50% of the absorbance at 675 nm, phosphorescence from $^1O_2$ is still observed Charge separation, and thus recombination to give the triplet state of P680, would have been completely inactivated by this time [5,9] Hence, the continued production of $^1O_2$ must be via the triplet states of the other chromophores present in the reaction centres, which are expected to be formed once energy transfer to P680 is prevented Therefore, as predicted above, it is highly likely that the bleaching of P680 and subsequently of the other chromophores of the PS II reaction centre is due to singlet oxygen The intensity of $^1O_2$ phosphorescence from the reaction centres increases up to 2-fold, during the time of excitation, implying that not only do the uncoupled chlorophylls generate $^1O_2$ but also they have a higher triplet yield than the yield of the P680 triplet

The steady-state intensity of the luminescence at 1270 nm is dependent on both the yield and the lifetime of $^1O_2$ and we estimate that the $^1O_2$ intensity from PS II reaction centres is three times less than the corrected signal from a reference sensitiser, chloroaluminium phthalocyanine tetrasulphonate The $^1O_2$ yield from this sensitiser is 0 34 [33], suggesting that the $^1O_2$ yield of reaction centres is approx 0 1 However, as mentioned already, the yield should be similar to the ^{3}P680 yield and therefore about 0 3 This would mean that the lifetime of $^1O_2$ in a D_2O solution of reaction centres is up to 3-times shorter than that of the reference sensitiser, in the 15 to 20 μs range, due to a dominant quenching term, $k_q[Q]$, resulting from the more complex nature of the reaction centre matrix (as compared to the reference sensitiser in solution) If this is the case, the observed 26 μs decay (see Fig 6), determined by time-resolved luminescence, appears to correspond to the lifetime of the P680 triplet state which has been partially quenched by oxygen (a lifetime of about 7 μs would be expected in a fully oxygen-saturated solution) The real $^1O_2$ decay rate would correspond to the rise in the luminescence signal (a rise is observed in Fig 6, but it is partially masked as a result of the intense fluorescence signal)

An interesting feature of our results was that the presence of singlet oxygen quenchers (azide, H_2O) suppressed the detection of $^1O_2$ but did not protect against the photodamage to the PS II reaction centre chromophores which is attributed to singlet oxygen This suggests that, although $^1O_2$ produced at P680, or the other pigments after inactivation of charge separation, causes damage at the site of its production, it can also diffuse into the surrounding medium In the bulk medium it presumably exists at a steady-state level which can be detected by its 1270 nm phosphorescence signal Although the $^1O_2$ in the medium can be quenched by azide or H_2O, these singlet oxygen quenchers are apparently unable to reach the site of $^1O_2$ production in the protein matrix (or cannot compete effectively with the intrinsic quenchers) of the reaction centre Hence, they are unable to protect against photodamage to the pigments

With isolated PS II reaction centres we seem to have detected singlet oxygen sensitised initially by ^{3}P680 and then, when charge separation has been inactivated, by triplets of the other chlorophylls bound to the complex ^{3}P680 formation has been detected by its spin polarised EPR signal in larger PS II complexes and in thylakoids after high light treatment which leads to 'acceptor side' photoinhibition [19,34] It was suggested

that when electron transfer away from the reaction centre was inhibited, the primary quinone acceptor (Q_A) which normally accepts only one electron might become doubly reduced and vacate its binding site on the D2 protein [34] It was proposed that ^{3}P680 would then be formed from the primary radical triplet and under aerobic conditions $^1O_2$ would be generated The toxic effect of singlet oxygen would destroy the chlorophyll of P680, and this has been proposed to be the trigger inducing degradation of the D1 protein [19,34,35] Triggering of D1 protein degradation may be related to conformational changes due to the loss of P680 or to some direct effect of $^1O_2$ on the protein environment around P680 This degradation leads to the production of specific fragments of D1 which are associated only with acceptor side photoinhibition [35,36]

It has previously been shown that carotenoid-containing reaction centres from purple photosynthetic bacteria are much more resistant to photodynamic damage than those isolated from carotenoid-less mutants [37,38] However, although PS II reaction centres bind two β-carotene molecules, they seem to be unable to protect against photodamage through the primary quenching of chlorophyll triplet states [9] This may be due to topological factors preventing a rapid triplet-triplet energy transfer from P680 to the carotenoids [39] and these factors could be a consequence of the isolation procedures Another possible explanation for the low yield of carotenoid triplet state (3% in Ref 9), is that it is only formed by energy transfer from the triplet state of the 6% uncoupled chlorophylls [5] However, our observation of an increase in the $^1O_2$ yield as the chlorophylls are uncoupled, suggests that this is not the case Carotenoids are known to be very efficient physical quenchers of singlet oxygen in homogeneous solution [40], but only 1 in 10^6 $^1O_2$ molecules chemically react with β-carotene [22] and this is obviously not a significant quenching mechanism occurring in PS II reaction centres

We have found that the β-carotene bound to the isolated PS II reaction centre can protect against photodamage by donating electrons to the highly oxidising species, $P680^+$, under conditions where charge recombination is prevented by the presence of secondary electron acceptors [12] The oxidised carotenoid is subsequently bleached irreversibly Indeed, it is possible that the very high oxidising potential of $P680^+$ (approx 1 1 V) causes the carotenoid bound to the PS II reaction centre to be unable to protect by the chlorophyll triplet quenching mechanism [16,22] because, if it is located close enough to undergo energy transfer to P680, it would always be susceptible to rapid oxidation which could compete with electron flow from water We have suggested, therefore, that the role of the β-carotene bound to the PS II reaction centre is to rereduce $P680^+$ if other electron donation pathways are inhibited [12]

Acknowledgements

A N M and T G T are very grateful to the following Cancer Research Campaign for a grant to construct the steady-state luminescence spectrometer, Scottish Home and Health Department for a studentship to ANM, Chris Lambert, David McGarvey and George Shaw for assistance in the construction of the steady-state luminescence spectrometer A T and J B thank the Agricultural and Food Research Council for funding and Caroline Woollin and Chris Barnett for expert technical assistance

References

1 Nanba, O and Satoh, K (1987) Proc Natl Acad Sci USA 84 109–112
2 Barber, J , Chapman, D J and Telfer, A (1987) FEBS Lett 220, 67–73
3 Danielius, R Y , Satoh, K , Van Kan, P J M , Nuijs, A M and Van Gorkom, H J (1987) FEBS Lett 213, 241–244
4 Crystall, B , Booth, P J , Klug, D R , Barber, J and Porter, G (1989) FEBS Lett 249, 75–78
5 Booth, P J Crystall, B , Barber, J , Klug, D R and Porter, G (1990) Biochim Biophys Acta 1016, 141–152
6 Okamura, M Y , Satoh, K , Isaacson, R A and Feher, G (1987) in Progress in Photosynthesis Research (Biggins, J ed) Vol II pp 379–381, Martinus Nijhoff, Dordrecht
7 Takahashi, Y , Hansson, O , Mathis, P and Satoh, K (1987) Biochim Biophys Acta 893, 49–59
8 Mathis, P , Satoh, K and Hansson O (1989) FEBS Lett 251, 241–244
9 Durrant, J R , Giorgi, L B , Barber, J Klug, D R and Porter, G (1990) Biochim Biophys Acta 1017, 167–175
10 Telfer, A and Barber, J (1989) FEBS Lett 246, 223–228
11 Telfer, A , He, W-Z and Barber, J (1990) Biochim Biophys Acta 1017, 143–151
12 Telfer, A , De Las Rivas, J and Barber, J (1991) Biochim Biophys Acta 1060, 106–114
13 Chapman, D J Gounaris, K and Barber, J (1989) Photosynthetica 23, 411–426
14 Shipton, C A and Barber, J (1991) Proc Natl Acad Sci USA 88, 6691–6695
15 Barber, J (1991) in Light in Biology and Medicine (Douglas, R H , ed), Vol II, pp 21–32, Plenum Press, New York
16 Cogdell, R J and Frank, H A (1987) Biochim Biophys Acta 895, 63–79
17 Kyle, D J (1987) in Topics in Photosynthesis, Photoinhibition (Kyle, D J Osmond, C B and Arntzen, C J eds) Vol 9, pp 197–226, Elsevier, Amsterdam
18 Mattoo, A K , Marder, J B and Edelman, M (1989) Cell 56 241–246
19 Barber, J and Andersson, B (1992) Trends Biochem Sci 17 61–66
20 McTavish, H , Picorel, R and Seibert, M (1989) Plant Physiol 89, 452–456
21 Knox, J P and Dodge, A D (1985) Phytochemistry 24, 889–896
22 Asada, K and Takahashi, M (1987) in Topics in Photosynthesis, Photoinhibition (Kyle, D J , Osmond, C B and Arntzen, C J , eds), Vol 9, pp 227–287, Elsevier, Amsterdam

23 Weishaupt, K R , Gomer, C J and Dougherty, T J (1976) Cancer Res 36, 2326–2329
24 Kanofsky, J R , Hoogland, H , Wever, R and Weiss, S J (1988) J Biol Chem 263, 9692–9696
25 Egorov, S Yu , Kamalov, V F , Koroteev, N I , Krasnovsky, A A , Toleutaev, B N and Zinukov, S V (1989) Chem Phys Letts 163, 421–424
26 Baker, A and Kanofsky, J R (1991) Arch Biochem Biophys 286, 70–75
27 Chapman, D J , Gounaris, K and Barber, J (1988) Biochim Biophys Acta 933, 423–431
28 Keene, J P , Kessel, D , Land, E J , Redmond, R W and Truscott, T G (1986) Photochem Photobiol 43, 117–120
29 Ogilby, P R and Foote, C S (1982) J Am Chem Soc 104, 2069–2070
30 Seibert, M , Picorel, R , Rubin, A B and Connelly, J S (1988) Plant Physiol 87, 303–306
31 Schmidt, R and Afshari, E (1990) J Phys Chem 94, 4377–4378
32 Land, E J (1991) J Photochem Photobiol , A Chem 61, 165–170
33 Rosenthal, I , Murali Krishna, C , Riesz, P and Ben-Hur, E (1986) Radiat Res 107, 136–142
34 Vass, I , Styring, S , Hundal, T , Koivuniemi, A , Aro, E -M and Andersson, B (1992) Proc Natl Acad Sci USA 89, 1408–1412
35 De Las Rivas, J , Andersson, B and Barber, J (1992) FEBS Lett 301, 246–252
36 Salter, A H , Virgin, I , Hagman, A and Andersson, B (1992) Biochemistry 31, 3990–3998
37 Boucher, F , Van der Rest, M and Gingras, G (1977) Biochim Biophys Acta 461, 339–357
38 Koka, P and Song, P-S (1978) Photochem Photobiol 28, 509–515
39 Gust, D and Moore, T A (1991) Advances in Photochemistry (Volman, D , Hammond, D and Neckers, D , eds), Vol 16, pp 1–65, John Wiley, New York
40 Conn, P F , Schalch, W and Truscott, T G (1991) J Photochem Photobiol , B Biol 11, 41–47