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Modification of pigment composition in the isolated reaction center of photosystem II

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Abstract The pigment content of isolated reaction centers of photosystem II was modified using an exchange protocol similar to that used for purple bacterial reaction centers. With this method, which is based on incubation of reaction centers at elevated temperature with an excess of chemically modified pigments, it was possible to incorporate [3-acetyl]-chlorophyll *a* and [Zn]-chlorophyll *a* into photosystem II reaction centers. Pigment exchange has been verified by absorption, circular dichroism and fluorescence spectroscopy, and quantitated by HPLC analysis of pigment extracts.

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Key words: Photosynthesis; Photosystem II; Reaction center; D1-D2-cytochrome *b*-559 complex; Pigment modification

1. Introduction

Photosystem II (PSII) is essential for oxygenic photosynthesis. Its reaction center (PSII-RC) generates, by light-driven charge separation, the highly positive oxidant required for the oxidation of water. Knowledge about PSII-RC has increased substantially after the first successful isolation of a PSII-RC complex consisting of only 5 (possibly 6 [1]) proteins [2,3]. Much of the current knowledge about the primary events occurring in this RC has been gained by spectroscopic methods or is based on assumed homologies with the purple bacterial reaction center (RC), the structure of which is known at atomic resolution [4–8].

There are two particular properties which severely hamper studies on PSII-RC in comparison to purple bacterial RC. The first is their lability: PSII-RC are quite unstable in most detergents [9–11] including LDAO and Triton X-100 (TX100), which impedes biochemical work, including crystallization. The second is the high degree of spectral overlap of the pigments' absorptions which renders it extremely difficult to separate and assign the absorption characteristics and functions of the individual chlorophylls (Chls) and pheophytins (Phes). Whereas the Q_y-absorption spectra of bacterial reaction centers are spread in the near-infrared over a range of as much as 3000 cm^{-1}, those of PSII-RC all crowd within only $\approx$500 cm^{-1} in the red spectral region. Furthermore, they contain a larger number of chromophores: there are six chlorophyll *a*-molecules (Chl) in PSII-RC, but only four bacteriochlorophylls (BChl) in the purple bacterial RC. Several attempts have been made to deconvolve the Q_y-absorption region [11–14], but the results obtained differ significantly.

PSII-RC, with modified pigment composition, could help to define specific pigment absorption spectra. Currently, the only preparation of PSII-RC with a generally accepted modified pigment content is one which contains only 5 Chls instead of 6 [15]. In this case, modification was accomplished by the use of a Cu affinity column for stripping out one of the Chls. Experiments to use high hydrostatic pressure as a tool for the modification of pigment content proved unsuccessful [16]. Pigment replacements have been more successful with bacterial RC where they were of considerable importance in the functional analysis. By replacing the amino acids liganding the central Mg, site-directed mutagenesis can yield RC in which BChl(s) are replaced by bacteriopheophytins (BPhes) [17–19]. More extensive variations are possible by exchange reactions with externally added pigments, which allow site-directed exchanges of the monomeric BChl binding sites B_A or B_B, and/or of the two BPhes at binding sites H_A or H_B [20,21].

We present here an approach to exchange pigments in PSII-RC following that developed with bacterial RC [20,21]. Adaptation of this method was previously hampered by the poor stability of PSII-RC, which was recently improved using detergents derived from bile acids, in particular with the use of the zwitterionic 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [9]. Exchange of pigments in PSII-RC is expected to expand the range of Q_y-absorptions and thus facilitate spectroscopic distinction of individual RC chromophores. Eventually, it may also yield PSII-RC containing defined pigments with specifically altered properties (e.g. redox-potentials, excited state lifetimes, etc.), which can be used in the functional analysis of this RC.

2. Materials and methods

2.1. Reaction centers

PSII-RC were isolated from pea leaves (Golf ZS, Bayerische Futtersaatbau) [9] using essentially the protocol of Braun et al. [11], which is based on the method of Barber et al. [3].

2.2. Pigments

Chlorophyll *a* (Chl) was isolated from *Spirulina geitleri* [22]. [3-Acetyl]-chlorophyll *a* (acetyl-Chl) was prepared from bacteriochlorophyll *a* (BChl) by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone [23]. [Zn]-chlorophyll *a* (Zn-Chl) was prepared by refluxing pheophytin *a* (Phe) with zinc acetate in acetic acid [24]. All pigments were purified using column chromatography.

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Abbreviations: Acetyl-Chl, [3-acetyl]-chlorophyll *a*; $B_{A,B}$, binding pockets of the monomeric BChls in the bacterial RC; BChl, bacteriochlorophyll *a*; BPhe, bacteriopheophytin *a*; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Chl, chlorophyll *a*; Chl′, 13^2 epimer of Chl; DM, dodecyl-β-D-maltoside; $H_{A,B}$, binding pockets of the monomeric BPhes in the bacterial RC; PSII-RC, photosystem II reaction center = D1-D2-cytochrome b_{559} complex; RC, reaction center; Zn-Chl, [Zn]-chlorophyll *a*

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2.3. Pigment exchange

Pigments (approx. 0.1 μmol) were dissolved in 30 μl MeOH containing 2% TX100. The solution was sonicated for 2 min and then dried in a stream of nitrogen. A mixture of PSII-RC (3.2 nmol) in 2 ml Tris buffer (50 mM, pH 7.2) containing 0.3% CHAPS was added. After sonication for 2 min, the mixture was incubated at 30°C for 45 min, then put on ice for 10 min, diluted with 2 ml Tris buffer and, after another 10 min on ice, centrifuged (10 min, 15 000×*g*, 4°C) to remove precipitated pigments and protein. The solution was put on a small column (0.5×2 cm) of Q-Sepharose FF (Pharmacia), which was equilibrated before use with Tris buffer (50 mM, pH 7.2) containing 0.2% TX100 and 30 mM NaCl. The adsorbed (modified) PSII-RC were washed with the same buffer (30 ml) and eluted with buffer containing 400 mM NaCl. The PSII-RC were diluted fivefold with Tris buffer (without salt and detergent), put on a second Q-Sepharose column and washed with 10 ml Tris buffer (50 mM, pH 7.2) containing 0.2% TX100 and 30 mM NaCl. TX100 was removed by washing with 4 ml of the same Tris buffer containing 0.6% CHAPS as detergent. Finally, the RC were eluted from the column with Tris buffer containing 0.6% CHAPS and 400 mM NaCl. After recording the absorption spectrum, preparations were frozen in liquid nitrogen and stored at −80°C.

2.4. Pigment quantitation

RC (1–2 nmol) were adsorbed onto a small column of Q-Sepharose. After washing with distilled water to remove detergent and buffer salts, most of the water was removed by flushing the column with a gentle stream of nitrogen. Pigments were then eluted by subsequent washing with methanol (0.5 ml) and chloroform (0.5 ml). The pigment solution was dried in a stream of nitrogen. Dried pigments were dissolved again in toluene and injected into the HPLC system. Pigment compositions of extracts were analyzed by HPLC (Econosphere Silica 5μ column (Merck), 5°C, 2 ml/min) using the following conditions: solvent A = toluene/methanol/2-propanol 100:0.05:0.1; solvent B = toluene/methanol/2-propanol 100:4.0:0.5; gradient: 0 min 0% B, 8 min 10% B, 10 min 50% B, 11 min 100% B. Pigment identity was checked by in situ spectroscopy using a diode array detector (Hewlett-Packard HP 8451) and by comparison with authentic pigments. For quantitation, peak areas of the elution traces for each pigment at the wavelength of its absorption maximum were integrated, and relative pigment ratios were determined from the peak areas. Extinction coefficients for the solvent mixtures used were determined with pure pigments, using those in well investigated organic solvents as standards [23,25,26]. To verify these extinction coefficients, HPLC runs were done with pigment mixtures prepared so that their compositions were close to those of the mixtures encountered in the extracts.

Absorption spectra were recorded on a Lambda 2 spectrophotometer (Perkin-Elmer). A Fluorolog 221 fluorimeter (Spex) was used for steady-state fluorescence measurements (spectra were not corrected), and a Dichrograph CD 6 (Yobin-Yvon) for CD measurements. Light-induced charge separation was determined according to [2].

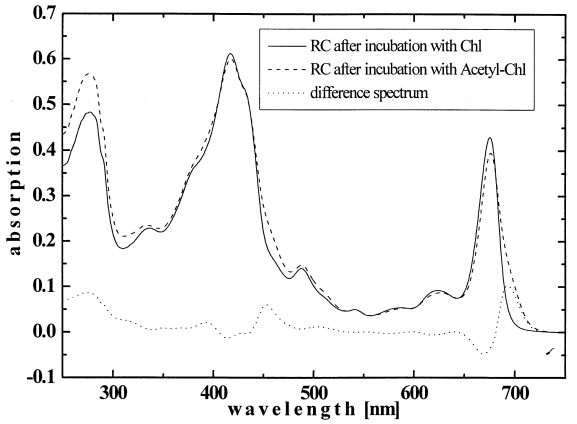


Fig. 1. Comparison of absorption spectra of PSII-RC after incubation with acetyl-Chl and Chl (30°C, 45 min); spectra were normalized to the Q_x-band of Phe (542 nm).

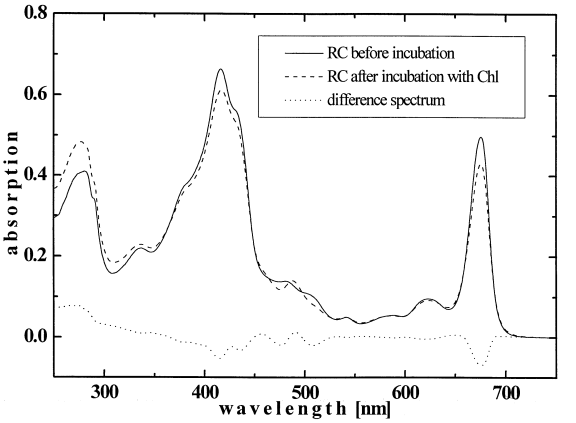


Fig. 2. Comparison of absorption spectra of PSII-RC before and after incubation with Chl (30°C, 45 min); spectra were normalized to the Q_x-band of Phe (542 nm).

3. Results and discussion

The present work was designed to find a method that allows modification of the pigment composition of PSII-RC. Analogous to experiments with bacterial RC [20,21], PSII-RC were incubated at elevated temperatures with a surplus of chemically modified pigments. Three such pigments were chosen: [3-acetyl]-chlorophyll *a* (acetyl-Chl), bacteriochlorophyll *a* (BChl) and [Zn]-chlorophyll *a* (Zn-Chl). Work with bacterial RC [21] and several other chlorophyll-proteins or enzymes of chlorophyll biosynthesis [27,28] has shown that pigments with a modified C-3 substituent or in which the central Mg of the macrocyclus was replaced with Zn are readily exchangeable. Acetyl-Chl was also chosen, because its structure is like an intermediate between that of Chl and BChl, and because of its red-shifted absorption, which allows its detection by absorption spectroscopy. The spectral features of BChl are even more distinct, but previous work suggests that an exchange of chlorins with bacteriochlorins may be more difficult (Paulsen, H., personal communication). Consequently, this work focusses on experiments with acetyl-Chl. In all cases, RC treated under identical conditions with Chl were used for comparison.

3.1. The exchange system

Attempts to exchange the native pigments with acetyl-Chl at 43°C, the temperature used with bacterial RC [20], failed: they resulted in an almost complete loss of PSII-RC. Better results were obtained at 30°C. Fig. 1 compares the spectrum of such a sample with a control treated with Chl. The presence of acetyl-Chl is obvious from the long-wavelength shoulders on both the Soret- and the Q_y-bands. The Q_y-maximum is shifted to 676.5 nm, and the difference spectrum (after normalization at the Phe Q_x-band at 542 nm) shows a loss of absorption at 670 nm and an increase at 693 nm. The changes in the Soret band are more complex with a major component also shifting to the red. These features indicate the presence of acetyl-Chl and a concomitant loss of Chl in the incubated sample. There is also a relative increase in protein absorption at 280 nm which is probably due to the presence of denatured proteins that have lost their pigments in the course of incubation. An increase in this region, together with a decrease of all major bands in the visible region, is already seen in the

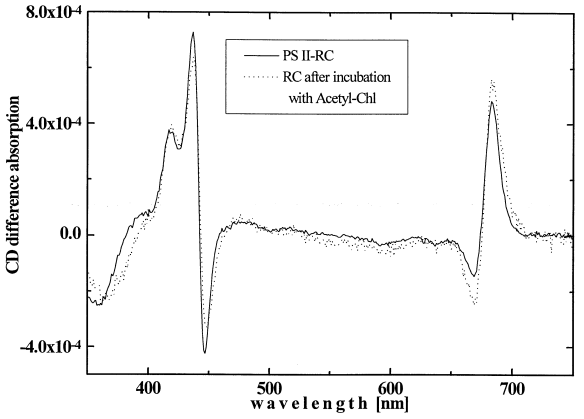


Fig. 3. CD spectra of PSII-RC (OD_{676} = 0.64/5.6 μg Chl) before and after incubation (45 min; 30°C) with acetyl-Chl (OD_{676} = 0.66/5.7 μg Chl).

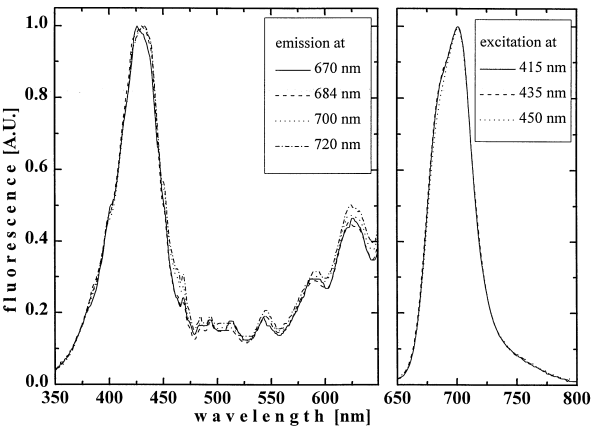


Fig. 4. Fluorescence excitation (left panel) and emission (right panel) of RC after incubation with acetyl-Chl; all spectra were normalized at the wavelength of the maximum signal.

self-exchange control (Fig. 2). Distinct changes in the control are also seen in the region around 500 nm and are very likely related to the carotenoids and independent of the pigment added (Fig. 2). However, the long-wavelength part of this region is also sensitive to changes in the pheophytins.

Two further spectroscopic methods were used for testing the modified RC. Circular dichroism is a particularly sensitive probe for pigment interactions. The shape of the CD-spectra is the same for RC before and after incubation (Fig. 3), suggesting that those pigments responsible for the CD signal are neither removed from the RC nor are their interactions significantly changed. There are, however, small intensity changes in the CD bands, which we cannot currently interprete. A noteworthy feature in the CD is the distinct red-shift at the positive extremum in the Q_y-region: this indicates that the acetyl-Chl introduced is involved in interactions with the other pigments. Such interactions are supported by fluorescence spectroscopy, which demonstrates the participation of the introduced acetyl-Chl in energy transfer within the RC (Fig. 4). If the external modified pigment participates fully in energy transfer, the shape of the emission spectra is expected to remain the same, irrespective of the excitation wavelength (and vice versa for excitation spectra). This is clearly seen in the emission spectra at 700 nm. The pigment is less obvious in the excitation spectra, but also distinct in the region around 460 nm. The shapes of all emission or excitation spectra are nearly identical after normalization.

To test the sensitivity of fluorescence measurements to detect unbound pigment, the same series of fluorescence spectra was obtained for a PSII-RC sample, to which a small amount of acetyl-Chl had been added at 5°C: this was enough to show absorption changes similar to those obtained after incubation at higher temperatures (Fig. 5). In these spectra excitation at the acetyl-Chl absorption peak of 450 nm causes increased emission from free acetyl-Chl (λ_{max} = 703 nm); however, excitation at shorter wavelengths caused mainly RC emission. Both emission and excitation spectra for different excitation or emission wavelengths showed marked differences after normalization. The same effect is obtained after denaturation of exchanged RC with TX100 (data not shown). Comparison of Figs. 4 and 5 clearly shows that acetyl-Chl is coupled to the native pigments in the exchanged samples, but uncoupled in the control.

Functional integrity of acetyl-Chl treated RC and controls was tested by light-induced accumulation of $Phe^{\bullet-}$ with dithionite as electron donor as a check of their photochemical activity. Treated RC showed about 80% of the activity of native RC.

HPLC-DAD (diode array detection) analysis shows for the self-exchanged sample only the three native pigments β-carotene, Phe and Chl (not shown). The sample treated with acetyl-Chl exhibits, in addition, a fourth signal with a retention time of 680 s and the absorption spectrum of acetyl-Chl (Fig. 6). The quantitative values for the pigment composition obtained by HPLC (Table 1) are relative and based on the assumption that there are 2 Phe present in all samples. This assumption is generally used for practical reasons, but not necessarily correct in all cases [29].

3.2. Experiments with other pigments

The results with Zn-Chl and with BChl are summarized in Table 1. As with acetyl-Chl, significant amounts of the non-

Table 1
Pigment composition of RC after exchange experiments with several pigments (mean values from two HPLC analyses)

	Native RC	RC after incubation with			
		Chl	Acetyl-Chl	Zn-Chl	BChl
β-Carotene	1.3	1.3	1.4	1.4	1.4
Phe	2	2	2	2	2
Chl	5.5	5.6	4.6	4.4	5.6
Modified pigment	–	–	1.6	4.3	0.7[a]
Σ tetrapyrrole/2 Phe	5.5	5.6	6.2	8.7	6.3

[a]Sum of BChl and its oxidation product, acetyl-Chl.
All experiments were performed in parallel with RC from the same batch under identical conditions.

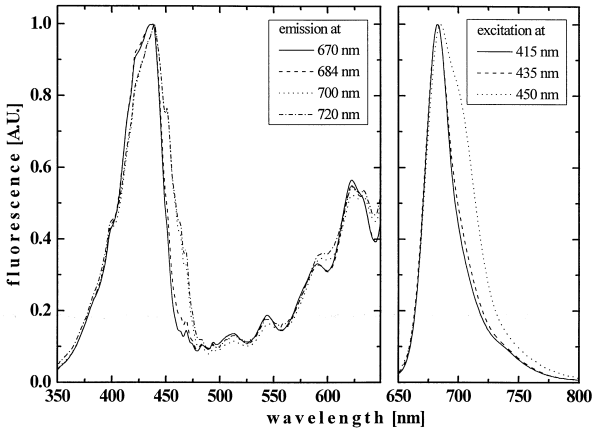


Fig. 5. Fluorescence excitation (left panel) and emission (right panel) of RC with added acetyl-Chl (RC corresponding to 1.7 nmol Chl+0.3 nmol acetyl-Chl; the absorption spectrum of this mixture resembles the one obtained after the incubation procedure); all spectra were normalized at the wavelength of the maximum signal, but not manipulated otherwise.

natural pigments are detected after the incubation procedure. However, the amount varies considerably and is much higher for Zn-Chl (4.5/2 Phe) and lower for BChl (0.8/2 Phe). With Zn-Chl, as with acetyl-Chl, there is concomitant loss of Chl, but not when BChl was used. This suggests that one Chl site is replaced with Zn-Chl or acetyl-Chl, respectively, and indicates that this site requires a chlorin type pigment. In no case, however, does the new pigment introduced quantitatively match the loss of Chl. Therefore, there is always more Mg (or Mg plus Zn) complex in the RC after the treatment. The carotene/Phe ratio is the same in all cases and serves as an internal control. If the changes mentioned above for the region around 500 nm (Fig. 2) are related to carotenoids, they probably represent a rearrangement rather than a loss.

The presence of additional pigment in the preparations might be explained by non-specific binding to the RC protein. To test this hypothesis, removal of the 'excess' pigment was attempted by washing with imidazole buffer which offers a surplus of ligand to bind external pigment. Alternatively,

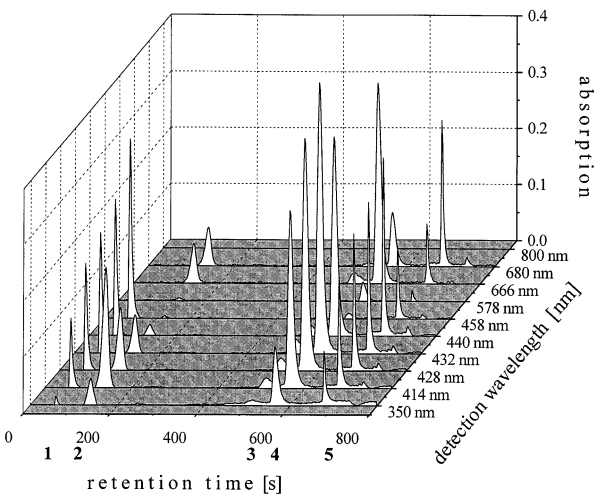


Fig. 6. HPLC analysis of RC after incubation with acetyl-Chl. Peak assignments: 1 = β-carotene; 2 = Phe; 3 = Chl′; 4 = Chl; 5 = acetyl-Chl.

washing was performed with buffer containing chaotropic reagents (urea or $LiClO_4$). All these attempts failed, and the additional pigments were not removed without destroying the RC. Currently, it is not clear how many binding sites exist for Chls in PSII-RC. It is conceivable that this number is higher than 6 because some sites may be so labile that their pigments are removed during isolation. The modified pigments present in addition may now bind more strongly and occupy these sites.

In the HPLC measurements pigment values are mostly non-integers. This points towards the presence of a mixture of RC with different degrees of modification. The same is generally true for bacterial RC after the first exchange [21]. Only repetition of the incubation procedure yields uniform RC material with an exchange rate close to 100%, indicating that all pigments in one or more site(s) are exchanged. Due to thermal lability, it is not yet possible to repeat the incubation with PSII-RC without losing most of the RC.

Collectively, the spectroscopic, photochemical and HPLC data show that the added chemically modified pigments are functionally incorporated into the PSII-RC during incubation. For acetyl-Chl and Zn-Chl there is a concomitant loss of Chl, as well as an appearance of the modified pigments in the fluorescence excitation spectra which proves that the external pigments take part in internal energy transfer. Therefore, it is most likely that in samples of PSII-RC prepared with acetyl-Chl and Zn-Chl a true pigment exchange has occurred: a fraction of the native pigments has been replaced and modified pigment has been functionally integrated into the RC.

3.3. Concluding discussion

When a native Chl from PSII-RC has been replaced, it is still unclear which of the 6 original Chl molecules was replaced. The negative peak at about 670 nm in Fig. 1 suggests the loss of a Chl molecule absorbing at a rather short wavelength. In the deconvolution approach of Konermann and Holzwarth [14] the two 'additional' Chl molecules (those missing in the bacterial RC) are thought to absorb at about 669 nm. According to the model of Michel and Deisenhofer [30], these Chls might be more accessible than the others, thus facilitating modification. On the other hand, we were unable to remove these Chl with Tris or imidazole buffer containing TX100 [31], which contradicts the view that these Chl are only loosely bound to the RC. Exchange with modified BChls in bacterial RC exclusively takes place at the binding pockets of the monomeric BChls, preferentially with B_B. If the analogy between bacterial and PSII-RC holds, one might expect modification of one of the presumably monomeric Chl in PSII-RC. Exchange of the dimeric BChl, the primary donor P870, was never observed in bacterial RC. However, the primary donor P680 in PSII-RC is significantly different from P870 [16,32–35] and might even resemble the monomeric pigments in the bacterial RC [36]; therefore, one can presently not even rule out an exchange at this position. To define where modification occurs within the RC requires application of more selective spectroscopies.

The experimental procedure for modification of PSII-RC was similar to that used for bacterial RC [20,21]. Incubation at elevated temperature was the only successful method for pigment exchange in PSII-RC; other approaches failed, e.g. use of high pressure [16] or pH-dependent dissociation and reassociation [37]. With bacterial RC, temperatures around

40°C were needed for exchange (although H_B exchange was reported at ambient temperature [38]). For PSII-RC 30°C was sufficient to achieve modification. This temperature is a compromise between exchange rate and RC recovery which is around 30% after the second purification step. Recent results of FT-IR measurements of PSII-RC in dodecyl-β-D-maltoside (DM) as detergent [39] showed that, beginning at about 30°C, denaturation increases rapidly (measured as a broadening of the amide I band). In CHAPS, the denaturation temperature is expected to be somewhat higher [9]. Therefore, pigment modification and denaturation in PSII-RC occur at about the same temperature. This supports the generally accepted explanation for pigment exchange [20], which postulates higher accessibility of pigment binding pockets at almost denaturing temperatures as the main principle for exchange. The recovery of fully-active RC was only possible when the bile salt detergent, CHAPS, was used in the incubation step and in the final step of the purification: CHAPS stabilizes PSII-RC much more than the commonly used detergent DM.

In bacterial RC it is also possible to exchange the native bacteriopheophytin (BPhe) with a variety of other (B)Phes [40], in one case already at room temperature [38]. Attempts to repeat this with PSII-RC have failed: exchange with pheophytins (BPhe *a*, [3-acetyl]-pheophytin *a*) was not successful. This was not expected, since PSII models [34] suggest a close similarity between the binding pockets for (B)Phes in PSII-RC and bacterial RC, and points to a considerably different binding situation.

In summary, the results presented here show that some of the pigments in PSII-RC can be reproducibly exchanged. By using CHAPS as detergent it is possible to isolate intact, active RC after incubation at 30°C. Absorption and fluorescence spectroscopy as well as HPLC analyses demonstrate the loss of native Chl, the presence of added pigment (acetyl-Chl or Zn-Chl) and the participation of the added pigment in energy transfer. Further investigations to determine the site of modification and the influence of pigment modification on the RC function are underway.

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