Biochemistry

 Volume 38, Number 26 June 29, 1999

Accelerated Publications

Carotenoid Oxidation in Photosystem II†

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Received March 18, 1999; Revised Manuscript Received April 29, 1999

ABSTRACT: The oxidation of carotenoid upon illumination at low temperature has been studied in Mn-depleted photosystem II (PSII) using EPR and electronic absorption spectroscopy. Illumination of PSII at 20 K results in carotenoid cation radical ($Car^{+\bullet}$) formation in essentially all of the centers. When a sample which was preilluminated at 20 K was warmed in darkness to 120 K, $Car^{+\bullet}$ was replaced by a chlorophyll cation radical. This suggests that carotenoid functions as an electron carrier between P680, the photooxidizable chlorophyll in PSII, and Chl_Z, the monomeric chlorophyll which acts as a secondary electron donor under some conditions. By correlating with the absorption spectra at different temperatures, specific EPR signals from $Car^{+\bullet}$ and $Chl_Z^{+\bullet}$ are distinguished in terms of their *g*-values and widths. When cytochrome b_{559} (Cyt b_{559}) is prereduced, illumination at 20 K results in the oxidation of Cyt b_{559} without the prior formation of a stable $Car^{+\bullet}$. Although these results can be reconciled with a linear pathway, they are more straightforwardly explained in terms of a branched electron-transfer pathway, where Car is a direct electron donor to $P680^+$, while Cyt b_{559} and Chl_Z are both capable of donating electrons to $Car^{+\bullet}$, and where the Chl_Z donates electrons when Cyt b_{559} is oxidized prior to illumination. These results have significant repercussions on the current thinking concerning the protective role of the Cyt b_{559}/Chl_Z electron-transfer pathways and on structural models of PSII.

Photosystem II (PSII)[1] is the photochemical reaction center which bears the water oxidizing and plastoquinone reducing activity (*1*, *2*). The high redox potential which is required in order to oxidize water renders the enzyme susceptible to damage, and elaborate protection mechanisms are present in order to minimize this intrinsic problem (*3*). The most oxidizing species present in PSII is the cation form of the photooxidizable chlorophyll, $P680^+$. It is estimated that it has an E_m of around 1.1 V. When the oxygen evolving enzyme is functional, $P680^+$ is reduced by a nearby tyrosine residue (Tyr 161 of the D1 protein, known as Tyr_Z). The oxidized Tyr_Z is reduced in its turn by electrons coming from the Mn cluster which is thought to be the active site for water oxidation.

Under a range of conditions, the delivery of electrons from the Tyr_Z to the highly oxidizing state $P680^+$ can be blocked or retarded, and thus $P680^+$ has the opportunity to undergo reactions with other components of the reaction center. These reactions are liable to be important under a range of physiological conditions: (1) during normal function of the enzyme where the natural equilibria allow $P680^+$ to be relatively long-lived in a statistical fraction of the centers (see ref *3* for a review, see also, e.g., ref *4*); (2) in the apo-

† This work was supported in part by HFSPO Grant RG 0349 and TMR Grants FMRX-CT 96-0031 and FMRX-CT98-0214. P.F. was supported by a fellowship from the Swiss National Science Foundation.

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[1] Abbreviations: Car, redox active carotenoid; Chl, chlorophyll; Chl_Z, redox active chorophyll in PSII; Cyt b_{559}, cytochrome b_{559}; EPR, electron paramagnetic resonance; Tyr_Z, kinetically competent redox active tyrosine residue; Q_A, the first of two quinones acting as electron acceptors in PSII.

10.1021/bi990633u CCC: $18.00
Published on Web 06/06/1999

enzyme and during the process whereby the Mn cluster is assembled (e.g., ref *5*); and (3) under stress conditions (reviewed in ref *3*).

When $P680^+$ is unreduced by Tyr_Z, the recombination of the $P680^+Q_A^-$ radical pair [200 μs−1 ms depending on the electrostatic environment of Tyr_Z (*1*)] is probably dominant under most circumstances. Nevertheless, $P680^+$ seems to be able to obtain electrons from a series of electron donors other than Tyr/Mn. Since these " side-path" donation reactions are thought to compete poorly with the usual forward reactions and with the charge recombination reactions at room temperature (see refs *1*, *4*), they have more often been studied at low temperature where they can be accumulated using continuous illumination.

It has long been known that reduced cytochrome b_{559} (Cyt b_{559}) will undergo oxidation when PSII is illuminated at 77 K (*6*, reviewed in ref *7*). When Cyt b_{559} is preoxidized, a chlorophyll molecule (recently termed Chl_Z) is oxidized instead of Cyt b_{559} (see refs *8*, *9*). This has led to the suggestion that a linear Cyt b_{559}−Chl_Z−P680 electron-transfer pathway exists (*9*), and this was supported by experiments which compared the efficiency of electron donation of Chl and Cyt b_{559} with the donation from the Tyr_Z/Mn cluster (*10*). It has been suggested that this may play a significant role at physiological temperatures, providing electrons to the hyper-reactive $P680^+$, thereby diffusing any potential for uncontrolled oxidative reactions (*10*). The possibility has been raised that oxidized Cyt b_{559} completes an electron-transfer cycle around PSII by obtaining electrons from reduced quinones (reviewed in ref *7*).

Carotene can also be oxidized by PSII under certain conditions. Oxidized carotenoid cation, $Car^{+\bullet}$, was detected by its absorption at 990 nm when PSII was illuminated at low temperature in the presence of ferricyanide (*11*, *12*). At room temperature, the $Car^{+\bullet}$ was detected in the presence of chemicals which perturb the usual electron-transfer reactions (*11*, *13*, *14*) and in isolated PSII reaction centers when an artificial electron acceptor is present (*15*).

Despite the literature showing $Car^{+\bullet}$ formation at low temperature, little information exists concerning the proportion of centers in which this reaction occurs, nor does much information exist distinguishing between $Car^{+\bullet}$ and $Chl^{+\bullet}$ formation at low temperature. Noguchi et al. (*12*) reported that 77 K illumination of Mn-containing PSII gave rise to $Car^{+\bullet}$ in at least a significant fraction of centers while 200 K illumination of Mn-depleted PSII resulted in $Chl^{+\bullet}$ formation with no contribution from $Car^{+\bullet}$. In other studies of PSII at low temperature, the possibility that $Car^{+\bullet}$ is present is occasionally considered (e.g., ref *9*), but in the majority of studies of low-temperature photochemistry in PSII, it is suggested, or at least implied, that $Chl_Z^{+\bullet}$ is the sole (or at least major) electron donor oxidized at low temperature (see, for example, refs *16*, *17*). In particular, no EPR studies have been reported of $Car^{+\bullet}$ in PSII although quite a lot of literature exists for carotenoid cation radicals in organic solvents (e.g., *18*, *19*). Given the results of Schenck et al. (*11*) and Nogushi et al. (*12*), it seems likely that some of the literature reports of $Chl^{+\bullet}$ should contain at least contributions from $Car^{+\bullet}$. Here we have specifically studied the temperature dependence of $Car^{+\bullet}$ and $Chl^{+\bullet}$ oxidation using EPR and optical methods, we clarify the conditions under which $Car^{+\bullet}$ and $Chl^{+\bullet}$ are observed, and we provide evidence that carotenoid may work as an electron carrier between P680 and Chl and that the carotenoid may be a branch point in electron transfer from Cyt b_{559} and Chl_Z.

MATERIALS AND METHODS

PSII-enriched membranes were prepared essentially by the method in (*20*) except that the pH was maintained at 6.3 after preparation of the thylakoid membranes. Mn was depleted from PSII by incubating the membranes (1 mg of Chl/mL) at 4 °C for 30 min in the dark in the presence of 1 mM freshly made NH_2OH. The membranes were then precipitated by centrifugation and washed 5 times in a buffer containing 5 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, 50 mM MES, pH 6.3, by successive resuspension and centrifugation steps in order to remove NH_2OH. The final pellet was resuspended in 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sucrose, 20 mM MES, pH 6.3. The Mn depletion and washing steps described above were done in total darkness.

In our early optical studies, PSII-enriched membrane samples were suspended in 60% glycerol at 0.8 mg of Chl/mL. However, although good spectra of $Car^{+\bullet}$ were obtained, spectra of $Chl_Z^{+\bullet}$ were poorly resolved. To improve the optical properties of the sample, the membranes (2 mg of Chl/mL) were solubilized for 1 h on ice in 0.5% (w/v) β-dodecyl maltoside (β-DM) in complete darkness. The samples were then centrifuged in a benchtop centrifuge in order to remove unsolubilized material. The supernatant was then taken and mixed with glycerol (to 60%, v/v). A range of detergent concentrations were tested, and the concentration used in the experiment was the lowest that solubilized PSII. Comparison of both the optical and EPR data with and without β-DM indicated that the reactions taking place were essentially unaffected by the detergent (not shown). It was also shown that glycerol at 60% had no significant influence on the EPR data. In some samples, potassium ferricyanide (5 mM) was added to the sample immediately prior to introduction of the sample into the optical cryostat; this had the effect of increasing the yield of $Car^{+\bullet}$ due to Q_A being fully oxidized prior to illumination at low temperature. Under the conditions of the experiment, little or no oxidation of the non-heme iron is expected.

EPR was done on samples (PSII membranes at ∼4 mg of Chl/mL, β-DM-solubilized PSII at ∼2 mg of Chl/mL) in 4 mm external diameter EPR tubes. The study of free radicals was done on both types of material. The data shown were from β-DM-solubilized PSII, but comparable results were found with PSII membranes. Ferricyanide was not required to maintain Q_A oxidized in the EPR experiments since samples could be manipulated in complete darkness. The data on Cyt b_{559} were obtained in Mn-depleted PSII membranes reduced with 2 mM ascorbate, incubated in the dark for 1 min, pelleted, and resuspended again in 2 mM ascorbate, similar to the methods used in, e.g., ref *10*. Spin counting of the radicals was done relative to the $TyrD^{\bullet}$ signal in the same samples previously used for the study of the Car and Chl radicals after 10 min illumination at 0 °C and 30 min dark adaptation. In some samples a small fraction (less than 10%) of $TyrD^{\bullet}$ was already present in dark-adapted NH_2OH-treated samples. This was subtracted from the light-induced spectra.

Absorption spectra were collected in an SMC-TBT flow cryostat (Air Liquide, Sassenage, France) cooled with liquid

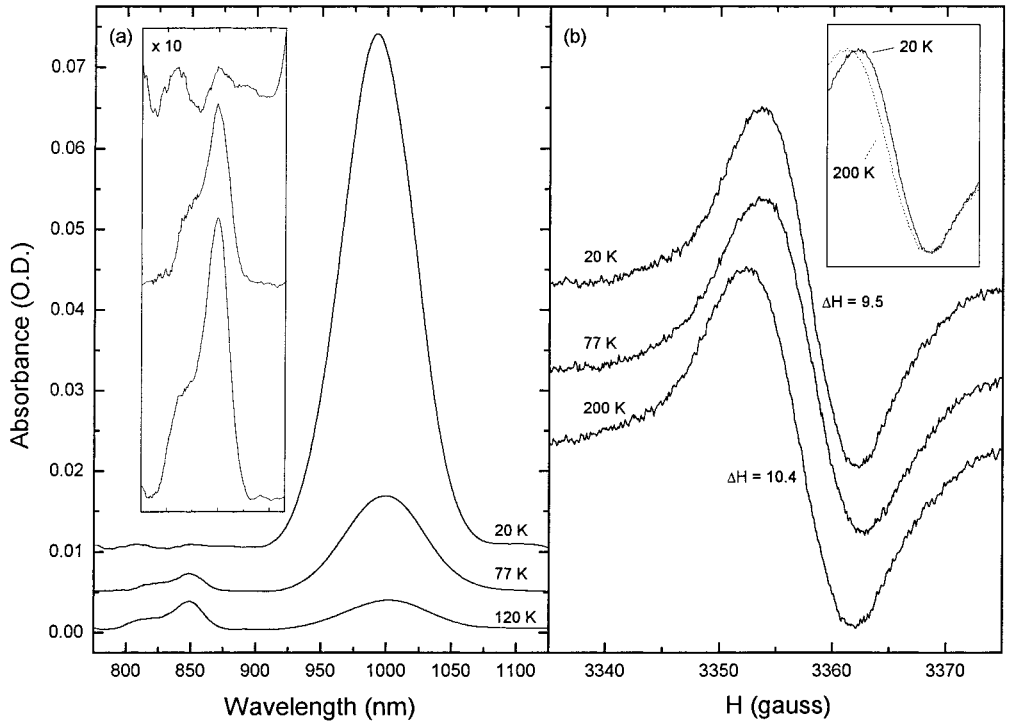


FIGURE 1: (a) Absorption difference spectra obtained at 20 K after illumination at 20, 77, and 120 K in Mn-depleted β-DM-solubilized PSII (see Materials and Methods). Inset: the spectral region 775−915 nm with gain multiplied by 10. Conditions were as described under Materials and Methods. (b) Light-minus-dark difference EPR spectra of Mn-depleted β-DM-solubilized PSII illuminated at 20, 77, and 200 K. The spectra were all recorded at 15 K. EPR instrument settings were as follows: microwave power, 45 dB; frequency, 9.4 GHz; modulation amplitude, 2 G. Inset in (b) the EPR signals generated by illumination at 20 and 200 K when overlaid to emphasize the differences in *g*-value and width.

helium, using a Varian Cary E5 double-beam scanning spectrophotometer. Illumination with white light was done in the cryostat using a Flexilux fiber optic illuminator. Illumination was continued until no further absorption changes occurred (approximately 10 min). The measuring beam was swept over the spectrum from longer to shorter wavelengths as far as 750 nm in order to minimize possible actinic effects of the measuring beam.

X-band EPR spectra were recorded with a Bruker ER 300 X-band spectrometer in an Oxford Instruments cryostat cooled with liquid helium. Illumination at 20 K was done in the cavity using a 800 W tungsten lamp filtered through 5 cm of water and infrared cutoff filters. Illumination at higher temperatures was done in unsilvered dewars containing liquid nitrogen (77 K) or a solid CO_2/ethanol mixture (200 K).

RESULTS

Figure 1a shows light-minus-dark absorption difference spectra taken at 20 K after illumination of PSII at three different temperatures. After illumination at 20 K, a strong absorption peak centered at 990 nm appears; this signal is similar to those arising from carotenoid cations in vitro (*21*, *22*) and has been reported before in PSII after illumination at 77 K (*11*, *12*). Accordingly, we attribute this spectrum to a carotenoid cation radical, $Car^{+\bullet}$. Figure 1a also shows that when illumination is performed at 120 K very little (∼5%) of the 990 nm band is formed while when illumination is given at 77 K 19% of the maximum $Car^{+\bullet}$ is formed. The inset in Figure 1a shows that illumination at 120 K gives rise to an absorption band with a maximum at 850 nm. By comparison to in vitro spectra (*23*), and in accordance with earlier reports in the literature (*8*, *16*), we attribute this peak to a chlorophyll cation monomer, $Chl^{+\bullet}$. The present $Chl^{+\bullet}$ spectrum is much better resolved than those published previously, and the peak position is somewhat different, being sharper and at 850 nm rather than 820 nm; however, it shows a greater similarity to the model chlorophyll cation in ref *23*.

The inset of Figure 1a also shows that as the illumination temperature is lowered the absorption from $Chl^{+\bullet}$ diminishes, being 72% (compared to the maximum intensity) at 77 K and less than 10% at 20 K. The data show that at 120 K Chl is predominantly oxidized while at 20 K Car is oxidized. Given the relative extinction coefficients for carotenoid and chlorophyll radicals [$Car^{+\bullet}$ 218 000 M^{-1} cm^{-1} (*22*), and $Chl^{+\bullet}$ 7000 M^{-1} cm^{-1} (*23*)], the relative yield of these two radicals (for identical samples illuminated at 20 K for $Car^{+\bullet}$ or at 120 K for $Chl^{+\bullet}$) is approximately 1:1. Furthermore, taking into account the PSII concentration and the pathlength (2 mm), we estimate that one carotenoid cation (or Chl cation) is formed per PSII. These approximations are backed up by the EPR studies described below.

Also shown in Figure 1 are the EPR spectra taken under conditions comparable to those in which the optical spectra were taken. For the EPR study, we show data for samples illuminated at 200 K rather than 120 K as used for the absorption studies. There are several reasons for this: (1) 200 K is more relevant since this is the more commonly used temperature in the literature and is easy to achieve for the EPR experiment; (2) the lower temperature had to be used in the optical study in order to avoid devitrification of the sample; (3) the higher temperature allows a more complete conversion of $Car^{+\bullet}$ to $Chl^{+\bullet}$, and this is important for the comparative EPR study.

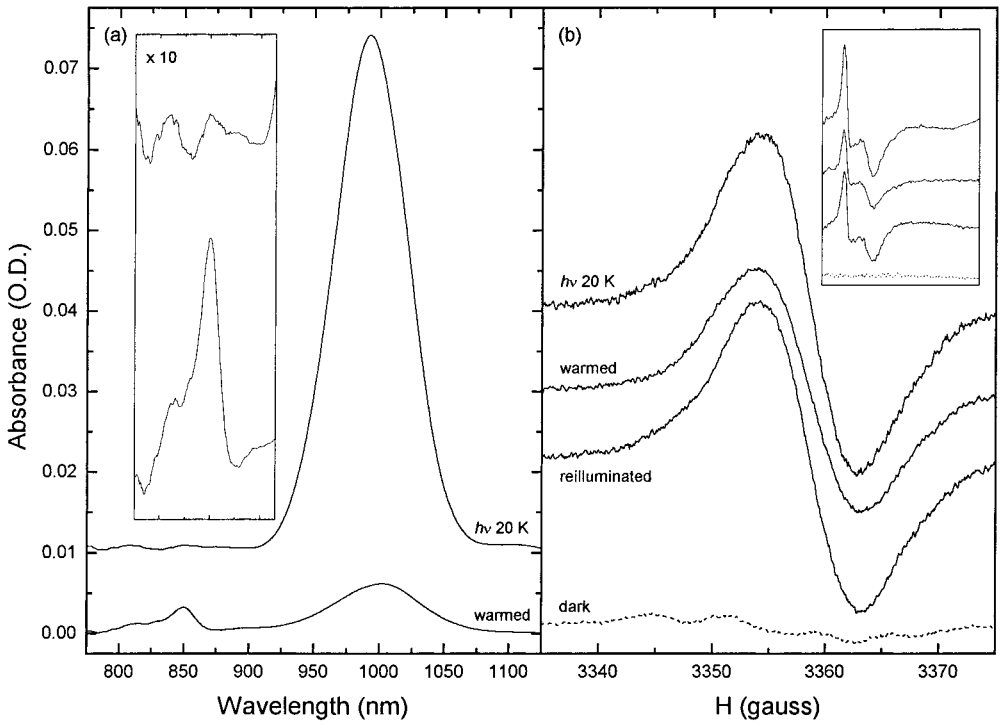


FIGURE 2: (a) Absorption difference spectra generated by illumination of Mn-depleted PSII at 20 K (upper spectrum) and after warming the same sample to 120 K for 30 min in the dark and then returning the temperature to 20 K (lower spectrum). Inset: the Chl cation radical region of the spectrum amplified 10 times. (b) EPR spectra recorded under conditions comparable to those in (a). A spectrum obtained after a second illumination and a spectrum recorded in the dark (dashed line) are also shown. The inset in panel b shows the $Q_A^-Fe^{2+}$ EPR signal in the samples shown in the main part of the figure. EPR instrument settings were as in Figure 1b for the free radicals while for the inset they were the following: temperature, 4.8 K; microwave power, 8 dB; modulation amplitude, 20 G.

Figure 1b shows that rather similar organic free radical spectra are generated at both illumination temperatures. The spectra, however, are distinguishable based on their *g*-values and line widths: $Car^{+\bullet}$, $g = 2.0024$, line width 9.5 G; $Chl^{+\bullet}$ radical, $g = 2.0026$, line width 10.4 G. When compared to the extent of the stable tyrosine radical generated by room-temperature illumination of a similar sample, the number of spins present in each case corresponds to 0.8 (±0.1) per reaction center for $Car^{+\bullet}$ and 0.8 for $Chl^{+\bullet}$. The results thus indicate that $Car^{+\bullet}$ formation takes place essentially in all centers at 20 K, while at 120 K and higher $Chl^{+\bullet}$ formation takes place instead.

Figure 2a shows an experiment in which a sample was illuminated at 20 K, warmed in darkness to 120 K, incubated for 30 min at this temperature, and recooled in the dark to 20 K. The upper spectra show Car oxidation at 20 K as manifest by the absorption at 990 nm as above. The lower spectra taken after the warming and cooling regime show that $Car^{+\bullet}$ is lost and is replaced by $Chl^{+\bullet}$.

The EPR spectra recorded under comparable conditions are shown in Figure 2b. Again the $Car^{+\bullet}$ absorption at 990 nm corresponds to a radical with $g = 2.0024$ and width 9.5 G, while the signal detected after the warming regime has $g = 2.0026$ and width 10.4 G, corresponding to $Chl^{+\bullet}$.

In Figure 2a the extent of the $Chl^{+\bullet}$ absorption at 850 nm is slightly smaller than seen when the signal was generated by illumination at 120 K (Figure 1a); it is about 20% smaller. This is also reflected in the size of the EPR radical signal attributed to the Chl^+ which represents 0.65 spin, i.e., a decrease of about 20%.

Also shown in Figure 2 is the $Q_A^-Fe^{2+}$ signal which is formed upon low-temperature illumination. The signal is seen to decrease upon undergoing the warming regime in the dark. Reillumination restored some of the $Q_A^-Fe^{2+}$ signal and resulted in the reappearance of the free radical signal (i.e., an increase of approximately 0.1 spin) in the majority of centers from which it had been lost upon warming. The extents of the changes in the radical signals should correspond with those of the $Q_A^-Fe^{2+}$ signal. In fact, after warming, the $Q_A^-Fe^{2+}$ signals are somewhat smaller than expected. This seems to be a problem in the temperature reading at the very low temperatures required for optimum $Q_A^-Fe^{2+}$ signals.

The samples from which $Q_A^-Fe^{2+}$ EPR spectra are shown contained sodium formate (100 mM). This chemical renders the $Q_A^-Fe^{2+}$ signal narrower, and thus the first-derivative signal is much bigger (*24*). Spectra were also obtained in the absence of formate, and the much smaller $Q_A^-Fe^{2+}$ signals showed comparable behavior (not shown), and the free radical spectra were also comparable to those shown.

These results are interpreted as follows. At 20 K, illumination results in $Car^{+\bullet}Q_A^-$ formation. Warming of the sample allows electron transfer from Chl to the higher potential $Car^{+\bullet}$. This electron donation does not occur in a fraction (approximately 20%) of centers where charge recombination of the $Car^{+\bullet}Q_A^-$ radical pair occurs as the temperature is raised. In this small fraction of centers, a second period of illumination regenerates the $Car^{+\bullet}Q_A^-$ radical pair.

It is of note that we found that the yield of the conversion from $Car^{+\bullet}$ to $Chl^{+\bullet}$ and the loss to charge recombination could vary from sample to sample in these experiments. This may be related to the rate of warming of the samples which was not well controlled in either of the two types of measurements: in the EPR experiments, samples were

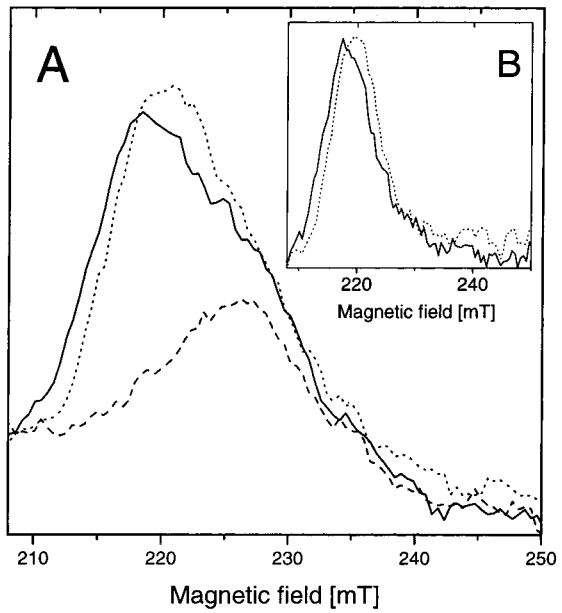


FIGURE 3: EPR spectra of the g_z of oxidized Cyt b_{559} heme: effect of illumination and subsequent warming. Mn-depleted PSII were reduced with ascorbate and dark-adapted (dashed line), illuminated (10 min) at 20 K (solid lines), and then warmed to 150 K for 5 min in an ethanol/liquid N_2 bath and then cooled to 20 K (dotted spectra). EPR instrument settings: power, 10 dB; modulation amplitude, 25 G; temperature, 15 K. Inset B shows the same data but after the subtraction of the spectrum from the dark-adapted sample.

warmed quickly by taking the sample out of the cryostat and incubating at the appropriate temperature followed by rapid cooling, while the optical setup necessitated that the sample was warmed and cooled in situ and, with the cryostat used, warming from 20 to 120 K took around 30 min, as did cooling over the same range, while equilibration at a given temperature also took up to 30 min.

Figure 3 shows an investigation of Cyt b_{559} oxidation after the low-temperature illumination and warming in the dark. The majority (~65%) of the Cyt b_{559} was reduced prior to illumination by incubation in darkness with sodium ascorbate. The dashed line shows the g_z peak from the remaining fraction (~35%) of oxidized Cyt b_{559} heme prior to illumination. The EPR spectra show that Cyt b_{559} is oxidized upon illumination at 20 K (solid line) and no increase in the extent of Cyt b_{559} oxidation occurs upon warming in darkness to 150 K (dotted line). The difference in the light-induced spectra (this can be seen more clearly in the light-minus-dark spectra shown in the inset to Figure 3) corresponds to the well-known structural "relaxation" of the conformation occurring upon warming the photo-oxidized heme (*7*). Similar results were obtained when illumination was done at 4 K. In the fraction of centers where the cytochrome was oxidized prior to illumination, changes in the free radicals occurred which were comparable to those reported in Figure 2.

DISCUSSION

The results show that carotenoid oxidation takes place in essentially all of the centres upon illumination at low temperature in Mn-depleted PSII. At higher temperatures, the stable electron donor is the well-known monomeric chlorophyll, Chl_Z. Warming of the sample containing the $Car^{+\bullet}$ leads to loss of this radical and the formation of $Chl^{+\bullet}$. The most straightforward interpretation of this effect is that the two donors are related in a sequential electron-transfer pathway with Car being the earlier donor to $P680^+$ and Chl_Z being the electron donor to $Car^{+\bullet}$ (i.e., Chl → Car → P680). This observation has important repercussions on structural and functional aspects of PSII.

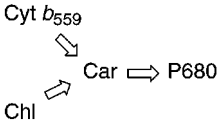
The current model for electron transfer in PSII involves Chl_Z acting as an electron carrier between Cyt b_{559} and P680 (*10*) (i.e., Cyt b_{559} → Chl → P680). One way of integrating the present data with that model is to propose that the same linear electron-transfer pathway exists but to insert Car as the electron donor closest to P680:

$$\text{Cyt } b_{559} \rightarrow \text{Chl} \rightarrow \text{Car} \rightarrow \text{P680}$$

In this model one might expect that the stable trapping of $Car^{+\bullet}$ at low temperature would also occur when Cyt b_{559} is reduced prior to illumination, and that oxidation of the heme itself would occur upon warming in the dark. This was not found to be the case (Figure 3). In fact, it was found that Cyt b_{559} was oxidized directly at 20 K with no intermediate trapping of $Car^{+\bullet}$.

This result can be rationalized in the linear electron-transfer chain model by assuming, for example, that the pre-oxidized Cyt b_{559} has an electrostatic effect on the adjacent Chl_Z, pushing up its redox potential, thus making it a less efficient electron donor. This would explain why $Car^{+\bullet}$ remains trapped at low temperature when Cyt b_{559} is oxidized but not when the Cyt b_{559} is reduced prior to illumination.

This is a fairly satisfactory explanation of the data, but the putative electrostatic effect is invoked purely to accommodate a linear pathway. A more straightforward explanation of the data is to assume a branched pathway in which both Cyt b_{559} and Chl donate to $Car^{+\bullet}$:



where the donation from Cyt b_{559} is much more rapid than that from Chl_Z. In this model, the trapping of $Car^{+\bullet}$ at low temperature is due to the fact that donation from Chl is slow and requires a thermal activation, while this does not occur when Cyt b_{559} is reduced prior to illumination simply because Cyt b_{559} is a more rapid donor under these conditions.

Given the two models (linear vs branched) described above, it is worth looking at the experiment which led to the suggestion that the linear Cyt b_{559}/Chl_Z/P680 pathway exists. The experiment in question was one in which electron donation to $P680^+$ from Cyt b_{559} or Chl_Z was compared under conditions where electron donation from Tyr_Z/Mn was progressively inhibited by lowering the temperature (*10*). The results showed that Cyt b_{559} and Chl_Z competed equally well with the Tyr_Z pathway, and thus it was concluded that the same electron donor to $P680^+$ was involved for both Cyt b_{559} and Chl_Z oxidation. Given the information available at that time (see, however, ref *8*), a linear pathway involving Cyt b_{559}/Chl_Z/P680 was proposed (*10*). In light of the present work, however, the data in Thompson and Brudvig (*10*) can be interpreted equally well in terms of the branched model with Car being the donor to P680 and the branch point for

both Cyt b_{559} and Chl_Z oxidation. Given that the more straightforward interpretation of the low-temperature illumination/dark warming experiments (Figure 2 and Figure 3) is in terms of a branched pathway, we favor the branched pathway over the linear pathway although the latter remains a reasonable alternative.

It is of relevance that Visser et al. (*8*) proposed a branched pathway for low-temperature electron transfer in which Cyt b_{559} and Chl were on different branches and both were able to donate to an unknown component which acted as the direct donor to P680. Our favored model seems to fit with this suggestion where we now identify the immediate donor to $P680^+$ as being Car. We need to extend our work to oxygen evolving PSII before we can make a detailed comparison with this earlier work, but the suggestion in Visser's thesis (*8b*) that the unknown component (Car?) is in redox equilibrium with P680 and may be reducible by the physiological donor is intriguing.

Cyt b_{559} can be reduced by electrons from the electron acceptor side of PSII (*4*, *25*, reviewed in *7*). A protective cycle has been invoked which could have importance in physiological conditions (reviewed in *7*). The present data can be incorporated into such a model with the Car being the direct electron donor to $P680^+$.

The present data have little influence on the hypothesised protective fluorescence quenching role of $Chl_Z^{+\bullet}$, as proposed in (*26*), except that we favor placing the Chl on a side-path and being oxidized by $Car^{+\bullet}$ rather than $P680^+$ itself. A question which is of interest is whether $Car^{+\bullet}$ itself could also play a quenching role. Its absorption maximum is further removed from the spectrum of $Chl_Z^{+\bullet}$, but it is very intense and quite broad so it may be worthwhile testing for fluorescence quenching under the conditions defined here.

It seems likely that the E_m of the Car/$Car^{+\bullet}$ couple lies between those of Chl/$Chl^{+\bullet}$ and P680/$P680^+$ (see *1*). In vitro, the E_m for the one-electron oxidation of β-carotene is 785 mV (e.g., *27*). Proteins commonly impose environments on the cofactors that markedly shift their redox potentials relative to their in vitro values; nevertheless, the value of around 800 mV is consistent with a role as an electron donor in the pathway proposed.

The data reported here showing $Car^{+\bullet}$ formation upon illumination at low temperature are consistent with earlier work (*11*, *12*). Schenck et al. (*11*) showed Car oxidation at 77 K and below in thylakoids, which were presumably functional in O_2 evolution, and in PSII particles which lacked activity. The relationship between the yield of $Car^{+\bullet}$ formation and the temperature was not addressed, nor was the overall stoichiometry elucidated. Noguchi et al. (*12*) also used Mn-containing PSII membranes and observed $Car^{+\bullet}$ formation at 80 K. Under these circumstances, we would predict that $Chl_Z^{+\bullet}$ was also formed, and indeed it seems likely from the FTIR spectra that contributions from $Chl^{+\bullet}$ could have been significant.

Much of the other work in the literature, however, has assumed that Chl_Z is oxidized after illumination at temperatures of 77 K and below. The present data indicate that under these conditions $Car^{+\bullet}$ is formed in some of the centers. Some reassessment of spectroscopic studies done under these conditions may thus be called for. It may, for example, be worth considering that $Car^{+\bullet}$ may contribute to the ENDOR spectra of the "Chl^+ radical" which was generated by illumination at 77 K in ref *17*. In cases where illumination was done at 77 K and the sample subsequently transferred to a cryostat for spectroscopic studies, the increase in the temperature could have allowed at least partial electron transfer from Chl to $Car^{+\bullet}$ to occur, resulting in the fortuitous generation of a nearly homogeneous $Chl^{+\bullet}$ spectrum (see, e.g., *28*). Indeed this occurred in our early studies of $Chl^{+\bullet}$ using pulsed EPR (unpublished).

At 20 K our data indicate that only $Car^{+\bullet}$ is formed. Spectroscopic changes reported under these conditions and assigned to Chl_Z^+ should be reassigned. A recent example concerns the shifted excitation Raman difference spectroscopy detected changes attributed to $Chl^{+\bullet}$ purported to be generated by 20 K illumination (*16*, *29*). To explain the vibrational changes apparently arising from chlorophylls, it may be suggested that $Car^{+\bullet}$ formation could perturb the environment of nearby chlorophyll molecules, or perhaps specifically the Chl_Z. In any case the conclusions drawn using site-directed mutants may require reassessment (*16*).[2]

Our data show the EPR spectra of $Car^{+\bullet}$ and $Chl^{+\bullet}$ to be unremarkable free radical signals; however, they can be distinguished from each other based on *g*-value and line width ($Car^{+\bullet}$, $g = 2.0024$, line width 9.5 G, $Chl^{+\bullet}$ radical, $g = 2.0026$, line width 10.4 G). In vitro, $Car^{+\bullet}$ is reported to have a *g*-value of $g = 2.0024$ in $C_2H_4Cl_2$, i.e., the same value as found here; however, its width in vitro was 13.4 G (*18*), a value which is significantly broader than found here in PSII. This width difference presumably reflects the environment imposed on $Car^{+\bullet}$ by the protein. Further information is required on the hyperfine couplings present in vivo before the potential significance of this narrowing can be commented on. Recently we have obtained ESEEM spectra of the two radicals, and these show very marked differences. These data will be dealt with in detail in a separate publication.

The differences in the 9 GHz EPR spectra are consistent with reports in the literature which have noted width differences in the free radical signals when generated at different cryogenic temperatures (e.g., *30*). It is now possible to assign these differences based on the current work. In a study of the effect of light-induced inhibition of PSII at physiological temperatures, two different free radical signals were distinguished (*31*). These were tentatively attributed to $Car^{+\bullet}$ ($g = 2.0042$, width 12 G) and $Chl^{+\bullet}$ ($g = 2.003$, width 10 G). Comparison of these values to those reported in the present work for $Car^{+\bullet}$ and $Chl_Z^{+\bullet}$ indicates that assignment of the signal to $Car^{+\bullet}$ in the earlier study is unlikely to be correct. The alternatives such as modified amino acid radicals etc. should be reconsidered.

The current work placing the carotenoid as an electron carrier between P680 and Chl_Z obviously provides constraints on structural models of the reaction center. The model favored here, which involves Cyt b_{559} as a more efficient donor to $Car^{+\bullet}$ than $Chl^{+\bullet}$ in a branched pathway, provides further constraints. Given the structure of β-carotene, a molecule which can be 25 Å long, its behavior as an electron

[2] A reviewer pointed out to us that these authors have reported low-temperature absorption spectra and resonance Raman spectra from carotenoid cation in PSII at a recent meeting. An abstract of that work has appeared since the submission of the present article (*40*). It seems then that these authors may have already reassessed their data in references *16* and *29* in line with the suggestions made here.

carrier will be mostly determined by its conformation in the protein. It can be readily imagined that this wire-like electron-transfer component could be very efficient in displacing electrons from one side of the membrane to the other. Thus, the carotenoid in an extended form could be adjacent to both the heme of Cyt b_{559} and P680 even though these components may be well separated, being on different sides of the membrane and located in different proteins.

The role of carotenoid as a donor in a potentially important electron-transfer pathway and having a close structural association with P680 may also explain the intriguing observations in the literature that carotenoid is obligatory for the synthesis of the PSII reaction center, while in other reaction centers, assembly can occur in the absence of this pigment (*32*, *33*). The present findings certainly provide a ready explanation for the early work on this subject which showed that carotenoid extraction prevents low-temperature light-induced oxidation of Cyt b_{559} and that reconstitution of the carotenoid restores this reaction (*34*−*36*).

It was reported that carotenoid is associated with the change between the low-potential and the high-potential forms of Cyt b_{559} (*34*−*36*). These old observations are intriguing in light of the following recent findings: (1) the heme in Cyt b_{559} is likely to be located close to the stromal side of the membrane (i.e., it is on the electron acceptor side of PSII) (see *7*); (2) carotenoid may be adjacent to both electron donor and electron acceptor side components (present work); (3) a specific change in the potential of Q_A occurs upon assembly of the oxygen evolving complex, i.e., Mn cluster assembly and Ca^{2+} binding (photoactivation) (*37*); (4) changes in the potential form (see *7*) and electron acceptor role of Cyt b_{559} occur during photoactivation (*38*); (5) calcium ion binding is important in determining the potential form of the Cyt b_{559} (*39*). Given the nature of the carotenoid molecule, it is interesting to consider the possibility that the carotenoid could play a role in the communication of structural information from one side of the PSII complex to the other.

NOTE ADDED IN PROOF

It has come to our attention that an electron transfer cycle involving carotenoid in PSII has been discussed elsewhere in the literature (*41*).

ACKNOWLEDGMENT

We thank R. Edge (University of Keele, U.K.), A. Boussac, P. Mathis, T. Mattioli, B. Robert, and S. Un (Saclay) for useful discussion.

REFERENCES

1. Diner, B. A., and Babcock, G. T. (1996) in *Oxygenic photosynthesis: the light reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 213−247, Kluwer, Dordrecht.
2. Britt, R. D. (1996) in *Oxygenic photosynthesis: the light reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 137−164, Kluwer, Dordrecht.
3. Andersson, B., and Barber, J. (1996) in *Photosynthesis and the Environment* (Baker, N. R., Ed.) pp 101−121, Kluwer, Dordrecht.
4. Buser, C. A., Diner, B. A., and Brudvig G. W. (1992) *Biochemistry 31*, 11449−11459.
5. Blubaugh, D. J., and Cheniae, G. M. (1990) *Biochemistry 29*, 5109−5118.
6. Knaff, D. B., and Arnon, D. I. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 963−969.
7. Stewart, D. H., and Brudvig, G. W. (1998) *Biochim. Biophys. Acta 1367*, 63−87.
8. (a) Visser, J. W. M., Rijgersberg, C. P., and Gast, P. (1977) *Biochim. Biophys. Acta 460*, 36. (b) Visser, J. W. M. (1975) Ph.D. Thesis, State University of Leiden.
9. De Paula, J. C., Innes, J. B., and Brudvig, G. W. (1985) *Biochemistry 24*, 8114.
10. Thompson, L. K., and Brudvig, G. W. (1988) *Biochemistry 27*, 6653−6658.
11. Schenck, C. C., Diner, B. A., Mathis, P., and Satoh, K. (1982) *Biochim. Biophys. Acta 680*, 216−227.
12. Noguchi, T., Mitsuka, T., and Inoue, Y. (1994) *FEBS Lett. 356*, 179−182.
13. Velthuys, B. R. (1981) *FEBS Lett. 126*, 272−276.
14. Mathis, P., and Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 217−222.
15. Telfer, A., De La Rivas, J., and Barber, J. (1991) *Biochim. Biophys. Acta 1060*, 106−114.
16. Stewart, D. H., Cua, A., Chisolm, A. D., Diner, B. A., Bocian, D. F., and Brudvig, G. W. (1998) *Biochemistry 37*, 10040−10046.
17. Rigby, S. E. J., Nugent, J. H. A., and O'Malley, P. J. (1994) *Biochemistry 33*, 10043−10050.
18. Grant, J. L., Kramer, V. J., Ding, R., and Kispert, L. D. (1988) *J. Am. Chem. Soc. 110*, 2151−2157.
19. Khaled, M., Hadjipetrou, A., and Kispert, L. D. (1990) *J. Phys. Chem. 94*, 5164−5169.
20. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
21. Mathis, P., and Vermeglio, A. (1972) *Photochem. Photobiol. 15*, 157−164.
22. Dawe, E. A., and Land, E. J. J. (1975) *J. Chem. Soc., Faraday Trans. 1 71*, 2162−2169.
23. Mathis, P., and Setif, P. (1981) *Isr. J. Chem. 21*, 316−320.
24. Vermaas, W. F. J., and Rutherford, A. W. (1984) *FEBS Lett. 175*, 243−248.
25. Whitmarsh, J., and Cramer, W. A. (1978) *Biochim. Biophys. Acta 501*, 83.
26. Schweiger, R. H., and Brudvig, G. W. (1997) *Biochemistry 36*, 11351−11359.
27. Jeevarajan, A. S., Khaled, M., and Kispert, L. D. (1994) *Chem. Phys. Lett. 225*, 340−345.
28. Kougliotis, D., Innes, J. B., and Brudvig, G. W. (1994) *Biochemistry 33*, 11814−11822.
29. Cua, A., Stewart, D. H., Brudvig, G. W., and Bocian, D. F. (1998) *J. Am. Chem. Soc. 120*, 4532−4533.
30. Nugent, J. H. A., Stewart, A. C., and Evans, M. C. W. (1981) *Biochim. Biophys. Acta 635*, 488−497.
31. Blubaugh, D. J., Atamian, M., Babcock, G. T., Golbeck, J. H., and Cheniae, G. M. (1991) *Biochemistry 30*, 7586−7597.
32. Margraf, T., and Oelmueller, R. (1991) *Planta 185*, 97−104.
33. Trebst, A., and Depka, B. (1997) *FEBS Lett. 400*, 359−362.
34. Sofrova, D., and Bendall, D. S. (1972) in *Proceedings of the 2nd International Congress of Photosynthesis Research* (Forti, G., Avron, M., and Melandri, A., Eds.) Vol. 1, pp 561−567, Dr. W. Junk, N.V. Publishers, The Hague.
35. Okayama, S., and Butler, W. L. (1972) *Plant Physiol. 49*, 767−774.
36. Cox, R. P., and Bendall, D. S. (1974) *Biochim. Biophys. Acta 347*, 49−59.
37. Johnson, G. N., Rutherford, A. W., and Krieger, A. (1995) *Biochim. Biophys. Acta 1229*, 202−207.
38. Mizusawa, N., Miyao, M., and Yamashita, T. (1997) *Biochim. Biophys. Acta 1318*, 145−158.
39. McNamara, V. P., and Gounaris, K. (1995) *Biochim. Biophys. Acta 1231*, 289−296.
40. Vrettos, J. S., Stewart, D. H., Cua, A., De Paula, J. C., Bocian, D. F., and Brudvig. G. W. (1999) *Biophys. J. 76*, A248.
41. Gruszecki, W. I., Strzalka, K., Radunz, A., Kruk, J., and Schmid, G. H. (1995) *Z. Naturforsch. 50C*, 61−68.

BI990633U