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Deactivation kinetics and temperature dependence of the S-state transitions in the oxygen-evolving system of Photosystem II measured by EPR spectroscopy

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The decay kinetics for the S_2 and S_3 states of the oxygen-evolving complex in Photosystem II have been measured in the presence of an external electron acceptor. The S_2- and S_3-states decay monophasically with half-decay times at 18°C of 3–3.5 min and 3.5–4 min, respectively. The results also show that S_3 decays via S_2 under these circumstances. The temperature dependence of the individual S-state transitions has been measured in single flash experiments in which the multiline EPR signal originating from the S_2 state has been used as spectroscopic probe. The half-inhibition temperatures are for S_0 to S_1 220–225 K, for S_1 to S_2 135–140 K, for S_2 to S_3 230 K and for the S_3-to-S_0 transition 235 K.

Introduction

Photosystem II catalyzes the light-driven oxidation of water to oxygen and the reduction of plastoquinone. PS II contains a chain of redox components of diverse chemical nature (for recent reviews, see Refs. 1 and 2). The absorption of a light quantum gives rise to a charge separation in the reaction center of PS II. The electron is rapidly transferred to the acceptor complex, which is comprised of two quinones, Q_A and Q_B, and a ferrous ion. Q_A accepts one electron during turnover while Q_B can accept two electrons. Q_B is firmly bound to its site in the semireduced state but exchanges freely with the plastoquinone pool in its fully reduced or oxidized states [3,4].

The oxidized primary donor, P-680$^+$, is reduced by a component Z which is, in its turn, reduced by the oxygen-evolving system, the electrons being ultimately derived from water. Under nonphysiological conditions (for example at very low temperatures or with an inhibited or destroyed water-splitting enzyme) other electron donors can take part in the reduction of P-680$^+$. These include cytochrome *b*-559 or, when the cytochrome is oxidized, a chlorophyll molecule [5,6].

The active site in the oxygen-evolving system is an entity comprised of 2–4 Mn atoms presumably organised in a cluster [7,8]. Successive charge separations in the reaction center results in the accumulation of highly oxidizing equivalents at least partially on the Mn-cluster. The intermediate redox states are designated S_0 to S_4 [9]. S_0 is the most reduced state while S_1, S_2 and S_3 represent sequentially higher oxidation states. The oxygen molecule is released in the S_3-S_0 transition in which S_4 is a transient state. In the light S_0 to S_3 are equally populated but S_2 and S_3 decays in tens

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Abbreviations: Chl, chlorophyll; D$^+$, the radical that gives rise to EPR signal II$_{slow}$; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; PPBQ, phenyl*para*benzoquinone; P-680, the primary electron donor chlorophyll(s) of PS II; PS II, Photosystem II; S_0–S_4, charge storage states of the oxygen-evolving enzyme.

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of seconds to S_1 when the light is switched of [10]. These decay reactions, which are known as deactivations, occur partially by backreactions with electrons from the reduced electron acceptors [11,12]. The result of the deactivation of S_2 and S_3 is that short dark adaptations give 75% S_1 and 25% S_0 which is the dark S-state composition normally observed. In more recent work [13] it was also observed that S_0 was oxidized to S_1 in the dark in a slow reaction. The oxidant in this reaction has been identified as D^+ (Signal II_{slow}) [14] which recently has been identified as a tyrosyl radical present on the donor side of PS II [15]. After the development of the S-state concept much effort has been put into measuring the stability of the S-states, the kinetics of the S-transitions and the thermodynamic characteristics of the S-states, etc. Knowledge of these parameters is important for mechanistic considerations and they also provide useful tools to produce samples with specific and well-defined S-state composition. Such samples are necessary in studies of the different S-states by spectroscopic techniques such as EPR, EXAFS, NMR or X-ray absorption-edge structure.

Until recently this type of information was mainly obtained by measurements of the flash-dependent release of oxygen with oxygen rate electrodes and to a lesser extent by thermoluminescence experiments. Another way to study the oxygen-evolving system was opened by the discovery of the so-called S_2-state multiline EPR signal [16,17] which is formed by treatments that allow only one turnover in the oxygen-evolving system such as a single flash or continuous illumination at 200 K. Furthermore, the amplitude of this signal oscillates with flash number and the signal is formed in high yield after five flashes [16,18]. This can sometimes provide an advantage over O_2 yield experiments, since fewer flashes are needed to reach the measured parameter in the second cycle. The multiline signal has been used as a spectroscopic probe to measure for example the deactivation kinetics of S_2 in the presence and absence of Cl^- [19]. In another type of experiments the multiline signal was used to determine the temperature dependence for the S_1-to-S_2 transition and partially for the S_2-to-S_3 transition [20].

Here this kind of EPR experiments has been taken further and the deactivation kinetics of S_2 and S_3 have been determined in PS II enriched membranes when the acceptor side is fully oxidized. In another set of experiments the temperature dependence of each S-transition is determined using single flashes at low temperatures.

Experimental

Materials. PS II-enriched membranes were prepared as in Ref. 21 with the modifications in Ref. 22. The membranes were stored at 77 K at 10 mg Chl/ml. For the experiments they were suspended and washed once before they were diluted to 1.5–2 mg Chl/ml in 20 mM MES-NaOH at pH 6.3, 1 mM $CaCl_2$ and 10 mM NaCl. The buffer contained 30% (v/v) ethylene glycol in the deactivation experiments and 50% ethylene glycol in the experiments designed to measure the temperature dependence of the S-transitions. PPBQ was added as external electron acceptor to a final concentration of 0.5 mM from a 20 mM solution in dimethyl sulfoxide.

EPR spectroscopy. EPR measurements were made on a Bruker ESR 200 D SRC spectrometer at 9.44 GHz using a 90 dB microwave bridge. The instrument was equipped with an Oxford Instruments Cryostat and temperature controller. The temperature was controlled from this unit at a constant flow rate of helium during all measurements to provide comparable temperatures at the sample level with a given meter reading.

The S_2-state multiline signal was recorded at 10 K with a microwave power of 20 mW and a modulation amplitude of 22 G. The amplitude of the multiline signal was estimated from the added amplitudes of the three peaks indicated in Fig. 1. The experimental variation in the measurement of the multiline signal amplitude within the same sample was approx. ±5%. The microwave power saturation of Signal II_{slow} was measured at 20 K with a modulation amplitude of 2.5 G as described in Ref. 23. The error in the measurement was estimated to ±0.5 dB.

Preflash treatment to prepare synchronized samples. PS II enriched membranes at 1.5–2 mg Chl/ml in calibrated EPR tubes were incubated on ice in the dark for 2 h and subsequently at room temperature for 1 min. Thereafter the sample was given one preflash from a Nd-YAG laser (15 ns, 300 mJ at 532 nm) and allowed to equilibrate for 15 min at 20 °C in total darkness. Then 0.5 mM PPBQ was added as external electron acceptor and 1 min after the addition the appropriate flash sequence (see below) was given. After the termination of the flash sequence the samples were rapidly immersed (within 2 s) in an ethanol-solid CO_2 bath (198 K) and then transferred to liquid nitrogen.

The preflash treatment was used to synchronize the centers in the D^+S_1 state. Centers present either as D^+S_0 or DS_1, 25% of the centers in total [14], are converted into D^+S_1 or DS_2, respectively by the preflash. The former centers are stable while the DS_2 centers rapidly deactivate to form D^+S_1 centers during the dark incubation after the flash. Centers present as D^+S_1 from the start (75%) are converted to D^+S_2 with the flash. In the absence of an exogenous electron acceptor S_2 decays to S_1 during the dark incubation mainly due to recombination with the electron on Q_B^- [11,12]. Thus this preflash treatment converts virtually all centers to the state D^+S_1.

Low-temperature flashes and continuous illumination. In the experiments designed to measure the temperature dependence of the individual S-state transitions the turnover was achieved by providing the sample with a laser flash at varying temperature. To perform this, the sample was immersed in a cooling bath at the desired temperature and allowed to equilibrate for 30 s. The cooling bath contained liquid nitrogen (at 77 K), isopentane (between 130 and 170 K) or ethanol (165–290 K). After temperature equilibration the sample was moved into the flash cell which was located immediately at the surface of the cold liquid. The flash was fired within 2 s after the sample had left the cooling bath. The maximal increase in temperature was measured with a thermocouple inserted in an EPR-tube containing a normal sample and was found not to exceed 3 K before the flash was fired. To provide a saturating flash at low temperatures it was necessary to keep the sample transparent. For this the lowest usable concentration of ethylene glycol was 50% (v/v). In addition it was necessary to approach the desired temperature from 273 K to keep the sample transparent. Samples in 50% ethylene glycol were slightly viscous at 220 K and remained liquid at temperatures down to 180–190 K.

Continuous illumination at 198 K was provided from an 800 W projector lamp for 2 min to a sample immersed in an ethanol-solid CO_2 bath in an unsilvered dewar.

Results

Flash saturation at different temperatures

The efficiency of the laser flash when given at low temperature was tested in the experiment presented in Fig. 1. Three similar EPR samples in 50% ethylene glycol were given the preflash treatment as described above to synchronize the centers in the S_1 state. After the addition of PPBQ one sample was given a flash at 293 K (Fig. 1A), the second sample was given a flash at 198 K (Fig. 1B) and the third sample was illuminated with continuous light at 198 K (Fig. 1C). The amplitude of the S_2-state multiline signal was virtually

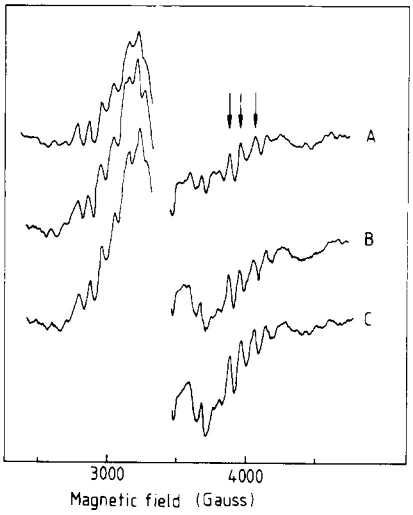

Fig. 1. The amplitude of the S_2-state multiline signal formed under various conditions. The amplitude was measured as the added sum of the three peaks marked with arrows in A. The S_2-state was formed by one flash at 293 K (A); one flash at 198 K (B); and continuous illumination at 198 K (C). PS II-enriched membranes were suspended in buffer containing 50% (v/v) ethylene glycol to a final concentration of 2 mg Chl/ml. The centers were synchronized and 0.5 mM PPBQ was added 1 min before the illumination. The large radical spectrum of S II_{slow} around 3370 G is omitted in the spectra.

identical in the two flashed samples while it was approx. 10% larger in the sample illuminated at 198 K. This shows that the flash was saturating to the same extent at both temperatures. The slightly larger signal after continuous illumination is reasonable taking in account the approx. 5–10% misses that are introduced in these experiments [14,23]. From these experiments it was concluded that the flash was saturating at least down to 198 K and there is little reason to believe that this was not the case also at lower temperatures, since the samples remained transparent.

Stability of S_2 and S_3 in the presence of PPBQ

Experiments with PS II-enriched membranes often demand the addition of an exogenous quinone acceptor to diminish limiting reactions on the acceptor side. Here the stability of S_2 and S_3 have been measured in the presence of PPBQ which is an often used electron acceptor in this type of preparation.

To measure the decay kinetics of S_2, synchronized (see Materials and Methods) samples were given one flash at room temperature. This results in the formation of S_2 in 90–95% of the centers. After the flash the samples were kept in darkness at 18 °C for different times before they were frozen and the amplitude of the S_2-state multiline signal measured. Fig. 2A shows the decay of the multiline signal (S_2) with time. The decay was monophasic (Fig. 2A inset) with a half decay time of 3–3.5 min.

It has earlier been observed that the formation of S_2 shortens the spin lattice relaxation time of Signal II_{slow} [23,24]. In EPR experiments this can be observed as an increase of the power for half saturation ($P_{1/2}$) for S II_{slow} (see Ref. 23 for details about this phenomenon). In the experiment in Fig. 2A, $P_{1/2}$ for S II_{slow} was also followed and it is clear that the decrease in $P_{1/2}$ was closely related to the disappearance of the multiline signal and consequently to the disappearance of the S_2 state.

The decay kinetics of S_3 at 18 °C in the presence of 0.5 mM PPBQ was also measured using the multiline signal as the spectroscopic probe. The outline of the experiment was as follows. The synchronized samples were given two flashes which resulted in the formation of about 85% S_3 centers. The flashes were followed by a dark incubation at room temperature for varying times before the samples were frozen. The EPR spectra were run and the amplitude of the multiline signal was measured (filled circles in Fig. 2B). These EPR measurements were followed by illumination at 198 K. This resulted in the formation of S_2 from any centers in the S_1-state while centers that were

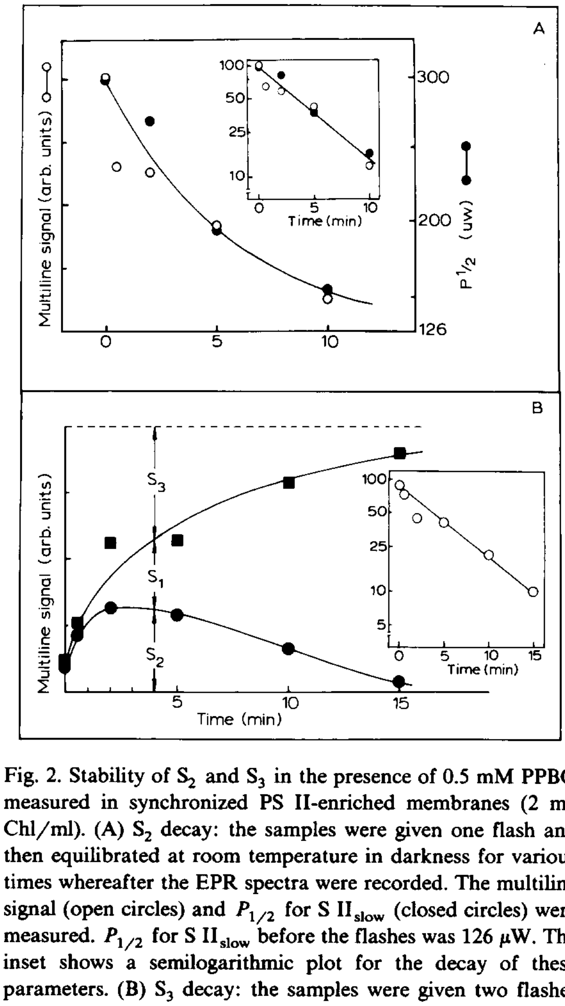

Fig. 2. Stability of S_2 and S_3 in the presence of 0.5 mM PPBQ measured in synchronized PS II-enriched membranes (2 mg Chl/ml). (A) S_2 decay: the samples were given one flash and then equilibrated at room temperature in darkness for various times whereafter the EPR spectra were recorded. The multiline signal (open circles) and $P_{1/2}$ for S II_{slow} (closed circles) were measured. $P_{1/2}$ for S II_{slow} before the flashes was 126 μW. The inset shows a semilogarithmic plot for the decay of these parameters. (B) S_3 decay: the samples were given two flashes and then dark incubated at room temperature for various times. The multiline signal was measured (filled circles). Then the samples were illuminated at 198 K and the multiline signal was measured again (filled squares). As indicated the multiline signal after the flash gave a measure of S_2, the change in the multiline signal obtained with the illumination at 198 K gave a measure of S_1. The remaining centers were assumed to be those in S_3 during the dark-incubation. The inset shows the decay of S_3.

already in the S_2-state were unable to advance to S_3 at this temperature (see below). Thus, when S_1 was present the illumination at 198 K resulted in an increase of the multiline signal (filled squares in Fig. 2B). From these measurements it was possible to estimate the fractions of centers that were in the S_2-state (after the flashes) or the S_1-state (after 198 K illumination), during S_3-deactivation. The rest of the centers, not accounted for by these two measurements, are assumed to be in the S_3-state and the data in Fig. 2B therefore permit the construction of a decay curve for S_3 (Fig. 2B inset). S_3 decays monophasically with a half-time of 3.5–4 min. Furthermore, the transient increase in the S_2-state after the dark incubation shows that S_3 decays via S_2 under these circumstances. This conclusion is strengthened by the observation of a lag in the formation of S_1. In addition the data could be accurately simulated (not shown) using the measured life-times of S_2 and S_3 (Fig. 2) and the known S-state composition immediately after the flashes.

Temperature dependence of the individual S-state transitions

The temperature dependence of the S_1 to S_2 transition was measured by providing one saturating flash to synchronized samples equilibrated at different temperatures. Fig. 3 (circles) shows the formation of S_2 at varying temperatures using the multiline EPR signal as probe. In the experiment all samples were equilibrated at 198 K after the flash before they were frozen in liquid nitrogen. This was done to permit transformation of any formed S_2-state $g = 4$ signal to the multiline signal [6,25]. The transition proceeds with high quantum yield above 200 K. The half inhibition temperature ($T_{0.5}$) was 135–140 K. The transition was partially uninhibited (10–15%) even at 80 K which was the lowest temperature investigated.

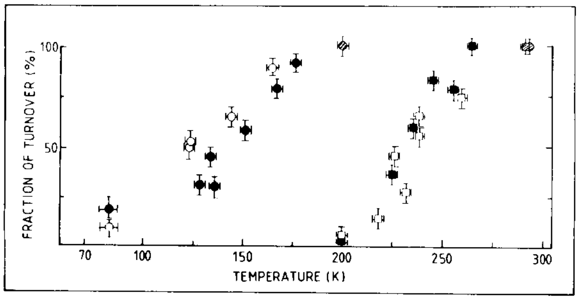

Fig. 3. The temperature dependence for the transitions S_1 to S_2 (circles) and S_2 to S_3 (squares) measured as described in the text. The data from two separate experiments are presented for each transition (open and filled symbols). The dashed points represent identical yield obtained in the two experiments. Flashes given at room temperature were considered to give the maximum yield and were set as 100%. The bars represent the estimated error in the temperature (±3 K) and in the amplitude of the multiline signal (±5%).

To measure the temperature dependence of the S_2-to-S_3 transition one flash was given to synchronized samples. This resulted in the formation of about 90–95% S_2 as judged from the amplitude of the multiline signal. Then the samples were thawed to 273 K for 10 s (to keep the sample transparent when the temperature was lowered) before they were given the low-temperature flash after which they were rapidly frozen. (The thawing to 273 K did not cause any significant disappearance of S_2 due to its slow decay in the presence of PPBQ (Fig. 2A)). The multiline signal was again recorded and the fraction that had passed from S_2 to S_3 was calculated from the decrease in the multiline signal. The data are presented in Fig. 3 (squares). The decrease in the multiline signal was maximal at room temperature; this point was therefore used as a 100% value. It should be pointed out that the flash at 198 K actually resulted in a small increase of the multiline signal (5–10%). This originated from centers that were left in S_1 due to misses in the first flash. This fraction of centers was assumed to form the multiline signal in all samples, and was therefore subtracted before the fraction that had actually formed S_3 was calculated. The transition was not inhibited above 265 K. $T_{0.5}$ was approx. 230 K and at temperatures below 200 K the transition was essentially blocked.

Fig. 4A shows the experimental protocol used to determine the threshold temperature for the S_3-to-S_0 transition. In this experiment the synchronized samples were given two flashes at room temperature before they were frozen and the EPR spectra run. From the amplitude of the multiline signal it was concluded that the two flashes had resulted in the formation of approx. 85% S_3 and 15% S_2 (to provide a good estimation of the miss factor a complete flash series at room tempera-

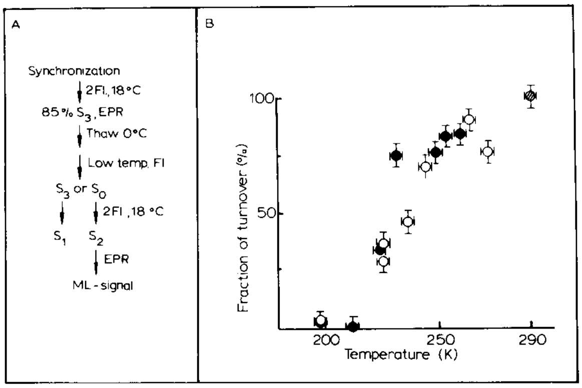

Fig. 4. The temperature dependence for the S_3-to-S_0 transition. (A) Outline of the experiment. (B) Experimental data from two separate experiments (open and filled symbols). The dashed point represents the fraction of turnover at room temperature which was considered to be 100% in both experiments. Error bars as in Fig. 3.

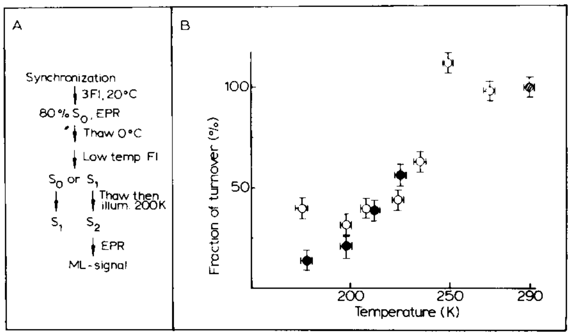

Fig. 5. The temperature dependence for the S_0-to-S_1 transition. (A) Outline of the experiment. (B) Experimental data from two experiments. The dashed point represent the room temperature flash which was considered to result in maximum turnover and was set as 100%. Error bars as in Fig. 3.

ture, 0–5 flashes, was run in this and the next experiment). Thereafter, the samples were thawed to 273 K for 10 s before the low temperature flash was given. Since S_3 deactivates slowly in the presence of PPBQ (Fig. 2B) the thawing procedure did not cause any significant disappearance of S_3. After the low-temperature flash the samples were thawed to room temperature and two more flashes were given (within 20 s) to form S_2 from the centers that had been converted to S_0 with the low-temperature flash. After the two room-temperature flashes the EPR spectra were recorded. The results are shown in Fig. 4B. The highest yield of the multiline signal was achieved when the variable temperature flash was given at room temperature. This is used as the value corresponding to a 100% passage in the graph. The transition is almost completely inhibited below 200 K. $T_{0.5}$ is approx. 235 K and there seems to be no inhibition of the transition above 270 K.

Fig. 5A shows the protocol used to measure the temperature dependence for the S_0-to-S_1 transition. Synchronized samples were given three flashes to form S_0 (approx. 80%). In this case no multiline signal could be detected (after three flashes 1–3% of the centers were in the S_2-state). After thawing to 273 K for 10 s the low-temperature flash was given which resulted in the formation of S_1 in a fraction of the centers. This fraction was estimated by measuring the S_2-state multiline signal formed by continuous illumination at 198 K. It should be mentioned that a thaw cycle (273 K for 10 s) was performed prior to the 198 K illumination to allow reoxidation of Q_A in the samples that had been flashed at low temperatures. The data are presented in Fig. 5B and show that the transition was essentially inhibited below 170 K. $T_{0.5}$ was 220–225 K and the transition was uninhibited above 250 K.

Discussion

In PS-II-enriched membranes the half-decay times for S_2 and S_3 have been measured to 35 and 40 s, respectively [26] but in a somewhat different preparation a longer time for S_3 deactivation (105 s [27]) was obtained. These kinetics have been attributed to recombination reactions with Q_B^- [11,28] and are much faster than the disappearance of S_2 (half-decay time about 3 min) and S_3 (decay half-time about 3.5 min) measured in the present work. The reason for the prolonged lifetime of the higher S-states here is the use of PPBQ as external electron acceptor. With this acceptor the first flash results in double reduction of PPBQ. First Q_A^- reduces PPBQ and the resulting semi-quinone oxidizes the Fe^{2+} of the acceptor side complex [29,30]. The net result is the formation of the state S_2-Q_A-Fe^{3+} in which there is no electron available on the acceptor side for recombination with S_2. The second flash then reduces the oxidized iron via Q_A [30] and results in the state S_3-Q_A-Fe^{2+} [29]. Also in this state the acceptor side remains oxidized after the flash, thus stabilizing S_3. From these experiments it can be concluded that the stability of the higher S-states is increased to a considerable extent when the acceptor side is oxidized and long lived S_2- and S_3-states have been observed earlier in leaves when Q_B was oxidized [28] or in experiments with PS II-enriched membranes where electron acceptors were used [31].

The source for the reducing equivalents needed to deactivate S_2 and S_3 and the reaction pathway are not known. It is possible that the reduction utilizes $PPBQH_2$ or PQH_2 and is mediated through the Q_B-site as suggested earlier in the case of intact leaves where a realtively high concentration of PQH_2 is present [28]. In the present study, however, a back-reaction with fully reduced $PPBQH_2$ is not expected to occur, since a high concentration of oxidized PPBQ is present. This probably has a higher affinity for the Q_B-site than the reduced form. Instead, deactivation of the S-states is more likely to be caused by direct reduction by exogenous electron donors, possibly $PPBQH_2$.

The increase in Fig. 2B of the S_2-state multiline signal also demonstrates very clearly that S_3 decays in a one-electron reaction via S_2 in PS II-enriched membranes. This has been demonstrated earlier by EPR [14] in an experiment where S_3 was rapidly reduced by the reduced form of D^+ and by oxygen yield experiments in chloroplasts [10,32]. On the other hand S_3-deactivation via S_2 was not observed in oxygen-yield experiments carried out in the absence of an electron acceptor in a preparation of this kind [27]. Another earlier attempt [18] to observe, using EPR, a transient increase in S_2 during the deactivation of S_3 was unsuccessful due to the use of non-synchronized samples and a non-saturating flash which resulted in a high concentration of S_2 (40–50%) also after the second flash. The experiment was also done in the absence of an electron acceptor and under these circumstances the decay reactions would have been dominated by the faster decay of S_2.

It should be pointed out that the long lifetimes of S_2 and S_3 formed in the presence of an electron acceptor is a useful property in experiments where mixing with reagents after the flashes is required (see, for example, Refs. 19 and 33). The stability of S_2 and S_3 also facilitated the temperature experiments in this study.

The temperature dependence of S-transitions has been measured earlier by thermoluminescence or by EPR spectroscopy. In thermoluminescence the efficiency at different temperatures to charge the so-called B-band has been measured. In early work [34,35] the complete illumination procedure was carried out at low temperature. This inevitably gives rise to acceptor-side limitations below 230 K [36] for all transitions except S_1 to S_2 (one electron only can be stored at the acceptor-side at low temperatures). In more recent work, however, single flashes at low temperature preceded by room-temperature flashes have been applied which eliminates this problem [37]. The interpretation of thermoluminescence measurements is complicated, since the B-band originates from recombination of Q_B^- with either S_2 or S_3 and that the intensity is double for $S_3Q_B^-$-recombination [38]. In addition, the previous studies have used non-synchronized samples which further complicates the data analysis.

In this study we have avoided the latter problem by the use of a preflash procedure that synchronizes all centers before the actual experiment is started. We have also used a spectroscopic probe, the S_2-state multiline signal which is only visible in centers in the S_2-state. The temperature data for the S-transitions obtained by this method are collected in Table I. The S_1-toS_2 transition has a half inhibition temperature of 135–140 K which is considerably lower than the other transitions. This value for the S_1-to-S_2 transition is lower than that earlier measured by an EPR-technique similar to ours (165–170 K [20]) or by thermoluminescence (178 K [37]). The reason for the discrepancy between the EPR measurements can partly be explained by the different experimental protocol used. In the earlier experiments [20] the illumination and freezing procedure at lower temperatures can be expected to have resulted in the formation of a substantial amount of S_2-centers exhibiting the $g = 4$ signal instead of the multiline signal

TABLE I

TEMPERATURE DEPENDENCE OF THE S-STATE TRANSITIONS IN SPINACH PS II-ENRICHED MEMBRANES

T_{open} is the temperature above which there is no inhibition of the transition; $T_{0.5}$ is the half-inhibition temperature; and $T_{\text{inhibition}}$ is the lower temperature limit for the transition.

	T_{open} (K)	$T_{0.5}$ (K)	$T_{\text{inhibition}}$ (K)
$S_0 \rightarrow S_1$	250	220–225	170
$S_1 \rightarrow S_2$	200	135–140	< 80
$S_2 \rightarrow S_3$	265	230	200
$S_3 \rightarrow S_0$	260	235	200

[6,25]. This was not known at that time but its formation would have led to an underestimation of the amount of S_2 formed below 170 K [6]. In the present study the formation of this signal was essentially avoided by the use of a warming cycle to 198 K before the samples were frozen in liquid nitrogen.

The half-inhibition temperature for the S_2-to-S_3 transition ($T_{0.5} = 230$ K) is slightly higher than that measured earlier by EPR (220 K [20]) but similar to that measured by thermoluminescence (228 K [37]. Also our measurement of the temperature needed to achieve total block of the transition (approx. 200 K) is in reasonable agreement with an earlier measurement [39]. The somewhat lower value in the earlier EPR-measurements [20] might be explained by the use of continuous illumination to transfer S_2-centers formed by a room-temperature flash to S_3. It is possible that, at the lower temperatures, the transition proceeds with a low quantum yield. Under such circumstances the continuous illumination used in Ref. 20 would favour the transition as compared to the single flash given in the present work.

The temperature needed to inhibit completely the S_3-to-S_0 transition measured here (200–210 K) is much lower than that measured by thermoluminescence when the complete illumination procedure was performed at low temperatures (255 K or 235 K [35,39]). These higher values could have been caused by inhibition of acceptor-side reactions. In the present work $T_{0.5}$ was measured to 235 K which is considerably lower than the measurement by thermoluminescence using room temperature preflashes ($T_{0.5} = 250$ K [37]). The reason for this difference might be the use of 50% ethylene glycol in the present study. This lowers the freezing point in the samples which might be of considerable importance, since the S_3-to-S_0 transition involves both the cleavage of water molecules and the expulsion of the formed O_2 molecule.

The temperature dependence for the S_0-to-S_1 transition is intermediate in its nature. $T_{0.5}$ is approx. 220 K which is slightly lower than $T_{0.5}$ in the transitions between S_2 and S_3 (230 K) or S_3 and S_0 (235 K). At 200 K a single flash results in S_0-to-S_1 turnover in 20–30% of the centers, while the S_2-to-S_3 and the S_3-to-S_0 transitions are nearly completely inhibited. The S_0-to-S_1 transition is essentially blocked below 170 K. To our knowledge this is the first measurement of the temperature dependence of this transition. It should be pointed out that, despite the complicated experimental protol applied to measure this transition, this experiment has one advantage over some of the other experiments in that the multiline signal could be developed by illumination at 198 K. This treatment avoids back-reactions from S_3-centers (present due to misses in the three flashes) to form the S_2-state multiline signal and it also is more quantitative than flash excitation.

It is very interesting that only S_1 to S_2 occurs at very low temperatures, while the other transitions require higher temperatures. This coincides with the proton release pattern in the S-cycle in which protons are released in all S-transitions except S_1 to S_2 [1]. It is probable that the release of protons is coupled to conformational changes and it is likely that these cannot occur at temperatures lower that approx. 200 K. Of importance is also the redox chemistry of the oxygen-evolving complex. Several lines of evidence indicate that S_0 to S_1 [23,40,41] and S_1 to S_2 [16,23,40–42] involve direct oxidation of Mn ions. The removal of an electron from the metal center can occur at low temperatures, since $T_{0.5}$ for S_1 to S_2 is about 140 K. The main difference between the S_0–S_1 and S_1–S_2 transitions could be explained by the proton release in the former. On the other hand several reports have been taken as indications that no oxidation of Mn ions occurs in the S_2–S_3 transition (Refs. 23, 41 and 43, but see Ref. 40). If this is the case this transition might involve oxidation of an, as yet, unidentified ligand. This might explain the similarities in the temperature dependence between the S_2-to-S_3 and the S_3-to-S_0 transitions. The latter is known to involve the oxidation of water and the formation and release of the oxygen molecule, reactions which almost certainly are temperature dependent. It is possible that also the binding or orientation (to its correct site) of the putative ligand oxidized in the S_2-to-S_3 transition involve temperature-sensitive rearrangements.

One further comment on the temperature data should be made. In several publications low-temperature illuminations have been applied to form certain S-states. For S_2, 198 K illumination is a standard method to reach this state. As seen from Table I this is appropriate, since the S_2-toS_3 transfer and the electron transfer from Q_A^- to Q_B^- [36] are blocked at this temperature. On the other hand attempts have been made to form S_3 specifically using continuous illumination at 235 [43] or 250 K [24]. The present data suggest that these treatments should result in a mixture of S-states and in fact nearly complete scrambling of the states was observed (not shown) with continuous illumination at 250 K in the presence of PPBQ (in the same buffer system as in Ref. 24). In this respect the illumination procedures applied in the present work might be more useful to prepare samples with well-defined S-state composition.

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