

Isolation and properties of PS II membrane fragments depleted of the non heme iron center

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Abstract The functional properties and the content of non heme iron and cytochrome *b559* were investigated by measuring flash induced transient changes of the relative fluorescence quantum yield and applying Mössbauer spectroscopy. It was found that untreated PS II membrane fragments contain a heterogeneous population of two types of non heme iron centers and about 2 cytochrome *b559* per PS II. Twofold treatment of these samples with a recently described 'iron depletion' procedure (MacMillan, F., Lenzian, F., Renger, G. and Lubitz, W. (1995) *Biochemistry* 34, 3144–3156) leads to a complete loss (below the detection limit of Mössbauer spectroscopy) of the non heme iron center while more than 50% of the PS II complexes retain the functional integrity for light induced formation of the 'stable' radical pair $Y_2^{\text{Ox}}\text{P680Pheo}Q_A^-$. This sample type deprived of virtually all non heme iron in PS II provides a most suitable material for magnetic resonance studies that require an elimination of the interaction between Fe^{2+} and nearby radicals.

Key words: Photosystem II; Non heme iron; Cytochrome *b559*; Mössbauer spectroscopy; Flash induced fluorescence

1. Introduction

Water cleavage in Photosystem II (PS II) by visible light leads to formation of molecular dioxygen and bound hydrogen in the form of plastoquinol PQH_2 (for a recent review see ref. [1]). The latter process takes place in a special protein pocket referred to as Q_B -site where a transiently and non-covalently bound molecule from the plastoquinone pool becomes reduced to plastoquinol via a sequence of two univalent redox steps (for a review see ref. [2]). This reaction is energetically driven with a special plastosemiquinone, Q_A^- , that is formed as a result of the stabilization of the primary charge separation (for a review see ref. [3]). In contrast to plastoquinone in the Q_B -site, Q_A attains only the redox states of quinone and semiquinone under normal conditions [4] and it is a permanently bound constituent of the PS II reaction center. The functional and structural organization of PQH_2 formation closely resembles that of ubiquinol formation in anoxy-

genic purple bacteria (for reviews see refs. [2,5]). A non heme iron center in the high spin form Fe^{2+} is located between Q_A and Q_B . The ligation of Fe^{2+} by four histidines is assumed to be similar in both types of organisms (two from each of the L and M subunits of the heterodimer forming the reaction center apoprotein of purple bacteria and of the D1 and D2 polypeptide of PS II) [5–7] while the glutamate ligand in the former type is probably replaced by bicarbonate in PS II [8]. Differences also exist with respect to the redox potential that is significantly lower in PS II for the couple $\text{Fe}^{2+}/\text{Fe}^{3+}$ [9–11].

The functional role of Fe^{2+} is not yet resolved. Regardless of this problem, the presence of a high spin transition metal center gives rise to magnetic interactions with the spin states of the nearby redox groups. This phenomenon profoundly affects the application of magnetic resonance methods as an analytical tool. Therefore, several attempts were made to eliminate the magnetic effect due to Fe^{2+} . In principle this goal can be achieved in two different ways: (i) transformation of the high spin ($S=2$) into the low spin diamagnetic state ($S=0$) of Fe^{2+} or (ii) extraction of the non heme iron. The former method was recently shown to be successful in PS II if the samples are incubated with high concentrations of CN^- that acts as a strong ligand to Fe^{2+} [12]. The second procedure was first developed for isolated reaction centers from anoxygenic purple bacteria where the Fe^{2+} could be reversibly removed [13]. An application of this method in PS II appears to be more difficult ([14] and references therein). Until now it was not even clear whether or not the Fe^{2+} is really removed from PS II or simply transferred to the low spin state by the 'extraction procedure'. The present study describes experiments to clarify this point and to optimize the preparation procedure. Based on Mössbauer spectroscopic data it is unambiguously shown that a complete removal of the non heme iron center from PS II can be achieved by using twice the previously developed 'iron depletion' method [14].

2. Materials and methods

2.1. Preparation of iron depleted PS II membrane fragments

PS II membrane fragments were isolated from market spinach according to the procedure of Berthold et al. [15] with slight modifications [16]. After the final isolation step the PS II membrane fragments were resuspended in 10 mM MES/NaOH (pH 6.5), 15 mM NaCl, 4 mM MgCl_2 , 400 mM sucrose to chlorophyll concentrations of about 5 mg/ml.

Iron depleted samples were prepared as described in MacMillan et al. [14]. After the final centrifugation step the samples were resuspended in 20 mM MES pH 6.5, 10 mM NaCl and 30% (w/v) sucrose to chlorophyll concentrations of about 5 mg/ml. If indicated, treat-

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Abbreviations: Chl *a*, chlorophyll *a*; CoRh, cobalt rhodium; Cyt *b559*, cytochrome *b559*; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; LED, light emitting diode; MES, morpholinoethane sulfonic acid; P680, photoactive chlorophyll of PS II; Pheo, pheophytin; Q_A , bound plastoquinone-9 acceptor of PS II; Q_B , plastoquinone-9 in Q_B -site of PS II; PQH_2 , plastoquinol-9; PS II, photosystem II; Y_2 , redox active tyrosine between P680 and the water oxidizing complex.

ment with lithium perchlorate and 1,10-phenanthroline and subsequent addition of conalbumin was repeated for a second time.

All samples were frozen in small aliquots in liquid nitrogen and stored at -80°C until use.

2.2. Measurements of the flash-induced changes of the fluorescence quantum yield

Flash-induced changes of the fluorescence quantum yield were monitored with a home-built equipment (Gleiter et al. [17]).

2.3. Mössbauer measurements

The samples for Mössbauer measurements were isolated from spinach grown hydroponically in a ^{57}Fe enriched medium. The ^{57}Fe -PS II membrane fragments were concentrated by centrifugation at $165000\times g$. Mössbauer experiments were performed at 14 K in a weak magnetic field perpendicular to the γ -beam using a $^{57}\text{CoRh}$ source as described in ref. [18]. All spectra were fitted by Lorentzians. The isomer shifts are given relative to metallic ^{57}Fe as a reference.

3. Results and discussion

Information on the properties of the non heme iron center can be obtained by measuring the transient changes of the fluorescence quantum yield induced by excitation with a train of laser flashes of dark adapted samples that were preincubated in the presence of $\text{K}_3[\text{Fe}(\text{CN})_6]$. The quantum yield of the fluorescence emitted from the chlorophylls of PS II de-

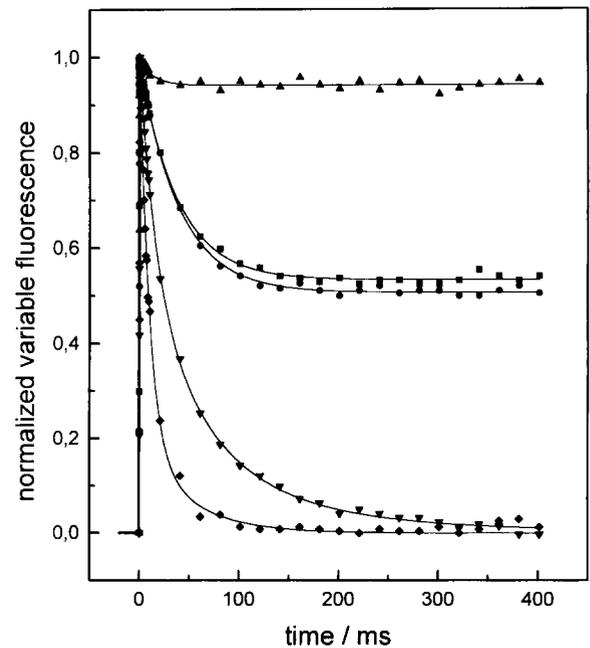


Fig. 2. Normalized variable fluorescence yield induced by a laser flash in 'iron depleted' PS II membrane fragments (double treated) illuminated by a preflash. The suspension contained: sample material (20 μg Chl/ml), 10 mM NaCl, 5 mM MgCl_2 and 50 mM Mes/NaOH pH = 6.5. Control: squares; addition of 10 μM $\text{K}_3[\text{Fe}(\text{CN})_6]$: inverted triangles; addition of 100 μM $\text{K}_3[\text{Fe}(\text{CN})_6]$: diamonds; addition of 5 μM DCMU: circles; addition of 4 mM NH_2OH : triangles.

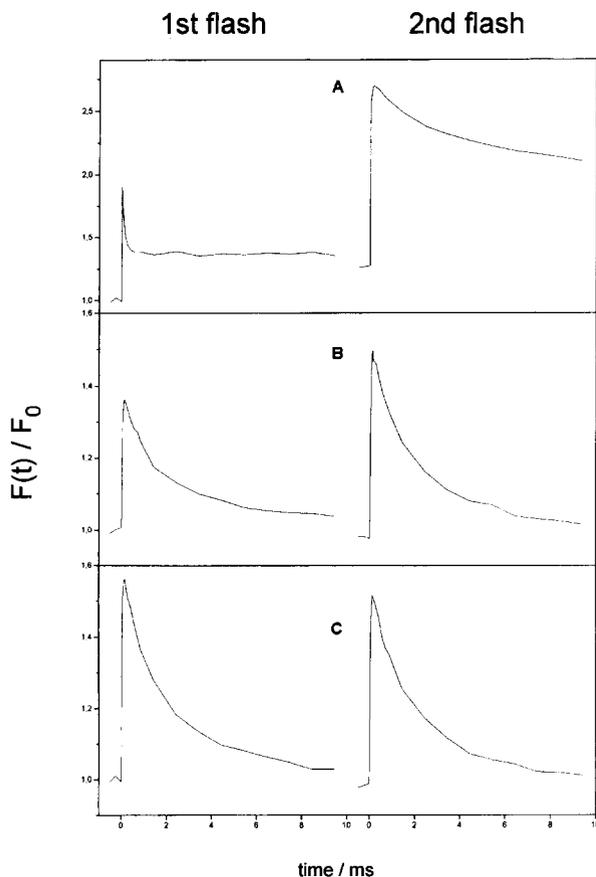


Fig. 1. Laser flash induced change of the relative fluorescence quantum yield as a function of time in dark adapted untreated PS II membrane fragments in the presence of $\text{K}_3[\text{Fe}(\text{CN})_6]$. A: control, B: samples subjected once and C: twice (bottom traces) to the 'iron depletion' procedure described in ref. [14]. The suspension contained: sample material (20 μg Chl/ml), 10 mM NaCl, 5 mM MgCl_2 , 50 mM Mes/NaOH pH = 6.5 and 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$. Time between the flashes 0.5 s. Other conditions as described in section 2.

pends on the redox state of the functional components P680, Pheo and Q_A . These components act in certain redox states as fluorescence quenchers (P680^{2+} and $\text{Pheo}^{\cdot-}$ as non-photochemical and Q_A as photochemical quencher) [19]. After excitation of the sample with a laser flash giving rise to the formation of the primary radical pair $\text{P680}^{2+}\text{Pheo}^{\cdot-}$ the fluorescence quantum yield (monitored as emission due to a train of LED pulses) rises with kinetics that are determined by the rate of P680^{2+} reduction by tyrosine Y_z and of $\text{Pheo}^{\cdot-}$ reoxidation by Q_A . The former process exhibits kinetics within the ns- and μs -range that depend on sample treatment [20] while the latter process is characterized by a reaction time of about 300 ps [21]. About 50 μs after the flash the non quenching state $\text{P680PheoQ}_A^{\cdot-}$ is attained in the vast majority of the PS II complexes and the Chl *a*-fluorescence emission reaches its maximum. The subsequent decay mainly reflects the reoxidation of $\text{Q}_A^{\cdot-}$, leading to the quenching state P680PheoQ_A (for further details see ref. [22]). Measurements of the absolute fluorescence yield are rather difficult. However, in almost all studies it is sufficient to determine the relative fluorescence yield. In this case the flash induced change referred to as variable fluorescence $F_{\text{var}}(t) = F(t) - F_0$ is normalized to its maximum extent $F_{\text{max}} - F_0$, where F_0 is the fluorescence level in dark adapted samples owing to the measuring LED pulses before the actinic flashes and F_{max} the maximum level obtained. This quotient $(F(t) - F_0)/(F_{\text{max}} - F_0)$ is referred to as normalized variable fluorescence. In the oxidized form (Fe^{3+}) the non heme iron rapidly abstracts an electron from $\text{Q}_A^{\cdot-}$. The kinetics of this process partly overlap with those of P680^{2+} reduction by Y_z . Furthermore, the reaction is too fast for complete detection at the limited time resolution of our

Table 1
Mössbauer parameters of the iron centers gathered from a numerical fit of the spectra depicted in Fig. 4

Sample	Type of Fe-center	Isomer shift (mm/s)	Quadrupole splitting (mm/s)	Normalized absorption area (%)	Line width (mm/s)
Control	Cyt <i>b559</i>				
	Fe ²⁺ low spin	0.46 ± 0.01	1.07 ± 0.01	62 ± 1	0.36 ± 0.01
	non heme iron				
	Fe ²⁺ high spin				
'Fe-depleted' (double treatment)	specie 1	1.21 ± 0.01	2.96 ± 0.02	25 ± 4	0.34 ± 0.04
	specie 2	1.16 ± 0.06	2.42 ± 0.18	13 ± 5	0.55 ± 0.14
	Cyt <i>b559</i>				
	Fe ²⁺ low spin	0.44 ± 0.01	1.05 ± 0.01	100	0.39 ± 0.01
	non heme iron signals not detectable				

equipment. Therefore, the measurable extent of the flash induced fluorescence yield is smaller in the presence of the state Fe³⁺ than of the state Fe²⁺. Furthermore, the oxidation of Fe²⁺ by exogenous K₃[Fe(CN)₆] is rather slow so that the Fe³⁺ reduced after the first flash by Q_A⁻ cannot be restored during a dark time of 0.5 s [23]. As a consequence, the flash induced fluorescence yield is large after the 2nd flash and the ratio of the detectable maximum amplitudes after the second and first flash, respectively, can be used as a qualitative measure of a K₃[Fe(CN)₆]-oxidizable non heme iron centers in PS II, as outlined in detail in ref. [24]. This general feature is illustrated in Fig. 1 for untreated dark adapted PS II membranes that are excited with two laser flashes separated by a dark time of 500 ms (top traces).

The pattern is drastically altered in PS II membrane fragments that were subjected to the 'iron depletion' procedure either once (middle traces) or twice (bottom traces). In comparison with the untreated control three phenomena are discernible: (a) the transients induced by the first and second flash exhibit a closer similarity (middle traces) or are virtually identical (bottom traces), (b) the extent of the ratio $F(t)/F_0$ is markedly diminished and (c) the very fast decay after the first flash disappears and the relaxation after the second flash is accelerated in 'iron depleted' samples.

With respect to the topic of this study the first phenomenon is the most interesting feature. This finding indicates that the 'iron depleted' samples do not contain a non heme iron center which can be oxidized by K₃[Fe(CN)₆] to Fe³⁺ thereby acting as electron acceptor for very fast Q_A⁻ reoxidation. In perfect agreement with this conclusion, the same sample type was recently shown to lack any magnetic interaction of a non heme iron center with Q_A⁻ [14]. Two basically distinct explanations can be offered for this effect: (i) the 'iron depletion' procedure really extracts the non heme iron from PS II or (ii) the Fe²⁺ remains still bound but the micro environment is markedly changed so that the iron center is transferred from the high spin into the low spin ($S=0$) diamagnetic state concomitant with a drastic increase of the redox potential that prevents its reoxidation by K₃[Fe(CN)₆]. In order to check for these two possibilities, Mössbauer spectra were measured because they permit an unambiguous differentiation between different types of iron centers. Before addressing this most important point, phenomena (b) and (c) will be briefly discussed leading to further information on the functional properties of the 'iron depleted' PS II membrane fragments.

Phenomenon (b) is owing to the trypsin treatment step of

the 'iron depletion' procedure that generally leads to a decrease of the normalized variable fluorescence, also under actinic cw-illumination [25]. Phenomenon (c) is also caused by the trypsin treatment step. It leads in addition to the fluorescence quenching to an interruption of the electron transfer from Q_A⁻ to Q_B (Q_B⁻) and a drastic increase of the susceptibility of Q_A⁻ towards direct oxidation by K₃[Fe(CN)₆] [26]. The kinetics of the latter reaction was shown to be strongly dependent on the K₃[Fe(CN)₆]-concentration [27]. The properties of Q_A⁻ reoxidation in 'iron depleted' (doubly treated) PS II membrane fragments were analyzed by measuring at different K₃[Fe(CN)₆] concentrations the transient changes of fluorescence yield induced by the second flash. Typical results are depicted in Fig. 2 at an extended time scale. In the absence of

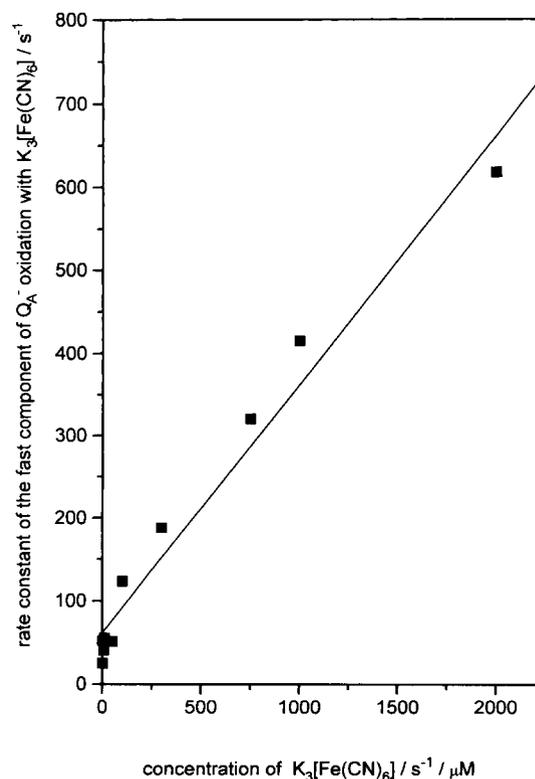


Fig. 3. Rate constant of the fast component of Q_A⁻ reoxidation after the second flash as a function of K₃[Fe(CN)₆] concentration in 'iron depleted' PS II membrane fragments (treated twice). Experimental conditions as in Fig. 2.

$K_3[Fe(CN)_6]$ the relaxation kinetics are at least biphasic (squares) and almost invariant to DCMU (circles) as expected for samples with a completely blocked electron transfer from Q_A^- to Q_B (Q_B^-). The fast phase is characterized by a life time of the order of 50 ms which is indicative for the reoxidation of Q_A^- by Y_z^{ox} in samples deprived of their oxygen evolution activity, e.g. by Tris-washing [28] or trypsin treatment. This idea is highly supported by the suppression of this reaction in the presence of 4 mM NH_2OH (triangles) that acts as an efficient electron donor for Y_z^{ox} reduction in dark adapted samples [29]. Likewise, the remaining part of the flash induced fluorescence of the control sample is explainable by the existence of an endogenous donor that competes with Q_A^- as reductant of Y_z^{ox} . This interpretation gains support by the disappearance of this remaining fluorescence yield at a concentration of only 10 μM $K_3[Fe(CN)_6]$ (inverted triangles) that does virtually not affect the life time of the decay but is assumed to oxidize the unknown donor component.

A quantitative evaluation of the data requires a transformation of the normalized variable fluorescence into $[Q_A^-(t)]$. Both quantities are connected via a non-linear relationship as described previously [30]. Based on this relation the rate constants of Q_A^- reoxidation were evaluated as outlined in [24]. The process in the ms time domain was found to be biphasic ($t_1 \sim 20$ ms and $t_2 \sim 50$ ms in the absence of $K_3[Fe(CN)_6]$). At $K_3[Fe(CN)_6]$ concentrations of $>100 \mu M$ the faster kinetics dominate (normalized amplitude $>70\%$) and therefore only

this component is further analyzed. Fig. 3 shows the rate constants for these kinetics of Q_A^- reoxidation gathered from the numerical evaluation of fluorescence measurement at different $K_3[Fe(CN)_6]$ concentrations in doubly treated PS II membrane fragments (data not shown). This data can be described by a linear relation $k = k_0 + k_A[A]$ where k is the rate constant of the overall decay, k_0 reflects the rate of Q_A^- reoxidation by endogenous redox components other than Q_B (vide infra) and k_A is the rate constant of the reaction mediated by $K_3[Fe(CN)_6]$ and $[A]$ its concentration. Based on the results of Fig. 3 a second order rate constant of $k_A = 3.0 \cdot 10^5 M^{-1} \cdot s^{-1}$ is obtained for the oxidation of Q_A^- by exogenous $K_3[Fe(CN)_6]$. This value is comparable with that obtained previously for trypsin treated thylakoids [27].

After characterization of the functional properties it remains to be shown whether or not the non heme iron is really removed from PS II by the 'iron depletion' procedure. This question can be answered by Mössbauer spectroscopy. The experiments were performed in the presence of $Na_2S_2O_4$ in order to reduce the low potential cytochrome *b559*. Fig. 4 compiles the results obtained in ^{57}Fe enriched control PS II membrane fragments (top trace) and in samples subjected twice to the 'iron depletion' procedure (bottom trace). A comparison of the spectra measured at 14 K in samples without and with $Na_2S_2O_4$ clearly shows that all cytochrome *b559* attains the reduced ferrous state in the latter case (data not shown). The isomer shifts and quadruple splittings gathered from the data fit by Lorentzians are compiled in Table 1. The interpretation of the Mössbauer spectrum of the control sample as three symmetrical quadrupole doublets and their assignment to different species of iron centers summarized in Table 1 are in agreement with data reported in the literature [31,32]. Interestingly, the contributions of the two distinguishable non heme irons are modified by $Na_2S_2O_4$ in favor of species 2. This shift was found at different temperatures. A similar sensitivity of the relative area of the two types is also mentioned in [31]. It seems to be in analogy to features of the two ferrous low spin non heme iron species that were found in the reaction centers of the anoxygenic purple bacterium *Rhodospirillum rubrum* [33]. The origin of the existence of two types of non heme iron is not yet clear. It is beyond the scope of this study and will not be further discussed.

Another interesting information can be obtained from the normalized area of the signals attributed to the different iron forms. The ratio r of the values for cytochrome *b559* and the non heme iron centers is 1.63 which closely resembles with a cytochrome *b559* content of 1.8 per PS II detected in the same sample by optical difference spectroscopy (data not shown) and using a difference extinction coefficient of $17.5 M^{-1} \cdot cm^{-1}$ [34]. Based on these data and taking into account the possibility of a loss during the isolation procedure [35] PS II membrane fragments are inferred to contain two cytochrome *b559* per PS II. However, it has to be emphasized that the Mössbauer results could be affected by contamination by a small fraction of PS I which is rich in non heme iron due to the Fe-S centers. Recent studies on PS I of cyanobacteria have shown that the Mössbauer parameters of oxidized Fe-S centers could be the same as those of low spin Fe(II) in Cyt *b559* at 80 K [36]. Therefore, contamination by PS I of about 5% would be sufficient to reduce the ratio r down to 1.0. This would require that: (i) more than 90% of these Fe-S are oxidized and remain unaffected by addition of $Na_2S_2O_4$,

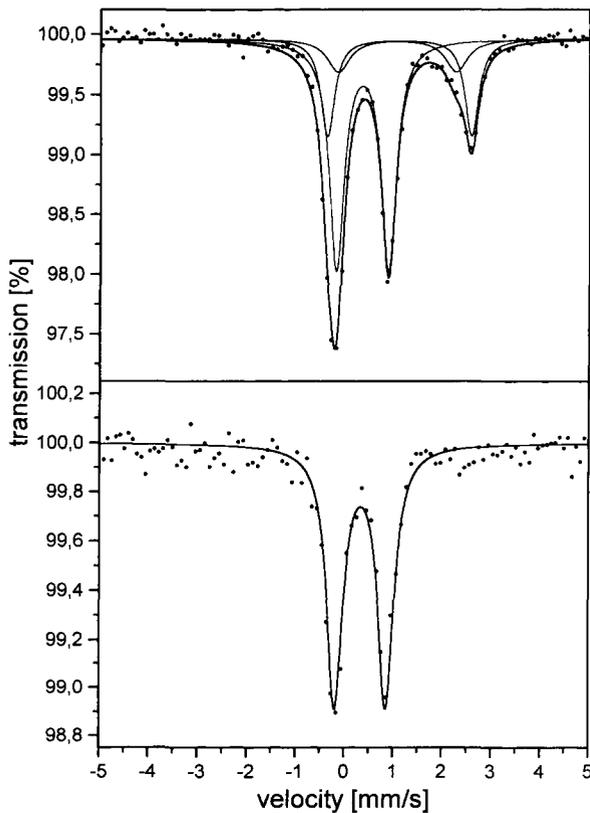


Fig. 4. Mössbauer spectra of untreated ^{57}Fe enriched PS II membrane fragments (top trace) and samples subjected twice to 'iron depletion' procedure (bottom trace). The thin-lined curves represent the spectra of the individual components simulated by Lorentzians, the heavy-lined curves are the composite spectrum of these components. The parameters used are compiled in Table 1.

and (ii) the Mössbauer parameters of the oxidized Fe–S centers exhibit an identical dependence on temperature as those of the low spin Fe(II) of Cyt *b559*. These properties are assumed to be less likely and therefore a ratio *r* of 2 is favoured. Experiments are in progress to clarify this point.

The most striking feature of Fig. 4 is the drastic change of the Mössbauer spectrum in PS II membrane fragments that were subjected twice to the 'iron depletion' procedure. A numerical fit of the data readily reveals that the spectrum cannot be described by the doublet found after a high to low spin transition of the non heme iron center caused by cyanide treatment [12]. This low spin form is characterized by an isomer shift of 0.26 mm/s and a quadrupole splitting of 0.36 mm/s. The spectrum can be well described by using the parameters for cytochrome *b559* summarized in Table 1. These findings provide clear and unambiguous evidence for the conclusion that the double treatment with 'iron depletion' procedure leads to a practically complete loss of the non heme iron centers from PS II membrane fragments. In forthcoming studies this material will be used for attempts to reconstitute the 'empty' site with other metal ions. Experiments of this kind were recently successfully performed in PS II membrane fragments deprived of the manganese of the water oxidizing complex ([37] and references therein).

4. Conclusions

The most important result of this study is the finding that the non heme iron centers can be fully extracted from PS II. These samples retain more than 50% of PS II centers with unaffected functional activity in forming the radical pair $Y_Z^{OX} P680Pheo Q_A^-$. For a high extent of the non heme iron depletion it is necessary to treat the sample twice with the 'iron depletion' procedure. The properties of these iron depleted PS II membrane fragments are most suitable for magnetic resonance studies where the effect of the non heme high spin Fe^{2+} is disturbing. Furthermore they provide an invaluable material for comparative studies with samples where Fe^{2+} is transferred into the diamagnetic low spin state ($S=0$) by incubation with high concentrations of CN^- .

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