Biochimica et Biophysica Acta 894 (1987) 573–577
Elsevier

BBA 40205

BBA Report

A low-temperature-sensitive intermediate state between S_2 and S_3 in photosynthetic water oxidation deduced by means of thermoluminescence measurements

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(Received 17 August 1987)

Key words: Oxygen evolution; Photosystem II; S state transition; Thermoluminescence; (Spinach chloroplast)

The temperature dependence of S-state transitions in Photosystem II was measured by means of thermoluminescence using two different protocols for low-temperature flash excitation: protocol A, "last flash at low temperature", and protocol B, "all flashes at low temperature". Comparison of the temperature-dependence curves obtained by these two protocols revealed a marked difference particular for the three-flash experiments. The difference was attributed to the formation of a low-temperature sensitive precursor state between S_2 and S_3. The state is formed by two flash illumination given at −5 to −50 °C, spontaneously transforms to normal S_3 on dark warming, and is not converted to S_0 by the 3rd flash. The precursor state was tentatively assigned to an S_3 in which H^+ release is not completed.

Kinetical analysis at low temperatures is a useful tool for elucidation of the mechanism of chemical reactions. In PS II, the S-state advancement of the oxygen-evolving complex becomes inhibited step by step from the higher oxidation state to the lower on lowering the ambient temperature [1–6]. The half inhibition temperature of the S_1-to-S_2 transition estimated by charging efficiency of the thermoluminescence B-band or by generation of EPR multiline and $g = 4.1$ signals is reported to be around −100 °C or lower [3–6]. On the other hand, the transitions beyond S_2 are far more sensitive to low temperatures [2–4]. This may be possible because the S-state advancement from S_2 and from S_3 is more complex in molecular mechanisms than that from S_1: the former two transitions involve some additional process(es) other than abstraction of one electron. Here we report a possible intermediate precursor state between S_2 and S_3 deduced from the comparison of two temperature-dependence curves obtained by two different protocols of low-temperature illumination.

Spinach thylakoids were prepared as in Ref. 7, suspended in 25% (v/v) glycerol, 10 mM $MgCl_2$ and 20 mM Hepes-NaOH (pH 7.0), and stored in liquid N_2 until use. After thawing the thylakoids were diluted (0.25 mg Chl/ml) with 25% (v/v) glycerol, 10 mM $MgCl_2$ and 40 mM Mes-NaOH (pH 6.5), preilluminated with continuous orange light (0.7 mW/m^2) for 45 s, and then dark adapted at 25 °C for 5 min to attain even distribution of

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCN, ferricyanide; PS II, Photosystem II; P-680, primary electron donor of Photosystem II; Q_A and Q_B; primary and secondary quinone acceptors of Photosystem II; respectively; Z, secondary electron donor of photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

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$Q_B : Q_B^-$ [2,3,8,9]. Thermoluminescence was measured as in Ref. 10. The samples were illuminated with xenon flashes (5 μs, 4J, white light), rapidly cooled to −196 °C, and then heated at a rate of 0.8 °C/s. Low-temperature illumination was given according to the following two different protocols.

Protocol A: the samples are preilluminated with flashes (0–2) at room temperature (10 °C) to effect normal electron transport. The preilluminated samples are then cooled to various low temperatures where the last single flash is given. After the flash illumination, the samples are cooled to −196 °C and then subjected to thermoluminescence measurements. The charging efficiency by the last flash is estimated by comparing the height of thermoluminescence B-band (arising from $S_2Q_B^-$ or $S_3Q_B^-$ charge recombination) with that after the same number of flashes given at room temperature. This protocol enables us to estimate the threshold temperatures of individual S-state transitions free from the low-temperature-induced inhibition of electron transport on PS II acceptor side [3].

Protocol B: the samples are cooled to various low temperatures, where all flashes (1–3) are sequentially given. After the flash illumination, the samples are cooled to −196 °C and then subjected to thermoluminescence measurements. The charging efficiency is estimated by comparing the B-band height with that after the same number of flashes given at room temperature. This protocol provides us with information about any of the low-temperature-induced limiting steps involved in the S-state transitions, including electron transport on the acceptor side.

In single-flash experiments, the above-mentioned two illumination protocols are eventually equivalent. Solid triangles in Fig. 1 show the temperature dependence of the S_1-to-S_2 transition estimated from the B-band height after excitation by one flash at the indicated temperatures. The height showed a constant maximum level above −40 °C, but decreased gradually with lowering the excitation temperature to a very low level at −190 °C. The half-inhibition temperature was estimated to be −95 °C [3], which roughly agreed with those reported for the formation of the EPR multiline S_2 signal [4–6]. In order to rule out the possibility that the decrease in B-band height is caused by the competition in electron donation between cytochrome *b*-559 and Mn (in the S_1 state), the same experiments were done in the presence of DCMU (10 μM) and FeCN (0.5 mM), which chemically oxidizes cytochrome *b*-559 in PS II. Note that PS II preferentially oxidizes cytochrome *b*-559 at low temperatures [11–13] and charge separation between cytochrome *b*-559 and Q_B (or Q_A) is non-thermoluminescent [8,10]. DCMU was added to minimize the loss of negative charge by the action of FeCN, and the charging efficiency in this case was estimated from the height of the Q-band which arises from $S_2Q_A^-$ charge recombination: in the absence of DCMU about 60% of B-band ($S_2Q_B^-$) is lost by oxidation of Q_B^- by FeCN, whereas only less than 10% of the Q-band ($S_2Q_A^-$) is lost by FeCN in the presence of DCMU [10]. As shown by open triangles, there was no significant difference between the absence and presence of FeCN. This indicates that oxidation of cytochrome *b*-559 does not affect the temperature dependence of S_2 formation. Thus, we can conclude that the observed temperature dependence is not due to the competition of electron donation between Mn in S_1-state and cytochrome *b*-559, but is due to the low-temperature-induced inhibition of the S_1-to-S_2 transition.

When the two-flash experiments were done according to protocol A, the temperature dependence shown by the broken curve was obtained. The flat line below −80 °C is due to the $S_2Q_B^-$ charge pair generated by the 1st flash at room temperature, and the gradual decline between −20 to −80 °C reflects the low-temperature limitation of the S_2-to-S_3 transition [3]. The half inhibition temperature was −45 °C, which roughly agrees with the value reported for the disappearance of the multiline EPR signal [4,5]. When the experiments were done according to protocol B, the profile shown by solid circles was obtained. The B-band height showed a constant maximum level above −20 °C, but decreased steeply at lower excitation temperatures. Below −50 °C it coincided with the curve obtained by one flash. Clearly, the B-band height obtained by protocol B is more sensitive to low temperatures than that obtained by protocol A. This difference is interpreted as follows: the electron transport from Q_A^- to Q_B is retarded below −30 °C [15,16], and the 1st flash

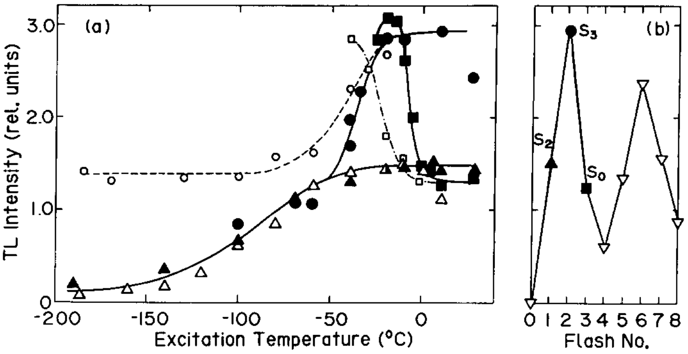


Fig. 1. Effect of low temperature on the S-state transitions measured by means of thermoluminescence. Panel b; normal oscillation of thermoluminescence B-band after a series of flashes given at room temperature (10 °C). Panel a; temperature dependence of S-state transitions measured by two different protocols for low-temperature illumination. Protocol A, "last flash at low temperature", protocol B, "all flashes at low temperature". One-flash experiments (S_1-to-S_2) in the absence (▲) and presence (△) of 0.5 mM FeCN and 10 μM DCMU. Two-flash experiments (S_2-to-S_3) by protocol A (○), and protocol B (●). Three-flash experiments (S_3-to-S_0) by protocol A (□), and protocol B (■). Note that efficiencies for the S_1-to-S_2 (△, ▲) and the S_2-to-S_3 (○, ●) transitions are expressed by the height of B-band (or Q-band for △) after one and two flashes, respectively, whereas the efficiency for the S_3-to-S_0 (□, ■) transition is expressed by the decrease in B-band height from its maximum level after two flashes. The B-band heights after full conversion of individual S-states in panel a are correlated to those in panel b with the same symbols.

excitation below −30 °C results in $S_2Q_A^-$ charge pair (instead of $S_2Q_B^-$), so that the 2nd flash will generate no stable charge pair. This situation will result in an apparent inhibition of S_3 formation. Thus, the steeper decline observed under protocol B is ascribed to the inhibition of Q_A^--to-Q_B electron transport rather than the inhibition of the S_2-to-S_3 transition. The half-inhibition temperature estimated from the data in Fig. 1 (−35 °C) coincides well with the reported values for Q_A^- to Q_B electron transport measured by different techniques [15–17].

The dot-dashed curve in Fig. 1 shows the plot of the three-flash experiments done according to protocol A, which reflects the temperature dependence of the S_3-to-S_0 transition. The half-inhibition temperature was estimated to be −23 °C, which is consistent with the delayed light data reported by Velthuys and Amesz [18]. When the three flash experiments were done according to protocol B, the dependence curve shown by solid squares was obtained. The B-band height showed a steep increase below 0 °C to reach a maximum at −20 °C, indicating the complete inhibition of the S_3-to-S_0 transition below −20 °C. The half-inhibition temperature was −7 °C, which is markedly higher (by more than 15 °C) than that determined by protocol A.

In protocol A experiments, two preillumination flashes were given to the sample at 10 °C in order to convert fully all the S_1 centers to S_3, then the 3rd flash was given at various low temperatures. Thus, the profile determined by protocol A reflects a more plausible temperature dependence of the S_3-to-S_0 transition. At −10 °C, almost all S_3 centers could be converted to S_0 by the 3rd flash in protocol A, whereas the three flashes at −10 °C by protocol B converted only 30% of S_3 centers to S_0, and the other 70% centers remained in S_3 state. Note that the efficiency for S_3 to S_0 conversion is measured by the decrease in the B-band height from its maximum level after two flashes. Since the 3rd flash at −10 °C in protocol A is fully effective in converting S_3 to S_0, as evidenced by the dot-dashed curve, the incapability of the 3rd flash in protocol B must be attributed to some special property of the product generated by the preceding two flashes given by protocol B. The possibility of interference due to inhibition of the Q_A^--to-Q_B electron transport can be ruled out, since this inhibition is negligibly weak at −10 °C (see solid circles).

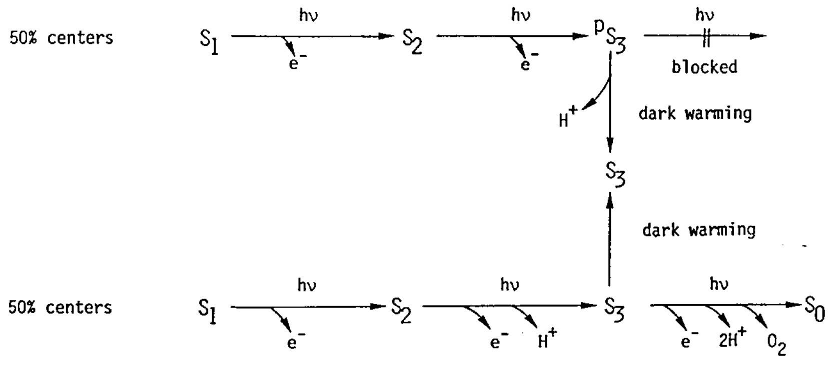
A possible explanation for this difference can be given, if we take into account that in thermoluminescence measurements, we warm the illuminated sample to effect charge recombination.

Thus, this technique does not always directly measure the product of a photoreaction, but rather the product of some reaction which may spontaneously occur in darkness during warming after the photoreaction. In other words, even if the photoreaction by two flashes at low temperatures produces an intermediate metastable S_3 state, it is detected as a normal S_3 state if the intermediate state can be transformed to normal S_3 during warming.

Applying this consideration to the three-flash experiments done by protocol B, the above differences in temperature dependence can be explained by the mechanism shown in Scheme I, in which a precursor state (denoted as $^{P}S_3$) is assumed to precede the normal S_3 state. The following properties of this precursor state are postulated: (1) the state is formed by two flashes at low temperatures below −10 °C (more correctly between −5 and −50 °C, see later discussion); (2) the state can be transformed to S_3 on dark warming; (3) the state cannot be converted to S_0 (or S_4) by the 3rd flash even if the flash is given in its effective temperature range. Scheme I illustrates the S-state transitions in all the S_1 centers during three sequential flashes at −7 °C, the half inhibition temperature. By the first two flashes, half of the centers are converted to normal S_3, whereas the other half are converted to the precursor $^{P}S_3$. If we measure thermoluminescence after these two flashes, both states are detected as $S_3Q_B^-$ charge recombination, since $^{P}S_3$ is transformed to S_3 by the warming procedure inherent in thermoluminescence measurement. Thus, the precursor state cannot be monitored after two flashes even if it has been formed. On the 3rd flash, however, the S_3 centers are converted to S_0 (or S_4), whereas the $^{P}S_3$ centers are not and remain in $^{P}S_3$. Since these $^{P}S_3$ centers remaining after the 3rd flash emit normal $S_3Q_B^-$ thermoluminescence, the extent of decrease in B-band height by the 3rd flash declines, and the efficiency of S_3-to-S_0 transition appears inhibited.

According to this scheme, we can regard the temperature dependence curve obtained by three flashes by protocol B as reflecting the temperature dependence of $^{P}S_3$ to S_3 transformation rather than that of S_3-to-S_0 transition. Similarly, we can regard the temperature dependence measured by two flashes by protocol A (two flash experiments, broken curve) as reflecting the temperature dependence of S_2 to $^{P}S_3$ transition, because thermoluminescence measurements do not distinguish $^{P}S_3$ from S_3, and no normal S_3 is expected below −20 °C (see solid squares). According to this consideration, the temperature range for generation of $^{P}S_3$ is between −5 and −50 °C (cf. $^{P}S_3$ property no. 1 in the previous paragraph). When we apply the same consideration to S_3-to-S_0 transition, it may be possible to assume another intermediate $^{P}S_4$: both $^{P}S_4$ and S_4 are detected as S_0 in thermoluminescence measurements as a loss of $S_3Q_B^-$ charge pair.

We have no information at present as to the



Scheme I. A scheme of possible S-state transitions during three-flash illumination by protocol B at −7 °C, the apparent half inhibition temperature for S_3-to-S_0 transition estimated from the solid curve with (■) in Fig. 1, where only half of the S_1 centers are normally converted to S_0 after three flashes, whereas the other half of the S_1 centers are converted to the precursor $^{P}S_3$ state after the 2nd. Note that the 3rd flash is abortive for $^{P}S_3$ centers, and $^{P}S_3$ is measured as S_3 in thermoluminescence measurements. S_0 centers in the initial dark-adapted conditions are neglected. H^+ release pattern was assumed according to Förster and Junge [19].

chemical identity of $^{P}S_3$. However, it seems possible that $^{P}S_3$ is an S_3 state in which H^+ release is blocked by low temperature, in other words, the product of one electron abstraction from S_2 with no H^+ release. This hypothesis is consistent with the observation that $^{P}S_3$ easily transforms to S_3 by dark warming. We have preliminary data on the pH dependence for $^{P}S_3$ to S_3 transformation, suggesting that the half inhibition temperature slightly shifts to higher temperature with lowering the pH (−8, −7 and −5°C at pH 7.5, 6.5 and 5.5, respectively) in support of the above hypothesis.

We have attempted to give an alternative explanation for the difference in temperature dependence due to different illumination protocols from another point of view: by taking Z^+ into account, the oxidized secondary donor of PS II, which may function as a positive charge reservoir similar to $^{P}S_3$ in S-state transitions. For the S_1-to-S_2 transition, the formation of the intermediate state, Z^+S_1, is clearly ruled out, since S_2 has been measured directly by the multiline EPR signal, and the temperature dependence of the generation of this signal agrees with our thermoluminescence data. For the S_2-to-S_3 transition, the expected intermediate state would be Z^+S_2. It is not known whether or not the multiline EPR S_2 signal is affected by the positive charge on Z^+. If we assume Z^+S_2 shows the signal (this appears more plausible), the existence of Z^+S_2 can be ruled out, since the temperature dependence estimated from disappearance of the signal by the 2nd flash (strictly saying, −30 to −70°C continuous light after one flash at 0°C [4]) agrees with our thermoluminescence data. If not, on the other hand, and if the lifetime of Z^+ is sufficiently long at around −10°C, the assumption in Scheme I have to be reconsidered. As to the S_3-to-S_0 transition, no relevant information is available at present other than the fact that Z^+ shows a longer lifetime. However, no matter how stable the Z^+S_3 is, it does not affect our assumption in Scheme I, since the precursor $^{P}S_3$ is assumed between S_2 and S_3, but not between S_3 and S_0 (or S_4).

This work was supported by a grant for Solar Energy Conversion by means of Photosynthesis at The Institute of Physical and Chemical Research (RIKEN) given by The Science and Technology Agency of Japan.

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