Reaction centers of photosystem II with a chemically-modified pigment composition: exchange of pheophytins with 13^1-deoxo-13^1-hydroxy-pheophytin *a*

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Abstract **Isolated reaction centers of photosystem II with an altered pigment content were obtained by chemical exchange of the native pheophytin *a* molecules with externally added 13^1-deoxo-13^1-hydroxy-pheophytin *a*. Judged from a comparison of the absorption spectra and photochemical activities of exchanged and control reaction centers, 70–80% of the pheophytin molecules active in charge separation are replaced by 13^1-deoxo-13^1-hydroxy-pheophytin *a* after double application of the exchange procedure. The new molecule at the active branch was not active photochemically. This appears to be the first stable preparation in which a redox active chromophore of the reaction center of photosystem II was modified by chemical substitution. The data are compatible with the presence of an active and inactive branch of cofactors, as in bacterial reaction centers. Possible applications of the 13^1-deoxo-13^1-hydroxy-pheophytin *a*-exchanged preparation to the spectral and functional analysis of native reaction centers of photosystem II are discussed.**

Key words: Photosynthesis; Photosystem II; Reaction center; Pheophytin *a*; 13^1-deoxo-13^1-hydroxy-pheophytin *a*; Pigment exchange

1. Introduction

The reaction center of photosystem II (PS II RC), consisting of the D1 and D2 proteins, the α- and β-subunits of cytochrome *b*-559 (cyt *b*-559) and the product of the *psbI* gene, was first isolated by Nanba and Satoh [1]. Subsequently, this complex has been extensively studied by biochemical and spectroscopic methods (for a review see [2,3]). It is generally accepted that PS II RC, also denoted as the D1-D2-cyt *b*-559 complex, binds several pigments: chlorophyll *a* (Chl), pheophytin *a* (Pheo), carotenoids and the heme group(s) of cyt *b*-559. There is convincing evidence that highly-purified preparations contain six Chls and 1–2 β-carotenes per two Pheo molecules [4–6]. The isolated RC complex lacks a quinone acceptor but it is still able to carry out fast electron transfer from the excited primary electron donor P680* to the pheophytin acceptor to form the radical pair $P680^+Pheo^-$ (for a review see [2,3]).

Spectroscopic and functional studies of the PS II RC are severely limited by the fact that the reaction center Chls and Pheos have strongly overlapping absorption spectra. The spectral congestion, especially in the Q_y region at 670–680 nm, makes it difficult to resolve the absorption bands belonging to the individual chromophores as well as to assign the photo-induced absorbance changes. A promising approach to address this problem is modification of the pigment composition of the RCs either by selective removal of constituent chromophores or by their chemical treatment. Several preparations of PS II RCs with an altered pigment content have been recently described in the literature [7–9]. The first modified preparation of the PS II RC was obtained by isolation of the RC on a Cu affinity column [7]. The preparation contained five Chls (instead of six) per two Pheos, with the missing Chl apparently having been lost from a peripheral position and serving a role in energy transfer to P680. Another type of modification has been achieved by irreversible reduction of the Pheo molecule not involved in charge separation (hereafter referred to as $Pheo_{inactive}$) to 13^1-deoxo-13^1-hydroxy-Pheo (13^1-OH-Pheo) by reaction with $NaBH_4$ [8].

Of particular interest is the application to the PS II RC of a pigment exchange technique based on replacement of the native chromophores with exogenous pigments having different structural, spectral and redox characteristics [10–12]. Recently, it has been shown [9] that one of the six Chl molecules present in PS II RC could be replaced by (3-acetyl)-Chl or Zn-Chl using the chemical exchange procedure following that developed with the bacterial RCs [10–12]. The first attempts to exchange Pheo against pheophytin derivatives (bacteriopheophytin *a*, (3-acetyl)-Pheo) were, however, not successful [9]. This may indicate a higher specificity of the Pheo binding pockets that limits those alterations in the structure of Pheo which can be accepted by the pockets.

Here, we report that both photosystem II reaction center pheophytins can be replaced by 13^1-OH-Pheo using an exchange procedure generally similar to those described for bacterial [10–12] and PS II [9] RCs. After double application of the exchange procedure, 70–80% of the Pheo molecules involved in primary charge separation ($Pheo_{active}$) were found to be replaced by 13^1-OH-Pheo. We compare absorption spectra and photochemical activities of the modified and control preparations and discuss possible applications of 13^1-OH-Pheo-exchanged RCs to the spectral and functional analysis of native RCs of PS II.

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Abbreviations: Chl, chlorophyll *a*; cyt, cytochrome; DM, *n*-dodecyl β-D-maltoside; P680, primary electron donor in PS II; Pheo, pheophytin *a*; $Pheo_{active}$, Pheo active in charge separation; $Pheo_{inactive}$, Pheo inactive in charge separation; PS II, photosystem II; RC, reaction center; TM buffer, 50 mM Tris HCl (pH7.2)/0.05% DM; 13^1-OH-Pheo, 13^1-deoxo-13^1-hydroxy-pheophytin *a*

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2. Materials and methods

PS II RCs were isolated from sugar-beet chloroplasts by the method of Nanba and Satoh [1], essentially as described earlier [8] except that the column chromatography step was performed using Fractogel EMD DEAE 650 (S) cellulose rather then Fractogel TSK DEAE 650 (S).

Pheo was obtained from nettle and purified as described in [8]. 13^1-OH-Pheo was prepared from Pheo as a mixture of stereoisomers [13,14] by treatment with $NaBH_4$ in MeOH [15] followed by purification on a powdered sugar column [16]. The purity of pigment preparations was checked by absorption spectroscopy.

Exchange of Pheo with 13^1-OH-Pheo was performed by a procedure generally similar to those recently described for bacterial [10–12] and PS II [9] RCs. Briefly, a solution of 13^1-OH-Pheo in acetone containing 1% of *n*-dodecyl-β-D-maltoside (DM) was added to 1.5 μM PS II RCs solubilized in 50 mM Tris-HCl (pH 7.2)/0.05% DM buffer (TM buffer) to achieve a 25–30-fold excess of the pigment (relative to the RC concentration) with a final acetone concentration of 1.5%. The mixture was incubated for 15–20 min at 27°C in the dark and afterwards, the modified preparation was purified on a Fractogel EMD DEAE 650 (S) column.

Three types of preparations were used in the study: native RCs, 13^1-OH-Pheo-modified RCs and control RCs. The control RCs were subjected to exactly the same incubation and purification procedures as the modified preparations but in the absence of 13^1-OH-Pheo. All three types of preparations were dissolved in TM buffer containing 300 mM NaCl and stored at −196°C until use.

For quantification of photochemically active pheophytin, the photoaccumulation of a reduced pheophytin acceptor was measured with a phosphoroscope at 10°C in the presence of methyl viologen and sodium dithionite [1] as described previously [8].

Absorption and absorption difference spectra were obtained using a UV-1601PC spectrophotometer (Shimadzu).

3. Results and discussion

Our recent study [8] has indicated that 13^1-OH-Pheo that was formed in the PS II RC structure by treatment of native Pheo with $NaBH_4$ did not leave the RC matrix. This observation suggests that 13^1-OH-Pheo, which differs from Pheo by reduction of the carbonyl group located in the isocyclic ring (C-13^1 position, IUPAC) [13–15,17], has a strong affinity for the binding site(s) of Pheo. In the present work, 13^1-OH-Pheo was used to replace the natural Pheos by a chemical exchange procedure based on incubation of PS II RCs with an excess of the externally-added free pigment.

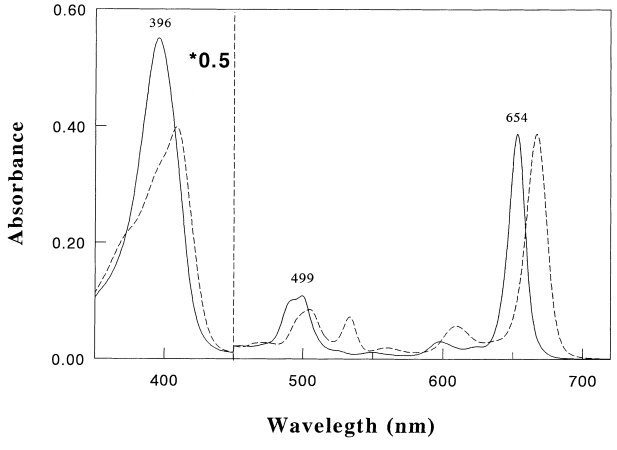

Fig. 1. Room temperature absorption spectra of Pheo (dashed line) and 13^1-OH-Pheo (solid line) in diethylether. Spectra were normalized at their Q_Y absorption maxima (667 and 654 nm, respectively). Here and in Fig. 2–4, the numbers show the wavelength positions of the maxima in absorption spectra or extrema in absorption difference spectra.

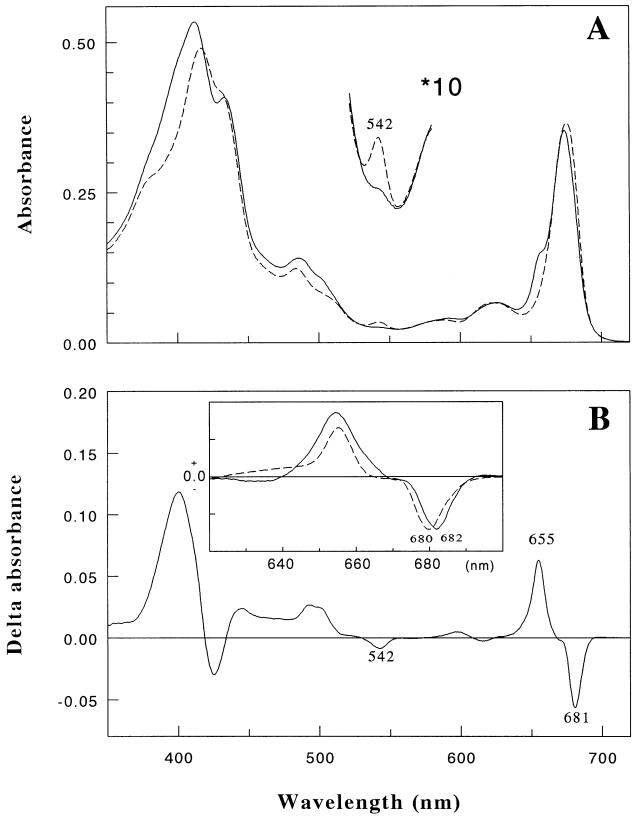

Fig. 2. A: Comparison of absorption spectra of PS II RCs, twice incubated at 27°C for 15 min with (solid line) and without (dashed line) externally added 13^1-OH-Pheo. Spectra were measured at 5°C and normalized at 623 nm. The Pheo Q_X region is shown separately with the absorbance axis 10-fold enlarged. B: Difference between absorption spectra presented in A, 'RCs exchanged with 13^1-OH-Pheo (solid line) minus control RCs (dashed line)'. Inset: Comparison of the absorption difference spectra for $NaBH_4$ treatment of native RCs (dashed line, taken from [8]) and for selective exchange of $Pheo_{active}$ with 13^1-OH-Pheo (solid line). Spectra were normalized at their long wavelength minima, 680 and 682 nm, respectively. The ΔA scale is given in arbitrary units. See Section 3 for details.

The room temperature absorption spectrum of 13^1-OH-Pheo in diethylether used in this study is shown in Fig. 1 (solid line). For comparison, the normalized absorption spectrum of Pheo is presented by the dashed line. The spectrum of 13^1-OH-Pheo displays the Q_Y, Q_X and Soret bands at 654, 499 and 396 nm, respectively. The Q_x band at 499 nm shows a shoulder at 490 nm. These absorption bands, as well as minor maxima at 528, 551, 598 and 624 nm, are all characteristic of the 13^1-OH derivatives of Pheo [14] and methyl pheophorbide *a* [13,15]. Notably, there are significant differences in wavelength positions of the Q_Y and Q_X absorption bands of 13^1-OH-Pheo and Pheo that potentially provide a simple spectroscopic verification of the exchange reaction between these pigments.

Introduction of 13^1-OH-Pheo into the incubation medium (a micellar solution containing 50 mM Tris-HCl (pH 7.2)/0.065% DM/1.5% acetone) does not effect the absorption spectrum of the pigment except that the main absorption bands are shifted for 2–4 nm to the red due, probably, to a solvent effect (data not shown). It is very likely, therefore, that

13^1-OH-Pheo exists in the incubation medium in the monomeric form. The 13^1-hydroxy substituent seems to prevent aggregation in water-detergent micellar solution, as it has been previously observed with 13^1-OH-Chl [17].

Fig. 2A compares the 5°C absorption spectrum of PS II RCs twice incubated with 13^1-OH-Pheo at 27°C and purified on a DEAE-cellulose column (solid line) with the normalized spectrum of the control RCs treated in the same way, but without addition of extraneous pigment (dashed line). The spectrum of control RCs was practically identical to the normalized absorption spectrum of native RCs (not shown). It is seen (Fig. 2A) that treatment of RCs with 13^1-OH-Pheo results in a pronounced decrease of the Q_X absorption band of Pheo at 542 nm and an appearance of the well-resolved shoulder near 655 nm due to absorption of 13^1-OH-Pheo. The red absorption band of RCs is shifted to the blue from 675.5 nm to 674 nm and the absorption difference spectrum (Fig. 2B) shows a prominent absorption decrease at 681 nm reflecting an expected loss of the Q_Y absorption band of Pheo. The absorption increases at 655, 500 and 400 nm (Fig. 2B) confirm the presence of 13^1-OH-Pheo in the incubated RCs. These data indicate clearly that incubation of PS II RCs with 13^1-OH-Pheo leads to a loss of the major fraction of the native Pheo molecules from RCs and a concomitant appearance of 13^1-OH-Pheo due to a pigment exchange reaction. A closer inspection of the spectra in Fig. 2A shows that about 85% of the dipole strength of the absorption band at 542 nm was lost after double application of the exchange procedure, which corresponds to replacement of about 85% of the total Pheo. Currently, it is generally accepted that two Pheo molecules are present in each PS II RC of which only one, $Pheo_{active}$, takes part in charge separation [2,3]. Based on this stoichiometry, one can conclude that both types of Pheo were replaced by 13^1-OH-Pheo in the double-exchanged preparations.

Previous experiments performed on native PS II RCs have indicated that $Pheo_{inactive}$ could be distinguished by its ability to react readily with $NaBH_4$ to form 13^1-OH-Pheo inside RCs [8]. In the present study, we employed this reaction to estimate

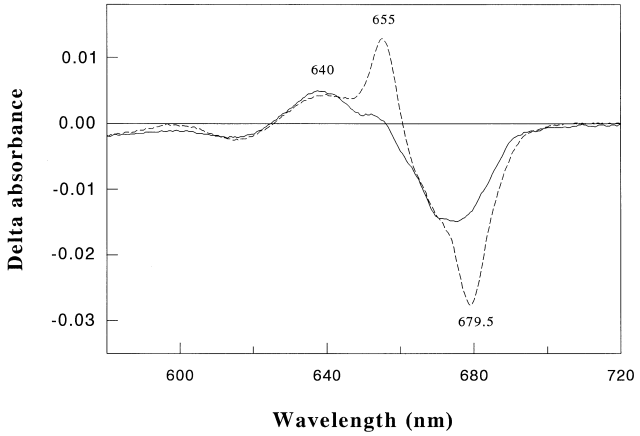

Fig. 3. Absorption difference spectra for reactions of $NaBH_4$ with the pigments in control (dashed line) and 13^1-OH-Pheo-exchanged (solid line) PS II RC preparations. Spectra were measured at 5°C at equal concentrations of the RC complex obtained by using samples with an equal absorbance at 623 nm [8]. The absorbance changes were taken at 60 min after addition of $NaBH_4$ (~0.5 mg/ml). About 60% of the total Pheo was replaced by 13^1-OH-Pheo in the exchanged preparation.

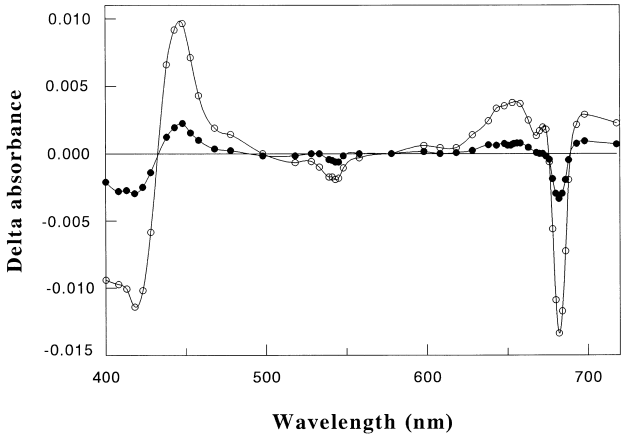

Fig. 4. Light minus dark difference spectra of reversible absorbance changes in control (open circles) and 13^1-OH-Pheo-exchanged (closed circles) PS II RCs in the presence of 6 mM sodium dithionite and 1 μM methyl viologen. Spectra were measured at 10°C. The Chl concentration was about 3 μg/ml. Samples were excited with red actinic light ($\lambda > 630$ nm; ~7 mW/cm^2).

the exchange rates of $Pheo_{active}$ and $Pheo_{inactive}$ in the double-exchanged preparations. We found that the absorption difference spectrum for the reaction of $NaBH_4$ with Pheos and Chls in control preparations (Fig. 3, dashed line) was very similar to the spectrum observed for native RCs [8]. In the red region, the spectrum is dominated by an absorption increase at 655 nm and a decrease at 679.5 nm due to formation of 13^1-OH-Pheo from, most likely, $Pheo_{inactive}$ [8]. In sharp contrast, the modified RCs in which 58% of the total Pheo was exchanged for 13^1-OH-Pheo exhibit a strongly diminished (< 10%) amplitude of the absorbance increase at 655 nm (Fig. 3, solid line) whereas the amplitudes of an increase at 640 nm and a decrease near 670 nm caused by formation of 13^1-OH-Chl [8] are comparable in both preparations. According to [8], these data indicate that more than 90% of $Pheo_{inactive}$ undergoes pigment exchange under conditions when the total pheophytin exchange rate is only about 60%. It means that $Pheo_{inactive}$ is exchanged considerably faster then $Pheo_{active}$. Assuming that in the double-exchanged RCs 90–100% of $Pheo_{inactive}$ was replaced, the exchange rate of $Pheo_{active}$ in this preparation can be estimated to be 70–80%.

In complementary experiments, the ability of control and double-exchanged RCs was compared for photoaccumulating reduced $Pheo_{active}$ ($Pheo^-_{active}$), by measuring the light-induced absorption difference spectra (Fig. 4) in the presence of sodium dithionite and methyl viologen [1]. The spectra were obtained from samples having an equal absorbance at the isosbestic point near 623 nm to provide equal concentrations of RC complexes [8]. As can be seen in Fig. 4, the shape of the difference spectrum is maintained, but there is a dramatic decrease of its amplitude in the modified preparation (closed circles) compared to control RCs (open circles). The native and control samples exhibit nearly identical photoaccumulation spectra (data not shown). Notably, the difference spectrum measured in modified RCs (Fig. 4, closed circles) does not show a bleaching near 655 nm which would reflect a photoaccumulation of a reduced form of a newly introduced 13^1-OH-Pheo. It seems likely therefore that absorbance changes observed in modified preparations are due to photoaccumulation of $Pheo^-_{active}$ in the residual non-exchanged RCs

or, more probably, in those modified RCs in which only $Pheo_{inactive}$ was exchanged with 13^1-OH-Pheo. Hence, a ratio of the amplitudes of absorbance changes in modified preparations to those in control or native RCs can be used as a measure of the exchangeability of $Pheo_{active}$. An inspection of Fig. 4 showed that this ratio varied slightly through the spectra being equal to about 0.2 in the region of 650–660 nm where the anion radical of Pheo absorbs in PS II RCs [18,19]. This value corresponds to about 80% of $Pheo_{active}$ having been replaced by 13^1-OH-Pheo in double-exchanged RCs and is in good agreement with the estimates made in the preceding paragraph.

These results suggest that the absorption difference spectrum in Fig. 2B (we will refer to it as spectrum 1) carries contributions from pigment exchange of both $Pheo_{inactive}$ ($\sim$100% exchange) and $Pheo_{active}$ ($\sim$70% exchange). On the other hand, the ΔA spectrum resulting from $NaBH_4$ treatment of native RCs (dashed line in the inset of Fig. 2B, referred to as spectrum 2) seems to be due to modification of 100% of $Pheo_{inactive}$ [8]. We used these two spectra to calculate the double difference ($\Delta\Delta A$) spectrum shown in the inset to Fig. 2B (solid line) which is expected to represent a 'pure' spectrum for exchange of $Pheo_{active}$. For this purpose, spectra 1 and 2 were first normalized at their minima at 681 and 680 nm, respectively, and then, the normalized spectrum 2 was subtracted from spectrum 1 with the amplitude reduced by a factor of 1.7 in accordance with afore mentioned rates of replacement of $Pheo_{inactive}$ and $Pheo_{active}$ in the two types of RCs. The red region of the calculated spectrum is compared with spectrum 2 (dashed line). The most characteristic features of the $\Delta\Delta A$ spectrum are the positive peak at 655 nm which is contributed by the introduction of 13^1-OH-Pheo and two minima at 682 and 543.5 nm (the latter is not shown) reflecting, respectively, a loss of the Q_Y and Q_X absorption bands of a pheophytin molecule, most likely $Pheo_{active}$. A comparison of the $\Delta\Delta A$ spectrum ($Pheo_{active}$) with the spectrum 2 ($Pheo_{inactive}$) allows one to suggest that the Q_Y and Q_X transitions of the two pheophytins in native RCs are located at nearly the same wavelengths, the transitions of $Pheo_{active}$ being slightly ($\sim$2 nm) shifted to the red. It is worth noting that the suggested approximate locations of the absorption bands of $Pheo_{active}$ (682 and 543.5 nm) are close to the absorption maxima found at 4.2 K in the hole-burning measurements (681.6 and 545.7 nm, respectively) [20].

It is tempting to relate these results to a functional asymmetry similar to that in bacterial RCs, namely an inactive branch of cofactors with a readily exchangeable Pheo ($=Pheo_{inactive}$), and an active branch with a Pheo which is more difficult to exchange (this paper) and inaccessible to modification with $NaBH_4$ ([8]).

The question arises why 13^1-OH-Pheo incorporated into the binding site of $Pheo_{active}$ is not photo-accumulated in a reduced state under conditions where photoaccumulation of $Pheo^-_{active}$ is easily observed in control and native RCs. According to the literature data [19], the redox potential for the couple Pheo/$Pheo^-$ in PS II is about -610 mV (versus NHE) and the gap between free energy levels of the excited primary donor P680* and the state $P680^+$ $Pheo^-$ was calculated to be equal to $\sim$0.06 eV. Our preliminary electrochemical measurements (Kozlov Y.N., Shkuropatov A.Y., unpublished results) showed that $E_{1/2}$ for the first one-electron reduction wave of 13^1-OH-Pheo in dimethylformamide (-0.97 V) is $\sim$0.3 V more negative then that of Pheo (-0.68 V). If this same difference is maintained for 13^1-OH-Pheo in the reaction center protein, the free energy level of $P680^+$ 13^1-OH-$Pheo^-$ is expected to lie well above the level of P680*. This could explain the inactivity of 13^1-OH-Pheo-exchanged RCs in the photoaccumulation reaction. If this is the case, femtosecond measurements of photo-induced absorbance changes in modified RCs might help to test a possible involvement of accessory Chl(s) in the charge separation, similar to what has been observed in Pheo-exchanged RCs from *Rhodobacter sphaeroides* R-26 [21,22]. Another intriguing application of modified RCs is to test the idea that P680 in native PS II RCs is composed of a multimer [23] of several excitonically-coupled pigments [24]. Indeed, the introduction of 13^1-OH-Pheo, in which the Q_Y transition is shifted to a higher energy by $\sim$560 cm^{-1}, may influence excitonic interactions in a putative multimer and, as a consequence, result in alteration of the spectral properties of the P680. Experiments to address these problems are under way.

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