Highly purified D1/D2/cyt *b*559 preparations from spinach do not contain the non-heme iron center

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Abstract ^{57}Fe enriched D1/D2/cyt *b*559 preparations were isolated from spinach grown hydroponically on a ^{57}Fe containing medium. In terms of polypeptide and pigment composition these samples are of high purity and functional integrity of P680. Mössbauer spectra measured in D1/D2/cyt *b*559 complexes revealed that these preparations are completely deprived of the non-heme iron center. Possible implications of this finding are discussed for the electron transfer from Pheo$^{-\bullet}$ to exogenous electron acceptors.

Key words: Cytochrome b559; Non-heme iron center; *Spinacea oleracea*

1. Introduction

The isolation of reaction centers from purple bacteria with full activity was a milestone in unraveling the functional and structural organization of these anoxygenic organisms. These preparations not only enabled the application of spectroscopic techniques with high sensitivity and resolution to study the elementary processes of light induced charge separation (for a recent review see [1] and papers cited therein), but also permitted the crystallization and resolution of the structure by X-ray diffraction pattern analysis (for a recent review see [2] and papers cited therein). Therefore numerous efforts have been made to obtain comparable preparations from PSII, which is the unique machine for water cleavage in all oxygen evolving photosynthesizers (for a recent review see [3] and papers cited and references therein).

PSII complexes that retain the full oxygen evolution capacity consist of at least eight polypeptides (CP47, CP43, D1, D2, the two subunits of cytochrome *b*559, the psbI gene product and the extrinsic PSII-O protein, see [4]). Smaller complexes can be isolated that are still able to form the radical ion pair P680$^{+\bullet}$Pheo$^{-\bullet}$ but are deprived of the capability to evolve oxygen and to reduce PQ_A [5,6]. This complex, designated D1/D2/cyt *b*559 preparation, binds P680, Pheo, the heme group of cytochrome *b*559, accessory Chl a and carotenoids. However, it lacks Q_A [5–7] and therefore, in the absence of powerful exogenous acceptors like SiMo the radical pair cannot be stabilized and recombines dissipatively with kinetics of 20–50 ns [8–12].

Until recently the pigment composition of D1/D2/cyt *b*559 complexes was a matter of controversy [7,11,13,14]. Recent studies, however, have provided convincing evidence that highly purified preparations contain 6 Chl a, 2 Pheo and 1–2 β-carotenes [15,16] and permit an almost 100% capacity for formation of the radical pair P680$^{+\bullet}$Pheo$^{-\bullet}$ [17]. On the other hand, it is still not clear to what extent the non-heme iron located between Q_A and Q_B [18] remains bound to D1/D2/cyt *b*559 preparations. Mössbauer spectroscopy provides a very powerful tool to address this point. Therefore, the present study was performed to answer this question. The results obtained unambiguously show that the samples are completely deprived of their non-heme iron center.

2. Materials and methods

2.1. Isolation of ^{57}Fe enriched PSII preparations

Spinach grown hydroponically on ^{57}Fe containing medium was used to isolate PSII membrane fragments according to the procedure of Berthold et al. [19] with slight modifications [20]. D1/D2/cyt *b*559 complexes were prepared using a modified protocol reported by Seibert et al. [21]. In order to minimize the chlorophyll content the washing procedure was carried out until the OD_{670} was below 0.003.

2.2. Room temperature absorption spectrum

Absorption spectra at room temperature were recorded on a Varian Cary 5 UV-VIS-NIR spectrophotometer.

2.3. SDS-PAGE and silver stained gel

Denaturing SDS-PAG electrophoresis was performed as in [22] with 15% (v/v) acrylamide and 4 M urea. Silver staining was carried out according to the method of Oakley et al. [23].

2.4. Mössbauer spectroscopy

For Mössbauer spectroscopy the samples were concentrated by centrifugation at 165 000 × *g*. In the case of D1/D2/cyt *b*559 preparations the centrifugation was preceded by an incubation with 25% PEG 3350 for 60 min. Mössbauer experiments were performed at 155 K in a weak magnetic field perpendicular to the γ-beam using a ^{57}CoRh source as described in Parak and Reinisch [24]. All spectra were fitted by Lorentzians. The isomer shifts are given relative to metallic ^{57}Fe as a reference.

3. Results and discussion

The purity of the ^{57}Fe enriched D1/D2/cyt *b*559 preparations was checked by silver staining of an SDS-PAGE and by measuring the absorption spectrum. Fig. 1 shows the silver stained PAGE. The bands resolved are ascribed to the D1/D2 heterodimer, the D1 and D2 polypeptides and the α- and β-subunits of cytochrome *b*559 as marked in Fig. 1. The high sensitivity of silver staining also reveals faint bands in the region of CP43 and CP47. According to a recently reported

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Abbreviations: Chl, chlorophyll; CoRh, cobalt rhodium; CP, chlorophyll protein; cyt, cytochrome; OD, optical density; PEG, polyethylene glycol; Pheo, pheophytin; PQ, plastoquinone; PSII, photosystem II; P680, photochemically active Chl a of PSII; Q_A, Q_B, primary and secondary quinone acceptor; SDS, sodium dodecylsulfate; SiMo, silicomolybdate

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Fig. 1. Silver stained gel of the D1/D2/cyt *b*559 preparation.

analysis [17] these bands account for a contamination of less than 5%.

In a complementary test the room temperature VIS absorption spectrum was measured that is shown in Fig. 2. Two parameters are essential to characterize the purity and quality of the sample: (i) the peak position of the Q_Y transitions in the red, and (ii) the ratio of the absorbance at 416 and 435 nm: A_{416}/A_{435}. An inspection of the spectrum reveals that the red band exhibits a peak position at 675 ± 1 nm which is indicative of a functionally intact P680 [10,20,25]. Therefore the preparation is highly active with respect to formation of the radical pair $P680^{+\bullet}Pheo^{-\bullet}$.

The second parameter A_{416}/A_{435} has a value of 1.16. According to a recent detailed study on A_{416}/A_{435} as a sensitive indicator of sample purity [26], a ratio of 1.16 is characteristic for samples of high purity in terms of pigment composition. Therefore, as a result of the measurements in Figs. 1 and 2 the ^{57}Fe enriched D1/D2/cyt *b*559 preparation provides the most suitable material for an analysis of the non-heme iron content by Mössbauer spectroscopy.

In order to have a suitable standard for comparison, control experiments were performed with ^{57}Fe enriched PSII membrane fragments. Fig. 3 shows the Mössbauer spectra measured at 155 K in ^{57}Fe enriched PSII membrane fragments (top trace) and in ^{57}Fe enriched D1/D2/cyt *b*559 preparations (bottom trace). Even at the first glance marked differences are discernible between the spectra of both samples. The control spectrum exhibits three peaks while only two are observed in D1/D2/cyt *b*559 preparations.

A closer inspection of the data reveals that the spectrum of the control sample can be resolved into three quadrupole doublets as illustrated by the thin lines in Fig. 3. This deconvolution is in agreement with reports in the literature [27,28]. The isomer shift and quadrupole splittings gathered from the data fit by Lorentzians are compiled in Table 1. Two of the doublets shown in Fig. 3, top, exhibit characteristic features (isomer shifts and quadrupole splittings) that are typical for the low spin forms of Fe(II) and Fe(III) in cytochromes. Accordingly, these components are ascribed to two cytochrome *b*559 forms which differ markedly in their redox potential so that one of them attains the oxidized and the other one the reduced state under the experimental conditions used for recording the spectra of Fig. 3. On the basis of its isomer shift and quadrupole splitting the third doublet is assigned to the non-heme Fe^{2+} center.

The data analysis of the trace with markedly different shape measured in ^{57}Fe enriched D1/D2/cyt *b*559 preparations shows that this spectrum is well described by a single asym-

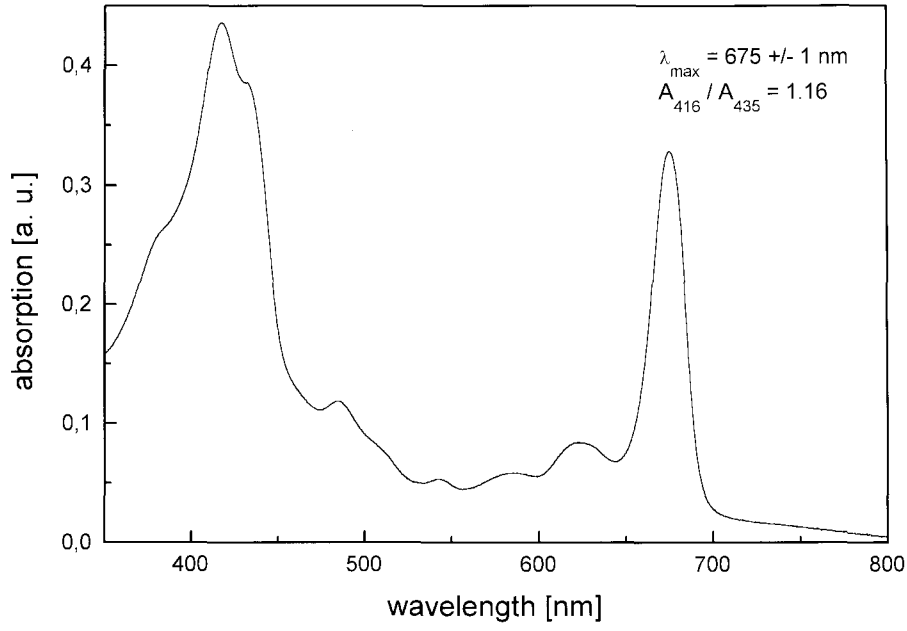

Fig. 2. Room temperature absorption spectrum of the D1/D2/cyt *b*559 preparation (a.u. = arbitrary units).

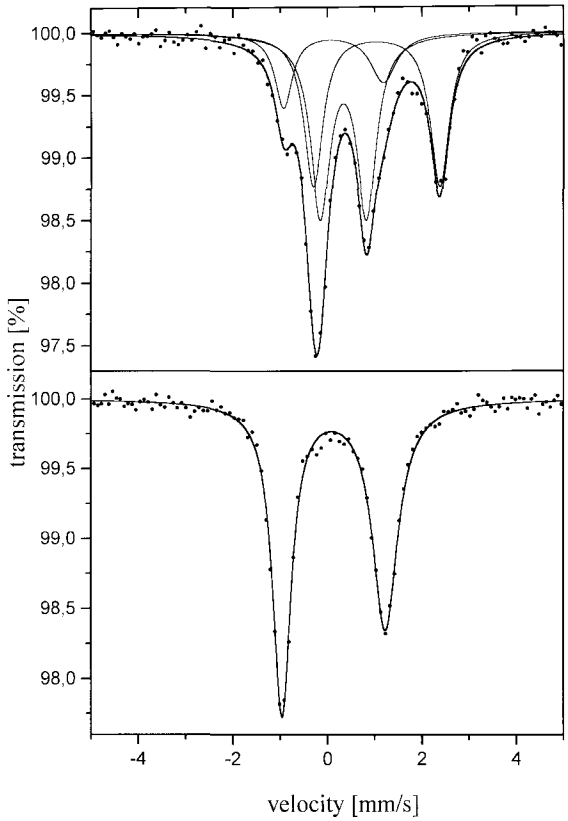

Fig. 3. Mössbauer spectra of ^{57}Fe enriched PSII membrane fragments (top trace) and D1/D2/cyt *b*559 preparations isolated therefrom (bottom trace). The thin lines represent the spectra of the individual components simulated by Lorentzians, the heavy lines are the composite of these components. The parameters used are compiled in Table 1.

metric quadrupole doublet. The characteristic features resemble those of the doublet in the control spectrum (see Fig. 3, top trace) that is ascribed to the low spin Fe(III) of oxidized cytochrome *b*559. This finding implies that D1/D2/cyt *b*559 preparations do not contain cytochrome *b*559 in the high potential form. An analogously structured Mössbauer spectrum has recently been found in ^{57}Fe enriched PSII membrane fragments that were treated twice with an iron depletion procedure [29]. In this case, however, the experiments were performed in the presence of $Na_2S_2O_4$ so that the doublet exhibits parameters (isomer shifts and quadrupole splitting, see Table 1) that are characteristic for low spin Fe(II), i.e. the reduced cytochrome *b*559.

Regardless of these differences of the cytochrome *b*559 Mössbauer spectrum (this interesting phenomenon and its implications will be outlined in detail in a forthcoming paper) the common feature of iron depleted PSII membrane fragments and of D1/D2/cyt *b*559 preparations is the absence of any detectable contribution of the non-heme iron to the overall absorption. Therefore, based on the present data and the comparison with iron depleted PSII membrane fragments the conclusion can be drawn that highly purified and active D1/D2/cyt *b*559 preparations are deprived of the non-heme iron center below the detection limit of Mössbauer spectroscopy.

This finding also has implications for the pathway of electron transfer from $Pheo^{-\bullet}$ to exogenous acceptors. To the best of our knowledge, SiMo seems to be the most powerful component to compete with the recombination reaction of the radical pair to $P680^{+\bullet}Pheo^{-\bullet}$ [30]. It was recently concluded that in PSII with an intact acceptor side SiMo binds in the neighborhood of the non-heme iron center and that the latter might be directly involved in the electron transport pathway [31]. This mode of reaction is certainly prevented in D1/D2/cyt *b*559 preparations. It is therefore concluded that $Pheo^{-\bullet}$ is directly oxidized by SiMo. Although the site of SiMo remains to be clarified, a close interaction giving rise to a very fast electron transfer is not achieved because the internal radical pair recombination was still found to dominate in the presence of this acceptor (data not shown). Attempts to use $Pheo^{-\bullet}$ as electron source to establish a light induced $NADP^+$ reduction in D1/D2/cyt *b*559 preparations by addition of an exogenous donor, ferredoxin and ferredoxin-$NADP^+$ reductase were successful, but the turnover rate is extremely low owing to inefficient electron transfer from $Pheo^{-\bullet}$ to the acceptor system [32].

In summary, it can be inferred that in D1/D2/cyt *b*559 preparations the acceptor side beyond Pheo is severely damaged and an efficient competition of electron acceptors with $P680^{+\bullet}Pheo^{-\bullet}$ recombination could not be achieved so far.

The large changes of the acceptor side most likely comprise a seriously altered structure of the D1/D2 heterodimer at its stroma side domain. This phenomenon is assumed to be the main reason for the failure to achieve a large scale reconstitution of a functionally competent Q_A. Therefore, it appears necessary to develop a procedure for isolation of D1/D2/cyt *b*559 preparations that avoids damaging structural changes. As a first step in this direction, experiments were performed

Table 1
Mössbauer parameters of the iron centers in PSII membrane fragments, iron depleted PSII membrane fragments and D1/D2/cyt *b*559 preparations

Sample	Type of Fe center	Isomer shift (mm/s)	Quadrupole splitting (mm/s)
PS II membrane fragments (T = 155 K)	cyt *b*559 Fe^{2+} low spin	0.44 ± 0.01	0.98 ± 0.01
	cyt *b*559 Fe^{3+} low spin	0.23 ± 0.02	2.12 ± 0.04
	non-heme iron Fe^{2+} high spin	1.14 ± 0.01	2.68 ± 0.01
Fe-depleted PSII membrane fragments reduced by $Na_2S_2O_4$[a] (T = 14 K)	Cyt *b*559 Fe^{2+} low spin	0.44 ± 0.01	1.05 ± 0.01
	non-heme iron signals not detectable		
D1/D2/cyt *b*559 (T = 155 K)	cyt *b*559 Fe^{3+} low spin	0.24 ± 0.01	2.19 ± 0.01
	non-heme iron signals not detectable		

[a]Data taken from [29].

to unravel the step(s) of the preparation procedure where Q_A becomes irreversibly lost. The functional integrity of the Q_A site was checked by measuring flash induced absorption changes at 320 nm that reflect Q_A^- formation [33]. It was found that treatment of PSII membrane fragments (final concentration: 0.82 mg[Chl]/ml) with 3.2% (w/v) Triton X-100 (conditions as for preparation of D1/D2/cyt *b*559 complexes) did not significantly affect the extent of flash induced Q_A^- formation even after a rather extended incubation period of 6 h on ice. However, when the Triton X-100 treated sample is put onto the chromatography column, washed and eluted, the capacity of electron transfer from $Pheo^{-\bullet}$ to Q_A is entirely lost (Karge, Kurreck and Renger, unpublished data). It is therefore necessary to find an alternative purification procedure in order to open the road for the preparation of Q_A containing D1/D2/cyt *b*559 preparations. In this respect it has to be taken into account that according to recent studies the structural requirements for Q_A binding are markedly different in anoxygenic purple bacteria [34]. As a consequence, it is important to clarify if other polypeptides of PSII (e.g. CP47, psb L gene product) and/or lipids (for further discussion see [35]) are essential constituents in protecting and/or establishing the structural determinants of a functional Q_A site in PSII.

4. Conclusion

The present study provides the first experimental evidence that D1/D2/cyt *b*559 preparations of high purity and competence of $P680^{+\bullet}Pheo^{-\bullet}$ radical pair formation are not only lacking the plastoquinone components Q_A and Q_B but are also deprived of the non-heme iron center. The lack of these non-covalently bound cofactors is very likely an indication of serious structural changes in stroma exposed regions of polypeptides D1 and D2. These modifications leave the primary charge separation virtually unaffected but it remains to be analyzed to what extent the properties of redox groups like P680 and Y_Z are altered via long distance structural effects of the protein matrix. The results obtained with Triton X-100 treated PSII membrane fragments, however, might open a new road for the isolation of D1/D2/cyt *b*559 complexes with a more intact acceptor side by searching for a mild separation procedure of the solubilized material that avoids the loss of Q_A. Apart from this perspective, the absence of the non-heme iron center in D1/D2/cyt *b*559 preparations is also of great advantage because it provides most suitable samples for detailed analyses of the cytochrome *b*559 ligation by using the very powerful technique of Mössbauer spectroscopy. Experiments are in progress to address this problem. The results will be presented in a forthcoming paper.

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