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Photosystem II reaction center with altered pigment-composition: reconstitution of a complex containing five chlorophyll *a* per two pheophytin *a* with modified chlorophylls

Andrea Zehetner [a], Hugo Scheer [a,*], Pavel Siffel [b], František Vacha [b]

[a] *Department Biologie I—Botanik, Universität München, Menzinger Str. 67, D-80638 Munich, Germany*
[b] *University of South Bohemia and Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, Ceske Budejovice, Czech Republic*

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Abstract

Pigment-depleted Photosystem II reaction centers (PS II-RCs) from a higher plant (pea) containing five chlorophyll *a* (Chl) per two pheophytin *a* (Phe), were treated with Chl and several derivatives under exchange conditions [FEBS Lett. 434 (1998) 88]. The resulting reconstituted complexes were compared to those obtained by pigment exchange of "conventional" PS II-RCs containing six Chl per two Phe. (1) The extraction of one Chl is fully reversible. (2) The site of extraction is the same as the one into which previously extraneous pigments have been exchanged, most likely the peripheral D1-H118. (3) Introducing an efficient quencher (Ni-Chl) into this site results in only 25% reduction of fluorescence, indicating incomplete energy equilibration among the "core" and peripheral chlorophylls.
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Keywords: Photosynthesis; Photosystem II; Reaction center; D1–D2–Cytochrome *b*-559 complex; Pigment modification; Energy transfer

1. Introduction

Photosystem II (PS II) uses light energy to oxidize water to oxygen, to release the protons, and to transfer the electrons across the photosynthetic membrane. The latter takes place in the reaction center (RC) complex of PS II (PS II-RC) consisting of the D1/D2 (=PsbA/D) protein heterodimer, the α- and β-subunits of cytochrome (Cyt) b_{559} (=PsbE and F), and several low molecular weight proteins. It binds the redox factors involved in the primary steps of light-induced charge separation across the thylakoid membrane. Nanba and Satoh [1] were the first to isolate a PS II-RC, the D1–D2–Cyt b_{559} complex. It contains six chlorophyll *a* (Chl), two pheophytin *a* (Phe), one or two β-carotenes (Car). However, it no longer binds the secondary electron acceptors, Q_A and Q_B. As a consequence, light-induced charge separation is restricted to the formation of $P680^{\bullet+}$ $Phe^{\bullet-}$, which, in the absence of external donors or acceptors, rapidly recombine to the singlet (^{1}P680*, P680) or the triplet (^{3}P680*) state [2–4].

The 3.8-Å structure of the PS II complex of the cyanobacterium, *Synechococcus elongatus*, has recently been published [5]. It underlines the previously noted [6–8] homology in protein structure and cofactor arrangement to the purple bacterial RC (b-RC) [9–11], but also the differences in pigment composition [six Chl vs. four bacteriochlorophyll *a* (BChl)] and pigment geometry. The mechanism of electron transfer in PS II-RC is still a matter of debate [12–15]. It is unclear which cofactors constitute the primary electron donor P680, and which cofactors have protective or energy-conducting functions [4,16–19]. A biochemical approach to gain information about the function of the different pigments is the preparation of RCs

Abbreviations: Ac-Chl, [3-acetyl]-chlorophyll *a*; b-RC, purple bacterial reaction center; Car, β-carotene; Chl, chlorophyll *a*; Cyt, cytochrome; DM, dodecyl-β-D-maltoside; HPLC-DAD, high-performance liquid chromatography combined with a diode array detection system; Ni-BChl, [Ni]-bacteriochlorophyll *a*; Ni-Chl, [Ni]-chlorophyll *a*; Phe, pheophytin *a*; PS II-RC, photosystem II reaction center (=D1–D2–cytochrome b_{559} complex); 5/6-Chl PS II-RC, photosystem II reaction centers containing five and six chlorophyll *a*, respectively, per two pheophytin *a*; PS II, photosystem II; Q_A/Q_B, plastoquinones; RC, reaction center; SiMo, silicomolybdate ($SiMo_{12}O_{40}^{4-}$); Zn-Chl, [Zn]-chlorophyll *a*

* Corresponding author. Tel.: +49-89-17861-295; fax: +49-89-17861-185.

E-mail address: scheer-h@botanik.biologie.uni-muenchen.de (H. Scheer).

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with an altered pigment composition. Several attempts have been made to lower the number of chlorophylls in RC preparations. It has been shown that one of the peripheral chlorophylls can be reproducibly extracted on an immobilized metal (Cu) affinity chromatography column [20]. This minimum complex, called 5-Chl PS II-RC, provides full electron transfer activity of conventionally isolated 6-Chl PS II-RC, but has a reduced thermal stability. Absorption spectroscopy at ambient temperature showed, that the removed Chl has its native absorption at 670 nm resulting in a red-shifted Q_y-band maximum and a reduced absorption at 670 nm of the 5-Chl vs. the 6-Chl RC.

An alternative approach to obtain RCs with modified pigment composition, is treating "conventional" 6-Chl PS II-RC with external pigments. Gall et al. [21] could show, that it is possible to insert a chemically modified pigment without losing full activity, and Shkuropatov et al. [22] developed a pheophytin exchange system, which was used to study energy transfer within the complex [23]. Vavilin et al. [24] have recently grossly changed the pigment composition of a cyanobacterium, *Synechocystis* sp. PCC 6803, by introducing plant-derived Chl oxidase and LHCII coding genes into the cyanobacterial genome. The resulting PS II-RC has almost three of its six Chl replaced by Chl *b*, and almost one of its two Phe by Phe *b*.

A critical question in all pigment modifications is an assignment of the sites involved. We present work comparing two modifications, viz. RC, prepared by reconstitution of various pigments into a 5-Chl PS II-RC, and RC prepared by exchange of native Chl with chemically modified Chl in "conventional" [1] 6-Chl PS II-RC. The results indicate that the same site is involved, and that this site is energetically only weakly coupled to the core pigments of the RC.

2. Materials and methods

2.1. Materials

PS II-RCs from pea leaves *Pisum sativum* (Erbi, BayWa, Germany) containing six Chl per two Phe (6-Chl PS II-RC) were prepared using essentially the protocol of Braun et al. [25] and Barber et al. [26] with slight modifications as described in Ref. [27]. All these preparations are based on the method of Nanba and Satoh [1], here referred to as "conventional" or 6-Chl PS II-RC.

PS II-RCs containing five Chl and one Car per two Phe, referred to as 5-Chl PS II-RC, were isolated from pea by using immobilized metal affinity chromatography [20].

Chl was extracted from *Spirulina geitleri* [28], [Ni]-chlorophyll *a* (Ni-Chl) and [Zn]-chlorophyll *a* (Zn-Chl) were prepared by refluxing Phe with nickel and zinc acetate, respectively, in acetic acid [29]. [3-Acetyl]-chlorophyll *a* (Ac-Chl) was prepared from BChl by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone [30] and purified using the method of Omata and Murata [28]. If not mentioned otherwise, all pigments were purified using column chromatography as described in the respective referenced literature cited.

2.2. Reconstitution of PS II-RC

Pigments (200 μM) were dissolved in 20 μl MeOH/1% Triton X-100 (TX100), sonified and dried under a stream of Argon. 5-Chl PS II-RCs (7.6 μM) in 1 ml Tris–HCl buffer (50 mM, pH 6.8) containing *n*-dodecyl-β-D-maltoside (DM, 1.0 mM) were added to the pigments. After sonication for 2 min, the mixture was incubated at 29 °C for 40 min, diluted 1-fold with 1 volume of Tris–HCl (50 mM, pH 6.8), put on ice for 5 min, and finally centrifuged for 10 min at 10 000 × *g* to remove undissolved pigment. The supernatant was loaded on a small column (0.5 × 2 cm) of Q-Sepharose FF (Pharmacia) which was equilibrated with Tris-buffer (50 mM, pH 6.8) containing NaCl (30 mM) and DM (1 mM). The adsorbed RC were washed with the same buffer to remove TX100; after that, the PS II-RCs were eluted with buffer containing NaCl (400 mM).

The resulting eluate (approx. 0.5 ml) was loaded on a linear sucrose density-gradient (10 ml, 0.1–1 M) in Tris–HCl (50 mM, pH 6.8) containing $MgCl_2$ (10 mM), KCl (20 mM) and DM (1 mM). After centrifugation (190 000 × *g*) for 16 h at 4 °C in a swing-out rotor, fractions of 1 ml were collected and their absorptions measured. The fractions containing RC were combined, resulting in a recovery rate of the PS II-RC of 70–90%. When required, the reconstituted RCs were concentrated using Centricon-100 membranes (Amicon) at 1000 × *g*.

2.3. Spectroscopy

Unless stated otherwise, all spectra were recorded in MES-buffer (50 mM pH 6.8, 1 mM DM).

Absorption spectra at room temperature were recorded with a UV300 spectrophotometer (Unicam, Cambridge, UK). Low-temperature spectra were recorded with a laboratory-built spectrophotometer composed of a microsecond Xe flash lamp (FX-1160, EG&G, Boston, MA) with a pulse duration of 1 μs, a model MS257 imaging monochromator (Oriel, Stratfort, CT) and an acquisition and triggering unit FL100 (Photon System Instruments, Prague, Czech Republic). The detector was composed of two large area photodiode arrays (S4111, Hamamatsu, Japan) for the sample and reference beams. The spectral resolution of this apparatus is 2.1 nm. Each absorbance spectrum represents an accumulation of 10 spectra measured within 1 s. The samples were diluted with glycerol to a final glycerol concentration of 60% (v/v) and an OD of about 0.5 (Q_y-transition), put in a Optistat bath cryostat (Oxford Instruments, Oxon, UK) and cooled down slowly to yield a transparent sample at 78 K.

Fluorescence emission spectra (78 K, 2 nm spectral bandwidth) were measured on a Fluorolog spectrofluorim-

eter (Jobin Yvon, Edison, NJ, USA). The excitation wavelength was 435 nm (bandwidth 4 nm). RCs were diluted to a concentration of 2.2 μM and mixed with an internal fluorescence standard (rhodamin B, 8 μM), filled into the depression of a metal holder which was then immersed in liquid nitrogen.

$Phe^{\bullet -}$ accumulation spectra were recorded with a TIDAS diode array spectrometer (J&M, Aalen, Germany) at room temperature. The sample (0.9 μM) was supplemented with 6 mM sodium dithionite and 1 μM methyl viologen and illuminated with red light (30 s, low-pass cut-off filter 620 nm, Schott, Mainz, Germany) at a light intensity at the cuvette surface of approximately 2200 μmol mol m^{-2} s^{-1}.

Photooxidation in the presence of silicomolybdate (SiMo) was measured at 4 °C, using the flash spectrophotometer described above. RCs (1.1 μM equivalent to 5 μg Chl ml^{-1}) with 200 μM of SiMo were illuminated for 8 s with white light (1500 μmol m^{-2} s^{-1}) provided by a halogen lamp coupled to a fiber bundle with a triggered mechanical shutter (VS14, Uniblitz, USA). The kinetics of P680 oxidation were measured by applying a logarithmically spaced sequence of measuring flashes during light exposure and the subsequent dark relaxation.

Circular dichroism (CD) spectra were recorded at 4 °C in a model Jasco V715 spectropolarimeter (JASCO, Tokyo, Japan). PS II-RCs were diluted to a final concentration of 11.1 μM; the path length was 1 cm.

2.4. Pigment quantitation

RC pigments were extracted as previously described [21]. Immediately following extraction, the pigments were re-dissolved in toluene and injected into the high-performance liquid chromatography (HPLC) system. Pigment compositions of extracts were analyzed by HPLC [Econosphere Silica, 5 μm (Merck), room temperature, 2 ml min^{-1}] using the following gradient: A = toluene, B = 90% toluene, 7% methanol, 3% 2-propanol; 0 min: 0% B, 1 min: 3% B, 8 min: 3% B, 10 min: 10% B, 18 min: 15% B, 21 min: 50% B, 23 min: 100% B, 28 min: 100% B, 30 min: 0% B, 35 min: 0% B. Samples containing Ni-Chl were analyzed using the following gradient: A = toluene, B = 85% toluene, 15% tetrahydrofuran; 0 min: 0% B, 5 min: 8% B, 15 min: 15% B, 20 min: 100% B, 28 min: 0% B, 37 min: 0% B.

Pigment identities were established by comparison with authentic pigments (retention time and absorbance) using in situ spectroscopy (diode array detector Tidas J&M). The data were evaluated with the Spectacle software Version 1.55 (LabControl, Köln, Germany). For quantitation, peak areas of the elution traces for each pigment at the wavelength of its absorption maximum were integrated, and pigment ratios were determined from these areas, based on calibration curves established for each pigment. The calibration curves were verified and regularly controlled with authentic pigment-mixtures of defined composition.

3. Results

As shown in previous works [21,31], it is possible to exchange one of the six Chl in conventional PS II-RC by incubation with several modified pigments, including Ac-Chl and Zn-Chl. Here we concentrate on the introduction of modified chlorins into the 5-Chl RC isolated according to Vacha et al. [20]. This 5-Chl PS II-RC preparation is treated with several modified Chl in an attempt to reconstitute the altered chlorins into the binding site of the missing peripheral chlorophyll. While exchange is expected to result always in an equilibrium between the originally contained and the added pigment, introduction of Chl (-derivatives) into an empty site may provide a preparative advantage, resulting in more uniform samples, and removing the need for multiple exchanges.

The following pigments were chosen: Chl, Ni-Chl, Zn-Chl and Ac-Chl. Chl was used as a standard to compare the reconstituted samples with conventional 6-Chl PS II-RC preparations [1,3,26]. Ni-Chl was chosen because (i) its bacterial analogue, [Ni]-bacteriochlorophyll *a* (Ni-BChl), readily replaces BChl in many sites, and (ii) it is capable to rapidly (< 100 fs) quench excited states by internal conversion [32,33]. This pigment can therefore be used as a probe to study excitation energy transfer and equilibration within the PS II-RC. Ac-Chl and Zn-Chl were used to compare the reconstituted samples of the present approach, with samples modified by the ‘‘exchange method’’ [31]. Both pigments can replace one of the six Chl in conventional PS II-RC preparations. In addition, RCs treated under identical conditions but without externally added pigment, were routinely used as controls (referred as 5-Chl control RC), as are the conventional 6-Chl PS II-RC preparations.

3.1. Reconstitution of 5-Chl PS II-RC with Chl

All attempts failed to reconstitute a 5-Chl PS II-RC using the protocol of Shkuropatov et al. [22], which is optimized to introduce chemically modified Phe into PS II-RC. Using this procedure, only complexes with an unaltered pigment content of five Chl/two Phe were obtained (data not shown).

Therefore, the protocols for pigment exchange were tested for their capacity to reconstitute the ‘‘missing’’ Chl into 5-Chl PS II-RC [21,22] by modifying the exchange protocol of Gall et al. [21] with an optimized cleaning procedure: instead of chromatographic purification of the complexes, a sucrose density-gradient centrifugation was performed to separate unbound, free pigment from the pigment protein-complex. This method also reduces RC degradation (as compared to chromatography), thereby increasing the yield of RC. The resulting preparations will

be referred subsequently as "reconstituted PS II-RC", in contrast to "pigment-exchanged PS II-RC".

3.2. HPLC-data analysis

Pigment contents in RC extracts have frequently been analyzed spectroscopically using a system of simultaneous equations [34–36]. This method becomes increasingly difficult if additional pigments are introduced, in particular if they have similar spectra as the native pigments. Since this is true for some of the modified pigments, all analyses were performed by column chromatography on silica combined with diode-array detection (HPLC-DAD). Different mobile phases and gradients were developed, based on a normal-phase (SiO_2) adsorbent, to separate the native plant pigments (Chl, Phe) from the modified ones. For each pigment and each mobile phase, calibration curves were generated with the individual pigments, based on the peak areas of traces at the Q_y-absorption maximum. They were linear for all pigments. These curves were also verified with pigment mixtures of defined stoichiometry (correlation coefficients better than 0.96 ± 0.03) and re-checked at regular intervals.

The pigment compositions of the different samples are summarized in Table 1. All values are relative to Phe, based on the assumption of a constant amount of two Phe per RC. According to these analyses, there are 5.2 Chl per 2 Phe in the 5-Chl PS II-RC preparation and 6.0 per 2 Phe in the "conventional" 6-Chl PS II-RC preparation; see also Refs. [37–39]. Reconstitution of the 5-Chl PS II-RC with externally added pigment results, in all cases, in an increase of Chl (if reconstituted with Chl) or the respective modified pigment. After treatment with Chl, the increase was nearly 1 (0.8). This value indicates that the pigment composition of the reconstituted sample is identical to that of a conventional 6-Chl PS II-RC preparation, within the experimental error limits. We therefore conclude that the pigment extraction by metal-chelation chromatography is not only selective and reproducible, but also fully reversible.

A similar amount of reconstitution was found for Ni-Chl (0.8 ± 0.2 per 2 Phe). In contrast, reconstitution with Ac-Chl results in an amount of "additional" pigment in the range of 0.5 ± 0.2, indicating only partial occupancy of the site. Reconstitution with Zn-Chl leads to a much higher incorporation of 4.0 ± 0.5 Zn-Chl per 2 Phe. We conclude that

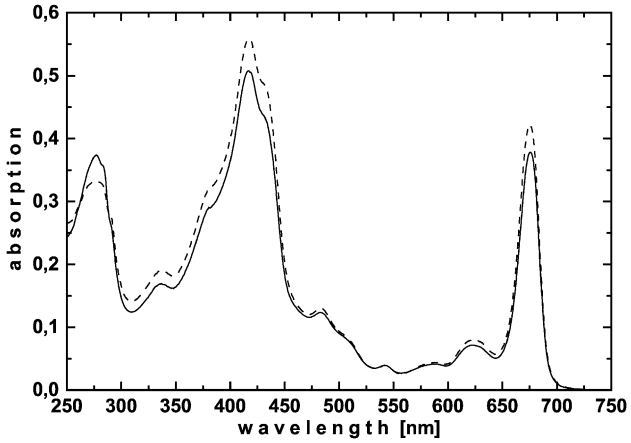

Fig. 1. Absorption spectra at 25 °C of 5-Chl PS II-RC after treatment without (control, solid line) and with Chl (dashes) at 29 °C for 40 min. The spectra were normalized to the Q_x-band of Phe (542 nm).

specific binding to the "empty" site occurs with Ac-Chl and Ni-Chl, while PS II-RCs bind Zn-Chl in excess. The latter result parallels those of previous exchange experiments with Zn-Chl and related Zn-chlorins: they always led to an increased number of pigments in the PS II-RC, which were not removable during purification [21]. This may be rationalized by the presence of additional binding sites in the complex, which can either be occupied by Zn-Chl only, or to which Zn-Chl binds much tighter than pigments with Mg or Ni as central metal. A discrimination for metals has been shown in reconstitution experiments of bacterial LH1-subcomplexes [40]. It should be noted that, irrespective of the modified Chl introduced, the Car/Phe ratio is the same in all cases. This indicates, that there is little interaction between carotenoids and the Chl binding site accessible to exchange. The non-integer stoichiometry of Ac-Chl reconstitution indicates only incomplete reconstitution after a single treatment, due to weaker binding. In bacterial-RC, complete replacement at a specific site can be generally achieved only by a repetition of the incubation procedure [41].

3.3. Spectroscopic analyses

Fig. 1 compares the spectrum of 5-Chl PS II-RC reconstituted with Chl, with that of a control sample treated in the same way but without the addition of extra pigment. The

Table 1
Pigment stoichiometry of different types of PS II-RC after incubation-treatment (29 °C, 40 min) with or without additional added pigment

Sample	Pigment added during treatment	Pigment contents (relative to Phe)			
		Chl	Phe	Car	Other
5-Chl PS II-RC	–	5.2 ± 0.2	2	1.2 ± 0.2	–
5-Chl PS II-RC	Chl	6.2 ± 0.4	2	1.2 ± 0.2	–
5-Chl PS II-RC	Ni-Chl	5.3 ± 0.2	2	1.2 ± 0.2	0.8 ± 0.1 Ni-Chl
5-Chl PS II-RC	Ac-Chl	4.7 ± 0.3	2	1.2 ± 0.2	0.5 ± 0.2 Ac-Chl
5-Chl PS II-RC	Zn-Chl	5.1 ± 0.2	2	1.2 ± 0.2	4.0 ± 0.5 Zn-Chl
6-Chl PS II-RC	–	6.0 ± 0.1	2	1.5 ± 0.1	–

Each value represents the average of three independent measurements.

spectra were normalized at 542 nm (Q_x-band of Phe). There is an increase of amplitude and a broadening of the Soret and Q_y-bands in the reconstituted sample, which is more prominent at the short-wavelength side of both bands. The Q_y-maximum is slightly shifted in the reconstituted sample as compared to the treated control, from 675.5 to 675.0 nm. The protein absorption at 280 nm in the reconstituted sample decreased, which is probably due to the additional purification after incubation, which removes denatured protein very efficiently. Small, but distinct differences are also seen in the region around 475 nm. In exchange experiments, we had previously observed an absorption decrease in this region although the Car/Phe ratio remained constant. If these changes in the 475-nm region are related to carotenoids, they probably represent a rearrangement rather than a loss during the incubation procedure [42,43]. Here, in contrast, we found an increase in absorption at this wavelength, while again the Phe/Car ratio remained constant. We cannot exclude, however, that these differences are artifacts of the normalization at a rather small band, or due to differences in light scattering. Alternatively, the changes may reflect different carotenoid conformations. The increase in the Q_y-region was quantitated by integration from 750 to 600 nm. In comparison to the control sample incubated without Chl, under otherwise identical conditions, the area increases by 11% in the sample treated with Chl. This corresponds reasonably well with the HPLC analysis (increase by 13%) and with the expected value of 14% (assuming complete reconstitution and the same oscillator strength for all six Chl and two Phe). The Q_y-region was further investigated by low-temperature (78 K) absorption spectroscopy (Fig. 2). The reconstituted sample shows an increase of absorption at around 672 nm, which is caused by the Chl introduced, and a broadening of the Q_y-band envelope of the PS II-RC at the short-wavelength side.

There is only little difference in the CD spectra (Fig. 3) of the reconstituted and control samples. This shows that (i) the inserted pigment contributes negligibly to the CD of the RC, and (ii) that the arrangement of those pigments, which are responsible for the CD signal, is not significantly disturbed by the reconstitution.

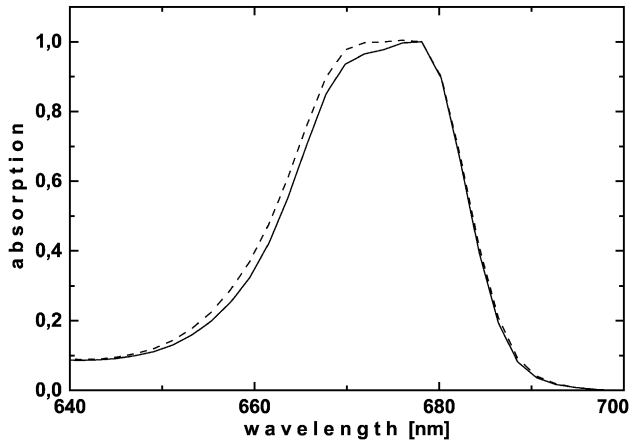

Fig. 2. Absorption spectra at 78 K of the Q_y-region of 5-Chl PS II-RC incubated at 29 °C for 40 min without (control, solid line) and with Chl (dashes). The spectra were normalized at 678 nm.

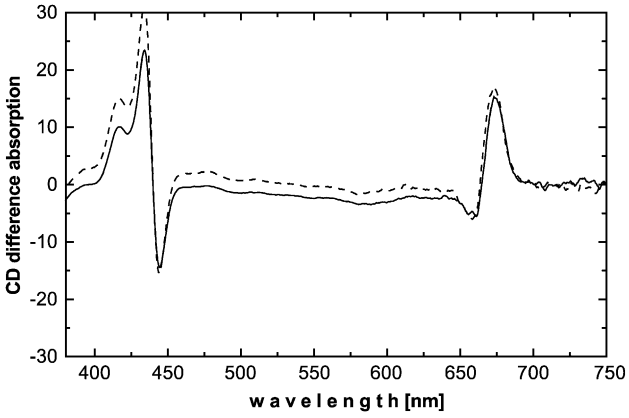

Fig. 3. CD spectra of 5-Chl PS II-RC incubated without (control, solid line) and with Chl (dashes).

Fluorescence emission and excitation spectra at different wavelengths were measured to test the functional integrity of the complex and to detect sample heterogeneities. Previous experiments [21] showed that the method detects sensitively unbound/uncoupled pigments, causing wavelength-dependent fluorescence excitation and emission spectra.

The shapes of the emission and excitation spectra of the Chl-reconstituted PS II-RC showed only slight variations of the excitation and emission wavelengths, respectively (data not shown). However, small but distinct differences become obvious on closer inspection, in particular a blue-shift of the emission upon changing the excitation from 415 to 450 nm. The most obvious explanation is that some pigment is bound nonspecifically. This is unlikely, however, in view of the reproducible stoichiometry <1. An alternative explanation is a specific binding into the vacant site, but incomplete energy equilibration among the pigments. This possibility will be explored in more detail below with the samples reconstituted with Ni-Chl.

There is also a quantitative agreement of the quantum yields of emission ($\lambda_{exc}=435$ nm) of the control-RC (6-Chl PS II-RC) and the Chl-reconstituted sample; the difference is $<3\%$, supporting again that the additional Chl is reconstituted into its original position.

Functional integrity of the Chl-reconstituted and control samples was tested by light-induced accumulation of $Phe^{\bullet -}$ and $P680^{\bullet +}$ in the presence of dithionite as an electron donor and SiMo as an acceptor, respectively. Both measurements showed spectra (Fig. 4) that are in agreement with previously published results [1,26]. The activities of untreated 5-Chl PS II-RC compared to untreated 6-Chl PS II-RC are practically the same. The $Phe^{\bullet -}$ accumulation of the reconstituted sample reached 90% of the untreated 5-Chl RC, whereas the control sample (treated without added pigment) reached only 57%. A loss of activity of approx. 30% after incubation (40 min, 25 °C) was described by Vacha et al. [20] for a 5-Chl PS II-RC preparation. This

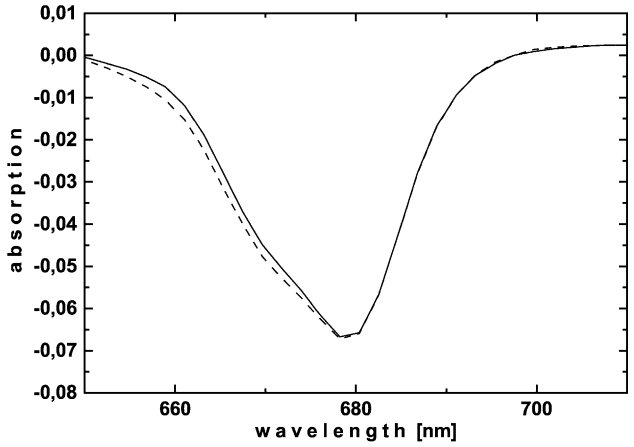

Fig. 4. Reversible absorption changes (light *minus* dark spectra in the presence of 200 mM silicomolybdate) measured at 4 °C. 5-Chl PS II-RC incubated without (control, solid line) and with Chl (dashes) at 29 °C for 40 min. Irreversible bleaching was subtracted.

indicates that the pigment-depleted sample suffers during the incubation at 29 °C, but is stabilized by pigment reconstitution.

Fig. 4 compares the $P680^{\bullet+}$ *minus* P680 absorption difference spectra of the reconstituted sample and the control RC. The spectra were normalized at their maxima. The spectra of the reconstituted sample shows a broadening at the short-wavelength side. Such a broadening has been related to irreversible oxidation of an accessory Chl caused by $P680^{\bullet+}$ within the RC [44,45].

3.4. Reconstitution with Ni-Chl

A comparison of the room temperature absorption spectra of the PS II-RC reconstituted with Ni-Chl, and of the treated control samples (Fig. 5), shows a similar spectral changes as were seen in the reconstitution of the 5-Chl PS II-RC with Chl. Besides the small absorption changes

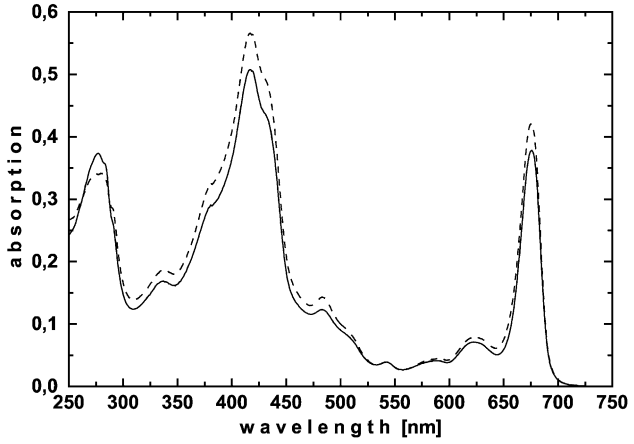

Fig. 5. Absorption spectra at 25 °C of 5-Chl PS II-RC after treatment without (control, solid line) and with Ni-Chl (dashes) at 29 °C for 40 min. The spectra were normalized to the Q_x-band of Phe (542 nm).

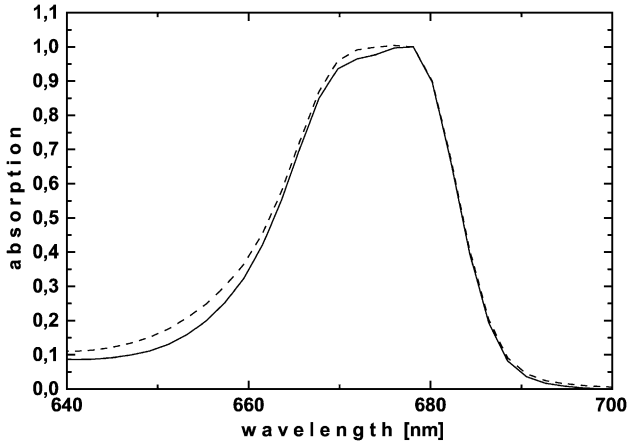

Fig. 6. Absorption spectra at 78 K of the Q_y-region of 5-Chl PS II-RC incubated at 29 °C for 40 min without (control, solid line) and with (dashes) Ni-Chl. The spectra were normalized at 678 nm.

around 500 nm discussed above, the Soret- and Q_y-bands are increased by 11%, the Q_y-maximum is blue-shifted from 675.5 to 675.0 nm, and the protein absorption at 280 nm is decreased. There is also a distinct broadening of the short-wavelength side of the Q_y-bands, which is probably related to the blue-shifted Q_y-absorption band of Ni-Chl as compared to Chl Q_y-band, in solution. This blue shoulder becomes more prominent in the absorption spectrum at 78 K (Fig. 6), where increased absorptions around 670 nm and 650 nm reflect the presence of Ni-Chl in the complex. The CD signal of the Ni-Chl reconstitution sample is shifted by 1 nm to the shorter wavelengths. This shift is more pronounced in the negative band of the spectrum, which again parallels the changes seen in the PS II-RC reconstituted with Chl (Fig. 7).

The energetic coupling of Ni-Chl to the other pigments in the RC was tested by fluorescence spectroscopy. The emission spectra remain unchanged, when excited at different wavelengths, and vice versa for excitation spectra (data not shown). Surprisingly, the fluorescence yield (relative to an internal standard) was still quite high in the sample containing Ni-Chl. Compared to the control, it is reduced

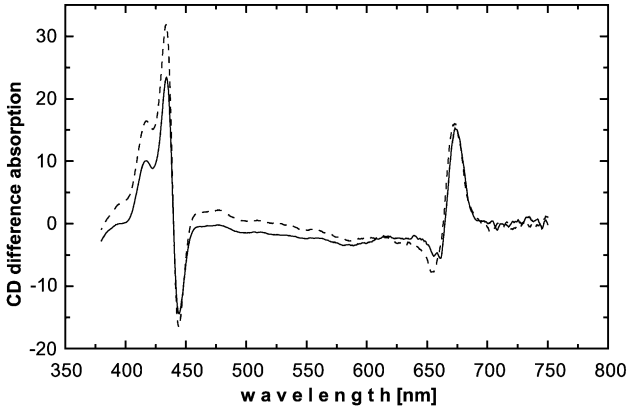

Fig. 7. CD spectra of 5-Chl PS II-RC incubated without (control, solid line) and with Ni-Chl (dashes).

only by 25%. Since Ni-BChl acts as a very efficient quencher which effectively drains all coupled excited states in pigment ensembles within equilibration times as short as 0.1 ps [32,33], this high fluorescence indicates a rather slow equilibration of excitation energy between the pigment in the accessible site (now assigned to D1-H118—see below) and the other five chlorophylls (see discussion). In agreement with this are the photochemical activities largely retained. The ability to accumulate $Phe^{\bullet -}$ under illumination amounts to 90% as compared with a non-treated sample. The normalized difference spectra of $P680^{\bullet +}$ formation of the Ni-Chl reconstituted sample are nearly identical with those of the sample reconstituted with Chl, except for a small but significant broadening of the blue side of the difference peak (data not shown).

4. Discussion

We show that it is possible to reconstitute a 5-Chl PS II-RC with Chl, as well as with modified Chls. Treatment with Chl leads to a 6-Chl PS II-RC with full activity and the spectroscopic characteristics of a ''conventional'' RC prepared by standard methods. Therefore, we conclude that the removal of the Chl from the D1-H118 binding site by Cu-affinity chromatography is fully reversible. (Based on the X-ray structure of PS II from *S. elongatus* [5], we favor this site for the exchange, since the D2-H118 position is probably shielded by Cyt b_{559} and the Psbl protein [14,46].)

Reconstitution with Ni-Chl led to a RC in which the D1-H118 site is occupied by the modified pigment. Most of the spectra and photochemical activities remained unchanged by the introduction of Ni-Chl, as compared to samples reconstituted with Chl. The most surprising feature is the preservation of high fluorescence, which indicates only slow energy transfer between the PS II-RC core pigments and the reconstituted modified pigment. In the purple bacterial antenna, LH I, quantitative quenching has recently been reported on a sub-picosecond time scale [47]. The reduction of the fluorescence yield by only $\sim$ 25% observed here, together with the known fluorescence lifetime of the PS II-RC of $\sim$ 100 ps, corresponds to a transfer time of $\sim$ 75 ps from the ''core''-pigments to the peripheral site occupied by Ni-Chl. Considering the fluorescence lifetime and the known distance of 32 Å [5], this is compatible with a Förster transfer mechanism. A detailed evaluation of this point is presently difficult, and beyond the scope of this work. The X-ray structure [5] is still not sufficiently resolved to include the orientation factor, and this analysis is probably also too simple, neglecting the possibility of charge recombination within the lifetime of the excited state.

Several groups have studied the energy transfer in PS II-RC and PS II-complexes (reviewed by Dekker and van Grondelle [48]). The interpretation of the observed time constants is still controversial, because of the spectral overlap of the pigments, their unknown orientations and the photochemical reactions after excitation (charge separation and recombination). A further complication is the possible contamination with extra pigments or other pigment–protein complexes due to the isolation procedure and degradation of the PS II-RC, which may contribute to the fluorescence [49–51]. The interpretation of steady-state measurements led to the conclusion that some of the RC pigments are energetically coupled at room temperature and that they are building an equilibrated excited state [52,53]. The multimer-model first described by Durrant et al. [54] is the most favored in explaining the kinetics of energy transfer, charge separation and fluorescence in the isolated PS II-RC. In this model, there is an efficient coupling of the ''core-pigments'' (four Chl and two Phe) which results in a very fast (sub-picosecond) equilibration of excitation energy, and which may include weak to medium excitonic coupling among the RC pigments. The two remaining peripheral Chl exchange energy with the ''core-pigments'' within a few tens of picoseconds [55–57]. The observed fluorescence reduction by only 25% upon replacement of one of these pigments with Ni-Chl is qualitatively in agreement with previous kinetic data (Refs. [20,41,49,50], reviewed by Dekker and van Grondelle [48]). Accordingly, there is rapid ($\sim$ 0.5 ps) excitation energy equilibration among the six central tetrapyrrole pigments that are equivalent to those of the bacterial RC, while much slower equilibration (20–30 ps) occurs between the two peripheral Chl and the core. The latter kinetics are slower than the fast trapping phase, and in fact one of the key arguments to assign it to the peripheral Chl was a reduction of its amplitude in a 5-Chl PS II RC preparation [20,37]. It should be noted, however, that different transfer directions may be probed by the latter authors and us, because in our case, it is mainly ''back'' transfer to the Ni-Chl which would lead to quenching.

Recently, measurements have been performed on whole thylakoid membranes (and isolated PS II-RC) of mutants in which the D2 binding site of one of the peripheral Chl was exchanged to threonine (D2-H117T) [58]. The excitation-energy transfer from the peripheral Chl to the core pigments is slowed down to 100 ps, whereas the rate constant of charge recombination in the RC is increased. The authors explain the effect by a changed orientation of the Chl bound to D2-117 followed by oxidation of this pigment to a Chl cation-radical which quenches the fluorescence. They rule out an incomplete equilibration in PS II within the time-window of the photochemically limited lifetime in another mutant (D2-H117N), the fluorescence decay kinetics of the isolated RC showed only a minor retardation (20–30 ps) of the excitation transfer from the peripheral Chl to the ''core-pigments'' [54]. New insights toward the pigment organization in the PS II-RC, derived from the X-ray structure [5,14], allows now to redefine the currently accepted models [55,56,59] of excitation-state dynamics, which underlines aspects that the peripheral Chl of the RC are not included into the equilibrated excited state of the PS II-RC and that the role in shuffling energy from the PS II core-antenna to

the RC "core-pigments" does not rely on the peripheral Chl. Pigments of the CP43 and CP47 subunits are much closer to the four "core"-Chl of the RC, than the two peripheral Chl of the RC.

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