Biochimica et Biophysica Acta, 1020 (1990) 269–277
Elsevier

BBABIO 43305

Abnormal redox reactions in photosynthetic O_2-evolving centers in NaCl/EDTA-washed PS II. A dark-stable EPR multiline signal and an unknown positive charge accumulator

Taka-aki Ono and Yorinao Inoue

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Saitama (Japan)

(Received 28 December 1989)
(Revised manuscript received 30 July 1990)

Key words: Oxygen evolution; ESR multiline signal; Calcium ion depletion; Photosystem II

Redox events that occur in photosynthetic O_2-evolving centers in NaCl/EDTA-washed PS II membranes were investigated by means of low temperature EPR and thermoluminescence. The following results have been obtained: (i) In the washed membranes, O_2 centers could maintain the S_2 state more than 3 h in darkness at room temperature. This dark-stable S_2 was modified as seen by a multiline EPR signal with reduced hyperfine line spacing and also by a thermoluminescence band with upshifted peak temperature. (ii) This modified S_2 state had an abnormally long life of $t_{1/2} = 7$ h at 20 °C, and its appearance required the presence of EDTA in the medium. On addition of exogenous Ca^{2+}, the modified S_2 was converted in darkness to normal S_2, and then decayed rapidly to be undetectable. (iii) On illuminating this modified S_2, an EPR signal centering at aroung $g = 2$ was newly induced at no expense of the dark-stable EPR multiline signal. This EPR signal was accompanied by a new thermoluminescence band peaking at around 5 °C, suggesting the presence of a new redox component whose oxidized form is capable of providing a positive charge for thermoluminescence in place of Mn. (iv) This new component was efficiently oxidized by illumination at −5 °C but much less at −60 °C, showing a half-inhibition temperature at around −40 °C. (v) Addition of various divalent cations in place of Ca^{2+} variously affected both thermoluminescence glow peaks arising from the dark-stable S_2 or from the new redox component, suggesting a cation-species-dependent modulation of the redox properties of both components. (vi) Both of these two thermoluminescence bands showed no dependency on flash number, suggesting interruption of further oxidation beyond their respective abnormal states. On addition of Ca^{2+}, all these abnormal properties were abolished and normal period-four flash pattern was restored. These abnormal properties of the redox events in NaCl/EDTA-washed PS II membranes were discussed in relation to the demand for exogenous Ca^{2+} in recovery of normal properties.

Introduction

O_2 evolution by thylakoid or PS II membranes prepared from higher plants shows no or very little demand for exogenous Ca^{2+}. However, when the membranes are treated with concentrated NaCl [1–3], low pH medium [4,5] or trypsin [6], O_2 evolution becomes markedly suppressed, and its maximum rate exhibits clear demand for exogenous Ca^{2+}. These phenomena are inferred as suggesting that Ca^{2+} originally serves as an indispensable cofactor for O_2 evolution, although the mechanisms of inhibition of O_2 evolution reported for various treatments are not always the same: NaCl wash blocks S_1–S_2 and/or S_3–S_4 transition(s), while low pH treatment blocks S_2–S_3 transition [5]. In fact, higher plant PS II membranes and PS II particles capable of O_2 evolution are known to contain one or two Ca atoms per unit of PS II [4,7,8]. Cyanobacterial PS II also contains one Ca atom, which is reported to be essential for electron transfer near the reaction center P680 [9,10].

Major part of the studies about the Ca demand have been done with so called NaCl-washed PS II membranes which were prepared by use of variously modified washing protocols. It turned out through these studies that the results derived differ considerably de-

Abbreviations: PS, photosystem; TL, thermoluminescence; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Q_A, primary quinone acceptor of Photosystem II; Chl, chlorophyll.

Correspondence: T-a. Ono, Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan.

pending on the washing protocol employed. When a NaCl wash is given in darkness, the resulted PS II membranes show heterogeneous Ca^{2+} demand among the O_2 centers: only a part of the centers are inactivated in darkness and the inactivation is enhanced during continuous illumination for O_2 measurements [7,11]. Similar enhancement of inactivation can be induced by inclusion of chelator in darkness [10]. These inhibitions are restored and/or protected by the presence of exogenous Ca^{2+}. The other part of the O_2 centers, however, remain non-inactivated and can evolve O_2 normally even in the presence of chelator [10]. The ratio between the inactivated and non-inactivated centers in these protocols fluctuates around 50–70% depending on PS II preparations [6,10,11]. When the NaCl wash is given in the light, O_2 evolution is more severely inactivated to 10–20% of the original activity regardless of the presence of chelator, and the activity is again well restored by the addition of Ca^{2+} [12]. The resulting PS II membranes, however, still show heterogeneous affinity for Ca^{2+} [13] and the enhanced inactivation by light during NaCl wash has been attributed to an S-state dependent change in the binding affinity of Ca^{2+} [14].

There have been reported two alternative inhibition sites in NaCl-washed membranes; S_1–S_2 [15,16] and S_3–S_0 [11,17,18] transitions. Although some of these results are attributable to partial Cl^- depletion brought about by an enhanced Cl^- demand due to the loss of 24 and 16 kDa extrinsic proteins [19] and/or an indirect effect on S_2 of high concentration of chelator [18], there still appear inconsistencies difficult to be reconciled. Conceivably, the large heterogeneity reflects different types of modifications among the O_2 centers, although all of these modifications are equally reversed by the addition of Ca^{2+} to restore O_2 evolution.

In this communication, we report EPR and TL properties of the O_2 centers in spinach PS II membranes treated with NaCl in the light. We employed a very stringent washing condition that is expected to effect a strict removal of Ca^{2+}: the PS II membranes were treated with NaCl under illumination, followed by an addition of relatively high concentration of EDTA to eliminate any free Ca^{2+} from the medium, which might re-ligate to a high affinity Ca^{2+}-binding site present or created in the washed membranes. The O_2 centers in thus treated PS II membranes showed characteristics that are completely different from those reported previously. An abnormal S_2, which is characterized by an altered EPR multiline spectrum and by an upshifted thermoluminescence (TL) glow peak, was stably observed in the washed membranes with no illumination, and exposure of this abnormal S_2 to light induced a new redox component which is characterized by a new EPR signal and a new TL band. All of these unusual properties were completely reversed to normal by the addition of exogenous Ca^{2+}.

Regarding the assessment of the status of knowledge related to this work, Boussac et al. [20,21], Sivaraja et al. [22] and Baumgarten et al. [23] reported the dark-stable multiline and flash-induced new EPR signals, and proposed that the new EPR signal originates from an oxidized amino acid [21]. Similarly, we have reported the dark-stable multiline signal at the 8th International Congress on Photosynthesis in Stockholm [24] and subsequently the flash-induced $g = 2$ signal at the 15th Annual Midwest Photosynthesis Conference in French Lick, U.S.A. [25]. The EPR signals we report here are very similar to those reported by the other two groups, but there appear some significant differences. In this paper, we discuss our results referring to the previous interpretation in particular to those in Ref. 21.

Materials and Methods

BBY-type Triton X-100 solubilized PS II membranes capable of O_2 evolution (> 600 μmol $\cdot$ O_2/mg Chl per h) were prepared from spinach [11], and stored in liquid N_2 until use. After thawing, the membranes were washed twice with 400 mM sucrose, 20 mM NaCl and 40 mM Mes-NaOH (pH 6.5), and then suspended in the same medium. For NaCl wash, the membranes were resuspended in 2 M NaCl, 400 mM sucrose and 40 mM Mes-NaOH (pH 6.5) to a sample concentration of 0.5 mg Chl/ml, and incubated under room light at 0 °C for 30 min (denoted NaCl-washed PS II). EDTA · 2Na was then added to the washed membranes to a concentration of 1 mM in the dark followed by further 5 min incubation at 0 °C (denoted NaCl/EDTA-washed PS II). The following procedures were all done under dim-green safe light when otherwise noted. NaCl-washed PS II (without EDTA addition) and NaCl/EDTA-washed PS II (with EDTA addition) were further washed with 400 mM sucrose, 20 mM NaCl, 0.2 mM EDTA · 2Na and 40 mM Mes-NaOH (pH 6.5), and then resuspended in the same medium. EDTA · 2Na was omitted from the washing and resuspending medium for NaCl-washed PS II. Both PS II membranes were dark-adapted for more than 3 h at 0 °C before EPR and TL measurements.

Low temperature EPR spectra were recorded at 6 K with a JEOL JES-FE1XG X-band EPR spectrometer equipped with an Oxford-900 continuous flow cryostat as reported previously [26]. A JEOL-ES-PRIT 23 EPR data system was used for averaging and subtraction of spectra. EPR samples in calibrated quartz tubes were illuminated with continuous light at -60 °C for 2 min or at -5 °C for 30 s. Sample temperature during illumination was controlled within ± 1 °C using a JEOL ES-DVT-1 temperature control system. In some experiments, 50 μM DCMU and/or 1 mM phenyl-*p*-benzoquinone was added to the sample. For TL measurements, the samples were excited with a saturating Xe flash, cooled quickly in liquid N_2 unless otherwise noted

and then the light emission during warming (approx. 1 C°/s) was recorded against sample temperature [11]. O_2 evolution was measured at 25°C with a Clark-type O_2 electrode with phenyl-*p*-benzoquinone (1 mM) as an electron acceptor as previously described [11]. DCMU and phenyl-*p*-benzoquinone were dissolved in dimethylsulfoxide as a stock solution.

Results

Fig. 1 shows the effect of NaCl/EDTA wash on EPR multiline signal arising from the Mn cluster in S_2 state [27] before (left panel) and after illumination at −60°C (right panel). In untreated control PS II, no S_2 multiline signal could be observed without illumination, and a normal multiline signal was generated by illumination at −60°C. In NaCl/EDTA-washed PS II, whose O_2-evolving activity was reduced to less than 15% of the control, however, a clear multiline signal was found without any illumination, and its amplitude was neither increased nor decreased by illumination at −60°C, indicative of the presence of a dark-stable multiline signal. The hyperfine structure of this dark-stable signal was modified, exhibiting a reduction in average hyperfine line spacing from 89 G to 65 G. This dark-stable signal was found regardless of the presence of inhibitor of electron transport (DCMU), electron acceptor (phenyl-*p*-benzoquinone) or sucrose in the medium, but strictly required the presence of chelator (0.2 mM EDTA · 2Na). The chelator at this concentration did not affect the formation of normal multiline signal in untreated control PS II (data not shown). In the absence of chelator, the NaCl-washed (without EDTA) PS II did not show the dark-stable signal, but a normal multiline signal could usually be induced by illumination at −60°C, although the O_2-evolving activity was largely inhibited by approx. 70% in these membranes (data not shown). When Ca^{2+} was added to the NaCl/EDTA-washed PS II, the dark-stable signal disappeared, and a normal multiline signal could be again generated by illumination at −60°C.

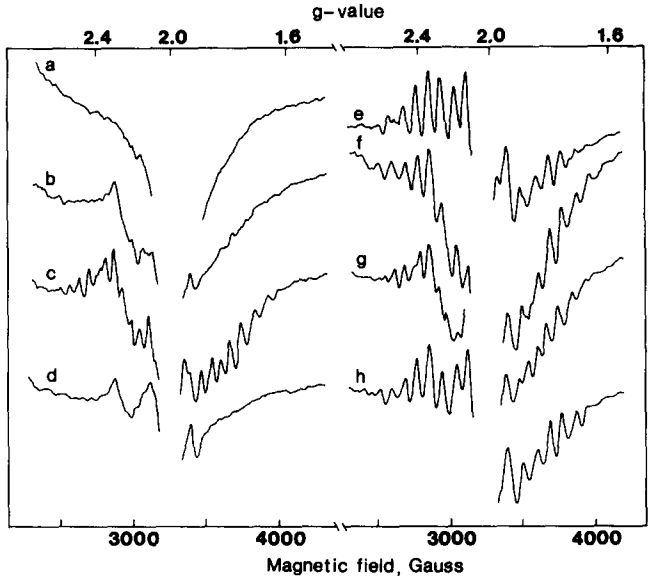

Fig. 1. Low-temperature EPR S_2 multiline spectra of NaCl-washed or NaCl/EDTA-washed PS II membranes before (left panel) and after (right panel) continuous illumination at −60°C for 2 min. Untreated control PS II membranes supplemented with 15 mM $CaCl_2$ (a, e); NaCl-washed (without EDTA) PS II supplemented with 30 mM NaCl (b, f); NaCl/EDTA-washed PS II supplemented with 30 mM NaCl (c, g) and NaCl/EDTA-washed PS II supplemented with 15 mM $CaCl_2$ (d, h). NaCl or $CaCl_2$ was added to the washed membranes followed by 30 min dark incubation at 0°C. Basal suspending medium contained 20 mM NaCl and 50 μM DCMU. Instrumental settings: temperature, 6 K; microwave power and frequency, 0.4 mW and 8.95 GHz; modulation frequency and amplitude, 100 KHz and 20 G, respectively. Chl concentration was 3.5 mg Chl/ml. See Material and Methods for detailed protocols for NaCl and NaCl/EDTA washing.

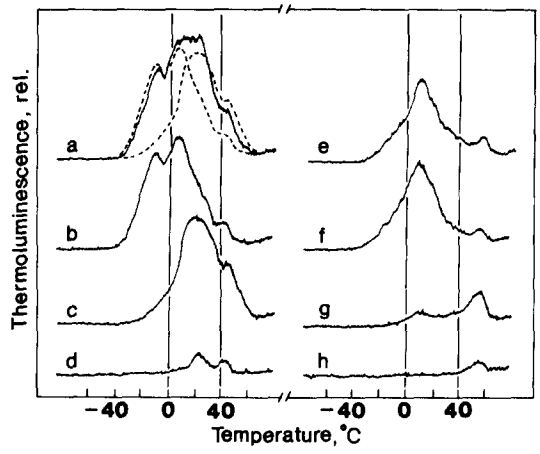

Fig. 2. TL glow curves ($S_2Q_A^-$ charge pair) of NaCl/EDTA-washed PS II membranes in the presence of 20 μM DCMU. Untreated control PS II membranes (right panel) and NaCl/EDTA-washed PS II membranes (left panel). One flash excitation at −10°C was followed by continuous illumination at 77 K for 1 min (a, e); one flash excitation at −10°C (b, f); continuous illumination at 77 K for 1 min without preceding flash illumination (c, g); no illumination (d, h). Broken glow curves on (a) are the reproductions of curves (b) and (c) in the same figure.

If we assume that this dark-stable EPR signal represents an oxidation state of the Mn cluster analogous to the normal S_2 state, a TL emission can be expected when we make this oxidized equivalent recombine with an electron on the acceptor side of PS II. As shown in Fig. 2, the untreated control PS II showed the TL Q-band arising from $S_2Q_A^-$ recombination [28] after a flash at −10°C in the presence of DCMU. But this Q-band was not generated by continuous illumination at 77 K, since S_1 to S_2 transition did not occur at 77 K [29,30]. In NaCl/EDTA-washed PS II, however, a TL band was generated by 77 K illumination without any preceding flash illumination. This is interpreted that 77 K illumination delivered one electron from cytochrome *b*-559 to Q_A [28], and a charge recombination occurred between the newly generated Q_A^- and the dark-stable S_2

which had been stably stored in NaCl/EDTA-washed PS II. Notably, this TL band was upshifted in its peak temperature, suggesting a decreased oxidation potential of the positive charge (the dark-stable S_2 state) [31,32]. The decreased oxidation potential may be correlated to the abnormally stable property of the dark-stable S_2. This TL emission did not occur unless the 77 K illumination was given. Obviously, no recombination is possible because of the absence of a reduced quinone acceptor as the negative counterpart for the dark-stable S_2, a positive equivalent.

Interestingly, flash excitation of the NaCl/EDTA-washed PS II could induce another TL band. This band showed almost the same peak temperature as that of normal Q-band ($S_2Q_A^-$) of untreated control PS II except for a broad shoulder below 0°C. A sharp dip around 0°C is ascribed to the change in heating rate due to melting of ice: the dip was prevented by inclusion of 30% ethylene glycol in the sample medium (data not shown). When the flash excitation was followed by 77 K illumination, the resulting TL band roughly overlapped on the imaginary envelope of the two independently induced TL bands, one induced by continuous illumination at 77 K and the other by flash illumination at −10°C. It seems that about 80% of the TL arising from the dark-stable S_2 is lost and replaced by the flash-induced TL. This result leads us to assume that these two TL bands originate from two different, distinguishable redox components both residing in the oxidizing side of the same PS II unit: if the two different O_2 centers are independently responsible for the respective two TL bands, the resulting glow curve will not be restricted within the envelope, but will be a simple sum of the two independent emissions. The partial survival of the TL component corresponding to the dark-stable S_2 after the flash implies that a portion (less than 20%) of the Q_A acceptor existed as its oxidized form even after the flash excitation at −10°C and was converted to Q_A^- by 77 K illumination. This is probably due to a rapid charge recombination between the flash-induced Q_A^- and the positive charge on the new EPR component, which would have occurred during cooling to 77 K. This is reasonably expected from the low peak temperature of the flash-induced TL component [31,32]. When NaCl/EDTA-washed PS II was supplemented with exogenous Ca^{2+}, no TL band was induced by 77 K illumination, but a normal Q-band ($S_2Q_A^-$) was induced by flash illumination as was observed in control PS II (see Fig. 7). Likewise in NaCl-washed (without EDTA) PS II, no TL band was induced by 77 K illumination, but a normal Q-band was induced by flash illumination (data not shown).

Fig. 3 shows the stability of the dark-stable S_2. The NaCl/EDTA-washed PS II membranes were incubated at 20°C in darkness for varying periods, and the amount of dark-stable S_2 remaining was estimated from the

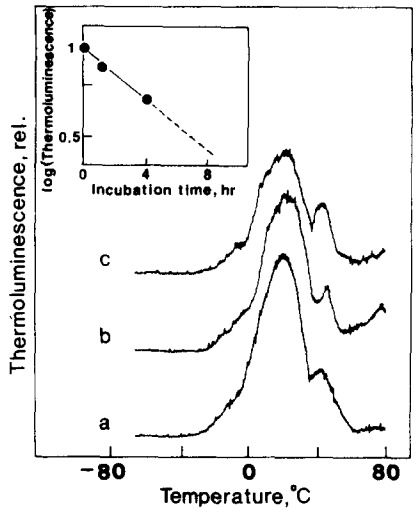

Fig. 3. Decay course of dark-stable S_2 state as measured by TL. NaCl/EDTA-washed PS II membranes were incubated at 20°C in darkness for varying periods, and then excited by continuous illumination at 77 K for 1 min for TL measurement. Inset: Semilogarithmic plot of the decay course. Broken line indicates extrapolation. DCMU (10 μM) was added immediately before TL measurements.

amplitude of the TL band induced by 77 K illumination. As expected from the observation that the EPR multiline signal could be found in the sample dark-adapted for more than 3 h at 0°C (see Figs. 1 and 2), the dark-stable S_2 was abnormally stable compared with the normal S_2 which decayed with a half decay time of approx. 1 min at 25°C [33]: even after a 4 h dark incubation at 20°C, approx. 70% of the signal was still preserved. The half decay time estimated was as large as 7 h at 20°C as shown by extrapolation of the semilogarithmic plot (Fig. 3, insert).

In Fig. 4, the temperature dependence for charging the new TL band additionally induced by flash excitation of NaCl/EDTA-washed PS II was compared with that of normal S_2 formation in untreated control PS II. In control PS II, the TL intensity showed a constant

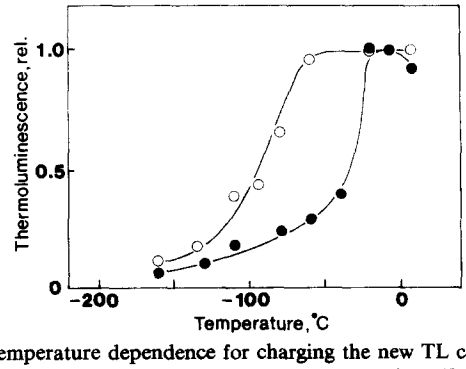

Fig. 4. Temperature dependence for charging the new TL component induced by flash excitation of NaCl/EDTA-washed PS II. One flash illumination of NaCl/EDTA-washed PS II membranes (●); one flash illumination of untreated PS II membranes (○). Samples were excited in the presence of 10 μM DCMU at varying temperatures and the amplitude of glow peak was plotted against excitation temperature.

maximum level when excited above $-60\,^{\circ}C$, and decreased gradually with lowering the excitation temperature to a very low level at around $-150\,^{\circ}C$, showing a half-inhibition temperature at around $-90\,^{\circ}C$ which agrees with the reported threshold temperature of S_2 formation [29,30]. In contrast, the new TL band showed a different temperature dependency; the band height became sharply suppressed below $-20\,^{\circ}C$, showing a half-inhibition temperature at around $-40\,^{\circ}C$, and this sharp decrease was followed by a gradual decrease down to around $-120\,^{\circ}C$. Presumably, the gradual decrease is due to the non-inhibited O_2 centers remaining in the washed sample (less than 15%). Judging from this difference in temperature dependence, the positive charge for this new TL component is not the normal S_2 state, although its peak temperature and shape of glow peak are similar to those of normal Q-band ($S_2Q_A^-$) as shown in Fig. 2. It is worthwhile to note that this temperature dependency resembles that for the transition from S_2 to S_3 in untreated membranes [29,30].

The origin of this new TL component was further studied by means of low-temperature EPR. The washed PS II retaining the dark-stable S_2 multiline signal was further exposed to continuous light at two different temperatures. As shown in Fig. 5A, the amplitude of the dark-stable multiline signal was not much changed by the continuous illumination either at -5 or at $-60\,^{\circ}C$. However, a new EPR signal was additionally induced between 3100 and 3500 G after illumination at $-5\,^{\circ}C$, although the whole feature of this new signal was difficult to be clearly recognized in this spectrum because of interference by Signal II. When illuminated at $-60\,^{\circ}C$, the new signal was much smaller (approx. 30%), and instead, photooxidation of cytochrome *b*-559 was clearer. This suggests that the new signal is attributable to a redox component which donates electron to Z^+ in competition with high potential cytochrome *b*-559. As shown in Fig. 5B, subtraction of dark spectrum from the light spectrum revealed a characteristic feature of the new signal, a broad, derivative-shaped signal centering at around $g = 2$. The feature of the new signal was more clearly seen when the microwave power was increased to a level at which both the multiline and Signal II were saturated. A $g = 1.9$ and 1.82 FeQ_A^- signal was also markedly induced under these conditions, indicating that an electron was delivered to Q_A from the donor side of PS II, although this illumination did not induce any change in the intensity of the dark-stable multiline signal. The untreated control and Ca^{2+}-supplemented NaCl/EDTA-washed PS II did not exhibit this new signal either after -5 or $-60\,^{\circ}C$ illumination (data not shown). Apparently, the spectral feature and temperature dependency for formation of this signal are similar to those reported by Boussac et al. [20,21] in which reconstitution of 16 and 24 kDa extrinsic proteins after NaCl wash is claimed to be required for observing both the two EPR signals.

As shown in Figs. 1 and 2, the dark-stable S_2 was not found when Ca^{2+} was externally added. This quenching by Ca^{2+} may be explained in two ways: (a) Ca^{2+} converts the dark-stable S_2 to normal S_2 which decays rapidly in darkness; (b) Ca^{2+} directly stimulates the

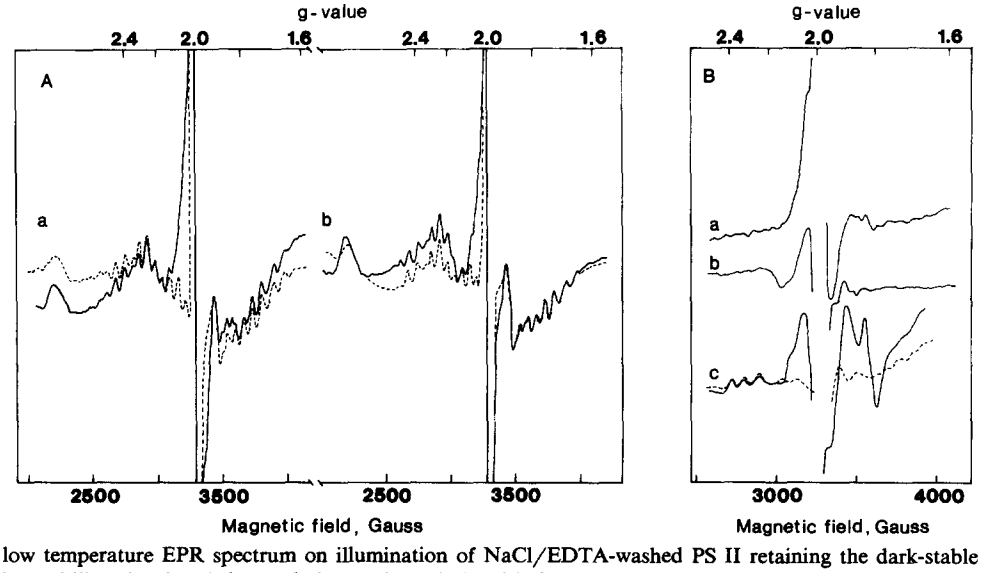

Fig. 5. Changes in low temperature EPR spectrum on illumination of NaCl/EDTA-washed PS II retaining the dark-stable multiline signal. (A) Whole features of the multiline signal and the newly induced $g = 2$ signal before (broken curves) and after (solid curves) illumination. Left panel (a), at $0\,^{\circ}C$ for 30 s; right panel (b), at $-60\,^{\circ}C$ for 2 min. (B) Light-minus-dark difference spectra of the newly induced $g = 2$ signal: (a) difference before and after illumination at $0\,^{\circ}C$ for 30 s (cf. A, left panel); (b) difference before and after illumination at $-60\,^{\circ}C$ for 2 min (cf. A, right panel); (c) the same spectra as for (a) measured with a higher microwave power of 100 mW before (broken) and after (solid) illumination at $0\,^{\circ}C$ for 30 s. Chl concentration was 3.5 mg Chl/ml. DCMU (50 μM) was added. Instrumental conditions are the same as those in Fig. 1 except for the two spectra in (B) c.

decay of the dark-stable S_2. Fig. 6 shows the effect of Ca^{2+} addition on the dark-stable EPR signal, in which the NaCl/EDTA-washed sample exhibiting the dark-stable multiline signal, was warmed to 0 °C, added with Ca^{2+} in darkness and quickly re-frozen, and then EPR signal was recorded. The dark-stable and normal multiline signals can be characterized by their respective typical superfine lines indicated by downward and upward arrows (Fig. 6A). On addition of Ca^{2+}, the lines with downward arrows diminished concomitantly with development of the lines with upward arrows, as a consequence, the resulting spectrum considerably resembled the normal multiline spectrum. The amplitude of the Ca^{2+}-supplemented signal amounted to 70–80% of that of the normal signal induced by 210 K illumination in untreated control PS II. This indicates that the dark-stable signal arose from the major population of the Mn cluster in NaCl/EDTA-washed PS II. Similarly, the effect of quick addition of Ca^{2+} on TL glow curve was examined (Fig. 6B). The abnormal TL band with an elevated peak temperature due to the dark-stable S_2 was transformed to a band showing a lower peak temperature similar to that of normal Q-band ($S_2Q_A^-$),

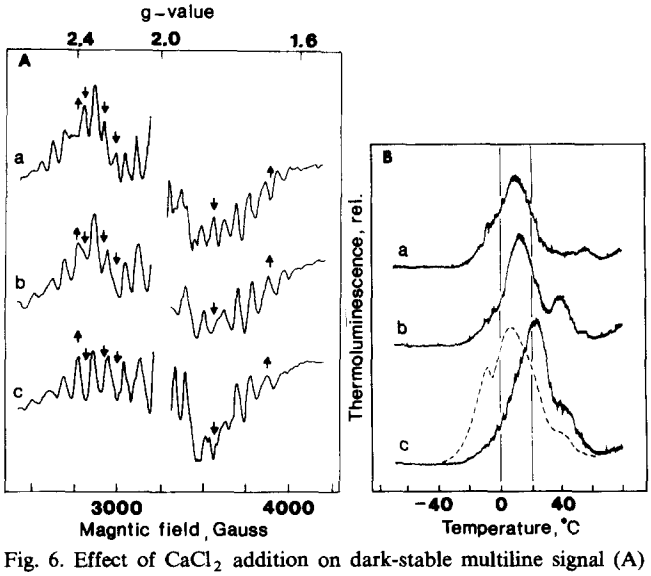

Fig. 6. Effect of $CaCl_2$ addition on dark-stable multiline signal (A) and TL glow curves (B). (A): NaCl/EDTA-washed PS II membranes, non-illuminated (a); NaCl/EDTA-washed PS II membranes supplemented with $CaCl_2$, non-illuminated (b); untreated control PS II membranes, illuminated for 2 min at 210 K (c). For (b), NaCl/EDTA-washed membranes in a sample tube were warmed in darkness to 0 °C, added with 20 mM $CaCl_2$, and then refrozen in liquid N_2 within 55 s. Chl concentration was 3.5 mg Chl/ml. Instrumental settings are the same as those in Fig. 1. (B): Untreated control PS II membranes were excited by 1 flash at 0 °C (a); NaCl/EDTA-washed PS II membranes were supplemented with $CaCl_2$ excited by 77 K illumination for 1 min (b); NaCl/EDTA-washed PS II membranes were excited by 77 K illumination for 1 min (c, solid curve) or by 1 flash at 0 °C (c, broken curve). For (b), the washed membranes were supplemented with 20 mM $CaCl_2$, incubated in darkness at 0 °C for 40 s, and then frozen in liquid N_2. DCMU (10 μM) was added immediately before freezing.

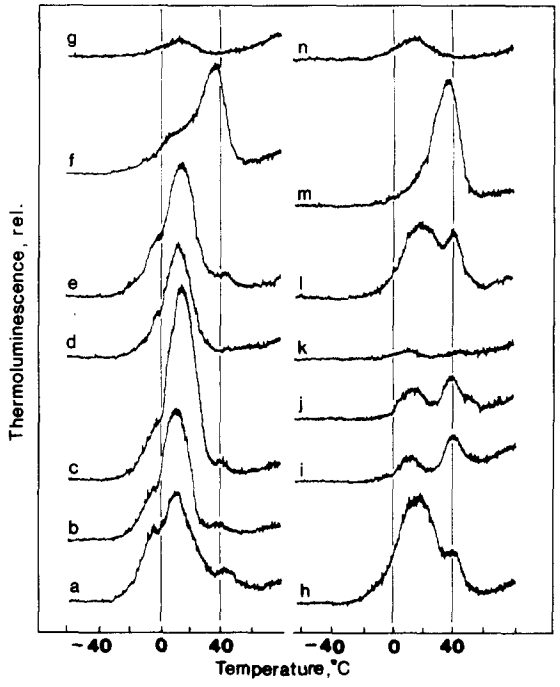

Fig. 7. Effects of various divalent cations on TL glow curves of NaCl/EDTA-washed PS II membranes. No addition (a, h), additions of $CaCl_2$ (b, i), $SrCl_2$ (c, j), $CdCl_2$ (d, k), $MgCl_2$ (e, l), $BaCl_2$ (f, m) and $MnCl_2$ (g, n). NaCl/EDTA-washed PS II membranes were supplemented with 20 mM of respective divalent salts, incubated at 0 °C for 30 min, and then excited by one flash at 10 °C (left panel) or by continuous illumination at 77 K for 1 min (right panel). DCMU (10 μM) was added.

indicative of reconstitution of normal S_2 by the addition of Ca^{2+}. Both these EPR and TL data indicate that the dark-stable S_2 in NaCl/EDTA-washed PS II is easily converted by Ca^{2+} to normal S_2, the mechanism (a) in the above-mentioned two alternatives.

Fig. 7 shows the effects of various cations on TL bands in the washed PS II membranes. As depicted in the right panel, NaCl/EDTA-washed PS II showed the TL band arising from recombination of dark-stable S_2 with Q_A^- at around 20 °C after 77 K illumination (see also Fig. 2). On addition of Ca^{2+}, Sr^{2+}, Cd^{2+} or Mn^{2+}, the glow peak of this band was markedly suppressed, leaving a small peak at around 40 °C in cases of Ca^{2+} and Sr^{2+}. In contrast, Mg^{2+} and Ba^{2+} did not show such quenching, but rather upshifted the peak temperature to 35 °C in case of Ba^{2+}. As already indicated by Fig. 6 experiments, the quenching effect by Ca^{2+} is due to reconstitution of normal S_2. By analogy, we may assume that a normal or an abnormal S_2 but with nearly normal oxidation potential was reconstituted by Sr^{2+} and Cd^{2+}. In this context, it is interesting to note that the upshifted TL peak arising from the abnormal S_2 formed in low-pH-treated PS II is converted by Ca^{2+}, Sr^{2+} or Cd^{2+} to an ordinary peak suggesting a normal oxidation potential of S_2, although the degree of restoration of O_2 evolution differs very much among the

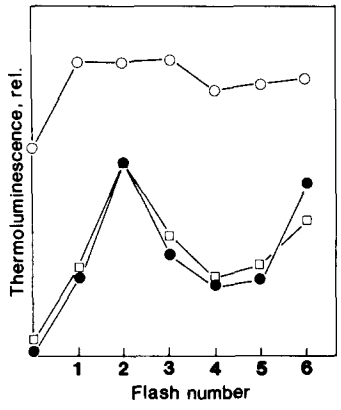

Fig. 8. Oscillation of TL from NaCl/EDTA-washed PS II membranes on illumination with a series of flashes. (●) untreated control PS II membranes; (○) NaCl/EDTA-washed PS II membranes; (□) NaCl/EDTA-washed membranes supplemented with $CaCl_2$. $CaCl_2$ (20 mM) was added to washed membranes followed by 30 min dark incubation at 0 °C. Dark-adapted memranes were excited by a series of flashes at 0 °C followed by 1 min continuous illumination at 77 K and total luminescence (integration of the area under the glow curve) was plotted as a function of flash number. DCMU (20 μM) was added immediately after flash excitation.

three cations [34]. In contrast to these, the glow curve arising from the new redox component (induced by flash excitation) was not much affected by the addition of Ca^{2+}, Sr^{2+}, Cd^{2+} and Mg^{2+}, although Sr^{2+} enhanced its amplitude and Ba^{2+} upshifted its peak temperature (left panel). Unlike these cations, the addition of Mn^{2+} resulted in strong suppression of both of the two TL components. This suppression could also be observed when Mn^{2+} was added immediately after flash excitation (data not shown). Presumably, Mn^{2+} binds to the Ca^{2+}-binding site, simultaneously approaches to the positive equivalent stabilized on the Mn cluster as dark-stable S_2 and neutralizes it by electron donation, as previously suggested for low-pH-treated PS II [34]. Incubation of untreated PS II with these cations did not induce any changes in TL peak temperature (data not shown).

Fig. 8 shows the flash pattern of TL band. Samples were illuminated with a series of flashes followed by DCMU addition and then by 77 K illumination for 1 min. In this protocol the negative charge on Q_A^- is provided by one electron delivery from cytochrome *b*-559 in all the PS II centers [28], so that the changes in TL amplitude can be a priori ascribed to the difference in the amount of positive charges in the Mn cluster. Untreated PS II showed typical period-four pattern with maxima at 2nd and 6th flashes as previously reported [26,28]. In NaCl/EDTA-washed PS II, an intense TL band arising from dark-stable S_2 was observed after 77 K illumination without any preceding flash illumination (flash number = 0). When one flash is given, the TL intensity was enhanced due to the additionally induced new TL band. However, the intensity of this composite band did not much change when illuminated with more flashes, indicating that neither the dark-stable nor the flash-induced bands show any flash dependency. On addition of Ca^{2+}, a normal period-four oscillation was reconstituted. These results suggest that the interruption of S-state advancement after the first flash is the main cause for the loss of O_2 evolution in NaCl/EDTA-washed PS II.

Discussion

The present study clearly showed that an abnormally stable S_2, modified EPR S_2 multiline signal, can be detected in NaCl/EDTA-washed PS II without illumination, and that Ca^{2+} addition reverses this abnormal signal to normal in complete darkness. Based on the finding that the amplitude of thus reconstituted signal amounted to 70–80% of that of normal signal induced in untreated control PS II, we may assume that the major population of the O_2 centers in NaCl/EDTA-washed PS II retains positive equivalent as a dark-stable form. Two alternative explanations may be possible for this phenomenon: (i) conformational structure of the Mn cluster is modified in NaCl/EDTA-washed PS II to make S_2 state more stable than S_1 state, in other words, S_1 is spontaneously oxidized in darkness by a bulk oxidant to yield S_2; (ii) an abnormal S_2 is formed from S_1 by the illumination during NaCl/EDTA wash, and this abnormal S_2 state remains after the treatment because of its abnormally high stability. We prefer the second possibility based on the facts that (a) the recombination between Q_A^- and the dark-stable S_2 occurs at an abnormally high temperature as shown in Fig. 2, and that (b) the dark-stable S_2 shows appreciable deactivation, although its rate is very slow in the absence of Q_A^- (see Fig. 3). We cannot, however, exclude the possibility that a portion of dark-stable S_2 is in equilibrium with S_1 state.

As shown in Fig. 2, an additional TL component was generated when the PS II retaining the dark-stable S_2 were further excited by a flash at room temperature. The TL features of this new component indicated that the positive charge responsible for this TL emission is trapped on a redox component in the same O_2 center as that exhibiting the dark-stable multiline signal. The temperature dependence for charging this TL component, furthermore, resembled that for S_2-to-S_3 transition [29,30]. At a simple glance, these appear to suggest that the dark-stable S_2 is transformed to S_3 to exhibit the flash induced TL component. However, this does not seem to be the case. The appreciable decrease in the TL component (emitting at 20–30 °C) arising from the dark-stable multiline signal as the positive counterpart is caused, in this case, by the significant loss in Q_A^- amount due to recombination (at around 0 °C) with another positive counterpart, the newly photoinduced

$g = 2$ EPR signal. Note that under the present conditions, one equivalent of negative charge (Q_A^-) shares two equivalents of positive charges in the same center, dark-stable multiline and the new $g = 2$ EPR component. This interpretation is clearly consistent with the observation that the amplitude of the dark-stable multiline signal did not change after illumination at $-5\,^\circ$C. Based on these considerations, the new TL component cannot be attributed to S_3 state. Whichever will be the case, we are forced to assume that the positive charge responsible for this TL band is stored on a redox component other than the Mn cluster, since almost all the Mn centers in NaCl/EDTA-washed PS II have been already stabilized as the dark-stable S_2.

An EPR signal was found to be photoinduced in NaCl/EDTA-washed PS II (Fig. 5). This broad signal at around $g = 2.0$ was efficiently induced by illumination at $-5\,^\circ$C, but much less at $-60\,^\circ$C. Notably, this signal could not be detected when Ca^{2+} was present. The good parallelism between this EPR signal and the flash-induced TL component led us to presume that the species responsible for this EPR signal is the positive counterpart for the new TL component.

Chemical entity of this new redox component is unclear at present. However, we can preclude Z^+ and D^+ from possible candidates, because they are completely different in their temperature dependence for formation and in EPR spectrum [35]. We may also preclude cytochrome *b*-559, since its oxidized form does not recombine with reduced quinones to emit TL (see Fig. 2). Padhye et al. [36] have proposed that an imidazole moiety of histidine residues can provide a redox-active ligand in place of Mn, and Tamura et al. [37] have suggested the involvement of histidine residue(s) of D1 protein as a ligand for Mn. Thus, oxidized cation radical of histidine could be one of the possible candidates for this redox component to exhibit the new EPR signal and the new flash-induced TL component. There may be two interpretations for such redox component: (i) the component is located on the main path of S-state transition, or (ii) an auxiliary component will be oxidized when the main path is impaired. As to the former interpretation (i), Boussac et al. proposed that this oxidant will be the formal S_3 state [21]. If we extend their speculation by taking into account the observation that the temperature dependence of oxidation of this component resembles that of S_2-to-S_3 transition, this component might be a precursor state for S_3. A speculative entity of such precursor could be a state in which electron abstraction from S_2 is completed but proton release is blocked. Presumably, the H^+-release accompanying the S_2-to-S_3 transition was interrupted in the NaCl/EDTA-washed PS II, and thereby further transition to normal S_3 was interrupted. However, in view of our observations that the amplitude of dark-stable multiline signal does not decrease on illumination, and that Ca depletion by low-pH treatment results in interruption of S_2 to S_3 transition [5], the latter interpretation (ii) is also possible: histidine oxidation is an auxiliary reaction which is manifested upon blocking of the S_2 to S_3 transition.

Ca^{2+} is clearly required for normal S-state transition as reported and discussed above, but the mechanism of Ca^{2+} action is not clear. The fact that the redox properties of both the dark-stable and flash-induced TL components were variously modified depending on the cation species (Fig. 7) seems to suggest that cation substitution at the putative Ca^{2+}-binding site causes these modifications. Of particular interest among these is Sr^{2+} substitution, which modifies the fine structure of multiline signal in a way similar to that found for the dark-stable multiline signal, although the S_2 in Sr^{2+}-substituted centers is not dark-stable (Fig. 7). This may imply that a moiety of Mn cluster responsible for the modification of the multiline signal is not directly related with its function in water oxidation which can be affected by NaCl wash. Our present results appear consistent with a view that NaCl/EDTA wash removes functional Ca^{2+} atom from PS II. However, the exact number of Ca^{2+} in PS II or the number of Ca atoms removed by NaCl wash is a matter of debate, and two contradictory results have been reported: loss of 1 Ca atom [7], or no change in Ca content [8]. In this relation, we note that the protocols of NaCl wash and/or Ca determination used in some of previous studies are not always compatible with the stringent treatment employed in this study. Thus, the conclusion has to await a more exact Ca determination with the NaCl/EDTA-washed PS II membranes that are prepared according to the protocol as we used in this study.

Our present results are significantly different from those reported by Boussac and Rutherford [18] who observed normal multiline signal by 210 K illumination in PS II membranes which were treated with NaCl under room light followed by addition of 50 μM EGTA. The contradictory observation in this case will possibly be due to incomplete Ca^{2+} depletion in Ref. 18 as already discussed in Ref. 24. Our results also differ significantly from those more recently reported by Boussac et al. [21]. The differences have mostly been mentioned in the above discussions, but will be summarized again as follows: (i) they claim that reconstitution of 16 and 24 kDa extrinsic proteins is closely related with the generation of dark-stable multiline signal and $g = 2$ signal, while we consider, as clearly shown in this study, that the crucial factor required for generation of both signals is not reconstitution of the extrinsic proteins, but rather the complete elimination of free Ca^{2+} by excess chelator; (ii) they claim that illumination of dark-stable S_2 at $0\,^\circ$C results in a loss of the dark-stable multiline signal concomitant with formation of the new $g = 2$ signal, while we observed that

the amplitude of the dark-stable multiline signal does not change after illumination. At present, it is difficult to reconcile the two contradictory results. Obviously, these are the points that should be clarified in future studies.

Acknowledgments

We would like to thank Dr. A. Boussac and Dr. A.W. Rutherford for valuable discussion at the meeting, and sending us their preprints. This study 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 (STA) of Japan, and partly by MESC Grants-in-Aid for Cooperative Research No. 1032064 and 01300009.

References

1 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130.
2 Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120.
3 Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286.
4 Ono, T. and Inoue, Y. (1988) FEBS Lett. 227, 147–152.
5 Ono, T. and Inoue, Y. (1989) Biochim. Biophys. Acta 973, 443–449.
6 Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
7 Cammarata, K. and Cheniae, G.M. (1987) Plant Physiol. 84, 587–595.
8 Shen, J.-R. Satoh, K. and Katoh, S. (1988) Biochim. Biophys. Acta 933, 358–364.
9 Kashino, Y., Satoh, K. and Katoh, S. (1986) FEBS Lett. 205, 150–154.
10 Satoh, K. and Katoh, S. (1985) FEBS Lett. 190, 199–203.
11 Ono, T. and Inoue, Y. (1986) Biochim. Biophys. Acta 850, 380–389.
12 Miyao, M. and Murata, N. (1986) Photosynth. Res. 10, 489–496.
13 Boussac, A., Maison-Peteri, B., Etienne, A.-L. and Vernotte, C. (1985) Biochim. Biophys. Acta 808, 231–234.
14 Boussac, A. and Rutherford, A.W. (1988) FEBS Lett. 236, 432–436.
15 Blough, N.V. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 377–381.
16 De Paula, J.C., Li, P.M., Miller, A.-F., Wu, B.W. and Brudvig, G.W. (1986) Biochemistry 25, 6487–6494.
17 Boussac, A., Maison-Peteri, B., Vernotte, C. and Etienne, A.-L. (1985) Biochim. Biophys. Acta 808, 225–230.
18 Boussac, A. and Rutherford, A.W. (1988) Biochemistry 27, 3476–3483.
19 Miyao, M. and Murata, N. (1985) FEBS Lett. 180, 303–308.
20 Boussac, A., Zimmermann, J.-L. and Rutherford, A.W. (1990) Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 713–716, Kluwer, Dordrecht.
21 Boussac, A., Zimmermann, J.-L. and Rutherford, A.W. (1989) Biochemistry 28, 8984–8989.
22 Sivaraja, M., Tso, J. and Dismukes, G.C. (1989) Biochemistry 28, 9459–9464.
23 Baumgarten, M., Tso, J., Marino, T., Sivaraja, M., Lin, C.P. and Dismukes, G.C. (1990) Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 953–956, Kluwer, Dordrecht.
24 Ono, T. and Inoue, Y. (1990) Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 741–744, Kluwer, Dordrecht.
25 Ono, T. (1989) Abstracts of the 15th Annual Midwest Photosynthesis Conference (Davis, D., Togasaki, R.K. and Yocum, C.F., eds.), p. 1.
26 Ono, T. and Inoue, Y. (1988) Arch. Biochem. Biophys. 264, 82–92.
27 Dismukes, G.C. and Siderer, Y. (1980) FEBS Lett. 121, 78–80.
28 Rutherford, A.W., Crofts, A.R. and Inoue, Y. (1982) Biochim. Biophys. Acta 682, 457–467.
29 Inoue, Y. and Shibata, K. (1978) FEBS Lett. 85, 193–197.
30 Styring, S. and Rutherford, A.W. (1988) Biochim. Biophys. Acta 933, 378–387.
31 Vass, I., Horvath, G., Herczeg, T. and Demeter, S. (1981) Biochim. Biophys. Acta 682, 140–152.
32 De Vault, D., Govindjee and Arnold, W. (1983) Proc. Natl. Acad. Sci. USA 80, 983–987.
33 Ono, T. and Inoue, Y. (1985) Biochim. Biophys. Acta 806, 331–340.
34 Ono, T. and Inoue, Y. (1989) Arch. Biochem. Biophys. 275, 440–448.
35 Koike, H., Siderer, Y., Ono, T. and Inoue, Y. (1986) Biochim. Biophys. Acta 850, 80–89.
36 Padhye, S., Kanbara, T., Hendrickson, D.N. and Govindjee (1986) Photosynth. Res. 9, 103–112.
37 Tamura, N., Ikeuchi, M. and Inoue, Y. (1989) Biochim. BIophys. Acta 973, 281–289.