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A marked upshift in threshold temperature for the S_1-to-S_2 transition induced by low pH treatment of PS II membranes

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The temperature dependence of the S_1-to-S_2 transition in low-pH-treated PS II membranes was investigated by means of low-temperature EPR spectroscopy and thermoluminescence. (1) continuous illumination at −60 °C (213 K) of low-pH-treated PS II induced neither the EPR multiline and $g = 4.1$ signals nor the thermoluminescence glow peak (B-band), but the same illumination at −5 °C appreciably induced both EPR multiline signal and glow peak, although the multiline signal was modified in its fine structures and the $g = 4.1$ signal was largely suppressed. (2) Temperature dependence analysis by use of thermoluminescence revealed that the half-inhibition temperature for S_1-to-S_2 transition is markedly upshifted (by about 70 °C) in low-pH-treated PS II. (3) The upshifted threshold temperature was returned to normal by the addition of exogenous Ca^{2+}. These results are discussed in relation to the roles of Ca in S-state transition, and in comparison with the abnormal S_2 state reported for Cl^--depleted PS II.

Ca is considered to be cofactor for photosynthetic O_2 evolution [1–4]. On treating PS II membranes with a low-pH medium for a short period, O_2 evolution is inhibited concomitant with liberation of weakly bound Ca (one of the two Ca per reaction center) without any loss of proteinaceous factors [5–7]. Since a simple addition of exogenous Ca^{2+} restores O_2 evolution, it has been inferred that removal of Ca is responsible for the loss of O_2 evolution. The treated PS II membranes show an abnormal thermoluminescence band with an elevated peak temperature after one flash illumination, and its amplitude does not show any oscillatory behavior on illumination with more flashes [6,7]. This abnormal thermoluminescence band has been tentatively assigned to arise from a recombination between the reduced quinone acceptor of PS II and the S_2 state (the first positively charged state of the Mn cluster catalyzing water cleavage) whose properties are modified by depletion of Ca^{2+}. The shape of the abnormal thermoluminescence band is variously modified when various cations are exogenously added to the treated membranes [7]. It was inferred that the redox properties of the S_2 state are regulated by Ca^{2+} and that O_2 evolution is inhibited due to interruption of S_2-to-S_3 transition in the absence of Ca. This interruption site of S-state transition contradicts some of the previous reports by other investigators, and a detailed discussion about this discrepancy has been made in Ref. 6.

During these studies we happened to find that in treated PS II, the EPR multiline signal is not induced by illumination at 200 K, which is the standard protocol to induce the signal in untreated normal PS II [8,9]. Here, we compared the temperature dependence of S_2 formation between normal and treated PS II. It was suggested that the threshold temperature of S_1-to-S_2 transition is markedly upshifted in low-pH-treated PS II membranes.

Spinach PS II membranes capable of O_2 evolution were incubated in darkness overnight at 0 °C for complete relaxation of both the donor and acceptor sides of PS II. The PS II membranes were subjected to low pH treatment by incubating the membranes with 400 mM sucrose, 20 mM NaCl and 20 mM citrate-NaOH (pH 3.0) in complete darkness at 0 °C for 5 min as described previously [5,6]. The pH of the sample solution was adjusted to 6.5 by dilution with an aliquot of 400 mM sucrose, 20 mM NaCl and 40 mM Hepes-NaOH (pH 7.5), followed by additional dark incubation for 30 min. For addition of exogenous Ca^{2+}, the treated membranes were supplied with 50 mM $CaCl_2$ followed by incubation for 30 min in darkness. Control membranes

Abbreviations: PS II, Photosystem II; Mes, 2-(*N*-morpholino)ethane sulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; Q_A, primary quinone acceptor of Photosystem II.

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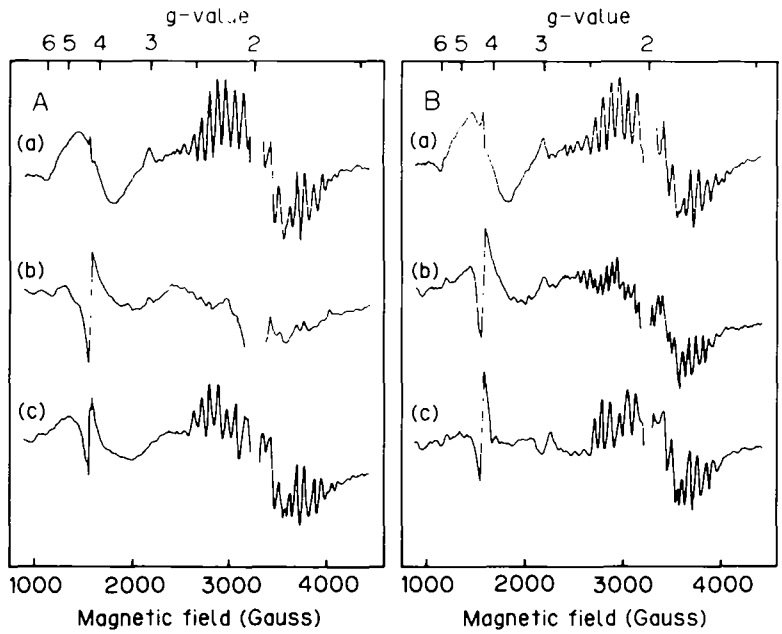

Fig. 1. Effect of low pH treatment on S_2 EPR spectra (light-dark) induced by continuous illumination for 2 min at −60 °C (A) and for 30 s at −5 °C (B). (a), untreated control membranes; (b), low-pH-treated membranes; (c), low-pH-treated and then Ca-supplied membranes. DCMU (50 μM) was included in all samples to ensure a single turnover of S-state. For (c), 50 mM $CaCl_2$ was added to the treated membranes followed by 30 min dark incubation at 0 °C. Instrumental settings: temperature, 6 K; microwave power, 0.4 mW; microwave frequency, 8.95 GHz; modulation frequency and amplitude, 100 kHz and 20 G, respectively. Sample concentrations were 3.6, 3.5 and 3.6 mg Chl/ml for (a), (b) and (c), respectively.

were suspended in the mixture of Hepes-NaOH buffer (pH 7.5) and citrate-NaOH buffer (pH 3.0) with a final pH of 6.5.

For EPR samples, the low pH treatment was preceded by one wash with 400 mM sucrose, 20 mM NaCl, 1 mM EDTA and 40 mM Mes-NaOH (pH 6.5) to eliminate free Mn. The control and treated membranes were suspended in the mixture of Hepes-NaOH (pH 7.5) and citrate-NaOH (pH 3.0) buffers with a final pH of 6.5, placed in calibrated EPR tubes, and then frozen in liquid N_2. All procedures were carried out within 30 min after the low pH treatment. For addition of exogenous Ca, the treated membranes were incubated in darkness for 30 min at 0 °C after the pH adjustment, supplemented with 50 mM $CaCl_2$, and then incubated again for 30 min in darkness.

Thermoluminescence was measured as described previously [6]: samples were illuminated with a single flash at varying temperatures and then quickly cooled in liquid N_2. The light emission during warming (about 1 C°/s) was recorded against sample temperature. Low-temperature EPR spectra were recorded at 6 K as described in Ref. 7. A JEOL ESPRIT23 EPR data system was used for averaging, subtraction and integration of the spectra. EPR samples in calibrated quartz tubes were illuminated with continuous light for 2 min at −60 °C or for 30 s at −5 °C using a JEOL ES DVT-1 temperature control system.

Fig. 1 shows the low temperature EPR spectra (light-dark) induced by continuous illumination at two different temperatures of −60 °C (panel A) and −5 °C (panel B). DCMU was included in all the samples to ensure a single turnover of the S state. In untreated control membranes (a), the multiline and $g = 4.1$ signals both arising from the Mn cluster in S_2 state were similarly induced by illumination both at −60 °C and at −5 °C. In low-pH-treated membranes (b), however, −60 °C illumination induced neither the multiline nor the $g = 4.1$ signals at all, whereas −5 °C illumination appreciably induced the multiline signal accompanied by a largely suppressed $g = 4.1$ signal. This indicates that the threshold temperature for S_2 formation is upshifted after low pH treatment. The shape of the multiline signal thus induced in treated sample was slightly modified as depicted by a reduced average hyperfine line spacing from 89 to 65 G. The relative spin concentration estimated by double integration for the modified multiline species was about 60% of that of the normal signal in control membranes. Although these modifications are similar to those reported for the multiline signal from Sr^{2+}-substituted PSII membranes, its temperature dependence is clearly different: the Sr^{2+}-substituted signal can be photoinduced at 200 K (−73 °C) [7,10], whereas this signal is not at −60 °C. Since no signal could be found to develop when the −60 °C-illuminated sample was warmed up to −5 °C (data not shown), we may preclude the possibility that −60 °C illumination generated a precursor state which could be converted to a multiline state at higher temperatures. When the low-pH-treated membranes were sup-

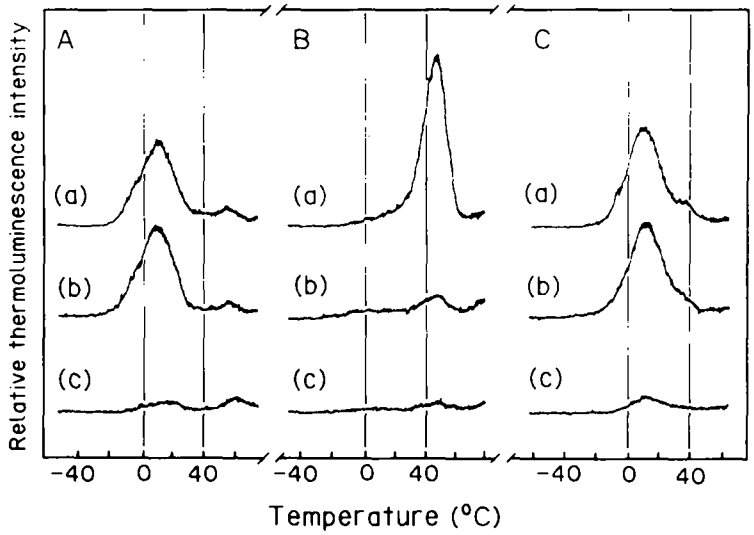

Fig. 2. Effect of low pH treatment on thermoluminescence glow curve ($S_2Q_A^-$ charge pair) induced by a single flash excitation at +10, −40 and −120 °C in the presence of 10 μM DCMU. (A), untreated control membranes; (B), low pH treated membranes; (C), low-pH-treated and then Ca-supplied membranes.

plemented with Ca^{2+} (c), a multiline signal with normal shape was restored and the restored signal could be induced both at −60 °C and at −5 °C. The relative spin concentration of the restored signal reached about 90% of that in control membranes. The $g = 4.1$ signal, however, was not restored by Ca^{2+} addition.

The upshift in threshold temperature due to low pH treatment was confirmed by means of thermoluminescence (Fig. 2). PS II membranes were illuminated with a single flash in the presence of DCMU at three different temperatures. In control membranes (panel A), the flash illuminations both at +10 and −40 °C similarly induced the Q-band at around +10 °C arising from $S_2Q_A^-$ charge recombination (Aa, Ab). At −120 °C, however, no band was induced (Ac), indicative of interruption of the S_1-to-S_2 transition below −120 °C [8,11]. In low-pH-treated membranes (panel B), a sharp band peaking at around +45 °C was induced by illumination at +10 °C (Ba). This band with unusually high peak temperature is equivalent to the abnormal band which we interpreted in our previous papers [6,7] as arising from charge recombination between Q_A^- and Ca-free S_2. Notably, this abnormal thermoluminescence band could not be generated by illumination at −40 °C (Bb), which is an excitation temperature high enough to allow S_1-to-S_2 transition in untreated membranes. On addition of Ca^{2+} to treated membranes (panel C), the elevated peak temperature of the abnormal band was reversed to normal temperature, and the restored band could be induced by illumination at −40 °C as well as at +10 °C.

When these results are compared with the EPR data in Fig. 1, a good parallelism can be pointed out between the abnormal thermoluminescence band and the modified multiline signal: if the excitation at low temperature was incapable of inducing the abnormal thermoluminescence band, it did not induce the modified EPR multiline signal either. Based on these parallel relationships, the positive charge responsible for the abnormal thermoluminescence band found in low-pH-treated membranes can be assigned to the positive charge accumulated on the Mn cluster as the modified EPR multiline signal.

In Fig. 3, the temperature-dependence of S_2 formation in low pH treated PS II is compared with that in normal untreated PS II. Samples supplemented with DCMU were illuminated with one flash at varying temperatures, and the thermoluminescence intensity was plotted against excitation temperature. In control membranes (solid circles), the thermoluminescence intensity showed a constant maximum level for excitations above

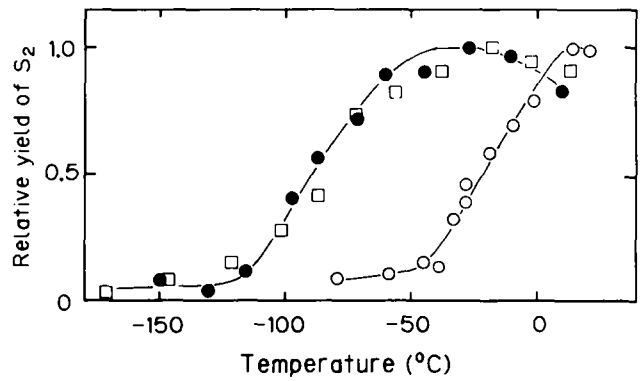

Fig. 3. Effect of low pH treatment on temperature dependence for S_1-to-S_2 transition measured by means of thermoluminescence. The samples were excited with one flash in the presence of 10 μM DCMU at varying temperatures, and amplitudes of glow curves were plotted against excitation temperature. (●) untreated control membranes; (○) low-pH-treated membranes; (□) low-pH-treated and then Ca-supplied membranes. Thermoluminescence amplitudes were normalized for respective maximum values of the three samples.

−60 °C, and decreased gradually with lowering the excitation temperature to nearly zero at around −120 °C. The half inhibition temperature estimated from this slope was −90 °C, which roughly agrees with the value previously determined by EPR multiline signal and thermoluminescence B-band [8,11]. In contrast, the temperature dependence of formation of abnormal glow peak in treated membranes (open circles) was markedly different: the intensity of the upshifted thermoluminescence band tended to be suppressed below 0 °C, showing a half inhibition temperature at around −20 °C, and practically no band was induced below −50 °C. When exogenous Ca^{2+} was added to treated membranes, the capability of charge pair formation showed the normal temperature dependence as found for untreated control membranes (open squares). These results clearly indicate that the threshold temperature for S_1-to-S_2 transition is markedly upshifted in low-pH-treated PS II membranes.

The present study has demonstrated that low pH treatment of PS II not only modifies the properties of S_2 state, but also alters the threshold temperature of S_2 formation. In our previous papers [5–7], we reported that low pH treatment inhibits S_2-to-S_3 transition by modifying the properties of S_2. The modified S_2 state was characterized by the absence of EPR multiline signal [7] and a lowered oxidation potential as deduced from the elevated peak temperature of thermoluminescence [6,7]. By analogy with the abnormal S_2 state in Cl^--depleted PS II, which lacks the multiline signal but exhibits the $g = 4.1$ signal after excitation at 200 K [12], we inferred that this S_2 stores a positive equivalent which is capable of recombination for thermoluminescence emission but incapable of exhibiting the multiline signal. In our previous experiments in Ref. 7, however, no alteration in temperature dependence was taken into account, and the excitation protocol to induce S_2 was different between EPR and thermoluminescence experiments: continuous illumination at 200 K for EPR spectroscopy, while one flash illumination at 0 to +10 °C for thermoluminescence measurements. When we reconsider our previous data in the light of the alteration in temperature dependence as revealed in this study, we have to change the interpretation as follows: at 200 K (−73 °C), the multiline signal could not be induced in low-pH-treated PS II because S_1-to-S_2 transition did not occur, whereas at 0 °C the modified multiline signal could be induced as well as the abnormal charge pair for thermoluminescence. This is clearly different from the abnormal S_2 in Cl^--depleted PS II, since in the absence of Cl^- the $g = 4.1$ signal and thermoluminescence were markedly induced by 200 K illumination, in spite of the absence of multiline signal [12], indicative of efficient charge separation even at 200 K.

The S_2 multiline signal induced in low pH treated PS II by −5 °C illumination was slightly but appreciably modified (Fig. 1). The relative spin concentration of the modified signal was as low as 60% of the normal signal in control PS II, but this value can be recalculated to exceed 80%, when we take into account the reduced yield of S_2 formation at −5 °C in low-pH-treated PS II, about 70% as shown in Fig. 3. The recalculated value is close to the relative spin concentration estimated for the signal amplitude in Ca-supplemented sample (Fig. 1). These data suggest that the modified multiline signal arises from the major population of the Mn cluster. In these calculations, we ignored the possibility that the acid treatment may modulate the equilibrium between the two EPR S_2 signals, the multiline and $g = 4.1$ signals. However, the above conclusion may not be affected by this possibility, since the enhancement of the multiline intensity at the cost of $g = 4.1$ signal is reported to be at most 20% (Ref. 9).

As we have already reported previously [5–7], most of the effects induced by the low pH treatment can be well reversed by exogenous Ca^{2+} with the only exception for $g = 4.1$ signal. In this study also, the upshift in threshold temperature of S_1-to-S_2 transition could be reversed by exogenous Ca^{2+}. Since the treatment halves the Ca abundance in PS II membranes [5], it was inferred that all of the effects induced by low pH treatment and restoration from them are caused by release and rebinding of Ca to its specific binding site in PS II, respectively. However, the present results that the interruption of the S_1-to-S_2 transition at −60 °C (213 K) in low-pH-treated PS II could be reversed without any Ca^{2+} by simply raising the excitation temperature above −5 °C seem to suggest that at least a part of the effects induced by acid treatment are of structural (conformational) nature that can be overcome by some physical factors (e.g., temperature). Whether this phenomenon is related to those reported by Shen et al. [13] and Völker et al. [14], who ascribed the Ca^{2+}-dependent reactivation of O_2 evolution to a non-specific cation effect, must be clarified in future studies.

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