Study of heme Fe(III) ligated by OH^- in cytochrome *b*-559 and its low temperature photochemistry in intact chloroplasts

Robert Fiege[a], Ulrich Schreiber[b], Gernot Renger[a], Wolfgang Lubitz[a], Vladimir A. Shuvalov[c,*]

[a]*Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Str. des 17. Juni 135, D-10623 Berlin, Germany*
[b]*Julius-von-Sachs-Institut für Biowissenschaften der Universität Wurzburg, Mittlerer Dallenbergweg 64, D-97082 Wurzburg, Germany*
[c]*Institute of Soil Science and Photosynthesis of Russian Academy of Science, Pushchino, Moscow Region 142292, Russian Federation*

Received 2 October 1995; revised version received 3 November 1995

Abstract EPR properties of Cyt *b*-559 have been investigated in intact chloroplasts that are functionally competent in O_2 evolution and in CO_2 fixation. After chemical oxidation of Cyt *b*-559 by 10 mM 2,3-dicyano, 4,5-dichloro-*p*-benzoquinone (DDQ) the major part of Cyt *b*-559 is found to be present in the high spin Fe(III) form. Only a small fraction of low spin heme Fe(III) (less than 5%) was formed by chemical or light-induced oxidation. This fraction increased during aging of intact chloroplasts. A comparison with the EPR signal of Fe(III) in myoglobin (Mb) reveals that the structure of the high spin signal in intact chloroplasts is indicative for the presence of an axial OH^- ligand at the heme Fe(III). This type of ligation comprised a considerable part (~40%) of the total Cyt *b*-559 content. Removal of the Mn-cluster caused a change of the EPR parameters of OH^- ligation. When in intact chloroplasts the heme Fe is chemically oxidized to Fe(III) ligated by OH^-, this OH^- ligation disappeared after a subsequent illumination at 80K by red light. Upon illumination at 140K this disappearance was accompanied by the formation of a high spin Fe(III) that is not ligated by OH^-. These results are discussed in terms of removal of OH^- from Fe(III) caused by structural changes or photooxidation at a complex of Cyt *b*-559 that could possibly also comprise the Mn-cluster. This photooxidation is assumed to be accompanied by the formation of a bound $OH^\bullet$ radical. The possibility is discussed that this process is related to photosynthetic water oxidation.

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

1. Introduction

The α- and β-subunits of Cyt *b*-559 are constituents of the reaction center complex of photosystem-II (RC-2) with heme groups exhibiting different redox and spectral properties [1]. A close proximity of Cyt *b*-559 to the RC-2 cofactors (chlorophyll, pheophytin, quinone etc.) is supported by the high quantum yield of Cyt *b*-559 oxidation at 80K in chloroplast membranes and in isolated D1D2Cyt *b*-559 complexes [1,2]. Both subunits of Cyt *b*-559 are oriented with their C-termini towards the lumenal side of the thylakoid membrane, whereas the histidine residues are close to their N-termini and therefore are located near the stromal side of the membrane (see [3]). Thus the suggestion of axial heme Fe ligation by two histidines [4] would require that the heme group of Cyt *b*-559 is located near the stromal side of the membrane. However, protein structure analysis of polypeptides D1, D2 and the Cyt *b*-559 subunits can indicate [5] that such a position of Cyt *b*-559 heme is not very well consistent with the oxidation of Cyt *b*-559 at low temperature.

It is generally accepted that in intact chloroplasts the major fraction of Cyt *b*-559 is present in a high potential form (E_m = +380 mV) [1]. Upon illumination of chloroplasts at 80K bleaching at 557 nm [1] and the formation of a low spin heme Fe(III) signal with $g = 3.08$ [6] are observed. These data strongly support the idea that this Cyt *b*-559 form is characterized by bis-histidine ligation (see also [7]). Accordingly, the fraction of Cyt *b*-559 with high spin EPR signal is believed to be a result of denaturation of membrane protein [8].

Optical and EPR studies revealed several forms of Cyt *b*-559 in D1D2Cyt *b*-559 complexes with different redox potentials at pH 7.2 and at pH 9.4 [7,9]. The optical and EPR (g = 5.9 and 6.4) properties of one form at pH 9.4 clearly show that its heme is ligated by OH^- at the 6th coordination position [7,9]. Although the 5th ligand of this form was suggested to be histidine [9], no convincing data are available to substantiate that proposal.

Since the Cyt *b*-559 forms are largely variable in RC-2 complexes [7] it seems likely that Cyt *b*-559 exhibits a high sensitivity to the integrity and state of the thylakoid membrane. In this work we present EPR properties of Cyt *b*-559 in intact chloroplasts that are functionally competent in O_2 evolution and in CO_2 fixation [10]. The chemical oxidation of Cyt *b*-559 by 10 mM 2,3-dicyano,4,5-dichloro-*p*-benzoquinone (DDQ) shows that a major part of Cyt *b*-559 is present in the high spin form in intact chloroplasts. Only a small fraction of low spin heme Fe(III) (less than 5%) is observed by chemical or light-induced oxidation in contrast to the previous observations [6]. A comparison with the EPR signal of myoglobin (Mb) shows that the structure of the high spin signal in intact chloroplasts is indicative for the presence of OH^- ligation of the heme Fe(III) in a considerable part (~40%) of Cyt *b*-559. Removal of the Mn-cluster of the water oxidizing complex (WOC) is accompanied by changes of EPR parameters characteristic for of OH^- ligation. When the heme Fe ligated by OH^- is oxidized by DDQ to Fe(III), the illumination of chloroplasts at 80K by red light absorbed by chlorophyll but not by Cyt *b*-559 leads to disappearance of 50%–100% of EPR signal characteristic of the OH^- ligation. Upon illumination at 140K this disappearance is accompanied by the formation of a high spin Fe(III) form that is not ligated by OH^-. These results are discussed in terms of

*Corresponding author. Fax: (7) (095) 939 3181.
E-mail: Shuvalov@issp.serpukhov.su

Abbreviations: Cyt *b*-559, cytochrome *b*-559; DDQ, 2,3-dicyano,4,5-dichloro-*p*-benzoquinone; Mb, myoglobin; PS-II, photosystem II; WOC, water oxidizing complex.

SSDI 0014-5793(95)01363-6

a structural modification of OH^- ligand upon illumination or photooxidation of heme Fe(III)–OH^- of the PS-II complex involving Cyt *b*-559 and possibly the Mn-cluster which is accompanied by the formation of a bound $OH^\bullet$ radical.

2. Materials and methods

Intact chloroplasts were isolated from spinach leaves as described earlier [10]. Approximately 40 g of leaves were briefly homogenized in a medium consisting of 300 mM sorbitol, 30 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 50 mM MES, pH 6.1 (buffer A). Coarse filtration of the homogenat, centrifugation at $4000 \times g$ for 4 min (4°C) and purification on a Percoll gradient in a buffer almost identical to A, except that MES was replaced by 50 mM HEPES, pH 7.5 (buffer B), were used. The intactness of the chloroplasts was approximately 90% as checked by measurements of the O_2 evolution rate in the absence and in the presence of $K_3[Fe(CN)_6]$.

The extraction of Mn from chloroplasts was done as described previously [11]. Chloroplasts were treated with 5 mM NH_2OH at a chlorophyll concentration of 100 mg/ml in buffer B for 30 min at 4°C in the dark followed by double washing in buffer B. Myoglobin (Mb) was purchased from Sigma (St. Louis, USA (No. M-0380)). The chlorophyll concentration of the chloroplasts used for the EPR studies was 10–14 mg/ml. The myoglobin concentration was 0.5 mM in 50 mM Tris buffer with a pH as indicated in the figure legend (Fig. 1). Chloroplasts in buffer B and Mb in Tris buffer were frozen to 77K in an argon atmosphere and then frozen to 10K for the measurements. The EPR spectra were measured at 10K using Varian spectrometer equipped with a Bruker TE102 cavity and an Oxford ESR 9 helium flow cryostate.

3. Results

Fig. 1a and 1b show the EPR spectra of Mb at pH 7.5 and 10.5, respectively. At pH 7.5 the spectrum is characterized by two lines with $g_\perp = 5.84(2)$ and $g_\parallel = 1.998(5)$. The feature is typical for high spin Fe(III) in the absence of a strong ligand at the 6th coordination position (tetragonal molecular field symmetry [19]). Increase of pH leads to a drastic change of the spectrum. The characteristic line with $g_\perp = 5.81(5)$ decreases in its amplitude and two new lines appear in the Fe(III) high spin region with $g = 6.87(5)$ and $g = 4.91(9)$. The corresponding parallel component with $g_\parallel = 1.994(5)$ is also present (marked by an asterisk in Fig. 1b). These changes are accompanied by the appearance of low spin Fe(III) signals with $g_1 = 2.54(2)$ and a low field shoulder at $g = 2.59(2)$, $g_2 = 2.17(1)$ and $g_3 = 1.88(1)$ as well as a high field shoulder at $g = 1.84(1)$ (Fig. 1b). Splitting of the high spin Fe(III) signal and appearance of low spin Fe(III) are due to ligation of Fe(III) by OH^- at high pH [12]. This ligation changes the symmetry of molecular field (appearing of octaedral symmetry with rhombic or tetragonal distortion [18]) and leads to thermal mixing of the high and low spin states [13]. The ratio of amplitudes multiplied by widths of the signals with $g = 6.87(5)$ at pH 10.5 and that with $g = 5.84(5)$ at pH 7.5 is ~1:2.5, respectively.

In Fig. 1c difference EPR spectrum of intact chloroplasts at pH 7.0 in the presence of 10 mM DDQ and without DDQ is shown. This spectrum exhibits signals typical for high spin Fe(III) with $g_\perp = 5.84(5)$ with two low field splitting components with a sharp shoulder at $g = 6.12(5)$ and a broad shoulder at $g = 6.8(1)$. Only one high field splitting component with $g = 5.62$ is clearly seen. Second component (should be around $g = 4.9$) is probably too broad and small to observe. Therefore we pay attention only to the high field component with $g = 6.8$. Features of low spin Fe(III) at $g = 3$ cannot be detected. The latter finding differs from results obtained previously in PS-II

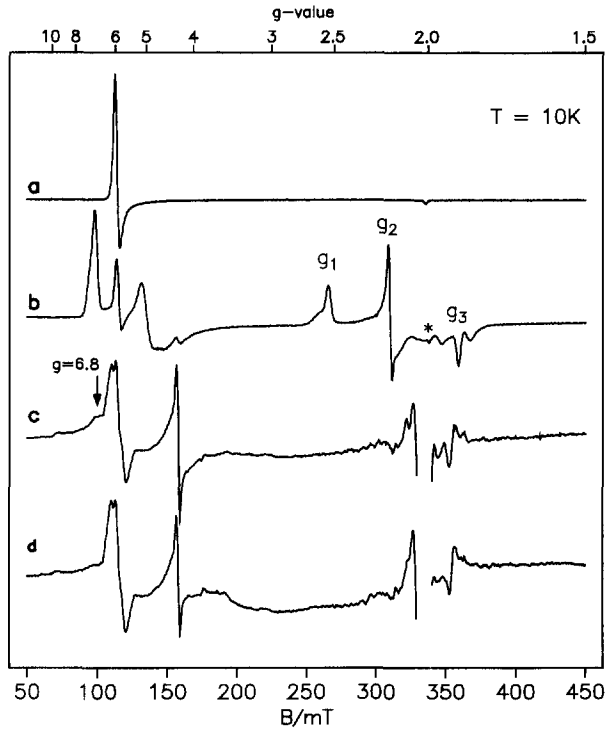

Fig. 1. EPR spectra of Fe(III) in myoglobin and in chloroplasts. (a) Myoglobin pH 7.5, (b) myoglobin pH 10.5; experimental conditions: concentration $5 \cdot 10^{-4}$ M, microwave power 25 mW, modulation amplitude 2 mT, recording time 168 s, 10K. (c and d) Difference spectra of chloroplasts in the presence of DDQ minus without DDQ, (c) chloroplasts in buffer B, (d) chloroplasts in buffer B in the presence of 100 mM NH_4Cl; experimental conditions: concentration 12 mg Chl/ml, microwave power 5 mW, modulation amplitude 1 mT, recording time 17 min, 10K.

membrane fragments [6,8,14]. An estimation of the integrals for high and low spin (with $g = 3$) components and a comparison with signals of D1D2Cyt *b*-559 complexes and Mb revealed that the latter form accounts for no more than 5% of the former one. In the spectrum of Fig. 1c several DDQ induced signals appear between $g = 2.15(1)$ and $g = 1.85(1)$. Our studies [9] and comparison with model systems like myoglobin or catalase as well as with different ligated cytochromes [15] clearly show that these signals cannot be assigned to a low spin Fe(III) cytochrome. The origin of the strong rhombic iron signal at $g = 4.26(2)$ is not clarified [16]. The high field splitting component with $g = 6.8$ is similar in g value to that observed for Mb at pH 10.5. It can be ascribed to OH^- ligation of the heme Fe(III) of Cyt *b*-559, in agreement with the previous assignment of similar EPR signals to the OH^- ligation of the heme Fe(III) of Cyt *b*-559 in the D1D2Cyt *b*-559 complex and of catalase [7,9].

This idea has been checked by experiments performed in the presence of NH_3 that is strong enough to replace a OH^- as a ligand [13]. In fact it was found that the high spin heme Fe(III) signal with $g = 6.8$ decreased in intact chloroplasts when 100 mM NH_4Cl was added before freezing (Fig. 1d) (see also [17] for effects of NH_4Cl on WOC).

The absence of any signal near $g = 8$ in Fig. 1c and 1d (see also Figs. 2 and 3) indicates that DDQ does not produce the Fe(III)-plastoquinone signal (described in [19,20]) in intact chloroplasts.

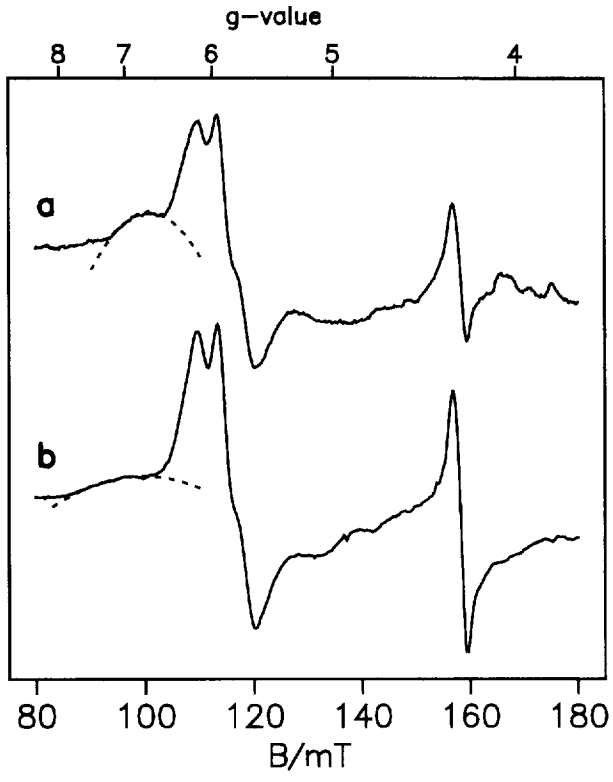

Fig. 2. Effect of NH_2OH treatment on high spin signals; (a) and (b) are difference spectra (in the presence of DDQ minus without DDQ); (a) untreated chloroplasts, (b) NH_2OH-treated chloroplasts; experimental conditions: concentration 12 mg Chl/ml, microwave power 5 mW, modulation amplitude 1 mT, recording time 8 min, 10K.

The high spin heme Fe(III) of Cyt *b*-559 gradually decreases when chloroplasts are kept in the dark at 4°C for several hours. In parallel a small low spin Fe(III) signal around $g = 3$ appears. This observation indicates that chloroplasts degradation causes the formation of low spin signal of Cyt *b*-559.

By comparison with the heme Fe(III) of Mb the amount of heme Fe(III) ligated by OH^- per PS-II reaction centers was estimated to be ~1:1, respectively, in intact chloroplasts while that of the low spin form with $g = 3.0$ is rather small (~0.1:1). Using the ratio of 1:2.5 for Mb heme Fe(III) signals (amplitudes multiplied by widths) with $g = 6.87$ and $g = 5.81$, respectively (see above) for calibration one can estimate from the ratio of ~1:4.5 for the chloroplast signals with $g = 6.8$ and $g = 5.8$, respectively, that in intact chloroplasts the OH^- ligated form accounts for about 40% ± 10% of the total amount of the high spin heme Fe(III) of Cyt *b*-559.

Fig. 2b shows that removal of the Mn-cluster participating in water oxidation [17], by NH_2OH treatment leads to a change of the *g* value of the heme Fe(III) ligated by OH^- from 6.8 to 6.9 and to broadening of this band. This finding could be considered as a hint for some interaction between the heme Fe of Cyt *b*-559 and the Mn-cluster (see also below).

In Fig. 1 and Fig. 2 results are presented that describe signals emerging from dark oxidation of the samples. Now it remains to analyse light induced phenomena. It is known that illumination of thylakoid membranes in chloroplasts at 80K leads to bleaching of the Cyt *b*-559 band at 557 nm [1] and to the appearance of low spin heme Fe(III) with $g_1 = 3.0$ [6]. In marked contrast to that, illumination at 80K of intact chloroplasts without added oxidants does not induce any significant signals, neither in the high spin (Fig. 3f) nor in the low spin (around $g = 3.0$) EPR regions (Fig. 3h). Only after several hours of ageing of intact chloroplasts at 4°C the appearance of a light-induced signal with $g_1 = 3.09(5)$ is observed (Fig. 3i). These data clearly show that Cyt *b*-559 in intact chloroplasts is not in the low spin form and the heme Fe(II) can not be photooxidized at 80K. Then the optical absorbance changes can reflect some changes of a ligand coordinating the heme Fe(II).

When the heme Fe(II) is preoxidized to Fe(III) light-induced EPR signals are observed the intact chloroplasts at low temperatures. Fig. 3a shows the light induced EPR signal in the $g = 6$ region upon illumination at 140K in the presence of 10 mM DDQ. The appearance of the splitted signal with $g = 5.8$ and

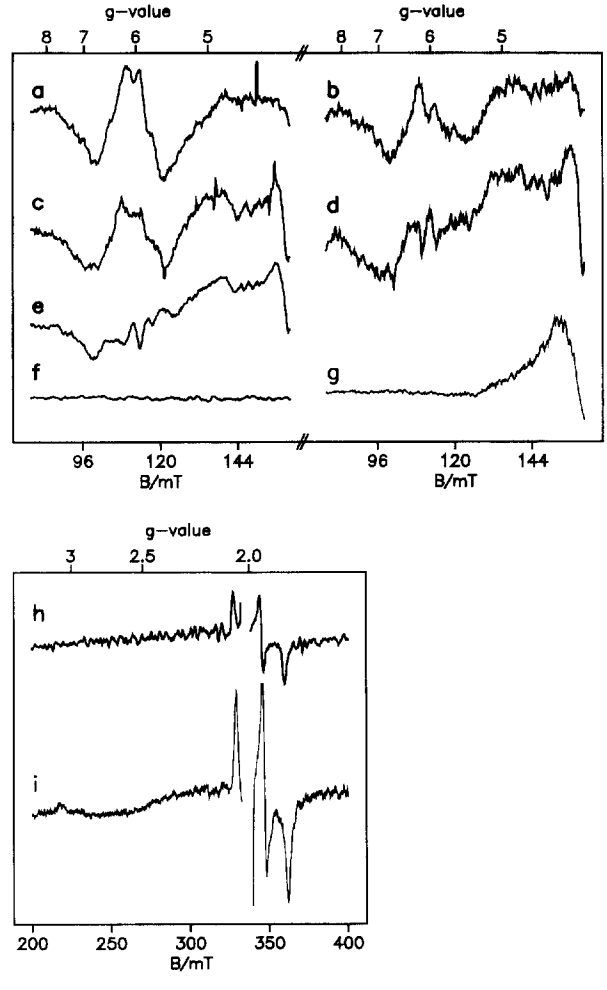

Fig. 3. Effect of illumination on EPR spectra under various conditions. All spectra are light-minus-dark difference spectra of chloroplasts; samples were illuminated by red light (cut off wavelength 600 nm); (a to g) Fe(III) high spin region, (h and i) Fe(III) low spin region, (a to e) chloroplasts preoxidized by DDQ, (f to i) not preoxidized. (a) Illumination at 140K, (b) illumination at 80K, (c) illumination at 140K of NH_2OH treated, (d) illumination at 80K of NH_2OH treated, (e) illumination at 140K and pH 5.9, (f and h) freshly prepared chloroplasts illuminated at 80K; (g and i) chloroplasts that were stored in the dark on ice for 6 hours and illuminated at 80K. Experimental conditions: concentration 12 mg Chl/ml, microwave power 5 mW, modulation amplitude 1 mT, recording time 4 min, 10K.

$g = 6.1$ and a concomitant decrease of the low field shoulder with $g = 6.8$ can be seen. The ratio of the amplitudes multiplied by widths of these two signals varied from sample to sample and is approximately 1:1. These EPR features are very similar to those seen in chemically oxidized chloroplasts (Fig. 1). The extent of the $g = 6.8$ signal loss is comparable with the amplitude of the corresponding dark EPR signal caused by dark oxidation with DDQ (Fig. 1c). This finding implies that 50–100% of the heme Fe(III) ligated by OH^- are modified upon illumination. Furthermore, the light-induced changes of the high spin EPR signal at low temperature indicate that this heme Fe(III) has to be close to P680. Accordingly, this heme is evidently Cyt *b*-559 in the high spin form (see [9]) and certainly not Cyt *f*. The decrease of the $g = 6.8$ signal is also observed upon illumination at 80K but in this case it is accompanied by the appearance of the $g = 5.8$ signal much smaller in amplitude (Fig. 3b).

Fig. 3c and 3d show that the removal of the Mn-cluster from chloroplasts by NH_2OH treatment changes the shape of the light-induced EPR signals that are generated at 140K and 80K. The light-induced decrease of the $g = 6.8$ signals is accompanied with the appearance of the $g = 5.8$ signal (but smaller amplitude than in untreated chloroplasts) at 140K, which is not seen at 80K (Fig. 3d). At pH 5.9 a decrease of the EPR signal with $g = 6.8$ is observed upon illumination at 140K (Fig. 3e). In this case the appearance of the $g = 5.8$ signal is not observed.

4. Discussion

The EPR signals induced by chemical oxidation of intact chloroplasts by DDQ and measured at 10K suggest that these signals are due to Cyt *b*-559 and not to Fe(III)–plastoquinone complex since the $g = 8$ signal [19,20] is absent. These signals also show the Fe(III) of Cyt *b*-559 in intact chloroplasts is predominantly in a high spin form. It is characterized by a splitted signal with g values at 5.8 and $g = 6.1$ and a shoulder at $g = 6.8$. In aged chloroplasts low spin heme Fe with $g_1 = 3.09$ is formed upon chemical or light-induced oxidation. The aging is accompanied by a dramatic decrease of the high spin heme Fe(III) signal. Based on these findings, the low spin EPR feature with $g_1 = 3.1$ observed in many kinds of PS~II preparations [6,8,14] is therefore assumed to be mostly due to some structural modification of PS~II. A comparison of the EPR signals of the high spin heme Fe(III) in intact chloroplasts with that in Mb (at different pH) (Fig. 1) where the 6th coordination position is not occupied by a strong ligand [12], leads to the conclusion that the signal with $g = 6.8$ is due to the ligation of heme Fe(III) by OH^- at the 6th coordination position (see also [9]). This ligation probably changes the symmetry of a molecular field (e.g. tetragonal symmetry is replaced by octaedral one with a rhombic or tetragonal distortion [18]) and gives rise to the splitting of the high spin EPR signal. This leads to appearance of the low field component with $g = 6.8$ seen for Mb and chloroplasts and of the high field component with $g = 4.9$ seen for Mb and not for choroplasts because it is too broad and small to be observed. The additional splitting of the high spin EPR signal into close components with $g = 5.6$ and $g = 6.1$ seen in all measured spectra of chloroplasts is probably not due to OH^- ligation and might be related to distortion of the axial symmetry of the molecular field by 5th axial ligand.

Quantitative estimations revealed that in intact chloroplasts about one Fe(III) ligated by OH^- is present per PS-II. A decrease of OH^- ligation is observed in the presence of NH_3 which is a stronger ligand than OH^- [13] (see also [17]).

The parameters of the EPR signal reflecting the heme Fe(III) ligated by OH^- are markedly changed when the Mn-cluster was removed by NH_2OH treatment. The g-value shift and broadening of the $g = 6.8$ signal are probably due to structural changes that modify the position of OH^- with respect to the heme Fe. This effect might suggest that the Cyt *b*-559 heme Fe ligated by OH^- could be in close proximity to the Mn-cluster.

Illumination at 80K with red light of intact chloroplasts which are preoxidized by DDQ before freezing leads to a considerable loss (50%–100%) of heme Fe(III) signal with $g = 6.8$. At 140K this disappearance is accompanied by appearance of the high spin heme Fe(III) signal with $g = 5.8$ (and $g = 6.1$) (Fig. 3a). The disappearance and appearance of the heme Fe(III) signals can be formally explained by reduction of the heme Fe(III) and oxidation of the heme Fe(II) which was not oxidized by DDQ, respectively. However, optical measurements (Shuvalov V.A. and Schreiber U., unpublished) show that at 80K no significant light-induced absorbance changes are observed around 560 nm when Cyt *b*-559 is preoxidized. This shows that at 80K Fe(II) is not formed by illumination in a significant extent. Based on these facts one can suggest that at 80K a light-induced modification of heme Fe(III) ligated by OH^- occurs. This modification can be due: (1) to removal of OH^- from Fe(III) leading to formation of intermediate states at 80K with broad EPR signal and to complete removal of OH^- at 140K when EPR signal with $g = 5.8$ appears; (2) to oxidation of Fe(III)–OH^- complex at 80K and to removal of $OH^\bullet$ radical from Fe(III) at 140K. The removal of OH^- at 140K can be monitored by EPR since the $g = 6.8$ signal reflects the OH^- ligation and the 5.8 signal indicates the absence of the 6th ligand. In fact, a ratio of ~1:1 was found for the amplitudes multiplied by widths of disappearing ($g = 6.8$) and appearing ($g = 5.8$) signals caused by illumination at 140K. This value is similar to the ratio of signals in Mb samples containing equal concentrations of Fe(III) with and without OH^- ligation, respectively, at the 6th coordination position (Fig. 1a and b). Therefore, it is most likely that at 140K red light absorbed by chlorophyll but not by Cyt *b*-559 induces a modification of Fe(III) ligation. The OH^- ligand is assumed to become modified and eventually released so that the Fe(III) attains a state without 6th ligand. One attractive speculation emerges from these findings, i.e. the possibility that heme Fe(III) ligated by OH^- becomes oxidized by $P680^+$ in a complex consisting of Cyt *b*-559 and the Mn-cluster. After oxidation the $OH^\bullet$ radical ligated to Fe(III) is transferred from the heme Fe to the Mn-cluster. In line with this idea, removal of the Mn-cluster leads to a change of the g-value parameters of the heme Fe(III) ligated by OH^- (Fig. 2) and to a decrease of the light induced formation of free heme Fe at 140K and 80K (Fig. 3c and 3d). The changes of the EPR spectrum induced by illumination at 80K and 140K correspond completely with g-value seen in a dark spectra (Fig. 1).

In contrast to pH 7 (Fig. 3a and 3b) the light-induced signal at pH 5.9 is characterized by the disappearance of the $g = 6.8$ signal without increase of the signal at $g = 5.8$ even at 140K (Fig. 3e). Thus the light-induced formation of the $g = 5.8$ signal is pH dependent. In view of the proposal that OH^- is removed from Fe(III) after its light induced oxidation at 140K one can

suggest that this phenomenon is due to the coulombic interaction of another OH^- with the Fe(III) which removes the presumed $OH^\bullet$ radical from the Fe(III) center at pH 7 but not at pH 5.9. However, at 140K the formation of a new OH^- ligation is not observed, probably due to freezing out diffusion processes that are required for this ligand exchange. The lower temperature at 80K can explain the fact that the removal of $OH^\bullet$ radical from the heme Fe(III) is hindered.

Thus, in freshly prepared intact chloroplasts oxidized by 10 mM DDQ almost all Fe(III) of Cyt *b*-559 attains the high spin form with ~40% of the hemes ligated with OH^- and ~60% of the hemes lacking a ligand at 6th coordination position. These findings might be indicative of the presence of two hemes in agreement with previous work on D1D2Cyt *b*-559 complex [17]. One of them is ligated by OH^- and another is not. The Cyt *b*-559 that is ligated by OH^- could be located proximal to the Mn-cluster and participate in water oxidation while the other heme participates in electron transfer processes. During aging of chloroplasts or preparation procedures applied for PS-II complexes the position of Cyt heme could be changed as a result of protein destortion in some fraction thus giving rise to formation of bis-histidine ligation [4].

The proposal that water is oxidized by a complex of Cyt *b*-559 ligated by OH^- in close proximity to the Mn-cluster is similar to models of heme-copper oxidases well known for prokariotic and eukaryotic organisms [21]. These oxidases reduce O_2 to water by electron transfer from the respiratory chain using a terminal complex consisting of two hemes and one atom of Cu. Four electron reduction of O_2 is accompanied by the change of valences of one heme Fe (ligated by O_2) and Cu (second heme is participating in the electron transfer). More detailed analysis of water oxidizing complex including Cyt *b*-559 is in progress.

Acknowledgements: We thank Prof. U. Heber, Prof. V.P. Skulachev and Dr. D.Sh. Burbaev for helpful discussion. Support by Deutsche Forschungsgemeinschaft (SFB 176 and 251 and Schwerpunktprogramm 'Bioanorganische Chemie: Übergangsmetalle in der Biologie und ihre Koordinationschemie'), by Russian Fund of Basic Research (V.S.) and INTAS (V.S.) is gratefully acknowledged.

References

[1] Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–172.
[2] Shuvalov, V.A., Heber, H. and Schreiber, U. (1989) FEBS Lett. 258, 27–31.
[3] Tae, G.-S. and Cramer, W.A. (1994) Biochemistry 33, 10060–10068.
[4] Babcock, G.T., Widger, W.R., Cramer, W.A., Opertling, W.A. and Metz, J.G. (1985) Biochemistry 24, 3638–3645.
[5] Shuvalov, V.A. (1994) J. Bioenerg. Biomembr. 26, 619–626.
[6] Bergstrom, J. and Vanngard, T. (1982) Biochim. Biophys. Acta 682, 452–456.
[7] Shuvalov, V.A., Scheiber, U. and Heber, U. (1994) FEBS Lett. 337, 226–230.
[8] Rutherford, A.W. (1985) Biochim. Biophys. Acta 807, 189–201.
[9] Shuvalov, V.A.., Fiege, R., Schreiber, U., Lendzian, F. and Lubitz, W. (1995) Biochim. Biophys. Acta 1228, 175–180.
[10] Asada, K., Neubauer, C., Heber, U. and Schreiber, U. (1990) Plant Cell Physiol. 31, 557–564.
[11] Tamura, N. and Cheniae, G.M. (1985) Biochim. Biophys. Acta 809, 245–259.
[12] Dickinson, L.C. and Symsons, M.C.R. (1983) Chem. Soc. Revs. 12, 387–414.
[13] Falk, J.E. (1964) Porphyrins and Metalloporphyrins, Elsevier, Amsterdam.
[14] Volker, M., Renger, G. and Rutherford, A.W. (1985) Biochim. Biophys. Acta 851, 424–430.
[15] Brautigan, D.L., Feinberg, B.A., Hoffman, B.M., Margoliash, E., Peisach, J. and Blumberg, W.E. (1977) J. Biol. Chem. 252, 574–582.
[16] Miller, A.-F. and Brudvig, G.W. (1991) Biochim. Biophys. Acta 1056, 1–18.
[17] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 262–352.
[18] Wertz, J.E. and Bolton, J.R. (1972) Electron Spin Resonance, McGraw-Hill Book Co, New York.
[19] Petrouleas, V. and Diner, B. (1986) Biochim. Biophys. Acta 849, 264–275.
[20] Zimmermann, J.-L. and Rutherford, A.W. (1986) Biochim. Biophys. Acta 851, 416–423.
[21] Trumpower, B.L. and Gennis, R.B. (1994) Annu. Rev. Biochem. 63, 675–716.