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Resolution of a long lived fluorescence component from D1/D2/cytochrome *b*-559 reaction centres

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D1/D2/cytochrome *b*-559 reaction centres rapidly degrade under continuous illumination. This is a problem in time-resolved spectroscopy experiments. A long lived fluorescence component assigned to radical pair recombination has been reported previously, but contributing to less than 2% of the fluorescence. This letter reports that sample stability is significantly improved under anaerobic conditions. Fluorescence lifetime measurements described here assign 44% of the total fluorescence yield to a 36.5 ± 2.5 ns lifetime.

Photosystem II; Fluorescence spectroscopy; Reaction center; Primary radical pair; Single photon counting

1. INTRODUCTION

Studies of photosystem II have been greatly advanced by the isolation of the D1/D2/cytochrome *b*-559 reaction centre complex [1,2].

Absorption spectroscopy of this preparation has indicated the presence of a component decaying with a lifetime of 35 ns, corresponding to the lifetime of the primary radical pair [3,4]. More recently, time-resolved fluorescence studies [5,6] have shown a lifetime of 25–35 ns which has been attributed to the recombination of the primary radical pair. However, the fluorescence from this component was observed to be less than 2% of the total light emitted.

This letter reports the observation of this long fluorescence component with a twenty-fold increased yield, in samples stabilised under anaerobic conditions. This suggests that charge recombination of the radical pair forms the largest contribution to the fluorescence of intact D1/D2/cytochrome *b*-559 reaction centres.

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2. MATERIALS AND METHODS

Photosystem II reaction centre complexes were prepared from pea chloroplasts as described in [7] with the following variations. Exchange to 2 mM β-lauryl maltoside was carried out after the first column purification in Triton X-100. Elution from the column was achieved using a NaCl gradient of 2 $mM \cdot ml^{-1}$. The preparation was stored at $-196°C$. Samples were then resuspended in a buffer of 50 mM Tris-Cl (pH 7.2 at room temperature) containing 2 mM maltoside to give a final chlorophyll concentration of 10 $\mu g \cdot ml^{-1}$. Anaerobic conditions were achieved by adding 5 mM glucose, 0.1 $mg \cdot ml^{-1}$ glucose oxidase and 0.05 $mg \cdot ml^{-1}$ catalase.

The state of the reaction centres was monitored during all measurements by noting the Q band absorption and fluorescence maxima. Steady-state absorption measurements were made using a Perkin-Elmer 554 spectrophotometer and fluorescence measurements with a Perkin-Elmer MPF-4 fluorimeter. Samples were maintained at 4°C while all measurements were taken and during time-resolved fluorescence experiments, the samples were also stirred.

Excited singlet-state lifetimes were measured using time-correlated single photon counting (SPC) [8]. The apparatus consisted of a mode-locked Coherent Antares Nd. YAG laser, synchronously pumping a cavity-dumped Rhodamine 6G dye laser. This provided a 4 MHz train of 8 ps pulses at 615 nm with an average power of 20 mW. Emission at 682 nm was selected with a Hilger-Watts monochromator and detected using a Hamamatsu R1564.U01 microchannel plate photomultiplier tube. The instrument response function was measured to be 120 ps (FWHM).

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Assuming multi-exponential decay kinetics, lifetimes were calculated by iterative re-convolution using a semi-linear Marquardt fitting algorithm. All decays were measured to at least 20000 counts in the peak channel (unless otherwise stated) and the quality of the fits was judged using a reduced Chi-square criterion and plots of the weighted residuals. Further details of the apparatus and the analysis technique can be found in [9].

3. RESULTS

Fig.1 shows the reaction centre steady-state absorption spectrum under aerobic conditions, exhibiting a maximum at 675.6 ± 0.1 nm. Anaerobic samples show an identical spectrum. Samples solubilised in maltoside have the same activity as that found with samples in Triton X-100 which exhibit an absorption maximum at 676.0 nm (Chapman, D., personal communication).

Fig.2 shows a typical series of fluorescence decays from an aerobic sample, each accumulated to 1000 counts peak channel. These were collected at 15 min intervals from a sample subjected to continuous illumination with laser light and took approximately 2 min each to collect. Insets are the accompanying steady-state absorption (I) and fluorescence (II) peaks. The blue shift of these maxima illustrate that the sample was degrading. The corresponding change in the decays is due to the increased fluorescence intensity of a 6.5 ns component and the loss of a 36.5 ns component. There is also a loss in the absorbance of the sample.

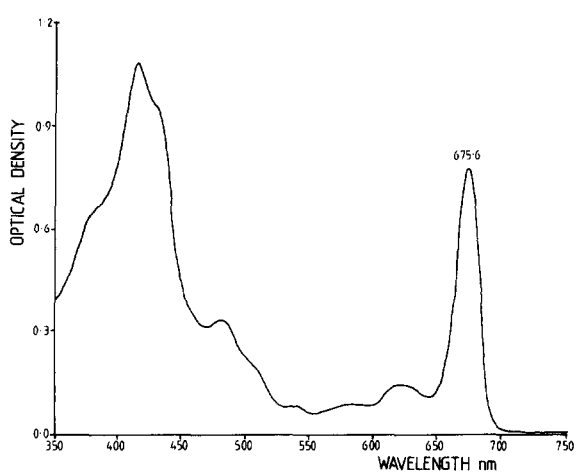

Fig.1. The steady-state absorption spectrum of D1/D2/cytochrome *b*-559 reaction centres solubilised in 2 mM lauryl maltoside measured at 4°C.

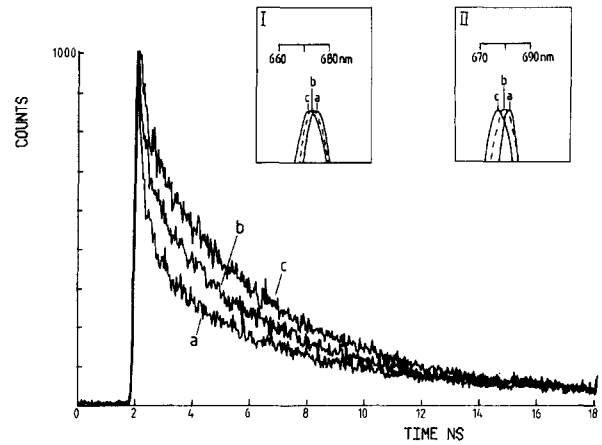

Fig.2. Fluorescence decays of D1/D2/cytochrome *b*-559 reaction centres under aerobic conditions in 2 mM lauryl maltoside measured after (a) 0, (b) 15 and (c) 30 min of continuous laser excitation at 615 nm, 20 mW average power. Insets are (I) the corresponding shifts in the absorption maximum: (a) 675.6 nm, (b) 674.3 nm and (c) 673.0 nm; and (II) the corresponding shifts in the fluorescence maximum: (a) 682 nm, (b) 680 nm and (c) 678 nm. Note: only peak shifts are shown, the accompanying loss in absorbance is not.

Fig.3 shows fluorescence decays from an anaerobic sample, collected to 1000 counts as described above. Under these conditions, the sample is more stable, as is illustrated by the offset decay (c), which was collected after 30 min of illumination. This decay remains unchanged for at least 1 h. The position of the steady-state absorp-

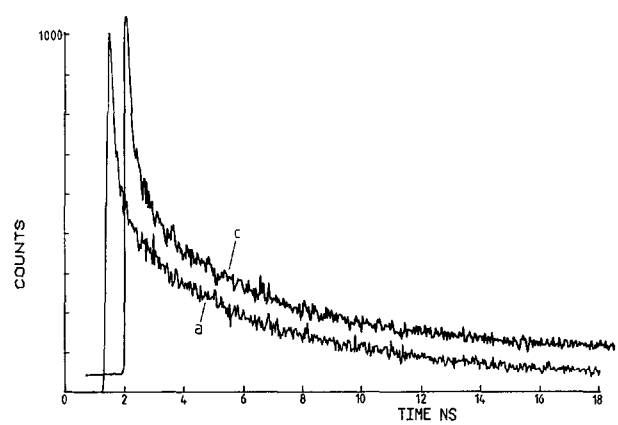

Fig.3. Fluorescence decays of D1/D2/cytochrome *b*-559 reaction centres under anaerobic conditions in 2 mM lauryl maltoside at 4°C, measured after (a) 0 min and (c) 30 min of continuous laser excitation at 615 nm, 20 mW average power. (For reasons of clarity, c is shown displaced.)

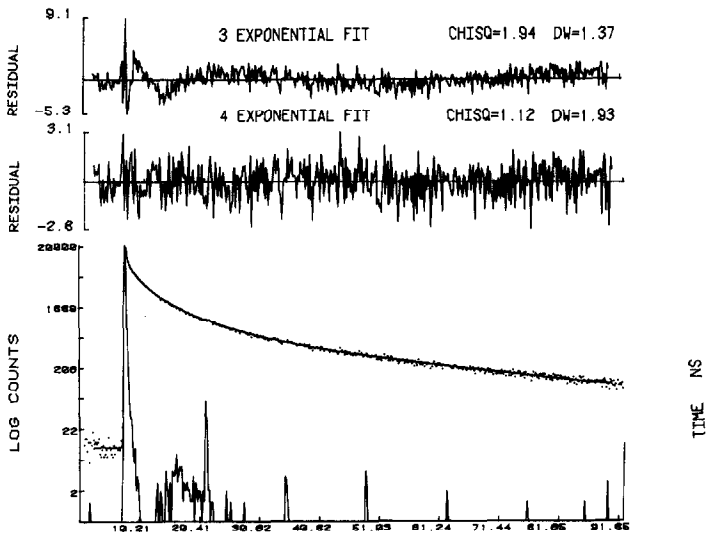

Fig.4. A typical fluorescence decay of D1/D2/cytochrome *b*-559 reaction centres in 2 mM lauryl maltoside under anaerobic conditions measured at 682 nm with 20000 counts in peak channel. Also shown are the residuals, Chi-squared and Durbin-Watson parameters for a three and a four exponential fit to the data.

Table 1

Fluorescence lifetime components from D1/D2/cytochrome *b*-559 reaction centres under anaerobic conditions at 4°C

T_x	Lifetime (ns)	Total fluorescence emitted (%)
T_1	36.5 ± 2.5	44 ± 4
T_2	6.5 ± 1.0	40 ± 5
T_3	1.5 ± 0.5	11 ± 2
T_4	0.1 ± 0.1	5 ± 2

All parameters were free running during analyses. Note: it is not suggested that the two shortest lifetimes are physically significant

tion and fluorescence peaks also remain stable during this period (not shown). However, these samples also show a slow loss of absorbance.

Fig.4 shows a typical fluorescence decay from an anaerobic sample, collected to 20000 counts peak channel. The residuals from a three and a four exponential fit to the data are also shown. The fluorescence decays from three different samples, each analysed to four exponentials, give the results shown in table 1.

4. DISCUSSION

It is clearly established that D1/D2/cytochrome *b*-559 reaction centres are extremely labile under the influence of light and heat when solubilised in Triton X-100 [7,10]. An improvement in stability results from the exchange of Triton X-100 with lauryl maltoside [10].

However, it can be seen from fig.2 that even with lauryl maltoside present and under the relatively low light intensities used in SPC experiments, the preparation is still subject to rapid degradation. This is shown by (i) the shift in both steady-state absorption and fluorescence maxima to the blue, (ii) the increase in the relative amplitude of a 6.5 ns lifetime (which corresponds to chlorophyll which is energetically uncoupled from the process of charge separation) and (iii) the concomitant loss of a 36.5 ns fluorescence lifetime component.

The data presented show that under anaerobic conditions and at identical excitation intensities, the sample stability is significantly improved.

Fig.4 shows that the fluorescence decay for the stabilised sample is best represented by a sum of four exponentials. However, no physical significance should be attached to the lifetime values assigned to T_3 and T_4 as they cannot be accurately resolved on the timescale used in the experiment. These two short components may be attributable to one or more fluorescence decays from active reaction centres. However, further

work on a shorter time scale is required if they are to be assigned accurately.

It has been suggested that a component with a fluorescence lifetime of 25–35 ns and a yield of less than 2% is due to the recombination of the primary charge separated state [5,6]. It can be shown (simulated data not presented) that the amplitude of this component is too low to obtain a reliable estimate of its lifetime from SPC experiments. More importantly, sample degradation occurring over a period of minutes makes it very difficult to collect fluorescence decays free from artifacts. This is because data collection to high counts in the peak channel requires at least 15 min. It is likely that the increased fluorescence yield for the 36.5 ns component reported in this letter is due to the improved stability of the sample.

In conclusion, this paper (i) confirms the correlation between the steady-state fluorescence and absorption red maxima of reaction centres and their photochemical activity, (ii) shows that significant protection against degradation by light can be achieved using anaerobic samples and (iii) suggests that the dominant contribution to the fluorescence decay is from charge recombination.

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