Volume 220, number 1, 67–73 FEB 04956 August 1987

Characterisation of a PS II reaction centre isolated from the chloroplasts of *Pisum sativum*

J. Barber, D.J. Chapman and A. Telfer

AFRC Photosynthesis Research Group, Department of Pure and Applied Biology, Imperial College, London SW7 2BB, England

Received 18 May 1987

A photosystem II reaction centre has been isolated from peas and found to consist of D1, D2 polypeptides and the apoproteins of cytochrome *b*-559, being similar to that reported for spinach by Nanba and Satoh [(1987) Proc. Natl. Acad. Sci. USA 84, 109–112]. The complex binds chlorophyll *a*, pheophytin and the haem of cytochrome *b*-559 in an approximate ratio of 4:2:1 and also contains about one molecule of β-carotene. It binds no plastoquinone-9 or manganese but does contain at least one non-haem iron. In addition to a light-induced signal due to $Pheo^-$ seen under reducing conditions, a light-induced $P680^+$ signal is seen when the reaction centre is incubated with silicomolybdate. In the presence of diphenylcarbazide, the $P680^+$ signal is partially inhibited and net electron flow to silicomolybdate occurs. This net electron flow is insensitive to *o*-phenanthroline, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea and 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene but is inhibited by proteolysis with trypsin and by other treatments. Fluorescence, from the complex, peaks at 682 nm at room temperature and at 685 nm at 77 K. This emission is significantly quenched when either the $P680^+Pheo$ or $P680Pheo^-$ states are established indicating that the fluorescence emanates from the back reaction between $P680^+$ and $Pheo^-$.

1. INTRODUCTION

PS II has a number of functional similarities to the photochemical reaction centre of purple photosynthetic bacteria [1]. Both complexes reduce primary and secondary quinone acceptors, Q_A and Q_B, via a route involving the initial reduction of a pheophytin molecule, and both contain non-haem iron which gives rise to characteristic EPR signals [2]. The functional unit which generates primary charge separation within the bacterial reaction centre consists of two polypeptides known as L and M which together bind four bacteriochlorophyll molecules, two bacteriopheophytin molecules, a non-haem iron and the two quinone molecules corresponding to Q_A and Q_B [3]. Two of the bacteriochlorophylls form a 'special pair' which functions as the primary donor. The L and M subunits have closely related amino acid sequences [4] and also show localized sequence homologies with the two thylakoid membrane proteins D1 and D2 of PS II-containing organisms [5]. D1 is a well characterised product of the *psb*A gene and appears to be the site of Q_B binding [6]. D2 is the product of the *psb*D gene and shows some similarities to the amino acid sequence of D1 [7]. The homology of the amino acid sequences of L and M subunits with those of D1 and D2 together with analysis of X-ray diffraction data of crystals of the reaction centre of *Rhodopseudomonas viridis*, led to the speculation that the latter

Correspondence address: J. Barber, AFRC Photosynthesis Research Group, Department of Pure and Applied Biology, Imperial College, London SW7 2BB, England

Abbreviations: Ant 2p, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DPC, diphenylcarbazide; PS, photosystem

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

two polypeptides form the reaction centre of PS II [8–10]. Prior to this suggestion the PS II reaction centre had been assigned to a 47 kDa polypeptide [11] which is the product of the *psb*B gene [12].

Experimental support for the concept that D1 and D2 form the reaction centre polypeptides has recently been published by Nanba and Satoh [13]. They isolated a chlorophyll containing complex from spinach which had some properties of a PS II reaction centre. The complex was free of 47 kDa protein but consisted of polypeptides of molecular masses indicative of D1 and D2 and also contained cytochrome *b*-559. For each haem there were 4–5 chlorophyll *a* molecules, 1–2 pheophytins and about 1 β-carotene. The complex contained no plastoquinone but could, under reducing conditions, accumulate reduced pheophytin. Okamura et al. [14] have shown that it will form a chlorophyll triplet on illumination, indicative of a radical pair formation, a conclusion also reached from the picosecond absorption studies of Danielius et al. [15] and nanosecond measurements of Takahashi et al. [16].

In this communication we report that the Nanba and Satoh complex [13] can be isolated from the thylakoids of pea chloroplasts and we provide evidence that it contains P680 and can support net electron flow from diphenylcarbazide to silicomolybdate.

2. MATERIALS AND METHODS

Membrane fragments enriched in PS II were isolated from greenhouse grown pea plants (*Pisum sativum* var. Feltham First) essentially according to the method of Berthold et al. [17]. These membrane fragments were suspended to 4 mg Chl $\cdot$ ml^{-1} in 5 mM $MgCl_2$, 15 mM NaCl, 50 mM Mes (pH 6.5) and 10% (w/v) glycerol, frozen at 77 K and stored at 190 K. PS II reaction centres were prepared from a 50 mg sample of the stored membrane fragments using a method developed from the approach suggested by Nanba and Satoh [13] in which solubilization in Triton X-100 is followed by ion-exchange chromatography. Briefly, the procedure was firstly to remove extrinsic membrane polypeptides by incubating the thawed sample on ice in the dark in 50 mM Tris, pH 9.0 (0.8 mg Chl $\cdot$ ml^{-1}), for 10 min followed by centrifugation at 40000 $\times$ *g* at 4°C for 20 min. The pellets were resuspended in 50 mM Tris, pH 7.2 (to about 50 ml), and 8 ml of 30% Triton X-100 added to give a final Chl concentration of 0.8 mg $\cdot$ ml^{-1}. A 60 min incubation with stirring, in the dark and on ice, was followed by centrifugation at 100000 $\times$ *g* for 60 min. The supernatant was then applied to a chromatography column (20 $\times$ 120 mm) containing Fractogel TSK DEAE-650(S) (Merck-BDH). The column was then extensively washed with 30 mM NaCl in a running buffer containing 0.2% Triton X-100 and 50 mM Tris-Cl, pH 7.2, until no more chlorophyll could be eluted. This procedure removed more than 98% of the chlorophyll applied, taking up to 3 h at 1.0 ml $\cdot$ min^{-1}. The column was then subjected to a linear NaCl gradient from 30 to 200 mM in the same running buffer and the fraction which eluted at about 110 mM NaCl collected as 2.0 ml samples. These samples were pooled, diluted 4-fold in running buffer and loaded onto a smaller column (10 $\times$ 60 mm) of the same DEAE-Fractogel. As before, the column was washed extensively with 30 mM NaCl (about 2 h at 0.5 ml $\cdot$ min^{-1}) and a linear NaCl gradient applied to obtain the PS II reaction centre as a sharp peak at about 110 mM NaCl.

The absorption spectra and ratio of Chl to cytochrome *b*-559 were measured immediately to judge the success of the isolation. If samples were not used immediately they were stored at 190 K after addition of glycerol to 10% (w/v). Chlorophyll concentrations were assayed by the method of Arnon [18] while cytochrome *b*-559 was determined by recording the difference spectrum between the oxidised and reduced state (ferricyanide vs dithionite) and applying an extinction coefficient of 15 mM^{-1}. Absorption spectra and light-induced redox changes were monitored using either a Perkin-Elmer 554 or 557 UV/Vis spectrophotometer (1 cm light path). Side illumination was provided by a quartz-iodide light source with an appropriate light guide and transmission filters (Calflex heat filter and 2 mm Schott RG 660 cut-off filter). The intensity of the actinic light at the cuvette surface was 20 W $\cdot$ m^{-2}. The photomultiplier was shielded by a 4 mm Schott B38 cut-off filter. Emission spectra were measured with a Perkin-Elmer MPF 44A fluorimeter and light-induced fluorescence yield changes (>700 nm) explored using a Heinz Walz chlorophyll fluorimeter (model PAM 1), employing a weak measuring

beam modulated at 100 kHz and an actinic light transmitted via a 2 mm Schott RG 645 nm cut-off filter at an intensity of 30 $W \cdot m^{-2}$.

Polypeptide composition was assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12–17% gel, or on a 10–20% gel containing 6 M urea, and stained with Coomassie blue [19]. Pigment and quinone levels were determined by extraction into chloroform and subjected to reverse-phase high-pressure liquid chromatography on a Spherisorb RP-18 column. Levels of Fe and Mn were analysed using the flameless attachment to a Perkin-Elmer atomic absorption spectrophotometer (model 2280).

3. RESULTS

With minor differences in the isolation procedure we have obtained a preparation from pea chloroplast thylakoids which seems to be the same as that reported by Nanba and Satoh for spinach [13]. Our preparation shows an identical absorption spectrum (see fig.1) to that given by the above workers. In addition we also confirm that, based on the presence of two pheophytin molecules, this preparation contains four to five chlorophyll *a* molecules, one cytochrome *b*-559 (as judged by an extinction coefficient of 15 mM^{-1}) and about one β-carotene molecule. As reported by Nanba and Satoh [13], the preparation contains no plastoquinone but we have detected at least one non-haem iron atom. As shown in fig.1, according to our SDS-PAGE procedures, the complex can be resolved to four protein bands. The 9 kDa band was shown by Nanba and Satoh [13] to be the α-subunit of cytochrome *b*-559 and in some gels we also detect the lower molecular mass (4 kDa) β-subunit. The two bands in the region of 30–32 kDa were attributed to D1 and D2. We have confirmed this by preferentially labelling D1 with ^{35}S and by immunoblotting with antibodies raised to the *psb*A and *psb*D genes [19], which have previously been shown to be monospecific to D1 and D2, respectively [20]. This earlier work also showed that in the absence of urea D2 runs with a lower apparent molecular mass than D1, but with 6 M urea present the reverse was true. Both radiolabelling and immunoblotting confirmed the speculation of Nanba and Satoh [13] that the higher molecular mass band, about 55 kDa on our

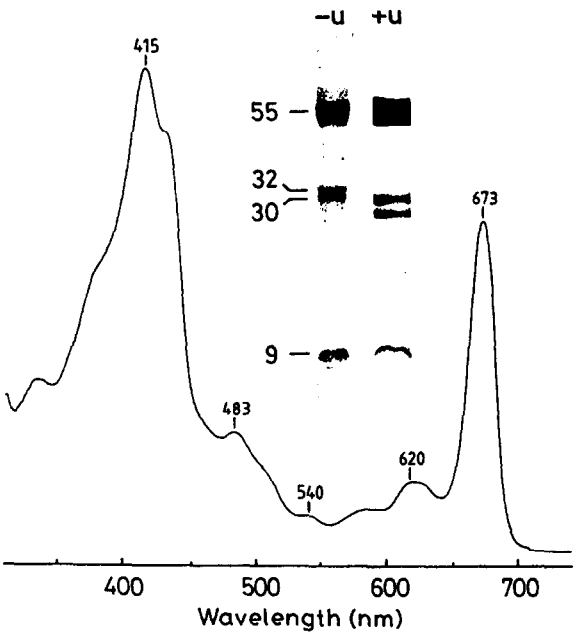

Fig.1. Absorption spectrum and SDS-PAGE profiles of purified pea PS II reaction centre. The absorption spectrum was measured after suspending in 60 mM Tris-Cl (pH 8.5) buffer at room temperature. Track −u was SDS-PAGE on a 12–17% polyacrylamide gel with no urea and track +u was SDS-PAGE using 10–20% gel containing 6 M urea. Approximate molecular masses of protein bands are indicated in kDa.

gels, is a dimeric aggregate of D1 and D2. Treatment with the lysine specific protease, Lys C [21], suggests that this aggregate is a heterodimer [19]. Immunoblotting with a monoclonal antibody raised to the 47 kDa polypeptide of PS II did not cross-react with any of the bands in agreement with the results of Nanba and Satoh [13].

As reported by Nanba and Satoh, in 60 mM Tris (pH 8.5), with dithionite and methyl viologen, a reversible light-induced signal is observed, indicative of the reduction of pheophytin [22]. A typical set of signals together with the light-dark difference spectrum are shown in fig.2. In our spectrum, however, the negative signal at 422 nm is larger than the positive signal at 445 nm which contrasts with that published by Nanba and Satoh. According to Klimov et al. [23] chlorophyll fluorescence is derived from the back reaction between $P680^+$ and reduced pheophytin. The PS II reaction centre does fluoresce and the room

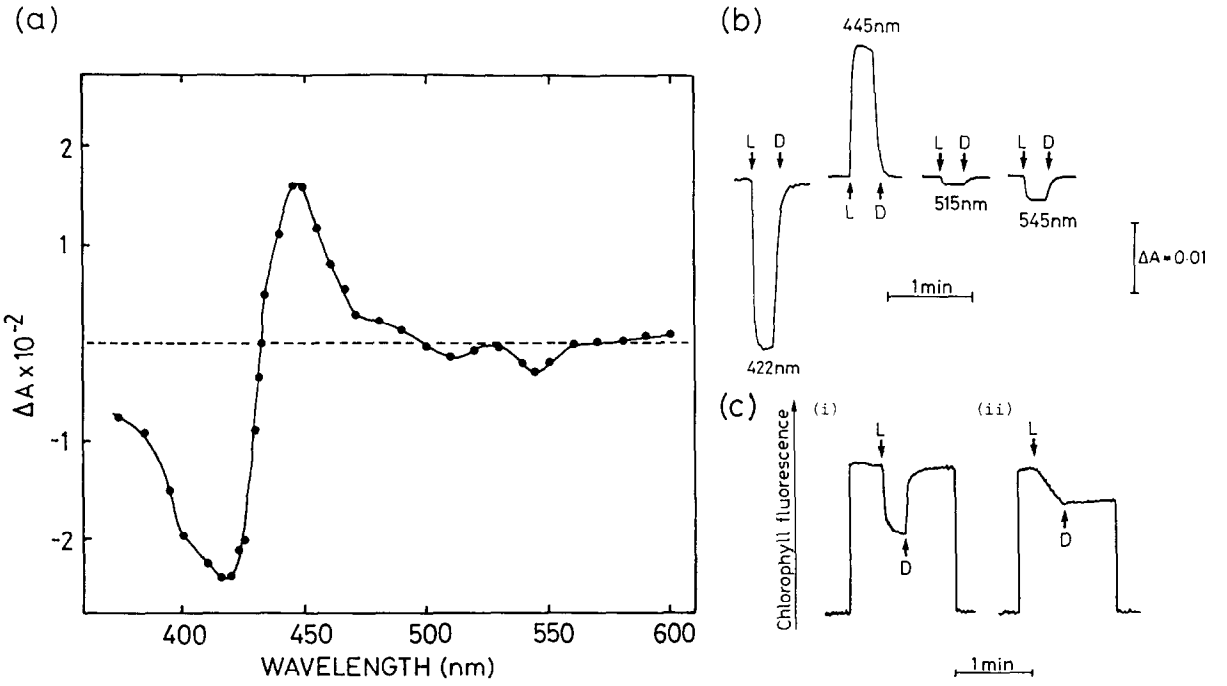

Fig.2 (a) Light-dark difference spectrum obtained in the presence of excess sodium dithionite plus 1 μM methyl viologen. The PS II reaction centre preparation was suspended in 60 mM Tris-HCl (pH 8.5) at 5 $\mu g \cdot ml^{-1}$ Chl and measurements were made at 4°C. (b) Light-induced absorption changes at four different wavelengths. (c) Light-induced chlorophyll fluorescence changes measured under the same conditions as those used for the absorption changes, with the exception of a slight modification in the actinic light (see section 2). Signal (i) with, and signal (ii) without, reductant.

temperature spectrum is shown in fig.3. If the Klimov et al. model is correct then the intensity of this fluorescence should decrease in the presence of dithionite and strong illumination when the $P680^+Pheo^-$ state is shifted in favour of $P680Pheo^-$. Such a decrease is observed with isolated thylakoids and with a PS II enriched preparation [23,24]. Indeed, as shown in fig.2 in the presence of dithionite and methyl viologen, strong light did cause a significant quenching of fluorescence from the D1/D2/cyt *b*-559 complex. This quenching reversed in the dark and showed approximately the same kinetics as the reversible light-induced $Pheo^-$ absorption signals. In the absence of reductant the strong actinic light also caused a quenching of chlorophyll fluorescence but this was much slower and did not reverse in the dark. Fig.3 shows the fluorescence spectrum at 77 K. It should be noted that the emission peaks at about 685 nm and that there is no additional peak at 695 nm. This observation contradicts a previous postulate that low temperature fluorescence at 695 nm emanates from pheophytin within the PS II reaction centre [23].

In addition to observing the light-induced signal

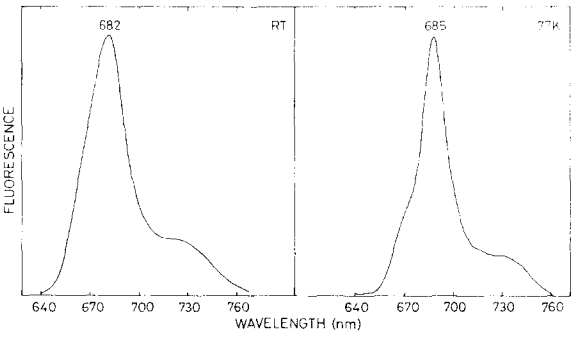

Fig.3. Fluorescence emission spectra of PS II reaction centres measured in 60 mM Tris-Cl buffer (pH 8.5) at room temperature (RT) and at 77 K. Excitation was at 420 ± 10 nm and emission was measured with a slit width of 2 nm.

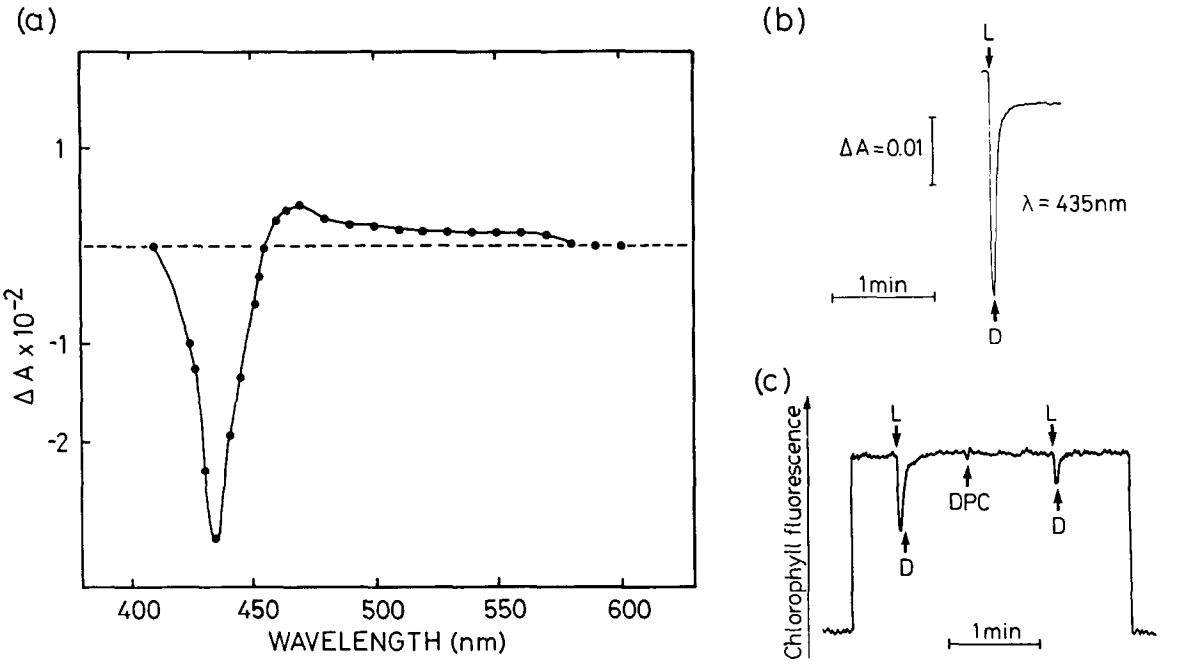

Fig.4. (a) Light-dark difference spectrum obtained in the presence of silicomolybdate (about 200 $\mu g \cdot ml^{-1}$). The PS II preparation was suspended in 60 mM Tris-HCl (pH 8.5) at 5 $\mu g \cdot ml^{-1}$ Chl and measurements were conducted at 4°C. (b) Light-induced absorption change at 435 nm. (c) Chlorophyll fluorescence signal measured with the same sample but with a slightly different actinic light (see section 2). Measuring temperature 4°C.

due to the accumulation of $Pheo^-$ we have also detected an absorption change indicative of $P680^+$ as judged from published spectra [26,27]. This was achieved by adding silicomolybdate to the suspension. In the presence of this acceptor, and with a relatively short exposure to actinic light (5 s), the light-induced signal shown in fig.4b was obtained which was almost totally reversible. The signal showed a light-induced spectrum as in fig.4a. Under the same conditions there was a light-induced quenching of chlorophyll fluorescence (fig.4c).

When DPC, as well as silicomolybdate, was present the light-induced $P680^+$ signal or chlorophyll fluorescence quenching was reduced. A net reduction of silicomolybdate occurred which was observed by recording an absorption increase at

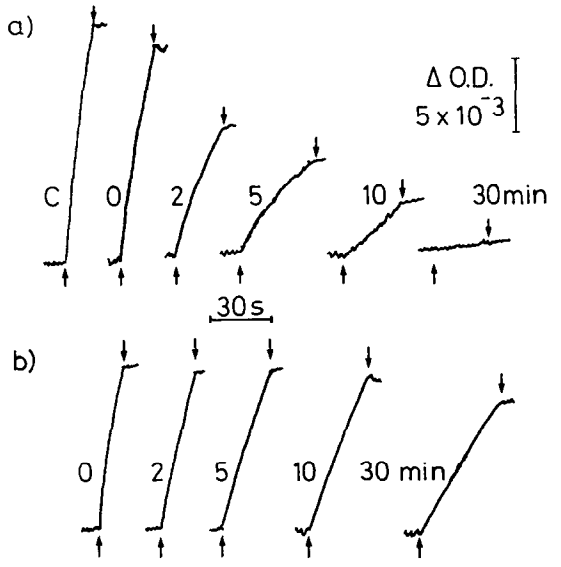

Fig.5. Light-induced net electron flow from diphenylcarbazide (30 $\mu g \cdot ml^{-1}$) to silicomolybdate (200 $\mu g \cdot ml^{-1}$). The same reaction medium as in fig.4 was used. Traces a: Sample treated with 10 $\mu g \cdot ml^{-1}$ trypsin for progressive periods of time as indicated in minutes at 4°C; c is control rate without trypsin. Traces b: Same as traces a, except 10 $\mu g \cdot ml^{-1}$ trypsin inhibitor has also been added. Upward arrows, light on; downward arrows, light off.

600 nm. For the experimental conditions used, the net rate of electron flow was in the order of 1000 μequiv. electrons · mg $Chl^{-1} \cdot h^{-1}$. It was not inhibited by DCMU, *o*-phenanthroline or Ant 2p whether added before or after the addition of silicomolybdate. On the other hand, the light-induced electron flow (and also the $P680^+$ signal) was inhibited by heating, by addition of 1% SDS and by proteolytic digestion. In the latter case the inhibition was induced by treatment with 10 μg/ml trypsin (see fig.5a). When trypsin inhibitor was also present in equal concentration to the proteolytic enzyme only a slight inhibition was observed (fig.5b). Any other degradation of the preparation (e.g. ageing) indicated by blue shifts in the red absorption and chlorophyll fluorescence peaks, also led to a loss of the light-induced signals including the net electron flow from DPC to silicomolybdate.

4. DISCUSSION

We have independently confirmed the findings of Nanba and Satoh [13] that a chlorophyll-binding complex can be isolated free of the 47 kDa PS II polypeptide and consisting of the D1 and D2 polypeptides and the apoproteins of cytochrome *b*-559. In our case we have used pea chloroplasts, although we have also isolated the same complex from spinach [19]. Not only have we shown, as did Nanba and Satoh, that the D1/D2/cyt *b*-559 complex accumulates $Pheo^-$ when illuminated in the presence of dithionite but also that a light-induced steady-state signal, indicative of $P680^+$, can be obtained when silicomolybdate is present. This compound presumably receives its electrons directly from pheophytin possibly by binding to the Q_A site. Such a notion is supported by the lack of effect of DCMU or *o*-phenanthroline on the reduction of silicomolybdate. The isolated complex seems to be unstable and the above light-induced signals must be measured at low temperatures (4°C). Even then their magnitudes are variable. At best the pheophytin signal corresponds to the reduction of almost half of the total pheophytin present as judged by the extinction coefficients published by Fuijita et al. [22]. The maximum size of the $P680^+$ signal may not have been determined since prolonged illumination (more than a few seconds) of the complex in the presence of silicomolybdate leads to a non-reversible signal, presumably due to photodamage. When DPC is present, however, the $P680^+$ signal is reduced and net electron flow occurs to silicomolybdate. Under these conditions the complex seems reasonably resistant to photodamage. The rates of net electron flow are not as high as might be expected possibly because DPC is an inefficient donor under the conditions used. The reversible quenching of chlorophyll fluorescence, when either the $P680 \cdot Pheo^-$ or the $P680^+Pheo$ state is accumulated, is in agreement with the original suggestion of Klimov et al. [23] that this emission is due to a back reaction between $P680^+$ and $Pheo^-$. Although the light-induced reversible quenching was a significant portion of the total emission from the sample, some background fluorescence remained. The origin of this emission could be 'prompt' fluorescence from any free chlorophyll present (i.e. not associated with the D1/D2/cyt *b*-559 complex) or directly from chlorophylls within the complex.

ACKNOWLEDGEMENTS

The above work was supported by grants from the Agricultural and Food Research Council and the Science and Engineering Research Council. We acknowledge the very valuable technical assistance of John De Felice and Kenneth Davis as well as constructive discussions with Drs Niki Gounaris and Jonathan Marder. The monoclonal antibody to the 47 kDa was supplied by T.M. Bricker.

REFERENCES

[1] Ke, B. and Shuvalov, V.A. (1987) The Light Reactions, vol.8, Topics in Photosynthesis (Barber, J. ed.) pp.31–43, Elsevier, Amsterdam, New York.
[2] Rutherford, A.W. (1986) Biochem. Soc. Trans. 14, 15–17.
[3] Okamura, M.Y., Feher, G. and Nelson, N. (1982) in: Photosynthesis: Energy Conversion by Plants and Bacteria (Govindjee, ed.) vol.1, pp.195–272, Academic Press, NY.
[4] Youvan, D.C., Bylina, E.J., Alberti, M., Bergusch, H. and Hearst, J.E. (1984) Cell 37, 949–957.
[5] Williams, J.C., Steiner, L.A., Feher, G. and Simon, M.J. (1984) Proc. Natl. Acad. Sci. USA 81 7303–7311.

[6] Marder, J.B., Mattoo, A.R. and Edelman, M. (1986) Methods Enzymol. 118, 384–396.
[7] Rochaix, J.-D., Dron, M., Rahire, M. and Malnoe, P. (1984) Plant Mol. Biol. 3, 363–370.
[8] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
[9] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[10] Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.
[11] Nakatani, H.Y., Ke, B., Dolan, E. and Arntzen, C.J. (1984) Biochim. Biophys. Acta 765, 347–352.
[12] Morris, J. and Herrmann, R.G. (1984) Nucleic Acids Res. 12, 2837–2850.
[13] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[14] Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) in: Progress in Photosynthesis Research, vol.1 (Biggins, J. ed.) pp.379–381, Martinus Nijhoff, Dordrecht.
[15] Danielius, R.V., Satoh, K., Van Kan, P.J.M., Plijter, J.J., Nuijs, A.M. and Van Gorkom, H.J. (1987) FEBS Lett. 213, 241–244.
[16] Takahashi, Y., Hansson, Ö., Mathis, P. and Satoh, K. (1987) Biochim. Biophys. Acta, in press.
[17] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[18] Arnon, D.I. (1949) Plant Physiol. 2, 1–15.
[19] Marder, J.B., Chapman, D.J., Telfer, A., Nixon, P. and Barber, J. (1987) Plant Mol. Biol., in press.
[20] Nixon, P.J., Dyer, T., Barber, J. and Hunter, C.N. (1986) FEBS Lett. 209, 83–86.
[21] Millner, P.A., Marder, J.B., Gounaris, K. and Barber, J. (1986) Biochim. Biophys. Acta 852, 30–37.
[22] Fuijita, F., Davis, M.S. and Fajer, J. (1978) J. Am. Chem. Soc. 100, 6280–6282.
[23] Klimov, V.V., Klevanik, A.V., Shuvalov, V.A. and Krasnovsky, A.A. (1977) FEBS Lett. 82, 183–186.
[24] Ke, B., Inoue, H., Babcock, G.T., Fang, Z.-X. and Dolan, E. (1982) Biochim. Biophys. Acta 682, 297–306.
[25] Breton, J. (1982) FEBS Lett. 147, 16–20.
[26] Van Gorkom, H.J., Pulles, N.P.J. and Wessels, J.S.C. (1975) Biochim. Biophys. Acta 408, 331–339.
[27] Allakhverdiev, S.I., Shafiev, M.A. and Klimov, V.V. (1986) Photobiochem. Photobiophys. 12, 61–65.