Correlated behavior of the EPR signal of cytochrome *b*-559 heme Fe(III) ligated by OH^- and the multiline signal of the Mn cluster in PS-II membrane fragments

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Abstract EPR signals of Cyt *b*-559 heme Fe(III) ligated by OH^- and the multiline signal of the Mn cluster in PS-II membrane fragments have been investigated. In 2,3-dicyano-5,6-dichloro-*p*-benzoquinone-oxidized PS-II membrane fragments the light-induced decrease of the EPR signal of the heme Fe(III)-OH^- is accompanied by the appearance of the EPR multiline signal of the Mn cluster. Addition of F^- ions, which act as a stronger ligand for heme Fe(III) than OH^-, decreases to the same extent the dark- and light-induced signal of the heme Fe(III)-OH^- and the light-induced multiline signal of the Mn cluster. These results are discussed in terms of the light-induced formation of a bound OH′ radical shared between the Cyt *b*-559 heme Fe and the Mn cluster as a first step of water oxidation.

Key words: Photosystem II; Cytochrome *b*-559; Water oxidation; Mn cluster

1. Introduction

In chloroplasts the major fraction of cytochrome *b*-559 is present in a high potential form ($E_m = +380$ mV) [1]. Upon illumination of chloroplasts at 80 K a bleaching at 557 nm [1] and the formation of a low-spin heme Fe(III) EPR signal at $g = 3.08$ [2,3] are observed. These data strongly support the idea that this Cyt *b*-559 form is characterized by bis-histidine ligation found also for isolated Cyt *b*-559 [4]. However, recently it was shown that in intact chloroplasts the oxidation of Cyt *b*-559 by 10 mM 2,3-dicyano,5,6-dichloro-*p*-benzoquinone (DDQ) leads to the appearance of an EPR signal that is characteristic of the high-spin heme Fe(III) while the extent of the low-spin EPR signal is comparatively small [5]. It was shown by EPR measurements that in intact chloroplasts the Cyt *b*-559 heme Fe(III) is present in two forms. Both are lacking a strong ligand at the 6th coordination position and one form at this site is ligated by OH^-. The EPR signal indicative of the heme Fe(III)-OH^- signal is considerably decreased upon chlorophyll illumination at 80 K and 140 K [5], showing that this heme is close to P680+ acting as an oxidant of the observed Cyt *b*-559 changes. The light-induced signal decrease was explained in two ways: (i) by the removal of OH^- from heme Fe(III); and (ii) by the oxidation of the heme Fe(III)-OH^- complex that could possibly comprise the Mn cluster.

The present work is an attempt to study more precisely the connection between the Cyt *b*-559 heme Fe(III) ligated by OH^- and the Mn cluster in PS-II membrane fragments. It was found that in DDQ-oxidized PS-II membrane fragments the light-induced decrease of the EPR signal of the heme Fe(III)-OH^- is accompanied by the appearance of the EPR multiline signal of the Mn cluster. Addition of F^-, which acts as a stronger ligand for heme Fe(III) than OH^-, decreases to the same extent the dark- and light-induced EPR signal of the heme Fe(III)-OH^- and the light-induced multiline signal of the Mn cluster. These results are discussed in terms of the interaction between Cyt *b*-559 heme Fe(III)-OH^- and the Mn cluster in a water-oxidizing complex.

2. Materials and methods

PS-II membrane fragments with high oxygen-evolving capacity were prepared according to the procedure of Berthold et al. [6] with some modifications [7]. Briefly, standard thylakoids were suspended (2 mg Chl/ml) in 400 mM sucrose/15 mM NaCl/5 mM $MgCl_2$/20 mM Mes-NaOH (pH 6.5) buffer and incubated with Triton X-100 (50 mg/ml) at 4°C for 30 min. After centrifugation (35000×*g*, 20 min) the precipitate was resuspended in the above buffer without Triton X-100 and centrifuged at 5000×*g* for 10 min. Then the supernatant was spun down at 35000×*g* for 20 min and pelleted PS-II membrane fragments were suspended in the same buffer and stored in liquid nitrogen. To oxidize Cyt *b*-559 10 mM DDQ (Sigma) was used. The chlorophyll concentration of the PS-II membrane fragments was 8–10 mg/ml.

The horse heart myoglobin (Sigma) concentration was 0.5 mM in 50 mM Tris buffer at pH 10.5. For EPR measurements, 200 μl of the samples were placed in oxygen-free argon-flushed quartz tubes (3 mm inner diameter). The EPR spectra were recorded at 10 K on a Varian E-9 spectrometer using standard Bruker TE102 cavity and an Oxford ESR 9 helium flow cryostat.

3. Results and discussion

Fig. 1 shows the EPR signal in high- and low-spin heme Fe(III) regions of PS-II membrane fragments. In untreated membrane fragments (spectrum a) only the rhombic iron signal at $g = 4.27$ is detected. The origin of this signal is not yet clarified [8]. After oxidation of PS-II membrane fragments by DDQ high-spin heme Fe(III) signals at $g = 7.0$, 5.9 and ~5.0 (small) as well as low-spin heme Fe(III) signals around $g = 3$ are observed (spectrum b). As earlier suggested for intact chloroplasts [5], the signals at $g = 7.0$ and ~5.0 represent the low- and high-field splitting components of the Cyt *b*-559 heme Fe(III) ligated by OH^-. For comparison Fig. 1f

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Abbreviations: Cyt *b*-559, cytochrome *b*-559; DDQ, 2,3-dicyano,5,6-dichloro-*p*-benzoquinone; Mb, myoglobin; PS-II, photosystem II

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also shows the EPR signal of the Mb heme Fe(III) ligated by OH^- at pH 10.5. This spectrum exhibits splitting components at $g = 6.9$ and 4.9 which are due to rhombic and tetragonal distortion of octahedral molecular field symmetry in the presence of OH^- as a ligand at the 6th coordination position [5].

The amplitude of the EPR signal at $g = 5.9$ that originates from the heme Fe(III) without a 6th ligand is relatively small with respect to the heme Fe(III)-OH^- signal in PS-II membrane fragments as compared with chloroplasts (see [5] for comparison). The ratio of the products, given as amplitude multiplied by width, of the $g = 5.9$ and $g = 7.0$ signals is about 0.01 in PS-II membrane fragments and 1.0 in intact chloroplasts. It was estimated [5] that the fraction of the Cyt *b*-559 that contains the heme Fe(III) ligated by OH^- is about 40% of the total amount of the high-spin heme Fe(III) in intact chloroplasts. In PS-II membrane fragments this factor is close to 95%. In contrast the low-spin heme Fe(III) signal at g=3.0 is relatively large in PS-II membrane fragments as compared with chloroplasts. Formally these facts would correspond to the appearance in PS-II membrane fragments of the strong ligand (probably histidine) at the 6th coordination position of the Cyt *b*-559 heme Fe(III), which has no ligand at this site in oxidized chloroplasts.

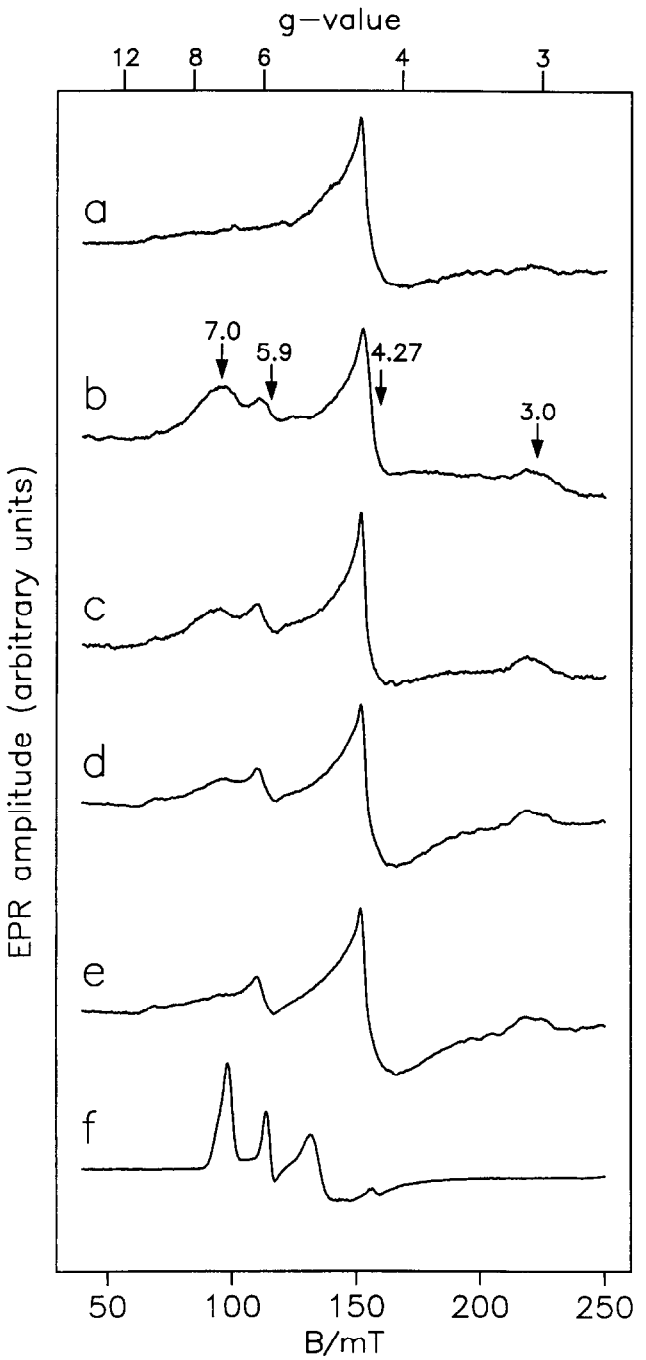

Fig. 1. The low temperature (10 K) EPR signals in the range of 40–240 mT including components of the high- and low-spin heme Fe(III) signals of PS-II membrane fragments and myoglobin. (a) Untreated PS-II membrane fragments. (b) PS-II membrane fragments in the presence of 10 mM DDQ. (c) The same as (b) but illuminated at 200 K for 10 min. (d) PS-II membrane fragments in the presence of 50 mM NaF and 10 mM DDQ. (e) The same as (d) but illuminated at 200 K for 10 min. (f) 0.5 mM Mb in 50 mM Tris buffer, pH 10.5. Experimental conditions: microwave power 10 mW, modulation amplitude 2 mT, recording time 30 min.

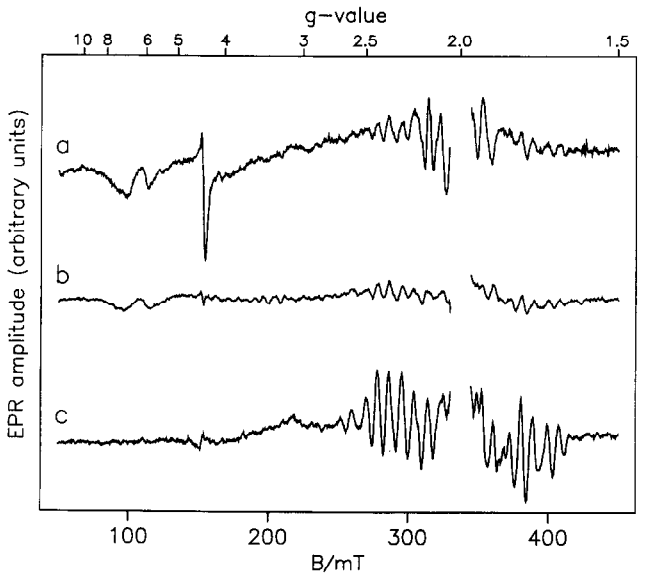

Fig. 2. The light-minus-dark difference EPR spectra measured in PS-II membrane fragments; illumination at 200 K for 10 min, (a) in the presence of 10 mM DDQ; (b) in the presence of 50 mM NaF and 10 mM DDQ; (c) in absence of additions. Experimental conditions: microwave power 10 mW, modulation amplitude 2 mT, 10 K, recording time 30 min.

Illumination of the PS-II membrane fragments at 200 K for 10 min leads to the considerable decrease ($\sim$50%) of the signal at $g = 7.0$ (Fig. 1c) similar to that observed for intact chloroplasts [5].

The addition of 50 mM F^- ions (Fig. 1d) leads to a decrease of EPR signals at g=7.0 and $\sim$5.0 by a factor of $\sim$2. It was shown [9] that this effect is due to a replacement of the OH^- ligand by an F^- ligand, which is a stronger ligand for the heme Fe(III). Illumination at 200 K for 10 min of the PS-II membrane fragments in the presence of 50 mM F^- causes a further decrease of the EPR signal at $g = 7.0$ (Fig. 1e).

Fig. 2 shows the difference (light minus dark) EPR signals in a wide range of magnetic fields (50–450 mT) measured in PS-II membrane fragments illuminated at 200 K for 10 min. One sees that for PS-II membrane fragments oxidized by 10 mM DDQ (curve a) the disappearance of the EPR signal at $g = 7.0$ is accompanied by the appearance of the $g = 6.0$ signal and of the multiline signal corresponding to the S_2 state of the

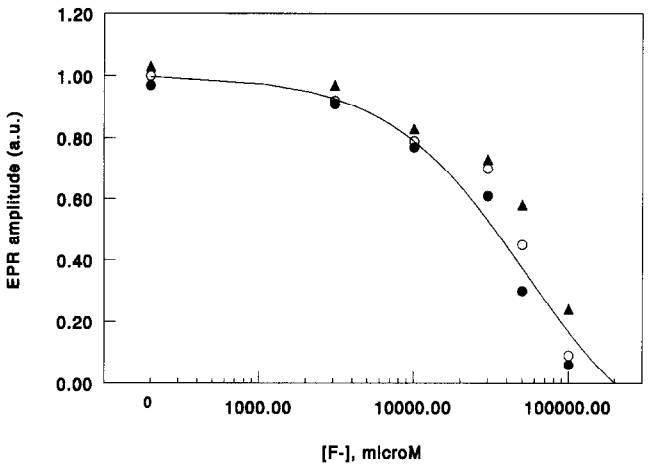

Fig. 3. EPR signal amplitudes in PS-II membrane fragments dependent on F^- concentration: high-spin heme Fe(III) signal at $g = 7.0$ of dark adapted PS-II membrane fragments (open circles), the light-induced decrease of the signal at $g = 7.0$ (closed circles) and the light-induced multiline signal (closed triangles). Experimental conditions: microwave power 10 mM, modulation amplitude 2 mT, recording time 30 min, T = 10 K, illumination at 200 K for 10 min. The solid line shows a modelling curve described in the text.

Mn cluster [10]. In the presence of 50 mM F^- both signals are decreased by a factor of ~2 (curve b). The light-induced multiline signal of PS-II membrane fragments in the absence of DDQ and F^- is depicted in Fig. 2c.

Fig. 3 shows the concentration dependence of the inhibitory effect of F^- on amplitudes of dark EPR signal at $g = 7.0$ (open circles), of the light-induced decrease of the signal at $g = 7.0$ (closed circles) and of the light-induced multiline signal (closed triangles). All these signals exhibit the same concentration dependence within experimental error of the data. The solid curve is calculated on the basis of the assumption that OH^- and F^- with different binding constants compete for the 6th coordination position of the heme Fe(III) and the concentration of F^- varies over a wide range. The steep slope of the curve at concentrations of F^- more than 50 mM is simulated by including a limited ion's volume of the heme Fe(III) site.

Since the decrease in the dark of the $g = 7.0$ heme Fe(III)-OH^- signal in the presence of F^- corresponds to the exchange of the presumed OH^- ligand by the F^- ligand [9], the similar dependence of all measured signals on F^- concentration implies that the loss of heme Fe(III)-OH^- ligation simultaneously leads to the disappearance of the multiline signal of the Mn cluster in the S_2 state. These results are consistent with a previous proposal [5] that the light-induced oxidation of the Fe(III)-OH^- complex comprising the Mn cluster leads to the formation of a bound OH′ radical as a first step of water oxidation. This radical is probably transferred to (or shared with) the Mn cluster. This would lead to a decrease of the $g = 7.0$ signal (since no more OH^- ligand is available), to an increase of the 5.9 signal (since the heme Fe(III) without ligand gives the $g = 5.9$ signal) and to the appearance of the multiline signal (since the OH′ radical distracts some electron density from the Mn cluster). As the light-induced formation of the $g = 5.9$ signal is smaller than required for a complete transformation of the heme Fe(III)-OH^- to the high-spin heme Fe(III) without a 6th ligand (see [5]) one can suggest that the OH′ radical is shared by both the heme FE(III) and the Mn cluster in some PS-II complexes. It is interesting that the light-induced decrease of the g=7.0 signal is observed even at 77 K [5] while the multiline signal is only induced upon illumination above 190 K [10]. This means that the Cyt changes precede the Mn cluster oxidation and the latter occurs when diffusional mobility is increased, in agreement with the above suggestion.

It should be noted that the described processes are observed when Cyt *b*-559 Fe(II) is oxidized to Fe(III). In the presence of the heme Fe(II) the light-induced formation of the multiline signal is observed as well (see Fig. 2c and [10]). Another important conclusion could be gathered if one assumes that the same mechanism is responsible for the appearance of the multiline signal in the presence of either heme Fe(II) or Fe(III). In this case the valency of the heme Fe ligated by OH^- (or H_2O) is not important for the light-induced production of the bound OH′ radical, i.e. the formation of the S_2 state is independent of the high or low potential form of Cyt *b*-559 as found previously [11]. This problem is now under study.

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