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Evidence for the photo-induced oxidation of the primary electron donor P680 in the isolated photosystem II reaction centre

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A steady-state reversible signal peaking at 680 nm in the red region of the light-dark difference spectrum has been generated in the isolated D1/D2 photosystem II reaction centre using silicomolybdate as an electron acceptor. The signal is dependent on the structural integrity of the reaction centre and is probably due to the formation of $P680^+$ resulting from oxidation of the primary donor, thought to be a chlorophyll *a* dimer. The spectral shape of the red band of the difference spectra, however, contains a shoulder at 672 nm which suggests that monomeric chlorophyll within the reaction centre may also be photooxidised. Prolonged illumination of the sample with bright white light causes a selective bleaching of the long-wavelength absorbing form of chlorophyll within the reaction centre. Protection against this photodestruction of P680 occurs when silicomolybdate is present during the preillumination.

Photosynthesis; Photosystem II; Reaction center; Primary donor; P680

1. INTRODUCTION

The isolation of a complex from the thylakoid membranes of higher plant chloroplasts consisting of the D1 and D2 polypeptides and binding a minimum number of chlorophyll molecules [1,2] fulfilled a prediction that these two polypeptides share functional and structural similarities to the L- and M-subunits of the reaction centres of purple bacteria [3,4]. Indeed, several lines of evidence are consistent with the notion that the D1 and D2 proteins form the heart of the photosystem II (PS II) reaction centre [5–8], and like their bacterial counterparts, bind six chromophores (four chlorophyll *a* and two pheophytin *a* molecules [1,9]). Unlike the purple bacterial reaction centre, however, the isolated PS II reaction centre complex does not contain an H-subunit but rather has three additional polypeptides, α- and β-subunits of cytochrome *b*-559 and the product of the *psbI* gene [1,10–12]. Moreover the isolated PS II reaction centre does not contain the two plastoquinone molecules which function as the secondary electron acceptors, Q_A and Q_B [1,2]. Because of this, the photochemical activity of the complex is restricted to primary electron transfer from a chlorophyll donor to one of the pheophytin molecules [13]. This primary charge separation is quickly reversed by a recombination reaction [6,14]. However, charge stabilization and light-induced net electron flow are possible if suitable electron donors and acceptors are added. In the presence of an efficient electron donor (sodium dithionite) it is possible to photoaccumulate reduced pheophytin [1,2]. If, on the other hand, certain quinones are added then electron transfer occurs to cytochrome *b*-559 which is mediated via quinone reduction [15,16]. Furthermore, in the presence of added quinone it is possible to observe a photoreduction of 2,6-dichlorophenolindophenol [16]. A more effec-

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Abbreviations: PS, photosystem; P680, primary electron donor in PS II; Q_A, Q_B, primary and secondary stable electron acceptors in PS II; SiMo, silicomolybdate

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tive acceptor, however, seems to be silicomolybdate [2,15,17] which shows a net rate of reduction in the light if suitable PS II electron donors are present such as diphenylcarbazide or manganese-(II). In the absence of an electron donor a light-induced absorption signal is observed in the presence of silicomolybdate having a difference spectrum in the blue indicative of the formation of an oxidised chlorophyll radical. This was speculated to be due to $P680^+$ [2].

Here, we explore in more detail the spectral properties of this putative $P680^+$ signal. The study is particularly important because there is considerable variation in the spectra previously reported in the literature. A transient light-induced absorption change attributed to the primary electron donor of PS II (chlorophyll a_{II}) was originally observed by Döring and co-workers [18,19] in isolated thylakoids. Since its discovery a number of very different spectra attributed to transiently observed $P680^+$ [20] and photoaccumulated $P680^+$ [21–23] in a variety of different PS II preparations have been reported. Key differences between the various published spectra are the wavelength of the maximum red absorption decrease (varying from 674 to 682 nm) and its half bandwidth (ranging from 17 nm [19] to 29 nm [23]). Because of the highly oxidising potential of $P680^+$ [7] it is possible that oxidised chlorophyll species in the antenna system contribute to the spectra obtained. This possibility can be overcome by using isolated PS II reaction centres which are free of antenna chlorophylls.

2. MATERIALS AND METHODS

The PS II reaction centre was isolated from pea (*Pisum sativum*) according to [15] and stored at −80°C. For experiments the preparation (in 50 mM Tris, pH 7.2, 0.2% Triton X-100) was thawed and resuspended in 50 mM Tris buffer (pH 8.0), resulting in dilution of the Triton level to <0.005%. Silicomolybdate, obtained from Pfaltz and Bauer, was added at 0.5 mM.

Light-dark difference absorption signals were recorded on a Perkin-Elmer model 554 UV-Vis absorption spectrophotometer equipped for side illumination with a 150 W tungsten light. For wavelengths below 600 nm the light was passed through a Schott RG 660 glass cut-off filter and a Calflex C heat filter (435 $\mu E \cdot m^{-2} \cdot s^{-1}$) and above 640 nm through a Corning 4-96 glass filter and heat filter (800 $\mu E \cdot m^{-2} \cdot s^{-1}$). Between 580 and 640 nm a Balzer K2 broad-band interference filter was used in addition to the Corning 4-96 (145 $\mu E \cdot m^{-2} \cdot s^{-1}$). The decreased excitation intensity reduced the size of the absorption signals in this region compared to wavelengths above 640 nm. To adjust for this the ΔA_{680} was measured plus and minus the K2 filter and the signals in the 500–640 nm region scaled up accordingly. The photomultiplier was protected from strong light by suitable glass cut-off filters. The optical path length was 10 mm and cuvette volume 1 ml. For measurement of the light minus dark difference spectrum, 3-s light pulses were given using a Uniblitz electronic shutter. Difference spectra for the effect of treatment of samples with white light (4000 $\mu E \cdot m^{-2} \cdot s^{-1}$) were recorded using a Shimadzu MPS 2000 absorption spectrophotometer. All absorption measurements were made with a half bandwidth of 1 nm and carried out at 4°C.

3. RESULTS

As can be seen in fig.1, when the isolated PS II reaction centres are incubated with silicomolybdate (SiMo) a light-induced decrease in absorbance is observed at 680 nm. No such signal is obtained in the absence of the artificial electron acceptor or in the presence of potassium ferricyanide. The decrease seen in the presence of SiMo is almost totally reversible if the illumination period is short but with longer exposure to light there is an additional irreversible decline in absorbance. The extent of this slower change varies with wavelength particularly below 450 nm where silicomolybdate absorbs strongly and seems to be due to the bleaching of this acceptor. Nevertheless, the size of the reversible component remains essentially constant as long as the oxidised silicomolybdate does not become limiting. In the presence of ferri-

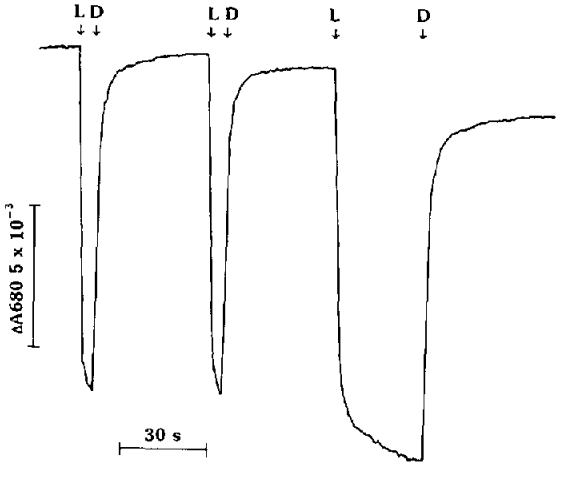

Fig.1. Light-induced absorption changes at 680 nm in the PS II reaction centre preparation in the presence of 0.5 mM SiMo. Chlorophyll, 2 $\mu g \cdot ml^{-1}$.

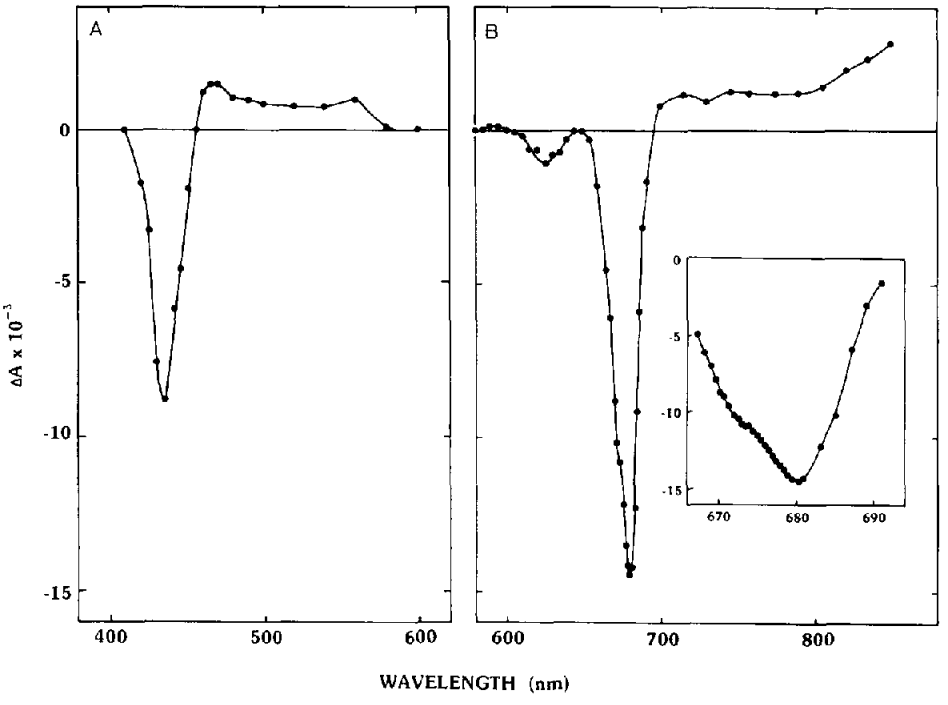

Fig.2. Light minus dark difference spectrum of the reversible absorption changes seen in PS II reaction centres in the presence of 0.5 mM SiMo. Chlorophyll, 2 $\mu g \cdot ml^{-1}$. Spectra (A and B) were measured with different actinic light intensities (see section 2).

cyanide, prolonged illumination leads to a slow irreversible decrease in absorbance (not shown).

A light minus dark difference spectrum of the reversible change observed in the presence of silicomolybdate is shown in fig.2. As demonstrated in fig.2B, the maximum absorption transient in the red is at about 680 nm. The main long-wavelength band of the absorption difference spectrum is however asymmetric, with a distinct shoulder at about 672 nm. Fig.2A shows the light-dark difference spectrum measured in the blue when silicomolybdate was present, which is very similar to that published previously [2]. It should be noted that the signal sizes in the red and blue regions of the spectrum are not directly comparable because of the difference in intensity of the excitation light (see section 2).

Fig.3 shows a double-reciprocal plot of the light intensity dependence of the 680 nm absorption decrease observed in the PS II reaction centre. Extrapolation to infinite light intensity indicates a maximal absorbance decrease equivalent to 1 $P680^+$ per 11 chlorophyll molecules (assuming all

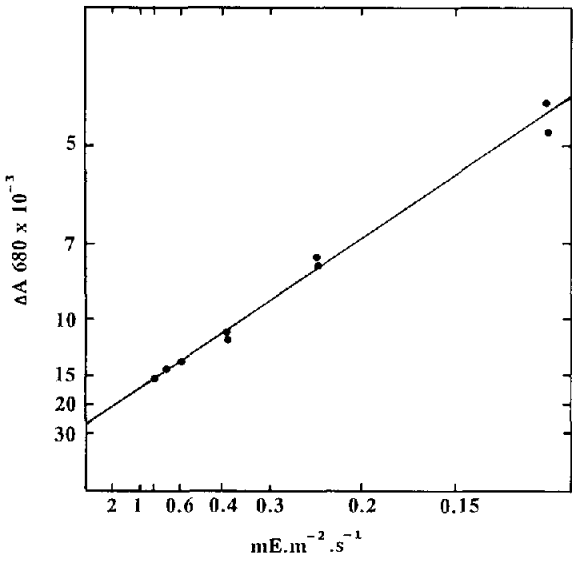

Fig.3. Double-reciprocal plot of the intensity dependence of the reversible light-induced absorption change of PS II reaction centres at 680 nm in the presence of 0.5 mM SiMo. Chlorophyll, 3.4 $\mu g \cdot ml^{-1}$.

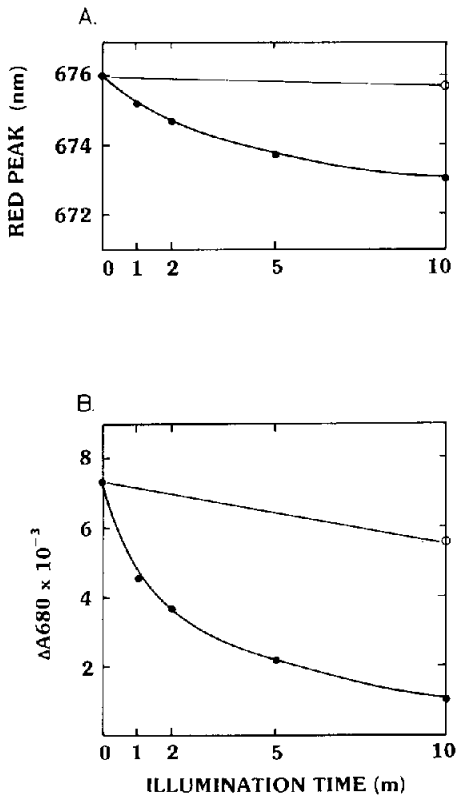

Fig.4. Effect of illumination of PS II reaction centres with white light in the absence of electron acceptor (●) on (A) wavelength peak of the red absorption maximum and (B) reversible light-induced absorption change at 680 nm seen in the presence of SiMo (added just prior to measurement). The effect of the presence of SiMo during illumination of PS II reaction centre with white light is also shown (○). Chlorophyll, 3.4 $\mu g \cdot ml^{-1}$.

the bleaching at 680 nm is due to $P680^+$ formation and an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ [21]). This is lower by a factor of three than would be expected if a functional PS II reaction centre consisted of four chlorophylls and may reflect heterogeneity in the activity of the sample. A similar low yield has been observed for primary charge separation in this type of preparation [6].

The isolated PS II reaction centre is inactivated by subjection to high light intensities in the absence of added electron acceptors [24]. This treatment is characterised by a blue shift and decrease in size of the red absorption band and also a loss of optical activity as measured by circular dichroism [25,26]. These spectral changes have been attributed partly to bleaching of some of the chlorophyll and to removal of exciton interaction between the special pair of chlorophylls which constitute P680, presumably by subtle conformational changes [25]. In fig.4 we show that the light-induced signal observed in the presence of silicomolybdate was significantly inhibited when the PS II reaction centres were pretreated with white light for 5 min (4000 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 4°C. This inhibition was matched by a shift of the red maximum absorption peak from 676 to 673 nm. However, if silicomolybdate was present during the preillumination period a protection against photodamage was observed (see fig.4). It appears that the chlorophyll species giving rise to the reversible light-induced signal at 680 nm is being removed by the strong preillumination. In fact, the difference spectrum in the red

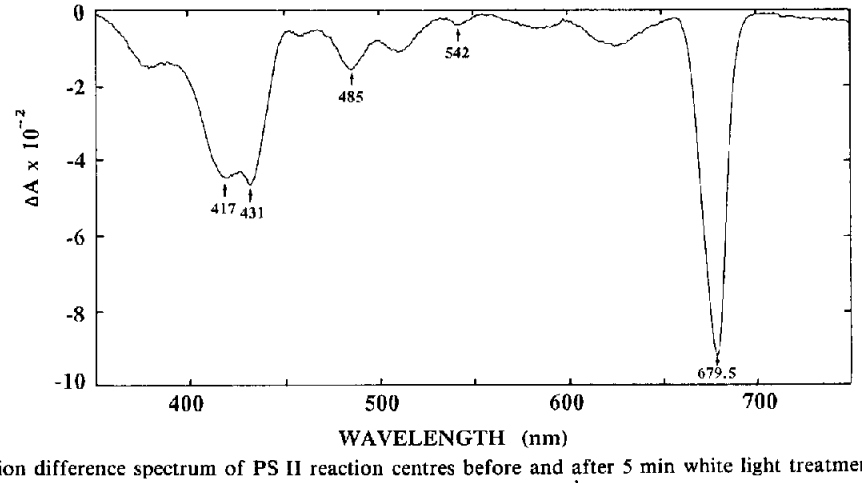

Fig.5. The absorption difference spectrum of PS II reaction centres before and after 5 min white light treatment in the absence of SiMo. Chlorophyll, 3.4 $\mu g \cdot ml^{-1}$.

between treated and untreated preparations as shown in fig.5 clearly indicates that a spectral form of chlorophyll which peaks at about 680 nm is being selectively removed by the photodamaging treatment. In the blue region of the spectrum, however, it can be seen that this treatment induces bleaching in addition to that of chlorophyll (413 nm), which can be attributed to pheophytin (417 and 542 nm) and carotenoid (485 nm).

4. DISCUSSION

The simplest interpretation of our results is that the light-dark difference spectrum shown in fig.2 is due to the conversion of P680 to $P680^+$. Such a spectrum is created presumably because silicomolybdate is able to accept electrons from reduced pheophytin (directly or possibly through the non-haem iron) thus allowing the photoaccumulation of $P680^+$. Recent experiments using EPR spectroscopy indicate that silicomolybdate can act as an electron acceptor in isolated PS II reaction centres even at 4 K [17]. In these experiments a light-induced narrow line radical (0.7 mT) at about $g = 2$ was observed indicative of the formation of $P680^+$. The narrowness of this signal suggests that $P680^+$ is a dimeric form of chlorophyll as is the case for the primary donor in the reaction centres of purple bacteria. Further evidence that P680 is a chlorophyll dimer comes from recent analyses of optical absorption and CD spectra [25,26]. The work in [25] indicated that within the isolated PS II reaction centre there are two chlorophyll *a* molecules which are excitonically coupled and which have a major absorption band at about 679 nm (at 4°C). The same analyses suggested that the remaining two chlorophylls and two pheophytins have ground-state absorption peaking at 672 nm. Harsh treatments which gave rise to a blue shift of the red absorption band of the complex from its initial position of 676 nm (at 4°C) also resulted in a loss of the CD spectrum. As can be seen in fig.4 the same blue shift was matched by a loss of the light-induced reversible signal peaking at 680 nm. Therefore, we conclude that the bleaching that we see at 680 nm is due to the photoinduced oxidation of the chlorophyll *a* dimer which acts as the primary donor of PS II.

Several different light-dark difference spectra for $P680^+$ formation have been reported in [19–23]. In fig.6 we compare our spectrum with the transient absorption spectrum of Döring et al. [19] and the steady-state spectrum of Van Gorkom et al. [22]. All three spectra differ; that of Döring et al. is asymmetric in favour of the red while that of Van Gorkom et al. is wider and asymmetric to the blue. Our spectrum is also asymmetric to the blue side but is significantly narrower than that of Van Gorkom et al. and clearly peaks at 680 nm. As emphasised in fig.2B, our difference spectrum possesses a shoulder at 672 nm. This could be due to the additional bleaching of an oxidised monomeric chlorophyll absorbing at a shorter wavelength than the special pair. The magnitude of the absorption difference of this component was consistently lower than the 680 nm bleaching. We have preliminary evidence for a kinetic difference in the re-reduction properties of ΔA_{672} and ΔA_{680} although we observed that the ratio between the two components was constant provided a full recovery was allowed between measurements.

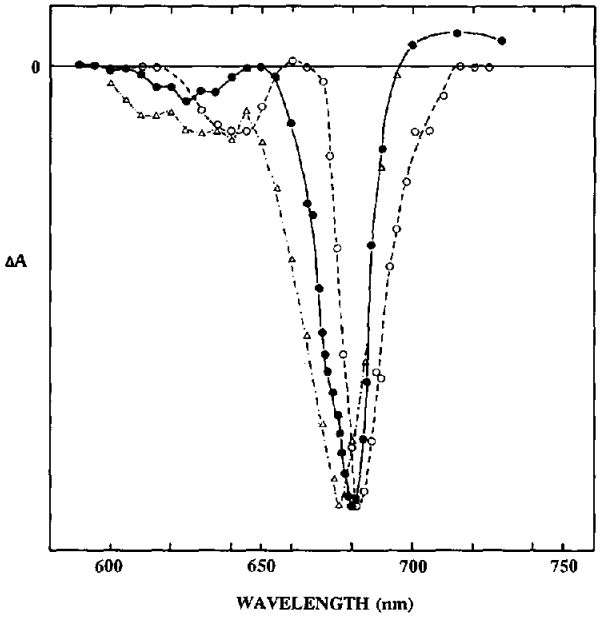

Fig.6. Comparison of light-induced difference spectra attributed to P680/$P680^+$. In isolated PS II reaction centres (from fig.2B; ●——●), in thylakoids (from [19]; ○---○), in a PS II-enriched preparation (from [22]; △ --- △). All spectra were normalised to give the same maximum absorbance decrease.

If indeed the short-wavelength component is due to photooxidation of monomeric chlorophyll, then the only candidates should be the two accessory chlorophyll *a* molecules thought to be in this isolated PS II complex. As pointed out by Thompson and Brudvig [27], $P680^+$ is unique in that it has a sufficiently high redox potential to oxidise chlorophyll. In their study they concluded that such oxidation can occur within the D1/D2 dimer of the PS II reaction centre. We suggest that the difference between the spectrum of Van Gorkom et al. [22] and that presented here is due to additional oxidation of antenna chlorophylls in the former. In contrast, the absence of a shorter wavelength component in the spectrum of Döring et al. [19] may be because this work was carried out with thylakoids which were able to generate a membrane potential and therefore induce additional electrochromic effects on the spectrum. Moreover, their spectrum was derived from signals induced by rapid flash excitation which may not cause production of monomeric chlorophyll radicals.

As clearly shown in fig.4, the isolated reaction centre is readily damaged by exposure to bright light. However, in the presence of silicomolybdate this effect of bright light was dramatically reduced. The reason for this effect is unclear but may arise because silicomolybdate can compete effectively with the recombination reaction and thus lower the level of chlorophyll triplet formed in the illuminated sample. Recently, we [15] and Seibert et al. [28] reported that the isolated PS II reaction centre could be stabilized by exchanging Triton X-100 with lauryl maltoside. However, this stabilization referred to dark treatments while the silicomolybdate effect we report here protects against photodamage.

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