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ESR spectroscopy demonstrates that cytochrome b_{559} remains low potential in Ca^{2+}-reactivated, salt-washed PSII particles

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Abstract. Cytochrome b_{559} in various Photosystem II preparations was studied by using low temperature ESR spectroscopy. This technique was used because it is able to distinguish high from low potential forms of the cytochrome owing to the g-value differences between these species. Moreover, by using low temperature irradiation to oxidize cyt b_{559} we have avoided the use of redox mediators. Previous work (Ghanotakis DF., Topper J.N. and Yocum, C.F. (1984) *Biochim. Biophys. Acta 767*, 524–531) demonstrated that reduction and extraction of manganese of the oxygen evolving complex, which might be expected to alter the redox properties of cyt b_{559}, occurs when certain PSII preparations are exposed to reductants. The ESR data presented here show that a mixture of high potential and lower potential cyt b_{559} species is observed in the oxygen evolving Photosystem II complex. Treatment of PSII membranes with 0.8 M Tris converts the high potential form(s) to those of lower potential. Exposure of the membranes to 2 M NaCl shifts a significant amount of high potential cyt b_{559} to lower potential form(s); addition of $CaCl_2$ reconstituted oxygen evolution activity but did not restore cyt b_{559} to its high potential form(s).

Abbreviations

Chl, chlorophyll; cyt, cytochrome; DCBQ, 2,5-dichloro-benzoquinone; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; ESR, electron spin resonance; OEC, oxygen evolving complex; PS, photosystem

Introduction

Despite considerable effort to elucidate the function of cytochrome b_{559}, its role in photosynthesis remains unclear [6]. The involvement of cyt b_{559} in the electron transport chain between the two photosystems was proposed early on by Cramer and Butler [5]. More recently, an accumulation of circumstantial evidence suggests a close association of cyt b_{559} with the oxidizing side of Photosystem II. It has been shown that at $-196\,^{\circ}C$ cyt b_{559} is photooxidized by Photosystem II [16]; in addition, treatments which inhibit oxygen evolution invariably cause a marked decrease in the midpoint potential of cyt b_{559} [10, 18]. A series of experiments carried out by

Dedicated to Prof. L.N.M. Duysens on the occasion of his retirement

Matsuda and Butler [20, 21] has clearly demonstrated that high potential cyt b_{559} requires the structural integrity of the photosynthetic membrane and that disruption of that integrity causes cyt b_{559} to be modified to lower potential forms.

Butler has pointed out that much of the confusion about the function of cyt b_{559} derives from the unstable nature of its redox properties [4]. Even though it has been common in the literature to speak of the high potential and low potential forms of cyt b_{559} as if they were well-defined chemical species, cyt b_{559} exists as a heterogeneous population which assumes a range of midpoint potentials [10]. Recent molecular biological, biochemical and spectroscopic evidence has revealed the likely basis for the ease with which the redox properties of cyt b_{559} can be modified and restored [1, 15, 28]. The heme iron of cyt b_{559} is apparently ligated in its axial positions by histidine nitrogens from two distinct polypeptide chains. The dihedral angle between the two histidines imidazole planes controls the ESR properties of the cytochrome. These had been shown previously to be diagnostic of the heme redox potential [2, 19, 24]. In lower potential forms the dihedral angle is close to 0° (coplanar imidazole rings) and g_z is close to 2.94; as the redox potential increases, the dihedral angle also increases and g_z approaches its high potential value of 3.08 [1]. Thus, simple shifts in the histidine geometry, presumably controlled by the relative orientation of the two different polypeptides involved, can be related to the redox properties of cyt b_{559}.

In this communication we have studied the effect of various inhibitory treatments on the state of cyt b_{559} in O_2-evolving PSII preparations. We have used ESR spectroscopy to characterize the redox properties of the protein, both because of the relationship between g-value and redox potential noted above and because the use of redox mediators may be avoided by using this approach. Our results show that treatment of PSII membranes with either high concentrations of Tris-buffer or 2 M NaCl converts high potential cyt b_{559} to a low potential form. Although addition of high concentrations of $CaCl_2$ to high-salt treated PSII membranes reconstituted oxygen evolution activity, conversion of the low potential form(s) to the high potential form(s) of cyt b_{559} was not observed.

Materials and methods

Photosystem II membranes were prepared and treated with 0.8 M NaCl as described in [12]. ESR measurements were carried out by using a Bruker ER 200D spectrometer. An Oxford ESR-9 liquid helium cryostat was used to maintain sample temperature; frequencies were measured with a Hewlett-Packard frequency counter and magnetic fields were determined with a Bruker gaussmeter accessory. Low temperature illumination was carried out by focusing the heat filtered output of a 300 W slide projector onto the ESR sample tube which was contained in a liquid nitrogen filled clear glass dewar.

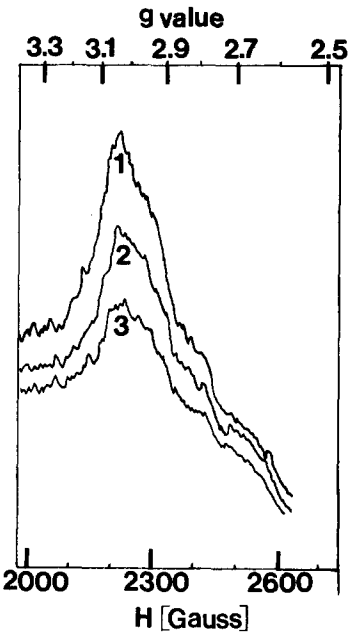

Figure 1. ESR spectra of intact Photosystem II membranes oxidized with 1 mM DDQ. The following conditions were used: Modulation amplitude, 20 G; time constant, 0.1 sec; sweeptime, 200 sec; gain 1.6×10^6; temperature, 18 K; microwave power, (1) 50 mW, (2) 20 mW and (3) 10 mW; each spectrum was obtained as the average of three scans. Chlorophyll concentration, 6.5 mg/ml

Results

Cyt b_{559} is usually described as being in a high or low potential form on the basis of whether it is reduced by hydroquinone. Optical spectroscopy in the α-band region of the cytochrome absorption spectrum is commonly used in this assay. As noted above, the state of cyt b_{559} can also be examined by use of ESR spectroscopy. High potential cyt b_{559} has characteristic g values ($g_x = 1.36$, $g_y = 2.16$ and $g_z = 3.08$) which are shifted upon conversion to the low potential form(s). The latter species have a range of g-values which have as asymptotes the following: $g_x = 1.50$, $g_y = 2.26$ and $g_z = 2.94$ [2, 19, 24]. The g_z component is the most convenient to monitor; g_y appears in a more congested region of the ESR spectrum and g_x, which occurs as a broad, weak feature at high field, has been observed only for the isolated protein [1].

As shown in Figure 1, after oxidation of cyt b_{559} by addition of 1 mM DDQ the ESR spectrum of the cytochrome in oxygen evolving PSII membranes shows a main peak around $g = 3.08$ along with a shoulder at $g = 2.94$; this spectrum is indicative of a high/low potential cyt b_{559} mixture. If we compare the ESR signal of cyt b_{559} shown in Figure 1 to that reported earlier

Table 1. Effect of various inhibitory treatments on oxygen evolution activity of photosystem II membranes

Treatment	Activity (μMol O_2/mg Chl• hr)*	
	$-CaCl_2$	$+CaCl_2$
None	720	730
0.8 M Tris, pH 8.0	0	0
2 M NaCl, pH 6.0	200	580

*Photosystem II membranes were assayed for oxygen evolution activity in a medium containing 50 mM MES, pH 6.0, 15 mM NaCl, ±15 mM $CaCl_2$. The chlorophyll concentration was 20 μg/ml and DCBQ (300 μM) served as an exogenous acceptor

by Bergström and Vanngård [2] for intact thylakoids, we observe an increased amount of low potential cyt b_{559} in the isolated PSII complex; this is probably due to the exposure of the photosynthetic membrane to the detergent Triton X-100 (see Discussion section). A titration of cyt b_{559} in an O_2-evolving PSII preparation has demonstrated that about 50% of the cyt b_{559} was in the high potential form(s) (hydroquinone reducible), while another 50% was reduced by ascorbate [12]. However, various values of the high potential/low potential ratio in untreated PSII preparations have been reported [11, 17, 20, 21, 22] (see below), and there is uncertainty in the overall cyt b_{559}/PSII stoichiometry in the resolved preparations. Several groups report two cyt b_{559} per P680 [e.g., 17, 22, 23, 26], whereas others report only one [8, 11, 12]. Figure 2a shows that upon inhibition of oxygen evolution activity by incubation with 0.8 M Tris-buffer (Table 1), cyt b_{559} is converted to the low potential form(s) ($g_z = 2.94$) as expected.

As indicated in Table 1, another treatment which inhibits oxygen evolution activity is exposure of the PSII complex to high ionic strength; this treatment is known to release two water soluble polypeptides (17 and 23 kDa) from the oxidizing side of Photosystem II without any release of functional manganese [13]. Previously, we have shown that the 17, 23 kDa-depleted PSII compex is very susceptible to exogenous reductants which reduce and destroy the Mn-complex [14]. Therefore, it is not possible to examine the state of cyt b_{559} in salt treated membranes reliably by use of hydroquinone because such an addition would result in release of functional manganese with concomitant conversion of any high potential cyt b_{559} to the low potential form(s) [10, 18]. To avoid this complication, we studied the state of cyt b_{559} in high salt-treated PSII membranes by use of ESR spectroscopy. As shown in Figure 2c, after exposure of the PSII complex 2 M NaCl a significant amount of high potential cyt b_{559} has been converted to the low potential form(s). Compared to the signal observed after Tris treatment (Figure 2a) however, we notice that after salt wash a fraction of the cytochrome still remains as the high potential form(s).

Since addition of high concentrations of $CaCl_2$ to salt washed PSII membranes is known to reconstitute high rates of oxygen evolution activity (Table

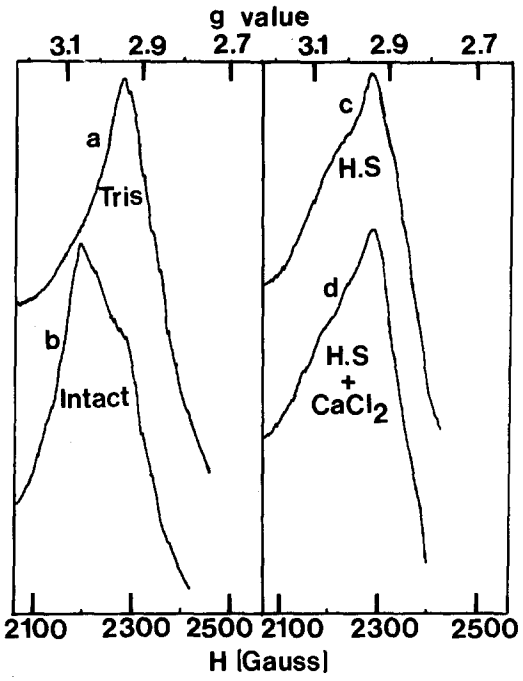

Figure 2. ESR spectra of (a) Tris treated; (b) intact; (c) 2 M NaCl-treated (H.S.), and (d) 2 M NaCl-treated plus 40 mM $CaCl_2$ PSII preparations oxidized with 1 mM DDQ. The following conditions were used: Microwave power, 30 mW; modulation, 20 G; time constant, 0.1 sec; sweeptime, 200 sec; gain, 1.6×10^6; temperature, 18 K; each spectrum was obtained as the average of six scans. The chlorophyll concentration was 6.5 mg/ml

1), we studied cyt b_{559} in 2 M NaCl-treated PSII membranes which had been reactivated by addition of 40 mM $CaCl_2$. In a separate series of time-resolved, room temperature measurements (not shown), we observed that addition of 40 mM Ca^{2+} to concentrated, salt-washed PSII membranes (~4 mg chl/ml) was sufficient to restore the rapid $Z^{\ddagger}$ reduction kinetics which accompany the reactivation of oxygen evolution [3, 13]. As shown in Figure 2d, even though $CaCl_2$ restores oxygen evolution activity, cyt b_{559} remains in its low potential form(s).

Since we cannot exclude the possibility that the reduced form of DDQ, which is present in the medium after addition of 1 mM of the oxidant as revealed by a strong ESR signal around $g = 2.00$ (data not shown), reduced and destroyed the Mn-complex [14] in the experiments in Figure 2, we repeated the experiment but this time we oxidized cyt b_{559} by illumination at −196 °C [16]. Figure 3b shows that in PSII membranes frozen in the dark, low potential form(s) of cyt b_{559} are oxidized while high potential form(s) are in the reduced state and thus ESR silent. Illumination of the PSII

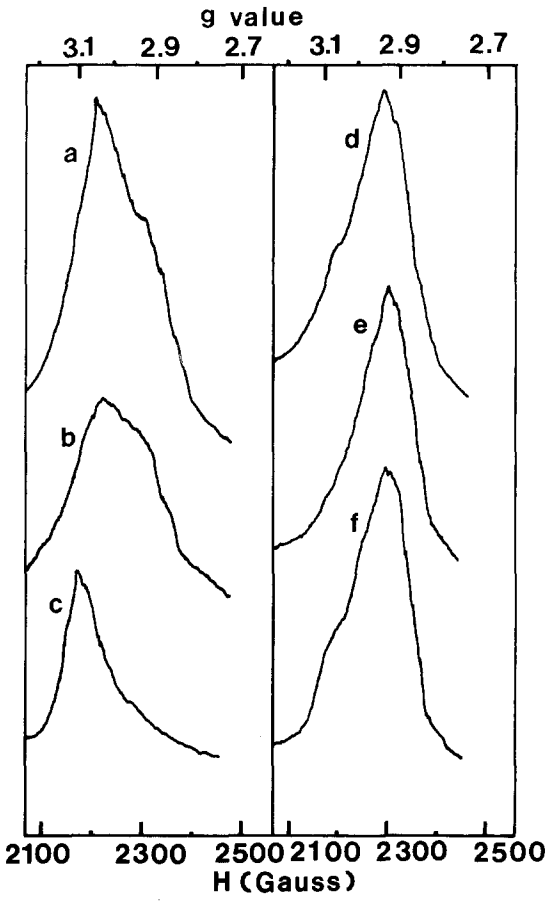

Figure 3. ESR spectra of (a) intact PSII membranes illuminated for 4 min at −196 °C; (b) dark-adapted PSII membranes; (c) spectrum (a) − spectrum (b); (d) salt-washed PSII membranes plus 40 mM $CaCl_2$ illuminated for 4 min at −196 °C; (e) dark-adapted, salt-washed PSII membranes plus 40 mM $CaCl_2$; (f) salt-washed PSII membranes plus 40 mM $CaCl_2$ illuminated for 4 min at 4 °C and then frozen and illuminated for another 4 min at −196 °C. Experimental conditions the same as those in Figure 2

complex at −196 °C results in photooxidation of the high potential form(s) (Figures 3a, 3c). Examination of cyt b_{559} in $CaCl_2$-reactivated salt-washed PSII membranes by the same method showed that only a small fraction of the cytochrome is in the high potential form(s) (Figures 3d, 3e). An attempt to expose the higher S-states to $CaCl_2$ before freezing, by illumination at 4 °C in the presence of $CaCl_2$, was also not successful in restoring cyt b_{559} to its high potential form(s) (Figure 3f).

Discussion

Although there are data in the literature which provide circumstantial links between high potential cyt b_{559} and the water-splitting capacity of PSII, elucidation of its function is problematic [4, 7]. Treatments which inhibit oxygen evolution activity (e.g. exposure to 0.8 M Tris, heptane extraction, etc.) cause a decrease in the midpoint potential of cyt b_{559}. In the case of heptane extraction, Okayama and Butler [25] showed that low potential cyt b_{559} was restored to the high potential form(s) upon reconstitution of oxygen evolution activity by addition of plastoquinone A and β-carotene. It has been suggested that high potential cyt b_{559} requires the structural integrity of the photosynthetic membrane and that disruption of that integrity, by use of treatments such as incubation with chaotropic reagents or detergents, causes modification of cyt b_{559} to the low potential form(s) and loss of oxygen evolution. Results which support this contention were provided by Matsuda and Butler [20, 21] who showed that low potential forms of cyt b_{559} in PSII preparations could be restored to the high potential form by incubation with digalactosyldiacylglycerol (DGDG)-containing liposomes. Accompanying the restoration of high potential cyt b_{559} in this system was in increase in rate of oxygen evolution [21]. Similarly, a comparison of the rates of O_2 evolution in PSII particles prepared in three different laboratories with the high potential/low potential ratio measured on the same preparations in the same laboratories shows that the higher the ratio the greater the O_2 rate [11, 12, 17].

Whether the link between high potential cyt b_{559} and O_2 evolution is causal, however, has been difficult to establish [6]. The results presented here provide some insights into this question. Exposure of the PSII complex to 2 M NaCl releases two water soluble polypeptides (17, 23 kDa) and decreases oxygen evolution activity to 20–30% of the control. The low temperature ESR data on salt-washed PSII membranes show that in 17, 23 kDa polypeptide-depleted membranes a significant fraction of high potential cyt b_{559} has been converted to the low potential form(s). The fraction of the cytochrome which remains in the high potential form(s) may be associated with the residual activity of the PSII complex (see Table 1). When we reconstituted high rates of oxygen evolution activity by addition of external calcium, however, we observed no restoration of the low potential cyt b_{559} to the high potential form(s). This observation suggests that high potential cyt b_{559} *per se* is not necessary for high O_2 evolution rates. It also rationalizes the correlation noted above between high potential cyt b_{559} and oxygen rates in PSII preparation as it is quite likely that the 17 and 23 kDa polypeptides may be removed to a greater or smaller extent during isolation. Loss of these polypeptides would decrease O_2 evolution and increase the concentration of low potential forms of the cytochrome. Evidence supporting this idea can be taken from the recent report by Preston and Critchley, who noted that the

low rates of O_2 evolution in their PSII preparations could be enhanced by addition of Ca^{2+} [27].

The situation is more complex than the above analysis indicates, however, as it is clear that Ca^{2+} reactivation of oxygen evolution in polypeptide-depleted PSII preparation does not reconstitute the native system. Dekker et al. have shown that even though $CaCl_2$ restores high rates of oxygen evolution activity the reactivated system differs from the intact system in that the $S_3 \rightarrow S_4 \rightarrow S_0$ transition is 2–3 times slower in the salt-washed system [9]. Similarly, the observations of Ghanotakis et al. [14] on the accessibility of the oxidizing side of PSII to exogenous reductants have demonstrated that the structural organization of PSII in calcium-reconstituted salt-washed membranes is different from that of the intact system. To these observations we now add our results on the differences between the properties of cyt b_{559} in untreated and polypeptide-depleted, Ca^{2+} reactivated preparations.

Figure 4 shows a model which summarizes the findings presented here and links them to recent suggestions as to the membrane and protein structural factors which control the properties of cyt b_{559} [1]. In both high and low potential cyt b_{559} the axial ligands are histidines, each one of which is contained in a separate and distinct polypeptide chain. The high potential form is achieved by structural factors which twist the plane of one of the two histidine imidazole rings out of a parallel orientation with respect to the second ring, i.e., the dihedral angle between the imidazole planes is not zero. In Figure 4a these structural forces are suggested to be the nonparallel orientation of the two histidine bearing polypeptide chains. This transmembrane organization is stabilized by the interaction of the two membrane polypeptides with the peripheral 17 and 23 kDa. Upon removal of the two extrinsic polypeptides, (Figure 4b), the transmembrane polypeptides relax to a more parallel orientation which allows the imidazole ring to assume the low energy, parallel (i.e., zero dihedral angle) conformation which is typical of low potential cyt b_{559} [1]. At the same time, the release of the peripheral polypeptides depletes the Ca^{2+} binding site and the OEC relaxes to an inactive configuration. Upon addition of high concentrations of Ca^{2+} in Figure 4c, the oxygen evolving complex assumes an active conformation, which is different from that of the untreated system; cyt b_{559} remains in low potential conformation.

Such a model is also able to rationalize the early observations of Butler and coworkers [4]. These data showed that cyt b_{559} existed as a heterogeneous population with a range of midpoint potentials after inhibition of O_2 evolution. In the model of Figure 4, this suggests that the dihedral angle between the two imidazole planes is somewhat labile and can assume a range of values all of which, however, should be fairly close to zero. Because of the link established between imidazole geometry and principal g-values for various forms of cyt b_{559}, this variability in imidazole orientation may be expected to be manifested in the ESR properties of the cytochrome. An

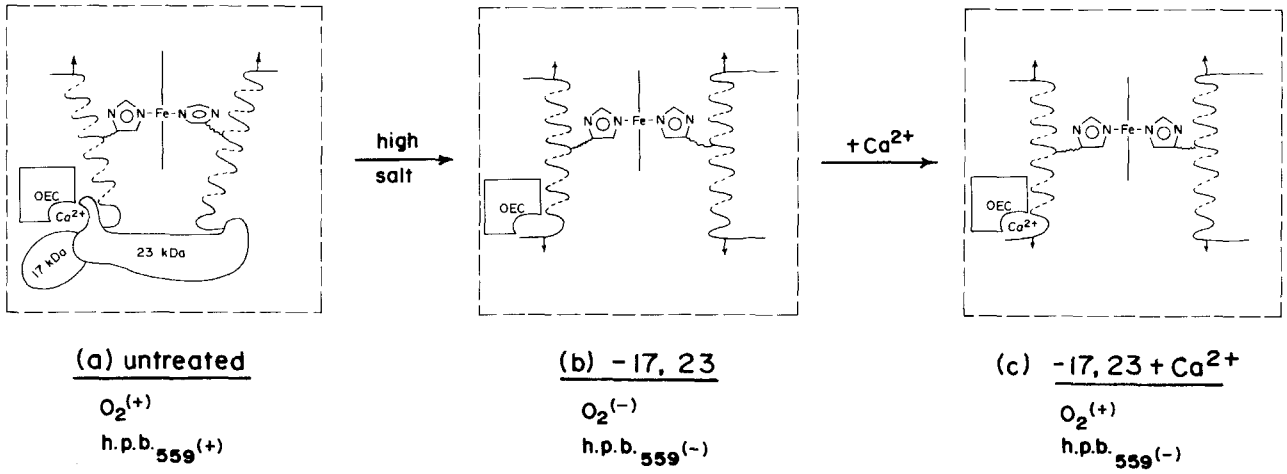

Figure 4. Model for the states of the oxygen evolving complex and cytochrome b_{559} in (a) untreated, (b) 17, 23 kDa polypeptide-depleted and (c) Ca^{2+} reactivated preparations. See text for details

example of this may be found in the recent work in inhibited systems by Matsuura and Itoh [22] which showed that g_z was 2.98 for Tris or heat-treated PSII preparations but 2.95 for the same preparation following a 1 M $CaCl_2$ wash.

Acknowledgements

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