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The accessory electron donor tyrosine-D of Photosystem II is slowly reduced in the dark during low-temperature storage of isolated thylakoids

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Storage of thylakoids for several months at 203 K in the dark changes the flash pattern of oxygen evolution by gradually shifting the first oxygen maximum from the third flash, where it is usually observed, to the fourth flash. This effect is accompanied with the increase of a fast phase ($t_{1/2}$ = 1.5 s) in the decay of the S_2 and S_3 states of the water-oxidizing complex in Photosystem II. In parallel to the changes in the oxygen flash pattern the EPR signal from the stable tyrosine-D$^+$ radical (Signal II_{slow}) completely disappears with a half-time of approx. 13 weeks. The normal oxygen yield sequence, showing the first maximum at the third flash, as well as the original amplitude of Signal II_{slow} can be restored by a single flash or by continuous illumination at room temperature. These data show that tyrosine-D$^+$ is reduced by an endogenous redox component in Photosystem II during dark storage of thylakoids at 203 K. In parallel with the reduction of tyrosine-D$^+$ we observed the oxidation of high potential cytochrome *b*-559, and it is proposed that at low temperature an electron can be transferred from high-potential cytochrome *b*-559 to tyrosine-D$^+$ in a slow reaction in most of the centers.

Introduction

Photosynthetic oxidation of water to molecular oxygen is catalysed by the Mn-containing water-oxidizing complex of Photosystem II (for reviews, see Refs. 1 and 2). Oxygen evolution, when induced by a train of short saturating flashes, reveals a characteristic period-four oscillation with the first maximum at the third flash [3]. This phenomenon was explained by the cycling of the water-oxidizing complex through five redox states denoted S_0–S_4 where molecular oxygen is released during the S_3–S_4–S_0 transition [4]. In dark-adapted plant material, the distribution of the S states is approx. 25, 75, 0, 0 and 0%, respectively [4].

Recent experimental findings suggest that the catalytic Mn cluster of water oxidation is bound to the D_1D_2 protein complex of the PS II reaction center [5–7], which carries the reaction center chlorophyll P-680 [8]. Electron transfer between P_{680} and the Mn cluster is mediated by a redox component, recently identified as the tyrosine-161 residue of the D_1 protein (Tyr-Z) [9]. Another redox component on the oxidizing side of PS II, the tyrosine-160 residue of the D_2 protein (Tyr-D) [10,11], is also involved in charge exchange with the Mn cluster of water oxidation. Photooxidation of Tyr-Z and Tyr-D induces characteristic EPR signals, Signal $II_{very\ fast}$ from Tyr-Z$^+$ [12] and Signal II_{slow} from Tyr-D$^+$ [13], which have very similar line shapes but different decay kinetics.

Although Tyr-D does not participate in the steady-state electron flow through PS II, several lines of evidence show its involvement in charge transfer events. Tyr-D is oxidized by the S_2 and S_3 states with a half-time of about 1–2 s [13–15]. On the other hand, Tyr-D$^+$ is slowly ($t_{1/2} \approx 10$ min) reduced by the S_0 state in the dark [16]. The participation of Tyr-D in electron transfer at cryogenic temperatures has also been established. At 77 K, both the photooxidation of Tyr-D [17] and the reduction of Tyr-D$^+$ via charge recombination with Q_A^- ($t_{1/2} \approx 5$ days) [17–19] take place.

Abbreviations: Chl, chlorophyll; cyt *b*-559, cytochrome *b*-559; DCPIP, 2,6-dichlorophenolindophenol; PS, Photosystem; P_{680}, primary electron donor of PS II; PS II, Photosystem II; Q_A, primary quinone acceptor of PS II; Q_B, secondary quinone acceptor of PS II;

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In a previous paper, we reported a characteristic change in the flash pattern of oxygen evolution during the storage of thylakoids at 203 K. The first oxygen maximum was shifted from the third flash, where it is usually observed, to the fourth one, indicating the formation of a reduced, one-electron donor to the Mn cluster [20]. Here we report further oxygen evolution and EPR data showing that this phenomenon reflects the slow dark reduction of Tyr-D^+. We have also identified the redox component involved in the reduction of Tyr-D^+.

Materials and Methods

Thylakoids were isolated from market spinach as described earlier [21], resuspended in 0.4 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, 1 mM $MnCl_2$ and 40 mM Hepes (pH 7.5) at 4–5 mg Chl/ml and stored at 203 K until use.

Flash-induced oxygen yield was measured with a home-built unmodulated Joliot-type O_2 electrode [22]. Thylakoids stored at 203 K were thawed in the dark, diluted to 0.75 mg/ml with the above medium and transferred to the Pt electrode in very dim green light. After a 3–5 min dark period, which is required for the equilibration of the O_2 electrode, the thylakoids were illuminated by a train of saturating Xe flashes (General Radio Stroboslave, 3 μs, 0.5 J).

For the measurements of the decay of the S_2 and S_3 states, thylakoids were preilluminated with one or two flashes (4 Hz), respectively, followed by various intervals of dark relaxation and then by a train of ten measuring flashes (4 Hz) [4]. The relative concentration of S_2 and S_3 was calculated as described previously [20]. The calculated S_2 and S_3 decay curves were resolved into exponential components by a least-squares fit program.

X-band low-temperature EPR spectra were recorded at 15 K with a Bruker ESP 300 spectrometer equipped with an Oxford Instruments cryostat and temperature controller. Low-temperature illumination of EPR samples (4–5 mg Ch/ml) was performed in an unsilvered dewar using a 1000 W projector lamp. In order to produce a single stable charge separation in the PS II centers, samples were illuminated at 198 K for 4 min using a solid CO_2/ethanol slurry. For the photooxidation of cyt *b*-559,the EPR samples were illuminated at 77 K for 20 min. The evaluation of the measured EPR spectra, such as baseline subtraction and integration of the cytochrome signals, were performed by the ESP 300 program of the EPR spectrometer. All EPR signals were measured under non-saturating conditions.

Chemically induced absorbance changes associated with cyt *b*-559 were measured between 559 and 570 nm in the dual-wavelength mode in an Aminco DW-2 or Shimadzu UV-3000 spectrophotometer. High- and low-potential cyt *b*-559 was oxidized by 1 mM ferricyanide and reduced by 5 mM hydroquinone and 4 mM sodium ascorbate, respectively. Redox compounds were added in the dark to stirred samples without interrupting the measurements.

Results and Discussion

Prolonged dark storage of thylakoids at 203 K induces a characteristic change in the oxygen flash pattern. The first oxygen maximum, which appears at the third flash in fresh samples (Fig. 1a, dashed line) is gradually shifted to the fourth flash when measured with 0.5 Hz flash frequency (Fig. 1a, b, solid line). However, when closely spaced flashes (4 Hz) were used, only a small increase in the ratio of the oxygen yields at the fourth and third flashes was observed during the 203 K storage (not shown). Preillumination of long-stored thylakoids, thawed in the dark, with continuous light or with a single flash completely restored the usual flash pattern observed in briefly dark-adapted samples (Fig. 1b, dashed line).

Chemical reduction of thylakoids by ascorbate in the presence of DCPIP resulted in a similar frequency-dependent change of the oxygen sequence [14,20,23] which was assigned to a fast electron donation from Tyr-D, reduced by the treatment, to the S_2 and S_3 states [14,20]. This process has been shown to result also in a fast

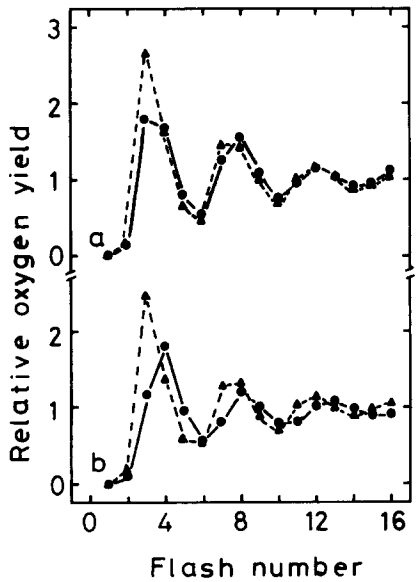

Fig. 1. Oxygen production as a function of flash number by isolated spinach thylakoids stored at 203 K for various periods of time. (a) Freshly prepared thylakoids (dashed line) and samples after 12 weeks of storage at 203 K (solid line). (b) Thylakoids after 20 weeks of storage at 203 K without preillumination (solid lines) and after a single-flash preillumination followed by 5 min dark adaptation (dashed line). Samples stored at 203 K were thawed in the dark before the oxygen measurements were performed with 0.5 Hz flash frequency. The data are normalized to the steady-state oxygen yield of 1.0.

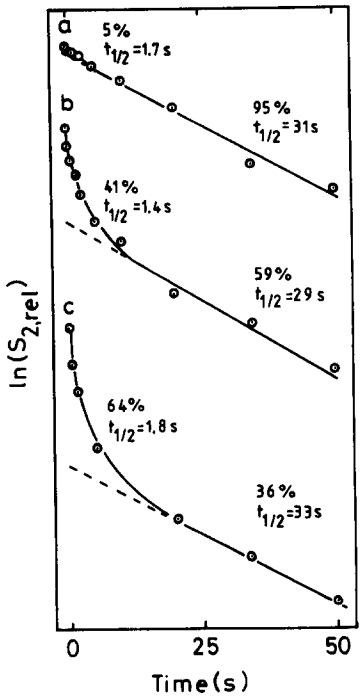

Fig. 2. Decay kinetics of the S_2 state in isolated spinach thylakoids stored at 203 K for various periods of time. (a) Freshly prepared samples; (b) thylakoids stored for 12 weeks; and (c) thylakoids stored for 20 weeks. The relative amount of S_2 was calculated from oxygen-yield sequences measured after one preflash as described in the Materials and Methods.

decaying phase of the S_2 and S_3 states [15,16,20]. As shown in Fig. 2, the amplitude of the fast phase in the decay of the S_2 state was indeed enhanced by the 203 K storage of thylakoids. A similar effect was obtained for the decay of the S_3 state as well (not shown). Preillumination of thylakoids, stored at 203 K, with a single saturating flash decreased the fast decaying phase of S_2 and S_3 to about 5% (not shown), indicating the oxidation of Tyr-D in the majority of centers [20].

The above data suggest that Tyr-D$^+$ is reduced in a large fraction of centers during storage at 203 K. This hypothesis was tested by measuring the EPR Signal II_{slow} from the Tyr-D$^+$ radical. The amplitude of the dark-stable Signal II_{slow} gradually decreased during the 203 K storage of thylakoids (Fig. 3a–c, solid line) confirming the reduction of Tyr-D$^+$. The amplitude of Signal II_{slow} was largely restored (Fig. 3a–c, dashed line) when the same samples were illuminated at 198 K, and then incubated for 3–5 min in the dark at room temperature. At 198 K the electron transfer between Q_A and Q_B is blocked [24]. Thus, during illumination at this temperature only the S_1 to S_2 transition takes place [25,26]. Then the S_2 state oxidizes Tyr-D during the room-temperature incubation.

The kinetics of Tyr-D oxidation after a single laser flash was measured by time-resolved EPR at room temperature by monitoring the formation of Signal II_{slow} at its low-field peak. As the inset in Fig. 3. shows, the increase of Signal II_{slow} is monophasic with an half-time of approx. 1.9 s. This observation agrees well with the earlier data of Babcock and Sauer [13] and Velthuys and Visser [14] suggesting the oxidation of Tyr-D by the S_2 (or S_3) state. The similarity of the respective half-times (Figs. 2 and 3) provides further support for the assignment of the fast decaying phase of S_2 (and S_3) state to the oxidation of Tyr-D by S_2 (and S_3) [15,20].

Fig. 4 shows the decay course of Tyr-D$^+$ during the dark storage of thylakoids at 203 K. The data were calculated from the relative amplitude of the slow decaying phase of the S_2 state (x), and from the amplitude of the dark stable Signal II_{slow} (o) relative to that measured after room temperature preillumination. The dark decay of Tyr-D$^+$ has a half-time of about 13 weeks at 203 K, and affects the whole population of the centers.

The question is then, which is the reducing component that interacts with Tyr-D$^+$? In briefly dark-adapted samples, the water-oxidizing complex is in the S_0 state in about 25% of the centers [4]. Thus, Tyr-D$^+$ can be

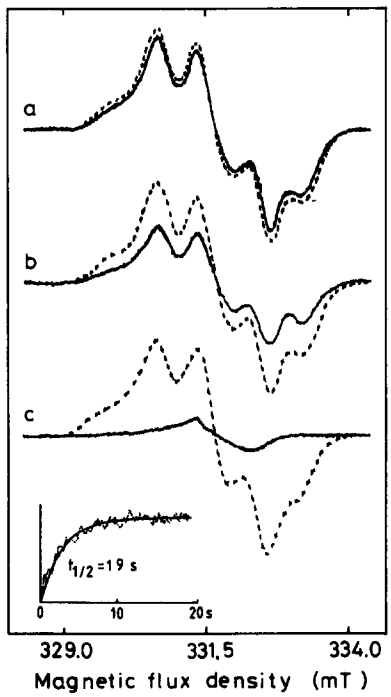

Fig. 3. EPR spectra of Signal II_{slow} in isolated spinach thylakoids stored at 203 K for various periods of time. (a) Freshly prepared samples; (b) thylakoids stored for 12 weeks; and (c) thylakoids stored for 20 weeks. The EPR spectra were recorded in the dark (solid lines) and after 3 min illumination at 198 K followed by 3 min dark adaptation at room temperature (dashed lines). EPR conditions: *T*, 15 K; microwave power, 56 dB below 200 mW; modulation amplitude, 0.32 mT; microwave frequency, 9.239 GHz. The inset shows the room-temperature kinetics of Signal II_{slow} formation after a single laser flash in a sample similar to sample c. EPR conditions: *T*, 293 K; field, 374.4 mT; microwave power, 10 dB below 200 mW; modulation amplitude 0.5 mT; microwave frequency, 9.779 GHz.

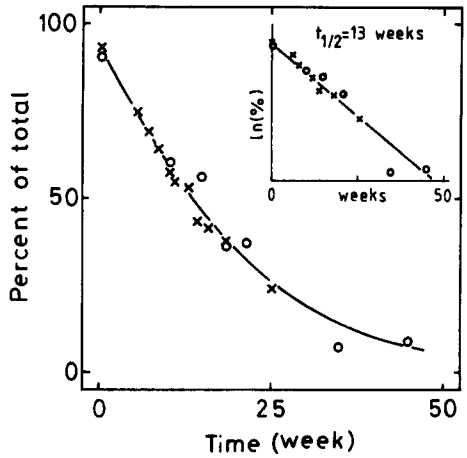

Fig. 4. The decay course of Tyr-D$^+$ as a function of the storage time at 203 K. The amount of Tyr-D$^+$ present in the dark was monitored by measuring the amplitude of Signal II_{slow} in the dark relative to the amplitude after room temperature illumination followed by 5 min dark adaptation (○). The amount of Tyr-D$^+$ was also estimated from the amplitude of the slow decaying phase of the S_2 state relative to that of the fast decaying phase (×).

reduced by the S_0 state ($t_{1/2}$ 10–20 min at room temperature [16,20]) in 10–15% of the centers during the approx. 10-min room-temperature manipulation of the samples before the oxygen and EPR measurements. Inspection of the decay curve in Fig. 4 shows that 10% of Tyr-D$^+$ was indeed reduced before the first measurement was made.

Tyr-D$^+$ can also be reduced via recombination with Q_A^- at 77 K [17–19]. Although in dark-prepared thylakoids the amount of Q_A^- is negligible, the slow reduction of a very large population of Tyr-D$^+$ at 203 K was still observed. In addition, the half-time of the recombination between Tyr-D$^+$ and Q_A^- is much shorter (about 5 days at 77 K [18]) than that of the reduction of Tyr-D$^+$ at 203 K (13 weeks, Fig. 4). Thus, recombination with Q_A^- cannot be the major pathway for the reduction of Tyr-D$^+$ at 203 K.

Another putative reductant of Tyr-D$^+$is reduced cyt *b*-559 that is a known electron donor to P-680$^+$ during low-temperature illumination [26,27]. This possibility was investigated by recording the changes in the redox state of cyt *b*-559 during the long dark storage at 203 K. In freshly prepared thylakoids the EPR signal that peaks at around *g* 2.94 (Fig. 5A curve a, solid line), indicates the presence of oxidized low-potential cyt *b*-559 [28]. Illumination of these samples at 77 K resulted in an increase of the cyt *b*-559 EPR signal at around *g* 3.05 due to the oxidation of one molecule of high potential cyt *b*-559 per reaction center [26] (curve a, dotted line). After 20 weeks of storage of thylakoids at 203 K, the peak of the cytochrome signal is shifted to higher *g* values due to the presence of a mixture of oxidized low- and high-potential forms (Fig. 5A, curve b, solid line). The amount of the oxidized low-potential form (at $g \approx 2.94$) is constant in the two samples. The existence of a large signal at $g \approx 3.05$ indicates the presence of an appreciable amount of oxidized high-potential cyt *b*-559 as well. Illumination of these samples at 77 K resulted in a further oxidation of the high potential cyt *b*-559 (curve b, dotted line), but to a significantly lower extent than in the fresh samples. Together these data suggest that the high-potential form of cyt *b*-559 progressively became oxidized in the dark during the long storage period at 203 K.

The dark oxidation of the high-potential cyt *b*-559 was confirmed by optical measurements. In freshly isolated thylakoids, the high-potential (hydroquinone-reducible [29]) form of cyt *b*-559 is mostly in the reduced state, whereas the low-potential (ascorbate reducible [30]) form is in the oxidized state (Fig. 5B, curve a). Storage of thylakoids for 20 weeks at 203 K resulted in an increase of about 50% in the dark-oxidized high-potential form of cyt *b*-559 (Fig. 5B, curve b).

The EPR and optical results are summarized in Table I. The percentage of the oxidized high-potential cyt

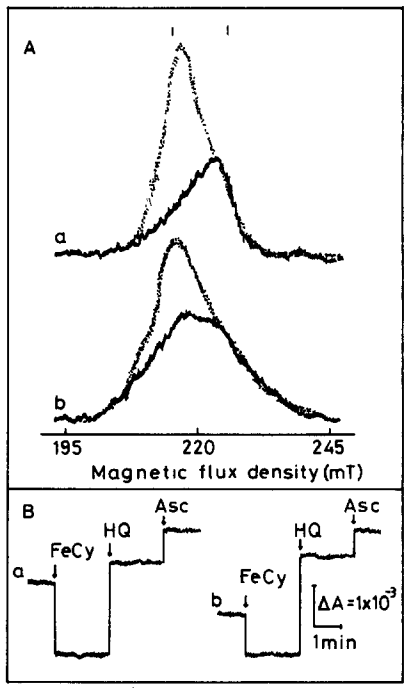

Fig. 5. The effect of 203 K storage of isolated thylakoids on the redox state of cyt *b*-559. A, EPR spectra of the g_z region of oxidized cyt *b*-559 recorded (a) in freshly prepared thylakoids and (b) after 20 weeks of storage at 203 K. Solid-line spectra were recorded in dark samples. Dotted-line spectra were recorded after 20 min illumination at 77 K. The bars indicate the field positions at $g \approx 2.94$ (224.5 mT) and $g \approx 3.05$ (216.5 mT). EPR conditions: T, 15 K; microwave power, 15 dB below 200 mW; modulation amplitude 1.25 mT; microwave frequency 9.239 GHz. B, Redox changes of cyt *b*-559 monitored by optical measurement of absorption difference changes between 559 and 570 nm. (a) in freshly prepared thylakoids and (b) after 20 weeks of storage at 203 K. Successive additions of 1 mM ferricyanide (FeCy), 5 mM hydroquinone (HQ) and 4 mM sodium ascorbate (Asc) were made as indicated.

TABLE I

The redox state of cyt b-559 and Tyr-D$^+$ in freshly prepared thylakoids and after 20 weeks of storage at 203 K

The percentage of oxidized high-potential and total cyt *b*-559 was calculated from the EPR and optical data of Fig. 5 as described in the text. The fraction of oxidized Tyr-D was measured by EPR as in Fig. 3.

		Oxidized cyt *b*-559 (%)		Tyr-D$^+$ (%)
		high potential	total	
Fresh	optical	21	43	91
	EPR		49	
Stored	optical	59	68	35
	EPR		70	

b-559 was calculated from the ratio of the absorption difference changes brought about by ferricyanide and subsequent hydroquinone additions (Fig. 5B). The fraction of the oxidized state of the total amount of cyt *b*-559 (high- and low-potential forms) was estimated in two ways: (i) from the ratio of the absorption difference changes observed after ferricyanide and ascorbate additions (Fig. 5B); (ii) from the EPR spectra of cyt *b*-559, shown in Fig. 5A, assuming that the 77 K illumination of freshly prepared thylakoids oxidized one molecule of high-potential cyt *b*-559 per reaction center [26]. The data show that 20 weeks of storage resulted in the oxidation of about 40% of high-potential cyt *b*-559, corresponding to 25% of the total cyt *b*-559. During the same period, 65% of Tyr-D$^+$ was reduced (Fig. 4, Table I). In thylakoid membranes two cyt *b*-559 molecules are considered to be present for each reaction center [26,31], while there is only one Tyr-D$^+$ [10,11,32]. Thus, the 25% oxidation of the total cyt *b*-559, can be responsible for as much as 50% reduction of Tyr-D$^+$. The remaining 10–15 of Tyr-D$^+$ was lost in the reaction between Tyr-D$^+$ and the S_0 state (see above) during the 5–10-min dark adaptation of the samples before the measurements (Fig. 4).

In conclusion, our results demonstrate the presence of a hitherto undescribed dark electron transfer in Photosystem II, i.e., the low-temperature reduction of Tyr-D$^+$ by cyt *b*-559. Thus, Tyr-D has been shown to participate in redox reactions with most of the redox components of PS II. It interacts with the water-oxidizing complex in the S_0 [16,20], S_2 and S_3 [13–15] states, with Q_A^- at 77 K [17–19] and, as shown here, with the high-potential form of cyt *b*-559 at 203 K. These results indicate that Tyr-D, although inactive in steady-state electron transport, plays an important role in the redox chemistry of PS II. The reaction between the S_0 state and Tyr-D$^+$ probably stabilizes the Mn cluster in the dark, and it could participate in Mn-binding to PS II [16]. The possible function of the slow reaction between Tyr-D$^+$ and cyt *b*-559 described here has to be clarified during further studies.

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