

Factors influencing the formation of modified S_2 EPR signal and the S_3 EPR signal in Ca^{2+} -depleted photosystem II

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NaCl/EGTA-washing of photosystem II (PS-II) results in the removal of Ca^{2+} and the inhibition of oxygen evolution. Two new EPR signals were observed in such samples: a stable and modified S_2 multiline signal and an S_3 signal [(1989) *Biochemistry* 28, 8984–8989]. Here, we report what factors are responsible for the modifications of the S_2 signal and the observation of the S_3 signal. The following results were obtained. (i) The stable, modified, S_2 multiline signal can be induced by the addition of high concentrations of EGTA or citrate to PS-II membranes which are already inhibited by Ca^{2+} -depletion. (ii) The carboxylic acids act in the S_3 -state, are much less effective in S_2 and have no effect in the S_1 -state. (iii) The extrinsic polypeptides (17- and 23-kDa) are not required to observe either the modified S_2 signal or the S_3 signal. However, they do influence the splitting and the lifetime of the S_3 signal, and they seem to have a slight influence on the hyperfine pattern of the S_2 signal. (iv) The S_3 signal can be observed in Ca^{2+} -depleted PS-II which does not exhibit the modified multiline signal. Then, it is proposed that formation of histidine radical during the S_2 to S_3 transition in Ca^{2+} -depleted PS-II [(1990) *Nature* 347, 303–306] also occurs in functional PS-II.

Photosynthesis; Photosystem II; Oxygen evolution; Ca^{2+} ; Water oxidase; Electron paramagnetic resonance

1. INTRODUCTION

Photosystem II catalyses light-driven water oxidation resulting in oxygen evolution. A cluster of 4 Mn located in the reaction centre of PS-II probably acts both as the active site and as a charge accumulating device of the water-splitting enzyme (reviewed in [1]). During the enzyme cycle the oxidizing side of PS-II goes through five different redox states that are denoted S_n , n varying from 0 to 4 [2]. Three extrinsic polypeptides are bound to the PS-II reaction centre on the inside of the thylakoid membrane. Two of these, the 17- and 23-kDa polypeptides, can be removed by NaCl-washing (reviewed in [3]). In the absence of these polypeptides, the Mn cluster is susceptible to attack by exogenous reducing agents leading to dissociation of the Mn from the enzyme [4]. Removal of these polypeptides results also in inhibition of oxygen evolution due to an increased requirement for chloride and calcium ions (reviewed in [5]).

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Abbreviations: PS-II, photosystem II; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; Mes, 2-(N -morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PPBQ, phenyl- p -benzoquinone; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea

Studies on the role of Ca^{2+} in oxygen evolution have led to controversies in the literature. These were mainly due to differences in the protocol used for Ca^{2+} -depletion [6]. Our results indicate that in NaCl-washed material, inhibition of the enzyme cycle occurs at the S_3 to S_0 transition [6,7]. A model was proposed in which a single Ca^{2+} binding site is associated with O_2 evolution but the Ca^{2+} binding affinity of the site varies with the intermediate state of the enzyme cycle (i.e. the S -state) [6]. In support of this model it was found that the effectiveness of Ca^{2+} -depletion by salt-washing done in each of the S -states was as follows: $S_3 > S_0 \approx S_2 > S_1$ [8].

Recently we developed a new salt-washing procedure in which PS-II membranes were depleted of Ca^{2+} and the 17- and 23-kDa polypeptides in the presence of high concentration of EGTA. Both polypeptides were then reconstituted. This preparation was incapable of O_2 evolution until Ca^{2+} was added. An EPR study revealed the presence of two new EPR signals. One of these is a modified S_2 -multiline signal which arises from a stable S_2 -state [9].

The other is an EPR signal attributed to S_3 which could be formed by continuous illumination [9] or by flash illumination [10]. Here, we demonstrate that this signal can be formed in Ca^{2+} -depleted preparations that do not exhibit the modified multiline. From earlier work we conclude that the charge stored in this S_3 -state can be used in O_2 evolution. The S_3 signal was suggested to arise from an organic free radical interacting

magnetically with the Mn-cluster [9,10] and from its UV spectrum was attributed to oxidized histidine [10].

However, in earlier work [6], in which Ca^{2+} -depletion resulted in an up to 80% inhibition of O_2 evolution, the S_2 -state was unmodified and the S_3 -state had not been detected. The factors responsible for the modification of the multiline signal and the ability to detect the S_3 signal remained unclear although it was suggested that the 17- and 23-kDa polypeptides and the high concentration of chelator could be involved [9].

Using different inhibitory treatments, both designed to remove Ca^{2+} , two other groups observed a modified S_2 -multiline signal. First, Ono and Inoue [11] used a NaCl-washing procedure in the presence of a high concentration of EDTA but without reconstitution of the 17- and 23-kDa polypeptides. Second, Sivaraja et al. [12] used a low pH-washing procedure buffered with citrate as first reported by Ono and Inoue [13]. This procedure led to Ca^{2+} -depletion without the removal of the extrinsic polypeptides. In this preparation, these authors [12] also detected a somewhat poorly resolved signal similar to the one we had earlier attributed to S_3 .

In this work, we looked for the factors required for the modifications of the S_2 -state and the observation of the S_3 signal using PS-II membranes already Ca^{2+} -depleted by the original procedure (a NaCl-washing in the light, in the presence of a low concentration of EGTA [6]).

2. MATERIALS AND METHODS

Photosystem II particles from spinach chloroplasts were prepared according to the method of [14] with the modifications of [15] and were stored at -80°C until used. The activity of these membranes was $\approx 600 \mu\text{M O}_2/\text{mg Chl} \cdot \text{h}$. NaCl-washing was done as in [6]. The PS-II membranes were incubated for 30 min in room light at 4°C at $\approx 0.5 \text{ mg of Chl/ml}$ in 1.2 M NaCl, 0.3 M sucrose and 25 mM Mes, pH 6.5. Then 50 μM ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) was added and the NaCl-washed PS-II particles were pelleted by 15 min centrifugation at $40\,000 \times g$, washed once in 30 mM NaCl, 25 mM Mes, pH 6.5 and 50 μM EGTA, pelleted again and resuspended at 8–12 mg Chl/ml in the same medium. For EPR measurements the samples were put into calibrated quartz tubes and, after 30 min dark adaptation on ice, when indicated, PPBQ dissolved in dimethyl sulfoxide, was added as electron acceptor to a final concentration of 1 mM.

EPR spectra were recorded at helium temperatures with a Bruker ESR 200 X-band spectrometer equipped with an Oxford Instruments cryostat. The samples were illuminated in a non-silvered Dewar flask in a solid CO_2 -ethanol bath at 198 K or in an ethanol bath cooled to 0°C with liquid nitrogen. In some experiments the sample was frozen under continuous illumination. In this case the temperature was between 0 and 10°C during illumination and the sample was directly cooled down to 77 K in liquid nitrogen. Illumination was done with a 800 W projector through infra-red filters.

3. RESULTS

It was pointed out earlier that high concentrations of EDTA [11] or EGTA [9] were required in order to generate the modified S_2 multiline signal. Fig. 1 shows

the effect of 10 mM EGTA added to salt-washed PS-II. Spectrum a shows the normal multiline signal generated by illumination at 198 K in a sample containing only 50 μM EGTA. As demonstrated previously such material exhibits the normal multiline signal, $\approx 80\%$ inhibition of oxygen evolution and an inhibition of the S-state cycle after S_3 formation [6]. The presence of 10 mM EGTA incubated in the dark (i.e. in the S_1 -state) had little effect (spectrum b). Dark incubation for 1 h with 10 mM EGTA had no further effect. The slight decrease ($\approx 20\%$) of the signal intensity was attributed earlier to a change in the signal saturation characteristics [6]. Under these conditions, no oxidation of a sidepath donor chlorophyll occurs. This chlorophyll is oxidized when the electron flow from the donor side of PS-II is inhibited [16].

It is known that continuous illumination of Ca^{2+} -depleted PS-II results in S-state advancement only as far as S_3 at room temperature [6,7,9] (see below). When the sample containing 10 mM EGTA was illuminated at 0°C for 2 min, the modified multiline signal was observed after a short dark-adaptation time and was stable in the dark for at least 1 h (Fig. 1, spec-

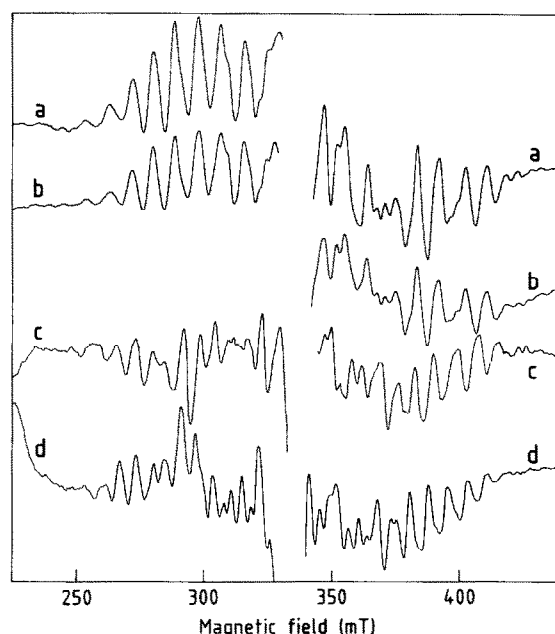


Fig. 1. Light minus dark spectra induced by a 198 K illumination in dark-adapted membranes further incubated for 30 min the dark with no addition (spectrum a), with 10 mM EGTA (spectrum b). PPBQ was added in each sample just before freezing. Spectrum c corresponds to the difference between the signal recorded after illumination followed by a further 60 min dark incubation at 0°C and the signal recorded on the same sample prior to the illumination. Illumination for spectrum c was done for 2 min at 0°C in the presence of PPBQ and 10 mM EGTA. Instrument settings: microwave frequency 9.44 GHz; modulation amplitude 20 G; temperature 10 K; microwave power 20 mW. Spectrum d is replotted from [9] and shows the stable, modified, multiline signal obtained in NaCl-EGTA-treated/polypeptide-reconstituted PS-II membranes (no normalisation of the amplitude of this signal versus the others).

trum c). In the presence of only 50 μ M EGTA no stable multiline signal was detected (not shown but see [6]). Fig. 1d shows the EPR spectrum of the stable modified S_2 multiline signal observed in Ca^{2+} -depleted/EGTA-treated membranes in which the 17- and 23-kDa polypeptides were reconstituted. In this spectrum additional features arise from the $g_z \approx 3$ and $g_y \approx 2.2$ of the oxidized cyt- b_{559} and perhaps from some contaminating Rieske protein ($g \approx 1.9$) which are all subtracted in the other spectra. Apart from these underlying differences some differences in the S_2 signal may be due to slight structural changes induced by the binding of the 17- and 23-kDa polypeptides.

The results in Fig. 1 show that the high concentration of EGTA in the presence of light is responsible for the modification of the S_2 multiline signal. However, Sivaraja et al. [12] apparently did not use chelators although a similar stable and modified S_2 multiline signal was reported after illumination of the sample at 4°C. It seemed possible that the use of a high concentration of citrate as a low pH buffer in this work might have had the same effect as EGTA. Fig. 2 shows that this is the case. Here again, the addition of citrate to a dark-adapted sample had no effect on the multiline signal generated by 198 K illumination (spectrum a) while an illumination for 2 min at 0°C resulted in the stable modified multiline signal (spectrum b). Citrate was used at pH 6.5. At this pH, citrate is unable to remove Ca^{2+} in untreated PS-II membranes [13]. Thus, it appears that EGTA and citrate have a common effect on Ca^{2+} -depleted PS-II, resulting in an increase in the stability of the S_2 -state and a modification of the shape of the multiline signal.

Amplitudes of spectrum c in Fig. 1 and spectrum b in Fig. 2 were approximately half that of the normal

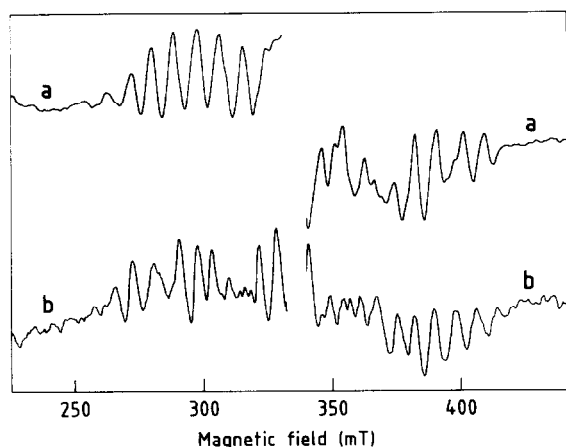


Fig. 2. The spectra correspond to the difference between the signal recorded after illumination and a further 60 min dark incubation at 0°C minus the signal recorded on the same sample prior to the illumination. Illumination was done for 2 min at 0°C in the presence of PPBQ and 10 mM EGTA (spectrum a) or 40 mM citrate (spectrum b). Instrument settings as in Fig. 1. In this figure the amplitude of the signal is multiplied by 2 in comparison to Fig. 1.

multiline signal. A further illumination at 0°C with DCMU present did not increase the amplitude of the multiline signal. This shows that the small signals are not due to deactivation of a part of the PS-II centres into the S_1 -state but more probably to the different splitting of the multiline signals giving an inherently smaller signal amplitude.

Illumination for 2 min at 0°C with 10 mM EGTA in the presence of DCMU instead of PPBQ gave rise to a small amount of stable and modified multiline signals (not shown). With DCMU only the S_1 to S_2 transition is allowed. Since illumination of salt-washed PS-II in the absence of DCMU allows S-state advancement only to the S_3 -state [6,7,9], this result indicates that EGTA (and citrate) preferentially act in the S_3 -state.

Using the same protocol, we were unable to generate the modified stable multiline signal with 200 mM acetate at pH 6.5 (not shown). This suggests the strict requirement for poly-carboxylic acids. Preliminary experiments using the rigid poly-carboxylic acids, phthalic or terephthalic acid, also had no effect (not shown). This may indicate that the modification of the multiline signal requires an interaction with poly-carboxylic acids in a given configuration obtained with flexible molecules such as EDTA, EGTA or citrate but not with poly-carboxy-benzoic acids.

In the NaCl-washed preparation which, as shown here, has been affected by the high concentration of

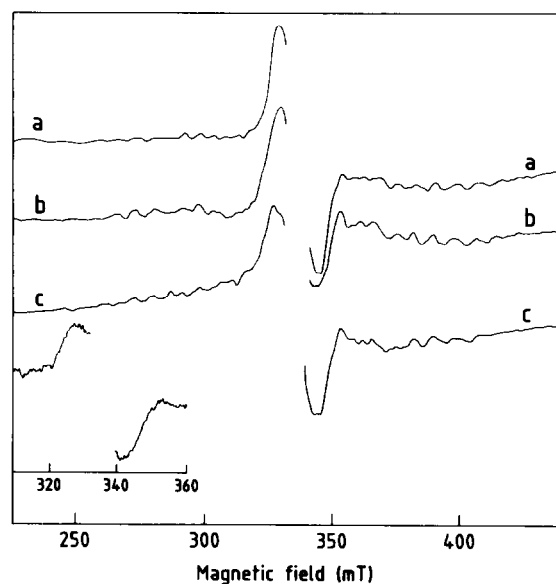


Fig. 3. The membranes were illuminated in the presence of PPBQ for 2 min at 0–10°C with 10 mM EGTA (spectrum a), 40 mM citrate (spectrum b) or with no addition (spectrum c). Then the samples were frozen under illumination directly in a 77 K bath. Instrument settings as in Fig. 1. Amplitudes of the signals were divided by 2 when compared with Fig. 1. The inset shows a better resolved spectrum of the light-induced signal around $g = 2$ in spectrum c. Instrument settings: temperature, 10 K; modulation amplitude, 3 G; microwave power, 2 mW.

EGTA or citrate in the light, we observed an S_3 EPR signal (Fig. 3) as in [9,10]. It is also shown that the S_3 EPR signal can be observed in the original Ca^{2+} -depleted preparation lacking the 17- and 23-kDa polypeptides when samples are frozen under continuous illumination. The S_3 signal was similar in the presence of 10 mM EGTA (spectrum a), 40 mM citrate (spectrum b) or 50 μM EGTA (spectrum c). The inset of Fig. 3 shows the S_3 signal of spectrum c with a better resolution. The width of the signal was 130 gauss. The appearance of the 130 gauss-wide signals was accompanied by the collapse of the multiline signals.

The S_3 signals are less well resolved in these preparations than in PS-II reconstituted with the 17- and 23-kDa polypeptides. This is due to the peak-to-trough width of the signal which is only 130 gauss compared to the 164 gauss reported earlier. The differences in the shape of the S_3 EPR signal shown in this report presumably reflect the influence of the 17- and 23-kDa polypeptides which were reconstituted in earlier work and not here. The S_3 -state seems also less stable. This agrees with an earlier report [7] and may reflect a protective role of the 17- and 23-kDa polypeptides against external reductants [4].

4. DISCUSSION

NaCl-washing [21] or citrate-washing [13] of PS-II results in depletion of 1 Ca^{2+} per PS-II centre. From this work it appears that Ca^{2+} -depletion is quite distinct from the situation generated by the subsequent addition of the carboxylic acids such as EGTA and citrate. When PS-II is depleted of Ca^{2+} it has the following properties: (i) the enzyme cycle is blocked after the formation of the S_3 -state [6,7,9], (ii) the S_2 -state exhibits the usual multiline signal [6], (iii) the S_3 EPR signal, attributed to an oxidized histidine radical interacting with the Mn-cluster [10], can be observed (this work). When, however, EGTA or citrate in high concentrations is added to the Ca^{2+} -depleted enzyme in the S_3 -state, the enzyme undergoes a further modification in that the S_2 -state becomes essentially stable in the dark and the multiline EPR signal is markedly modified (Figs 1 and 2). Ono and Inoue [18] have shown that low pH-washing, using citrate buffer, results in an inhibition of the S_1 to S_2 transition at 198 K but not at 0°C. We have made the same observation in the NaCl/EGTA-treated, polypeptides-reconstituted PS-II after a dark period long enough to allow the stable S_2 -state to deactivate (≈ 48 h at 0°C, not shown). This change in the temperature dependence of the S_1 to S_2 transition is a further consequence of the EGTA- or citrate-induced modifications.

De Paula et al. [19] reported some loss of S_2 multiline formation in salt-washed PS-II treated in darkness with high concentration of EGTA in contrast with our observations [6]. Previously, we rationalized this observation

as being due to an apparent signal decrease due to a change in the saturation characteristics of the multiline signal [6]. Although this does occur (see Fig. 1), it is also possible that, in a small fraction of centres, the EGTA-induced modification of the enzyme may have occurred. Stable S_2 would have been subtracted from the 198 K light-induced spectrum while any modified S_1 would not have undergone oxidation at 198 K.

We have considered three possible origins for the EGTA/citrate effect. (i) The carboxylic acids may result in the additional removal of Ca^{2+} . This seems unlikely for the following reasons. The simple Ca^{2+} -depleted PS-II differs from that with the EGTA/citrate modifications only with respect to the properties of the multiline signal and the photochemical inhibition occurs at the same step, after S_3 formation. Procedures designed to remove all the Ca^{2+} from PS-II are reported to block Mn-oxidation [20]. If a second Ca^{2+} was removed we would expect different inhibitory lesions. Moreover, Sr^{2+} reconstitution of the NaCl/EGTA-treated, polypeptides-reconstituted enzyme gives a modified multiline signal [9] which is essentially identical to that seen with the single Ca^{2+} -depleted enzyme [6]. If EGTA induced the depletion of a second Ca^{2+} one might expect Sr^{2+} reconstitution in both sites to give further spectral modifications. (ii) Ca^{2+} release may occur in S_3 in salt-washed membranes, but when high concentrations of carboxylic acids are present, the Ca^{2+} is trapped in solution, while in their absence, it rebinds upon deactivation. This implies that, in the salt-washed material used in [6], Ca^{2+} would still be present in S_1 and S_2 . If this were the case we would expect this material to behave like membranes salt-washed in the dark. In contrast, however, the latter material is virtually uninhibited while the dark-adapted material, which had been salt-washed in the light, is 80% inhibited [6,8], which argues against this explanation. (iii) The carboxylic acids may bind directly to the Mn. This seems possible, since both citrate and EGTA, although they have different chelation properties, would be expected to play a similar liganding role. If direct liganding of carboxylic acids to Mn does take place, then these results would show that Ca^{2+} prevents this from occurring, indicating that Ca^{2+} plays a role in controlling ligand binding to the Mn. It has already been suggested that Ca^{2+} may regulate the access of water to the substrate site [1] and it has been shown that Ca^{2+} protects the Mn-cluster from reduction by extrinsic electron donors [21].

The observation that the EGTA/citrate effect seems to occur specifically in the S_3 -state, may well be related to the earlier observations that Ca^{2+} -depletion [8] and Sr^{2+} (and Ca^{2+}) reconstitution (unpublished data) also occur much more efficiently in S_3 .

In this report, we show that the S_3 EPR signal can also be observed in Ca^{2+} -depleted PS-II that has not been further modified by EGTA/citrate nor by

reconstitution of the 17- and 23-kDa polypeptides. The somewhat smaller peak-to-trough width of the signal (130 gauss) compared to our earlier report (164 gauss) is attributed to the absence of the 17- and 23-kDa polypeptides in the present work. Using the same theoretical treatment as reported earlier [10] the 130 gauss-wide signal can be simulated using a slightly different isotropic exchange coupling constant, $J = 0.0038 \text{ cm}^{-1}$ with $\mathcal{H} = 2JS_1S_2$, between the radical and the Mn-cluster spins when compared to $J = 0.0053 \text{ cm}^{-1}$ for the 164 gauss-wide signal (N.B. in our earlier work the J value was found to be equal to 0.0064 cm^{-1} [10] but is found now to be equal to 0.0053 cm^{-1} . This is due to a slight improvement of the fitting procedure). Such a minor change in the J value from 0.0053 cm^{-1} to 0.0038 cm^{-1} may correspond to a slight conformational change, induced by binding of the 23- and 17-kDa polypeptides.

Ono and Inoue [11] have suggested that the lack of multiline modifications reported in our earlier work and the inhibition at the S_3 to S_0 transition, which we demonstrated in such preparations, was due to Ca^{2+} contamination. This assertion was contradicted (i) by the finding that the level of inhibition of oxygen evolution achieved in our earlier preparation was 80%, which is significantly greater than in most previous work [6], and (ii) by the observation of a quantitative formation of the same S_3 EPR signal in our more recent preparation which exhibited a modified S_2 multiline signal and no oxygen evolution [9] and in earlier NaCl-washed PS-II [6]. Ono and Inoue based their assertions on thermoluminescence studies both in low pH-(citrate) washed [22] and in salt-washed/EDTA-treated material [11] which showed that a single charge could be accumulated under these conditions. This was interpreted as a Ca^{2+} -depletion-induced block of the S_2 to S_3 transition [11,22]. In the light of our recent results [9] and here, it is almost certain that the S_2 signal was stable in the dark in these samples and that the one charge accumulated state was S_3 . Thus, the results of Ono and Inoue can be easily reconciled with our conclusion that Ca^{2+} -depletion blocks after S_3 formation.

There are 2 reasons why we did not see the S_3 signal in NaCl-washed PS-II membranes in our earlier studies. First, we did not make a detailed search for a new EPR signal in this state. Second, observation of this signal requires that the sample is frozen under illumination. This is probably due to the short lifetime of the S_3 state as reported earlier [7].

It was reported that the 164 gauss-wide signal was better resolved when formed under flashing light than under continuous illumination [10]. In the flash experiment, 30–40% of the centres showed a rapid decay of S_3 and the S_3 -state in these centres was not trapped in the EPR flash/freeze experiment [10]. It now seems likely that this can be at least partially attributed to centres lacking the 17- and 23-kDa polypeptides. The S_3 signal

in such centres is only 130 gauss wide compared to 164 gauss wide when the polypeptides are present. When samples are frozen under illumination, virtually all of the centres are trapped in the S_3 -state. The superimposition of the 130 gauss-wide signal on the 164 gauss-wide signal in such samples seems likely to be responsible for the poorer resolution of the S_3 signal compared to that generated by flash illumination [10].

In a study monitoring luminescence from $S_3Z^+Q_A^-$, we showed earlier that the rapid addition of Ca^{2+} to the S_3 -state in the NaCl-washed enzyme resulted in formation of the normal S_3 -state (as monitored by oscillation of the luminescence) [23]. We have demonstrated in this work that this kind of inhibited enzyme gives rise to the S_3 EPR signal. The histidine radical is therefore convertible to the normal S_3 -state upon Ca^{2+} addition. This indicates that the histidine must be at least as oxidizing as the S_3 -state in the functional enzyme. Indeed, we have proposed that S_3 in the functional enzyme may be a histidine radical [10]. It is of note that in the inhibited enzyme a band shift in the UV spectrum was observed which was taken as an indication that a positive charge was accumulated upon S_3 formation. Deprotonation occurs in this step in the functional enzyme. This difference may explain some of the properties of the inhibited enzyme and may be related to the role of Ca^{2+} .

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