Biochimica et Biophysica Acta 1837 (2014) 1870–1881

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Chemically modified reaction centers of photosystem II: Exchange of pheophytin *a* with 7-deformyl-7-hydroxymethyl-pheophytin *b*

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ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form 14 August 2014
Accepted 19 August 2014
Available online 27 August 2014

Keywords:
Photosystem II
Reaction center
Pheophytin *a*
7-Deformyl-7-hydroxymethyl-pheophytin *b*
Chemical exchange
Charge separation

ABSTRACT

The native pheophytin *a* (Pheo *a*) in isolated reaction centers of photosystem II (PSII RCs) has been chemically exchanged with extraneous 7-deformyl-7-hydroxymethyl-Pheo *b* (7^1-OH-Pheo *b*) which differs from Pheo *a* by the C-7 substituent (hydroxymethyl instead of methyl). The two pigments have similar reduction potentials in vitro [M. Meyer, Dissertation, Universität München, 1997], while their absorption spectra show small but distinct differences in the visible region. The resulting 7^1-OH-Pheo *b*-modified reaction center preparations were characterized by high-performance liquid chromatography, electronic absorption and light-induced Fourier transform infra red absorption difference spectroscopies, together with photoaccumulation of the reduced pheophytin electron acceptor and $NaBH_4$-treatment. About 70% of the total Pheo *a* molecules are found to be replaced by 7^1-OH-Pheo *b* molecules in modified preparations, indicating that both the photochemically active ($Pheo_{D1}$) and inactive ($Pheo_{D2}$) binding sites were subjected to pigment exchange. The 7^1-OH-Pheo *b* molecule located at the $Pheo_{D1}$ site is able to functionally replace the native Pheo *a*, participating in primary charge separation as an electron acceptor. The Q_x absorption band of this modified pheophytin molecule is localized at ~546 nm; its Q_y band is blue-shifted with respect to the absorption of other reaction center core pigments, being located at ~665 nm. The Q_y and Q_x optical transitions of the 7^1-OH-Pheo *b* molecule exchanged into the $Pheo_{D2}$ site are identified at 677 and 543.5 nm, respectively. The photochemically active double-modified PSII RCs additionally containing 7-deformyl-7-hydroxymethyl-13^1-deoxo-13^1-hydroxy-Pheo *b* at the $Pheo_{D2}$ site were obtained by treatment of the 7^1-OH-Pheo *b*-modified RCs with $NaBH_4$.

1. Introduction

The reaction center (RC) of photosystem II (PSII) of oxygenic photosynthesis of higher plants, algae and cyanobacteria is a transmembrane pigment–protein complex that performs initial charge separation via a series of fast electron-transfer reactions and provides the oxidant with the high positive redox potential required to drive water oxidation.

The X-ray crystal structures of the PSII core complexes from cyanobacteria that contain the RC complex together with two integral antenna proteins CP43 and CP47 and the oxygen-evolving complex are available [1–5] with the current resolution of 1.9 Å [6]. In its biochemically-isolated state, the PSII RC (D1/D2/cyt *b*559 complex) consists 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 [7]. The D1 and D2 polypeptides non-covalently bind six chlorophyll *a* (Chl *a*) molecules and two pheophytin *a* (Pheo *a*) molecules, as well as 1–2 β-carotene (β-car) molecules, depending on the preparation [8]. According to the structural data [1–6], four central chlorin pigments of the PSII RC are arranged in two symmetry-related branches: both branches share a dimer (P_{680}) of Chl *a* molecules P_{D1} and P_{D2}, and each branch is also composed of one monomeric Chl *a* (Chl_{D1} or Chl_{D2}) and one Pheo *a* ($Pheo_{D1}$ or $Pheo_{D2}$). In addition, there are two more distant Chl *a* molecules that are symmetrically located at the periphery of the RC. Typically, the secondary electron acceptors (plastoquinone molecules) are lost from PSII RCs during purification. As a consequence, electron transfer in the isolated D1/D2/cyt *b*559 RC complex is limited to the ultrafast primary charge separation leading to the formation of the transient radical pair $P_{680}^{+}Pheo^{-}$ [9–13]. Of the two cofactor branches, only the D1 branch

Abbreviations: ΔA, light-induced absorbance changes; BT-buffer, 20 mM Bis-Tris (pH 6.5) 20 mM $MgCl_2$, 5 mM $CaCl_2$, 0.03% (w/v) *n*-dodecyl-β-D-maltoside; Chl, chlorophyll; Chl_{D1} and Chl_{D2}, binding sites for monomeric Chl *a* at the active and inactive branches of the reaction center, respectively; cyt *b*559, cytochrome *b*559; β-car, β-carotene; D1 and D2, the D1 and D2 polypeptides of the reaction center; DM, *n*-dodecyl-β-D-maltoside; FTIR, Fourier transform infra red; Pheo, pheophytin; $Pheo_{D1}$ and $Pheo_{D2}$, binding sites for Pheo at the active and inactive branches of the reaction center, respectively; 13^1-OH-Pheo *a*, 13^1-deoxo-13^1-hydroxy-Pheo *a*; 7^1-OH-Pheo *b*, 7-deformyl-7-hydroxymethyl-Pheo *b*; 7^1-OH-13^1-OH-Pheo *b*, 7-deformyl-7-hydroxymethyl-13^1-deoxo-13^1-hydroxy-Pheo *b*; PSII, photosystem II; P_{680}, a dimer comprised of Chl *a* molecules P_{D1} and P_{D2}; HPLC, high-performance liquid chromatography; RC, reaction center; TX-100, Triton X-100.

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http://dx.doi.org/10.1016/j.bbabio.2014.08.004
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(P_{680}, Chl_{D1}, and $Pheo_{D1}$) is known [7,14,15] to be involved in electron transfer, while the D2 branch is normally photochemically inactive. The photogenerated $P_{680}^{+}Pheo_{D1}^{-}$ recombines to the ground singlet state (directly or via the excited singlet state) or to the chlorophyll triplet state (reviewed in Ref. [16]). In the presence of an externally added reductant to rapidly rereduce P_{680}^{+} in the $P_{680}^{+}Pheo_{D1}^{-}$ pair, the radical ion $Pheo_{D1}^{-}$ can be accumulated upon continuous illumination of PSII RCs [7,14,17].

Although spectral [18–22] and redox [23] properties of PSII RCs are subjected to partial changes due to the removal of the integral antenna polypeptides CP43 and CP47 upon purification, isolated D1/D2/cyt *b*559 RC complexes represent a valid model system for reaction centers in more intact PSII core complexes. It has been recently shown that pigment site energies obtained from experiments on isolated PSII RCs [24] could be applied to modeling of spectroscopic properties of RC pigments in PSII core complexes [25,26], with the site energies for Chl_{D1} and $Pheo_{D2}$ shifted by several nanometers to red. According to recent femtosecond transient absorption measurements and kinetic modeling [12,27–29], the mechanism and dynamics of the early electron-transfer processes in the isolated PSII RC correspond to those observed for the PSII core complexes. A close similarity between the time-resolved IR-difference spectra of the state $P_{D1}^{+}Pheo_{D1}^{-}$ found for PSII cores and the D1/D2/cyt *b*559 RC complex has indicated that the local structure of the primary reactants remained intact in the isolated RC complex [27]. It is noteworthy that assignment of spectral characteristics and functions of the individual PSII reaction center chlorophylls and pheophytins directly within core complexes is extremely complicated because, in addition to the inherent spectral congestion of the Q_y absorption region of the PSII RC, the absorption bands of the RC pigments strongly overlap the pigment bands of the core antenna complexes CP43 and CP47, which contain about thirty Chl *a* molecules. Recently, it has been reported on a possibility to prepare heterogeneous samples of isolated PSII RCs from *Chlamydomonas* (*C.*) *reinhardtii* containing (together with the standard RC with Q_A lost, referred to by authors as RC680) some fraction of more intact reaction centers (referred to as RC684), which in turn had a small subpopulation of RCs with the photoreducible primary plastoquinone acceptor Q_A present [21,22]. However, RC684 were shown to be not stable after isolation and were very sensitive to sample handling procedures [22].

In spite of the fact that the isolated PSII RC binds a minimal number of pigment cofactors, its spectroscopy is quite complex because the lowest-energy Q_y optical transitions of all the eight RC chlorins are located in the narrow wavelength region at about 675 nm at room temperature, and the Q_y absorption contour is only partially split at cryogenic temperatures, showing peaks near 670 and 680 nm and a shoulder at about 684 nm (see, for example, [30]). This extensive spectral overlapping makes it difficult to resolve the absorption bands of the individual chromophores and seriously complicates interpretation of both the steady-state and time-resolved light-induced absorbance changes for PSII RCs. An effective approach to address these problems and obtain information on the properties and functioning of the native PSII RC pigments is analysis of the effects of their selective replacement by other pigments with different spectral and/or redox characteristics, using site-directed mutagenesis or chemical methods (chemical treatment of RCs and pigment exchange).

By introducing His as a potential Mg ligand over the center of the macrocycle ring of $Pheo_{D2}$ or $Pheo_{D1}$, the mutant RCs of *C. reinhardtii* have been obtained in which the inactive-branch (mutation D1-L210H) [31] or the active-branch pheophytin (mutation D2-L209H) [21] has been replaced by a Chl *a* molecule. The kinetics of primary charge separation were not substantially altered in D1-L210H RCs, indicating that the substitution of inactive Pheo with Chl had not perturbed the energetics of the primary electron donor/acceptor pair [31], while D2-L209H mutant RCs were nonactive [21]. Vavilin et al. [32] have described mutant PSII RCs from a cyanobacterium *Synechocystis* sp. PCC 6803 in which two–three of six Chl *a* and one of two Pheo *a* were substituted by Chl *b* and Pheo *b*, respectively, as a result of incorporating higher plant genes coding Chl *a* oxidase and LHCII into the cyanobacterial genome [33]. Chl *b* and Pheo *b* were shown to participate in electron-transfer reactions in mutant RCs within the core complex; however, the amount of Pheo *b* in the isolated D1/D2/cyt *b*559 complex was found to be very low [32].

Vacha et al. [34] have obtained the modified PSII RCs containing only five (instead of six) Chls, in which one of the two peripheral Chl *a* molecules was removed by using immobilized metal affinity chromatography. The photochemically inactive pheophytin *a* molecule at the $Pheo_{D2}$ binding site of PSII RCs could be selectively transformed to 13^1-deoxo-13^1-hydroxy-Pheo *a* (13^1-OH-Pheo *a*) [14] by means of the chemical reaction of the Pheo *a* with $NaBH_4$ [35–39] inside the RC complex. Recently, a similar $NaBH_4$-treatment has been also used to modify the $Pheo_{D2}$ molecule to 13^1-OH-Pheo *a* in the spinach PSII core complex [40].

Selective chemical exchange of chromophores was originally applied to bacterial RCs [41–47]. Using this technique, the absorption characteristics of individual pigments could be selectively altered without affecting the other cofactors and the RC protein structure. The significant advantage of chemical exchange is that optical properties and/or redox potentials of the RC pigment cofactors can be changed in a wide range, since both natural and non-natural extraneous pigments can be introduced. Gall et al. [48] were the first to show that it is possible to exchange external pigments into the binding sites of chlorophyll cofactors in isolated PSII RCs. On the basis of the exchange protocol developed, several modified chlorophylls, including [3-acetyl]-Chl *a* [48], [Zn]-Chl *a* [48], Chl *d* [49], and [Cu]-Chl *a* [50], were introduced into native PSII RCs. Also, PSII RCs containing five Chls [34] were reconstituted with Chl *a*, [Ni]-Chl *a*, [Zn]-Chl *a*, and [3-acetyl]-Chl *a* under pigment exchange conditions [51]. It has been proposed that the exchangeable pigment was one of the two peripheral chlorophyll molecules (probably at the D1-H118 binding site [51]), which are not involved in charge separation but slowly transfers excitation energy to the central RC pigments. It has been reported that in the monomeric form of the Chl *d*-modified RCs the number of Chl *a* molecules decreased from six to four [49].

Shkuropatov et al. [15] have developed a pheophytin exchange system that allowed obtaining two kinds of PSII RC preparations chemically modified by 13^1-OH-Pheo *a*, one with selective exchange of only $Pheo_{D2}$ [30], and another one with full replacement of $Pheo_{D2}$ and partial (~50%) replacement of $Pheo_{D1}$ [30]. Taking advantage of the fact that the Q_x and Q_y transitions of 13^1-OH-Pheo *a* are well separated from those of Pheo *a*, the 13^1-OH-Pheo *a*-modified RCs were used as useful tools to study spectral properties and interactions of the Pheo cofactors by applying different spectroscopic techniques such as low temperature absorption, linear dichroism, circular dichroism, and triplet-minus-singlet absorption-difference spectroscopy [30,52], and resonance Raman spectroscopy [53]. A possible involvement of Pheo in a charge transfer state was proposed using Stark spectroscopy [54]. The effects of Pheo substitution on energy and electron transfer in PSII RCs have also been studied by femtosecond transient absorption-difference spectroscopy [55]. However, these measurements were limited to the $Pheo_{D2}$ only-exchanged preparations because introduction of 13^1-OH-Pheo *a* into the $Pheo_{D1}$ binding site resulted in modified RCs which were inactive in charge separation due most probably to a strongly negative reduction potential of 13^1-OH-Pheo *a* and an unfavorable mutual position of the free energy levels of P_{680}^{*} and $P_{680}^{+}13^1$-OH-Pheo a^{-} [15].

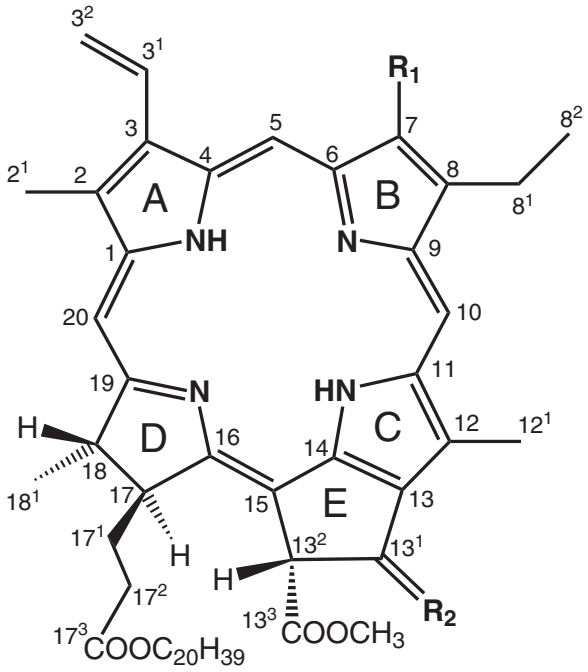
Here, we report on the preparation and characterization of pheophytin-modified PSII RCs from spinach, which contain an extraneous pigment, 7-deformyl-7-hydroxymethyl-pheophytin *b* (7^1-OH-Pheo *b*), inserted by the chemical exchange technique without loosing the photochemical activity of RCs. We show that 7^1-OH-Pheo *b* can replace both native Pheo *a* molecules in the RC. The modified pigment exchanged into the active-branch $Pheo_{D1}$ binding site participates in primary charge separation in modified RCs, functioning as an electron acceptor. In addition to this modification, PSII RCs containing another

derivative of Pheo *b*, 7-deformyl-7-hydroxymethyl-13^1-deoxo-13^1-hydroxy-pheophytin *b* (7^1-OH-13^1-OH-Pheo *b*), at the inactive Pheo$_{D2}$ binding site were also obtained.

2. Materials and methods

PSII RCs (D1/D2/cyt *b*559 complexes) were obtained from spinach PSII-enriched membranes [56] by the method of van Leeuwen et al. [57] and suspended in BT-buffer (20 mM Bis-Tris (pH 6.5), 20 mM $MgCl_2$, 5 mM $CaCl_2$, 0.03% (w/v) *n*-dodecyl-β-D-maltoside (DM)) containing 100 mM $MgSO_4$ and 200 mM sucrose.

Chl *a* and Pheo *a* and *b* were prepared from nettle using standard procedures and purified by column chromatography on sugar powder [58]. The modified pheophytins, 7^1-OH-Pheo *b* and 7^1-OH-13^1-OH-Pheo *b*, were obtained from purified Pheo *b* by irreversible chemical reduction of its carbonyl groups with sodium borohydride in methanol [35–39]. Of the two borohydride-reactive carbonyl groups in Pheo *b*, a formyl group at C-7 and a keto carbonyl group at C-13^1 (Fig. 1), the formyl group is more reactive with $NaBH_4$ [35,39], and its irreversible reduction yielded an intermediate product, 7^1-OH-Pheo *b*. A subsequent $NaBH_4$-reduction of the 13^1-keto group in 7^1-OH-Pheo *b* resulted in formation of 7^1-OH-13^1-OH-Pheo *b*. Both reaction products, obtained separately, were transferred into diethyl ether and then purified on powdered sugar columns [58]. 13^1-OH-Pheo *a* was prepared from Pheo *a* by $NaBH_4$-treatment as described earlier (see Ref. [15]). The purity of pigment preparations was checked by absorption spectroscopy in the visible and mid-infrared regions.



	R_1	R_2
Pheo *a*	$-CH_3$	=O
13^1-OH-Pheo *a*	$-CH_3$	−H,−OH
Pheo *b*	−CHO	=O
7^1-OH-Pheo *b*	$-CH_2OH$	=O
7^1-OH-13^1-OH-Pheo *b*	$-CH_2OH$	−H,−OH

Fig. 1. Structures of pheophytin *a*, pheophytin *b*, and their hydroxy-derivatives: 13^1-OH-Pheo *a*, 7^1-OH-Pheo *b*, and 7^1-OH-13^1-OH-Pheo *b*. Numbering is based on IUPAC-IUB notation [62].

Pigment exchange was performed using a procedure generally similar to that described in Refs. [15,30] with some modifications. Instead of adding a solution of the external pigment in an organic solvent directly to a PSII RC suspension, 7^1-OH-Pheo *b* was dissolved in acetone containing 2% Triton X-100 (TX-100) and 1% DM and then dried to form a layer (see [48]). After subsequent dissolving of the pigment layer in BT-buffer, the concentrated PSII RCs in BT-buffer/100 mM $MgSO_4$/200 mM sucrose were added to obtain a mixture containing ~0.6 μM RCs and ~20-fold excess of the pigment over the RC concentration; concentrations TX-100 and DM were 0.03 and 0.045%, respectively. The mixture was incubated at 28.5 °C for 20 min and then loaded on a column of Q-Sepharose FF (Sigma). The adsorbed RCs were washed with BT-buffer containing 0.05% TX-100. After that, TX-100 was removed by washing with BT-buffer, and purified 7^1-OH-Pheo *b*-modified PSII RCs were eluted from the column with BT-buffer containing 100 mM $MgSO_4$. RCs subjected to the same exchange conditions but in the absence of extraneous 7^1-OH-Pheo *b* were used as a control.

Treatment of 7^1-OH-Pheo *b*-modified and control PSII RCs with $NaBH_4$ was performed in BT-buffer containing 100 mM Bis-Tris (pH 6.5) essentially as described in [14], using Q-Sepharose FF columns for chromatographic purification of the resulting samples. RC samples subjected to the same procedures of incubation, dialysis, and additional purification as the $NaBH_4$-treated preparations but in the absence of $NaBH_4$ were used as 'untreated RCs'. Absorption difference spectra '$NaBH_4$-treated-minus-untreated RCs' were calculated from the absorption spectra normalized to the equal absorbance at the isosbestic points for the conversion of pheophytins into hydroxy-derivatives by $NaBH_4$ treatment in situ: 623 nm for control RCs [14] and 605 nm for modified RCs (this work, not shown).

If necessary, PSII RC preparations were concentrated in a pressure cell under argon gas and/or with Ultrafree-MC centrifugal filter units (Millipore) using 30 kDa cutoff membranes (Millipore). All preparations were stored at −70 °C until use.

HPLC analysis of pigment extracts from PSII RCs was performed essentially as described elsewhere [59]. The pigments were extracted with an acetone–methanol mixture (7:2, v/v), transferred into petroleum ether, and dried as a film under argon gas. The dried film was then re-dissolved in the acetone–methanol mixture (7:2, v/v), and the pigment extract obtained was injected into a column Agilent Zorbax SB-C18 4.6 × 250 mm (Agilent). A gradient of ethyl acetate (23–100%, v/v) in the acetonitrile/water/ethyl acetate mixture (69.3%:7.7%:23%, v/v) was used to elute the column at a flow rate of 1.0 ml/min. Pigment assignments were made using in situ absorption spectroscopy (diode array detector SPD-M20A (Shimadzu)). Pigment quantification was performed on the basis of peak areas of the elution traces taken at a selected wavelength and fitted by Gaussian functions. Extinction coefficients for Chl *a* and Pheo *a* in the solvent mixtures used were determined with pure pigments, using their known coefficients in diethyl ether (89.8 and 52.6 mM^{-1} cm^{-1} at the Q_y maximum, respectively [60]) as standards. To our knowledge, no extinction coefficient values for 7^1-OH-Pheo *b* are available in the literature. As a first approximation, we roughly estimated the extinction coefficient for 7^1-OH-Pheo *b* using the 7^1-OH-Pheo/Pheo *b* absorbance ratio determined in methanol in the course of the reaction of Pheo *b* with $NaBH_4$ (not shown) and assuming that this value is valid also for the pigments in diethyl ether. By adopting the known value of the extinction coefficient for Pheo *b* in diethyl ether (34.8 mM^{-1} cm^{-1} [60]), the extinction coefficient for 7^1-OH-Pheo *b* at its Q_y maximum was estimated to be 44.9 mM^{-1} cm^{-1} in diethyl ether and 38.7 mM^{-1} cm^{-1} in the solvent mixture.

Visible/near-IR electronic light-induced absorbance changes associated with photoaccumulation of $Pheo_{D1}^-$ were measured at room temperature as described earlier, using a vacuum-tight quartz cuvette with an optical pathlength of 1 cm [14]. RC samples (optical density of

~0.5–0.6 per cm at the Q_y maximum) solubilized in BT-buffer containing sodium dithionite (3 μM) and methyl viologen (1 μM) were degassed by a mild application of vacuum. Light-minus-dark $Pheo_{D1}^{-}$/$Pheo_{D1}$ difference spectra were obtained by subtracting the single-scan electronic absorption spectra measured in the dark from those collected under illumination of samples for 7 s with continuous red light through a water heat filter (λ > 600 nm; ~160 mW/cm^2).

For preparation of a sample for FTIR measurements of light-induced absorbance changes upon photoaccumulation of $Pheo_{D1}^{-}$, 1 μl of 4.6 μM water solution of methyl viologen was deposited on a CaF_2 plate (19 mm in diameter) and dried under a stream of argon gas. Methyl viologen was then re-dissolved with 3.5–4.0 μl of PSII RCs (optical density of 120–150 per cm at the Q_y maximum) solubilized in BT-buffer; the resulting mixture was lightly dried under a stream of argon gas and after the addition of 0.8 μl of freshly prepared 0.5 M sodium dithionite solution in 1 M Bis-Tris (pH 6.5), the RC film was sealed with another CaF_2 plate. Light-minus-dark $Pheo_{D1}^{-}$/$Pheo_D$ difference spectra were obtained at room temperature as a difference between the FTIR spectra (16 scans; accumulating time of 6 s) taken under and before illumination of the sample with continuous red light (λ > 600 nm; ~15 mW/cm^2). Cycles of illumination separated by dark intervals to allow photoinduced absorbance changes to relax were repeated to improve the signal-to-noise ratio.

Electronic absorption spectra in the visible/near-IR range were measured with Agilent 8453 or Shimadzu UV-1601PC spectrophotometers. For low-temperature (100 K) absorption measurements, a home-built liquid nitrogen cryostat and a Plexiglas cell with a path length of ~2 mm were used; 65% (v/v) glycerol was added to samples to obtain a clear glass.

Light-induced FTIR difference spectra were recorded on a Bruker IFS66v/S spectrophotometer equipped with an MCT detector (D313/6) and a KBr beam splitter at 4 cm^{-1} resolution. Reaction centers were protected from an actinic effect of the He–Ne laser light of the spectrophotometer by a Ge filter placed before the sample. Another Ge filter was placed at the front of the detector in order to protect it from the excitation red light.

FTIR absorption spectra of purified pigments dissolved in CCl_4 were measured at room temperature using a DGTS detector and a KBr beam splitter at 2 cm^{-1} resolution, in a cell with a pair of CaF_2 plates separated by a spacer of 50–75 μm.

3. Results

In the present work, a hydroxy-derivative of pheophytin *b*, 7^1-OH-Pheo *b*, was used to replace the native pheophytin *a* molecules in isolated PSII reaction centers by the chemical exchange procedure that is based on incubation of RCs with an excess of the externally added pigment at a slightly elevated temperature [15,30,48]. This modified pigment was chosen because its reduction potential in vitro is comparable to that of Pheo *a*: it has been shown [39] that one electron potential $E_{1/2}^{-}$ for Pheo *a*/Pheo *a*$^-$ and 7^1-OH-Pheo *b*/7^1-OH-Pheo *b*$^-$ couples in tetrahydrofuran are equal to −1.15 and −1.17 V, respectively. Thus, one might expect that the redox properties of 7^1-OH-Pheo *b*, at least as determined in solution, would be favorable for its participation in electron-transfer reactions in PSII RCs in lieu of the native Pheo *a*. The molecular structure of 7^1-OH-Pheo *b* differs from that of Pheo *a* by the presence of the CH_2OH group at C-7 in the former instead of the CH_3 group in the latter (Fig. 1). The infra-red absorption spectra of the pigment preparations used in this work, measured in CCl_4 at room temperature (Fig. S1, Supplementary data), confirmed the absence of the formyl C=O stretching mode for 7^1-OH-Pheo *b* [35], which is the mode observed for the Pheo *b* molecule at 1670 cm^{-1} (Fig. S1, Supplementary data; [61]). PSII RCs treated under identical exchange conditions but without externally added 7^1-OH-Pheo *b* were used as a control, in order to separate changes in the composition and properties of reaction centers induced by pigment replacement from those caused by the modification procedure itself.

3.1. Comparison of absorption spectra of 7^1-OH-Pheo b and Pheo a in solution

Fig. 2 shows electronic (370–720 nm) absorption spectra of 7^1-OH-Pheo *b* (solid line) and Pheo *a* (dotted line) in diethyl ether, measured at room temperature and scaled according to extinction coefficients of the pigments. In agreement with previous studies [35,39,63], spectra of the two pigments are similar but not identical to each other, differing in positions and relative intensities of the bands. The long-wavelength Q_y absorption band of 7^1-OH-Pheo *b* located at 664 nm is blue-shifted by 3 nm and is decreased in intensity with respect to the band of Pheo *a* (Fig. 2), giving rise to a prominent negative peak at 671 nm and a less intensive positive peak at 658 nm in the absorption difference spectrum '7^1-OH-Pheo *b*-minus-Pheo *a*' (Fig. 4C). The maxima of the Q_x (0–0) and Q_x (0–1) absorption bands of 7^1-OH-Pheo *b* are shifted to red as compared to those of Pheo *a* (from 534 to 536 nm, and from 505 to 507.5 nm, respectively), and the intensities of both bands are slightly decreased in 7^1-OH-Pheo *b* (Fig. 2). In the absorption difference spectrum (Fig. 4C), these differences are seen as a well-expressed feature with positive signals at 514 and 542 nm and a negative one at 532 nm. The difference in the peak position of the Soret absorption bands of 7^1-OH-Pheo *b* and Pheo *a* (413 vs 409 nm) (Fig. 2) leads to the appearance of a differential signal at 421/404 nm in the absorption difference spectrum (Fig. 4C). Taken together, these small but distinct changes between the absorption spectra of the two pigments can be used for a spectral verification of the pheophytin exchange reaction.

Solubilization of 7^1-OH-Pheo *b* in the incubation media (an aqueous micellar system containing 20 mM Bis-Tris (pH 6.5)/0.03% TX-100/0.045% DM) did not significantly affect the absorption spectrum of the pigment except that the main absorption bands were slightly broadened and red-shifted by 3–4.5 nm as compared to those in diethyl ether (not shown). These spectral changes are most probably due to a solvent effect, and it is likely that 7^1-OH-Pheo *b* was in the incubation medium in a monomer form.

3.2. HPLC analysis

HPLC analysis showed that pigment extract from the control PSII RC contained three native pigments, Chl *a*, Pheo *a* and β-carotene, as well as a small quantity of epimers of Chl *a* and Pheo *a* (Fig. 3A). A Chl *a*: Pheo *a* ratio of 5.8:2 was estimated assuming a constant amount of two pheophytins per RC (Table 1). The RC preparation treated with

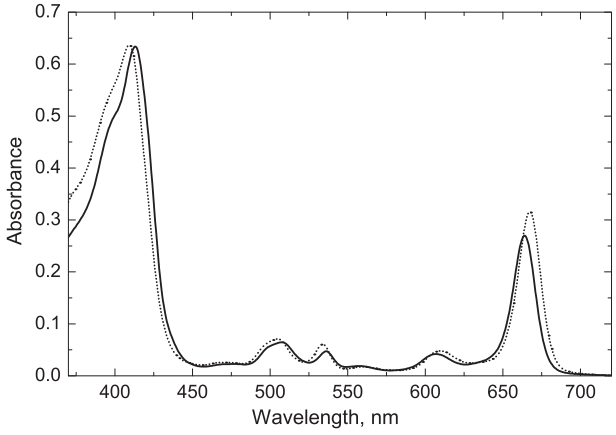


Fig. 2. Room-temperature electronic absorption spectra of 7^1-OH-Pheo *b* (solid line) and Pheo *a* (dotted line) in diethyl ether. Spectra are scaled according to extinction coefficients of the pigments.

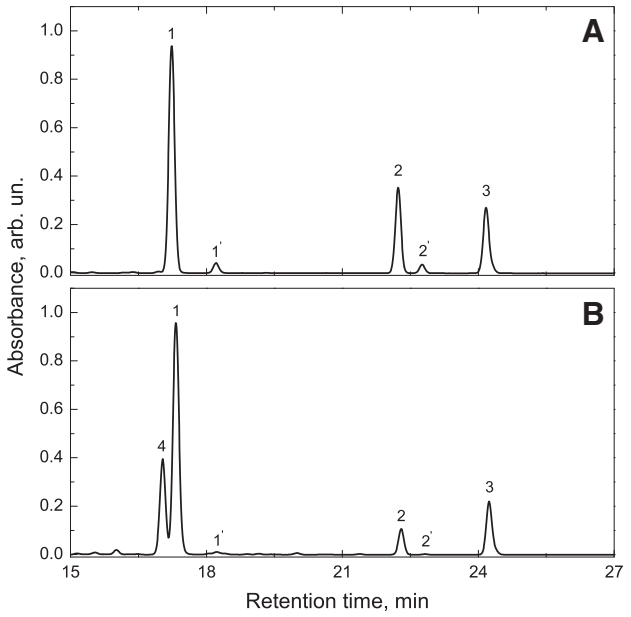


Fig. 3. HPLC analysis of pigment extracts from control (A) and 7[1]-OH-Pheo *b*-modified PSII RC preparations (B). Peak assignments: 1: Chl *a*; 2: Pheo *a*; 3: β-carotene; 4: 7[1]-OH-Pheo *b*. Peaks 1′ and 2′ correspond to C-13[2]-epimers of Chl *a* and Pheo *a*, respectively. Chromatograms were detected at 420 nm (at which wavelength the extinction coefficients for Chl *a* and Pheo *a* in the corresponding solvent mixtures were comparable) and normalized to the equal area of Chl *a* peaks (sum of areas of peaks 1 and 1′), assuming that the chlorophyll content was not altered upon pheophytin exchange. arb. un.: arbitrary units.

7[1]-OH-Pheo *b* under pigment exchange conditions showed the presence of a forth pigment (Fig. 3B, peak 4) with the absorption spectrum of 7[1]-OH-Pheo *b*. The appearance of this new pigment is accompanied by a partial loss of Pheo *a*. These observations suggest that incubation of PSII RCs with an externally added 7[1]-OH-Pheo *b* led to replacement of the native Pheos by the modified pigment. The stoichiometry of pheophytins in 7[1]-OH-Pheo *b*-modified preparations (Table 1) corresponds to the presence of ~0.6 Pheo *a* and ~1.4 7[1]-OH-Pheo *b* per modified RC, based on the assumption that the amount of Chl *a* per RC is not altered upon pheophytin exchange. Table 1 shows that the total content of Pheos (Pheo *a* plus 7[1]-OH-Pheo *b*) in modified preparations was only slightly higher than 2 per RC, suggesting the presence of a minor amount of non-specifically bound 7[1]-OH-Pheo *b*. Based on the HPLC data, the percentage of pheophytin replacement was estimated to be close to 70%. This implies that 7[1]-OH-Pheo *b* replaced Pheo *a* both at $Pheo_{D1}$ and $Pheo_{D2}$ binding sites of the RC. As the pigment exchange was only partial, 7[1]-OH-Pheo *b*-modified preparations apparently contained a mixture of RCs with different degrees of modification, including those in which either one or both original Pheo *a* molecules have not been exchanged with the modified pigment.

It is interesting to note that relative amounts of pigment epimers (peaks 1′ and 2′ for Chl *a* and Pheo *a*, respectively) and β-carotene (peak 3) were decreased in the pigment extracts after the exchange reaction (Fig. 3), but the reasons for these effects remain to be understood.

Table 1
Chlorophyll and pheophytin composition of control and 7[1]-OH-Pheo *b*-modified PSII RCs, determined by HPLC analysis of the pigment extracts. Average values of two determinations are shown. It is assumed that there is a constant amount of two Pheo *a* per RC in control preparations, and that the content of Chl *a* is not altered upon pheophytin exchange.

Sample	Chl *a*[a]	Pheo *a*[b]	7[1]-OH-Pheo *b*	Total Pheos (Pheo *a* plus 7[1]-OH-Pheo *b*)
Control	5.8	2	–	2
Modified	5.8	0.59	1.44	2.03

[a] Sum of Chl *a* and its epimer Chl *a*′.
[b] Sum of Pheo *a* and its epimer Pheo *a*′.

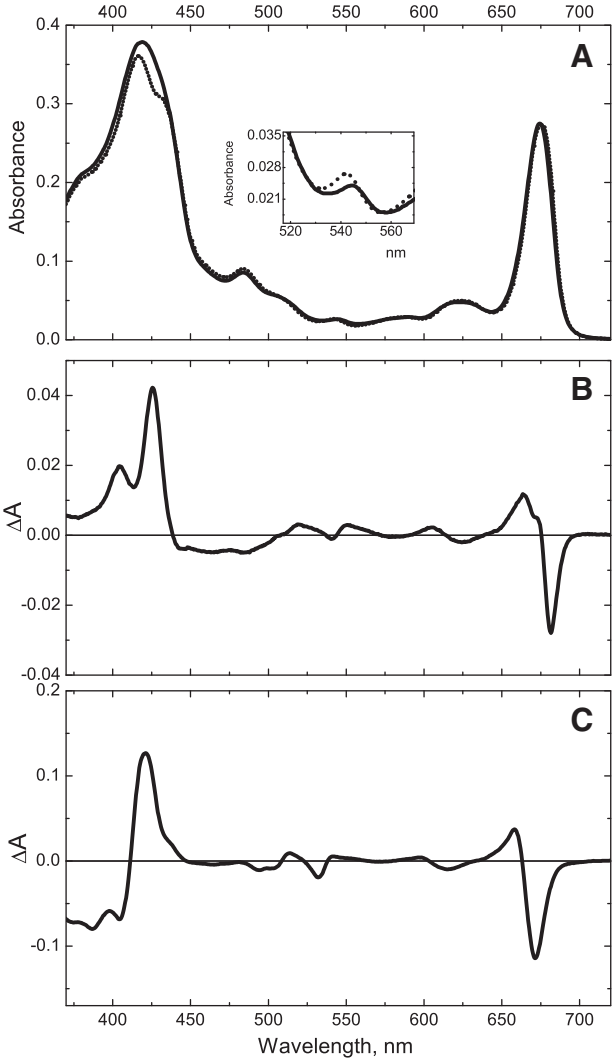


Fig. 4. (A) Room-temperature electronic absorption spectra of 7[1]-OH-Pheo *b*-modified (solid line) and control PSII RCs (dotted line). Spectra are normalized at the chlorophyll *a* Q_x band at 588 nm. The inset displays the Q_x spectral region of pheophytins in an expanded scale; the spectra are brought into coincidence in the range of 555–560 nm. (B) Difference spectrum 'modified-minus-control RCs', calculated from the absorption spectra in panel A. (C) Difference spectrum '7[1]-OH-Pheo *b*-minus-Pheo *a*', calculated from the room-temperature absorption spectra of the pigments in diethyl ether presented in Fig. 2.

3.3. Spectral effects of pigment exchange

Fig. 4A compares room-temperature electronic (370–720 nm) absorption spectra of 7[1]-OH-Pheo *b*-modified (solid line) and control PSII RCs (dotted line) normalized at the Chl *a* Q_x band at 588 nm, assuming that the chlorophyll content is the same in the two preparations. The pigment replacement is evident from an observation that the difference between these spectra (modified-minus-control RCs) (Fig. 4B) displays several features basically similar to those observed in the absorption difference spectrum '7[1]-OH-Pheo *b*-minus-Pheo *a*' for pure pigments in diethyl ether (Fig. 4C), but shifted to red by 4–10 nm due probably to environment-induced effects. In particular, the pheophytin Q_x band in the absorption spectrum of modified preparations shifted to about 545 nm and decreased in intensity as compared to the 542 nm band of control RCs (Fig. 4A, inset), and the difference spectrum 'modified-minus-control RCs' (Fig. 4B) shows a characteristic feature with two positive bands at 520 and 551 nm and a negative one at 541 nm, which is similar to that observed at 514/542/532 nm in the solution difference spectrum for purified pigments (Fig. 4C). Upon modification,

the Q_y absorption maximum of RCs shifted from 675.5 to 674.5 nm (Fig. 4A), and the difference spectrum (Fig. 4B) displays a loss of the Pheo *a* absorption at 681.5 nm, and an appearance of a band at 664 nm, which is apparently caused by the introduced 7^1-OH-Pheo *b*. Similar to what was observed for pure pigments (Fig. 4C), the intensity of the bleaching signal was larger than that of the appearing band (Fig. 4B). A notable feature in the difference spectrum 'modified-minus-control RCs' is the presence of a shoulder at ~672 nm (Fig. 4B), an analog of which is, however, not observed in the difference spectrum for pure pigments (Fig. 4C). In the Soret region, the difference spectrum 'modified-minus-control RCs' (Fig. 4B) shows a prominent peak at 425.5 nm which, according to Fig. 4C, is also attributable to the modified pheophytin. Small absorption decreases observed for modified preparations in the region around 500 nm (Fig. 4A, B) could be at least in part due to a loss of β-car molecules upon pigment exchange, in agreement with the HPLC data (Fig. 3).

Fig. 5 shows that the main differences between the normalized absorption spectra of 7^1-OH-Pheo *b*-modified and control preparations observed at room temperature (Fig. 4A, B) are also present at 100 K, but slightly better resolved. The 100 K absorption spectrum of control RCs (Fig. 5A and inset, dotted lines) is virtually identical to the low-temperature spectra previously reported for isolated PSII RCs in the literature (see, for example, Ref. [64]), exhibiting the two split peaks of the Q_y band at 672 and 678.5 nm, while the spectrum of modified preparations (Fig. 5A and inset, solid line) shows a slightly increased single peak at 673 nm and a weak shoulder at ~680 nm. The weak positive shoulder seen in the room-temperature difference spectrum at ~672 nm (Fig. 4B) is resolved at 100 K (Fig. 5B) as a prominent narrow peak at 674 nm

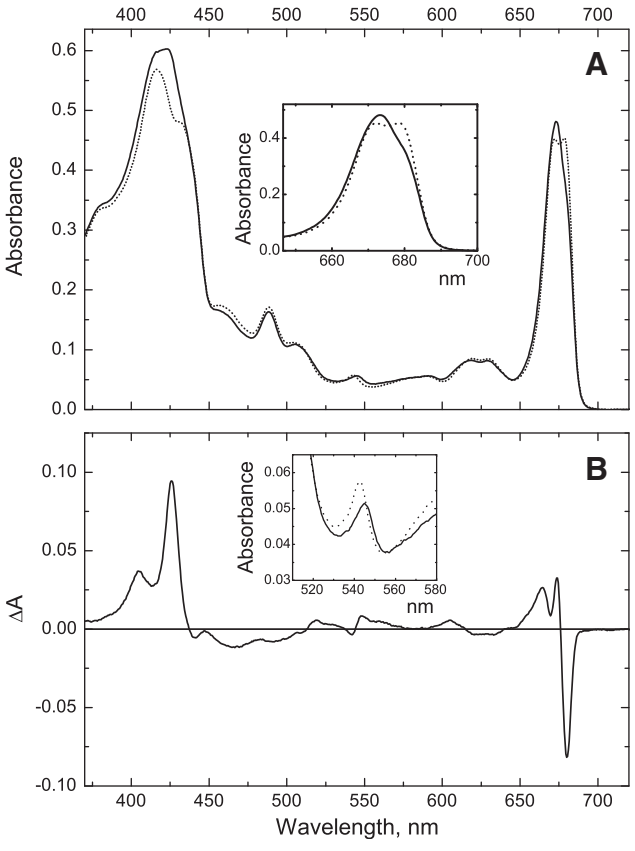


Fig. 5. (A) Low-temperature (100 K) electronic absorption spectra of 7^1-OH-Pheo *b*-modified (solid line) and control PSII RCs (dotted line). Spectra are normalized at the chlorophyll *a* Q_x band at 588 nm. The inset shows the Q_y spectral region in an expanded scale. (B) The difference spectrum 'modified-minus-control RCs', calculated from the absorption spectra in panel A. The inset displays the Q_x region of pheophytins in the absorption spectra in panel A plotted in an expanded scale; the spectra are brought into coincidence at 555 nm.

(together with a broader band at 664 nm). The maxima of the Q_x band in the 100 K absorption spectra of control and modified preparations are located at 542.5 and 545 nm, respectively (Fig. 5B, inset).

3.4. Photoaccumulation of reduced pheophytin

In order to test the photochemical activity of 7^1-OH-Pheo *b*-modified RCs, light-induced electronic and FTIR absorption difference spectra for photoaccumulation of $Pheo_{D1}^-$ were measured in the presence of a reductant (sodium dithionite) and a redox mediator (methyl viologen). FTIR difference spectroscopy, showing very high sensitivity to molecular changes associated with charge separation, provides also a possibility to probe structures and bonding interactions of the RC redox-active cofactors in their neutral and ionic forms [65,66]. The resulting $Pheo_{D1}^-/Pheo_{D1}$ electronic and FTIR difference spectra of the modified preparations were compared to those of the control PSII RCs.

3.4.1. $Pheo_{D1}^-/Pheo_{D1}$ electronic difference spectra

Fig. 6A presents room-temperature light-induced electronic (400–950 nm) $Pheo_{D1}^-/Pheo_{D1}$ difference spectra for the reversible reduction of the pheophytin electron acceptor in control and 7^1-OH-Pheo *b*-modified PSII RC preparations in the presence of sodium dithionite and methyl viologen. The difference spectra were calculated for comparable concentrations of RCs by normalizing them to the same absorbance of cyt *b*559 at 559 nm in the original absorption spectra. Cyt *b*559 was fully reduced by dithionite under the conditions used. The difference spectrum of control samples (Fig. 6A, dotted line) corresponds well to analogous spectra described in the literature [7,14,15,17]. Photoaccumulation of $Pheo_{D1}^-$ manifests itself by characteristic absorbance decreases at 544, ~515 and ~420 nm reflecting selective bleaching of $Pheo_{D1}$ absorption bands, together with a development of positive radical anion bands at ~900, ~800, ~700, 655, 598 and ~447 nm due to formation of $Pheo_{D1}^-$. There is also a prominent negative signal at 682 nm that was attributed to bleaching of the $Pheo_{D1}$ Q_y absorption band [67] and/or to a blue band shift of a Chl *a* pigment upon $Pheo_{D1}^-$ formation [24,67]. A small sharp peak at 672 nm is due to such a shift. The light-induced difference spectrum of 7^1-OH-Pheo *b*-modified preparations (Fig. 6A, solid line) also shows features characteristic of photoaccumulation of reduced pheophytin molecules. However, this spectrum noticeably differs from that of control samples, especially in the visible region, and the observed differences apparently reflect the reduction of 7^1-OH-Pheo *b* instead of Pheo *a* in modified RCs. Since pheophytin reduction is accompanied by bleaching of the Q_y, Q_x and Soret absorption bands (see [68]), analysis of absorbance decreases in the $Pheo_{D1}^-/Pheo_{D1}$ difference spectrum may provide information on absorption properties of a photoreducible pheophytin in modified preparations. Fig. 6A (inset) shows that one prominent difference between the photoaccumulation spectra is that the Pheo *a* Q_x bleaching band at 544 nm in control RCs is replaced by a negative band of a smaller intensity at 546 nm in 7^1-OH-Pheo *b*-modified preparations. This spectral change parallels a red shift and an intensity decrease of the $Q_x(0–0)$ absorption band of 7^1-OH-Pheo *b* compared to that of Pheo *a* in solution (Fig. 2) and in reaction centers (Fig. 4A, inset), confirming that the reduced 7^1-OH-Pheo *b* molecules are photoaccumulated in the modified RC. An expected contribution to the $Pheo_{D1}^-/Pheo_{D1}$ difference spectrum of modified preparations from a negative signal at 544 nm due to a fraction of unchanged and/or $Pheo_{D2}$ only-exchanged RCs seems to be hidden under the major bleach at 546 nm. Another clear change in the difference spectrum of modified preparations is an intensified and slightly red-shifted negative peak at ~425 nm in the Soret-band region (Fig. 6A), which is also consistent with 7^1-OH-Pheo *b* functioning as an electron acceptor. In the Q_y region, a striking feature in the $Pheo_{D1}^-/Pheo_{D1}$ difference spectrum of modified preparations is a distinct new negative signal centered at 667 nm. While the parental difference spectra of control and modified samples are quite complex in this spectral region, the double difference spectrum

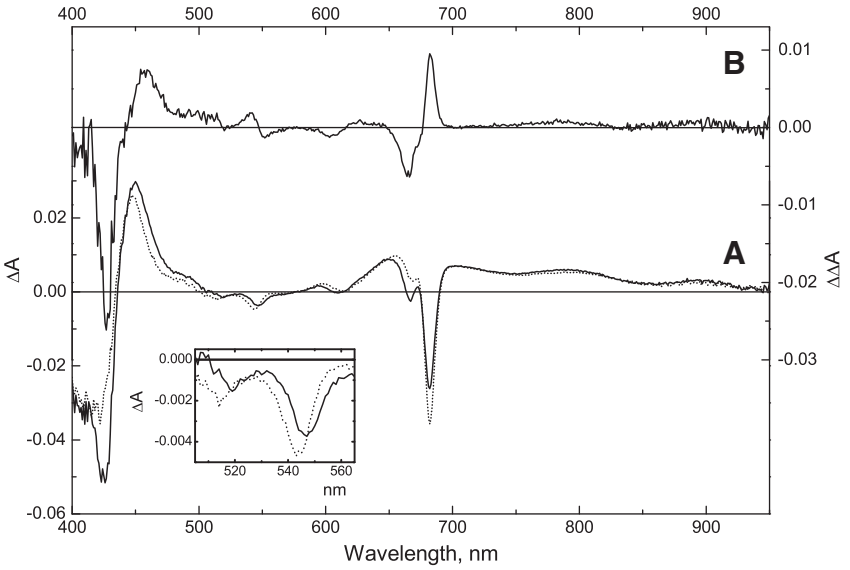


Fig. 6. (A) Room-temperature light-induced electronic $Pheo_{D1}^{-}/Pheo_{D1}$ difference spectra of 7^1-OH-Pheo *b*-modified (solid line) and control PSII RCs (dotted line) in the presence of dithionite and methyl viologen. Spectra are normalized to the equal absorbance of cyt *b*559 at 559 nm. The inset displays the Q_x region of pheophytins in an expanded scale. (B) Double difference spectrum between the spectra in panel A (modified-minus-control RCs).

(modified-minus-control RCs) (Fig. 6B) is relatively simple and can be readily explained by a decrease in the amplitude of the bleaching at 682 nm and an appearance of a bleaching signal at about ~665 nm. Notably, the peak position of this new bleach is close to that of the positive band at 664 nm in the absorption difference spectra for pigment exchange that is assigned to the Q_y band of the inserted 7^1-OH-Pheo *b* (Figs. 4B and 5B). It is likely therefore that the photoreducible 7^1-OH-Pheo *b* molecule in the modified preparations absorbs in the Q_y region around 665 nm. The red shift and intensity decrease of the pheophytin Q_x bleaching band in the difference spectrum of modified RCs compared to that of control RCs (Fig. 6A, insert) are seen in the double difference spectrum (Fig. 6B) as a differential signal at 541/552 nm. A positive signal observed in the double difference spectrum at ~458 nm originates from a small red shift (from ~447 to ~449 nm) and slight increase in intensity of the pheophytin radical anion band due to exchange of Pheo *a* with 7^1-OH-Pheo *b* (Fig. 6A).

3.4.2. $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectra

Fig. 7 compares light-induced $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectra of 7^1-OH-Pheo *b*-modified and control PSII samples in the mid-IR frequency region (1800–1100 cm^{-1}) measured in the presence of dithionite and methyl viologen at room temperature. Spectra are normalized at 1741 cm^{-1}. Both spectra correspond to the reversible absorbance changes. The spectrum of the control RCs is similar to the light-induced FTIR difference spectra for photoaccumulation of $Pheo_{D1}^{-}$ earlier obtained with PSII samples containing Glu at the D1-130 position: isolated pea RCs [69], PSII–PsbA3 core complexes from *Thermosynechococcus elongatus* and PSII-enriched membranes from spinach [70], as well as

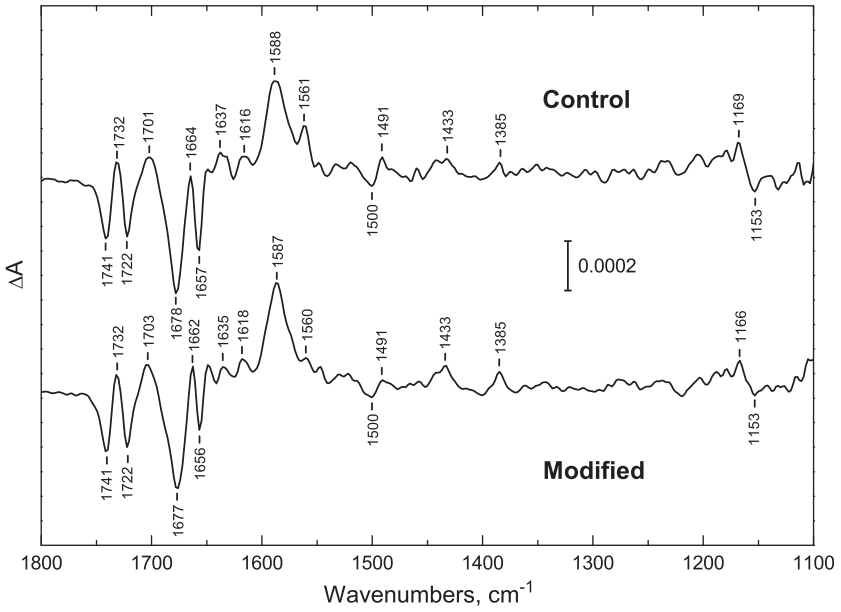


Fig. 7. Light-induced $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectra of 7^1-OH-Pheo *b*-modified and control PSII RCs in films in the presence of sodium dithionite and methyl viologen. Spectra were measured at room temperature; 608 interferograms were averaged for two separate films of each RC. Spectra are normalized at 1741 cm^{-1}. Spectral resolution was 4 cm^{-1}.

large spinach PSII particles [71]. Nabedryk et al. [69] have provided assignments of a number of bands in the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectrum on the basis of comparison with a redox-induced Pheo a^{-}/Pheo *a* FTIR difference spectrum in tetrahydrofuran, and Shibuya et al. [70] have proposed some additional attributions of the spectral features observed. According to these studies, the large negative band seen in Fig. 7 at 1678 cm^{-1} is assigned to the 13^1-keto C═O stretching mode of neutral $Pheo_{D1}$ [69] which shifts to lower frequencies upon $Pheo_{D1}^{-}$ formation showing the prominent positive peak at 1588 cm^{-1} [70]. The keto group is proposed to be hydrogen-bonded to D1-Glu130 both in neutral and anionic forms of $Pheo_{D1}$ [69]. The negative bands at 1741 and 1722 cm^{-1}, observed in the high frequency carbonyl stretching range of 1750–1700 cm^{-1} (Fig. 7), can be assigned to a carboxylic group of D1-Glu130 and to a protein-bound 13^3-ester C═O group of $Pheo_{D1}$, respectively [69]. By analogy with bacterial RCs [72], another possible interpretation is that the band pattern in the 1750–1700 cm^{-1} region may reflect the presence of two conformationally distinct populations of $Pheo_{D1}$ which differ in the hydrogen-bonding interactions between the 13^3-ester C═O group and the protein [70]. In one population, giving a negative band at 1722 cm^{-1}, the 13^3-ester C═O group is proposed to be hydrogen-bonded with D1-Tyr126, while in another one, a free 13^3-ester C═O contributes to the 1741 cm^{-1} band [70]. The corresponding positive peaks are located in the FTIR spectrum at 1702 and 1732 cm^{-1}, respectively (Fig. 7). A strong negative peak at 1657 cm^{-1} is probably due to the amide I vibrations of the protein backbone around $Pheo_{D1}$ perturbed upon $Pheo_{D1}^{-}$ formation. Several small negative and positive peaks in the ~1630–1100 cm^{-1} frequency region are attributable to the chlorin ring skeletal vibrations of $Pheo_{D1}$ in neutral and anionic forms, respectively.

Over the whole frequency region studied, the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectrum of the 7^1-OH-Pheo *b*-modified samples showed features very similar to those observed for control RCs, with only minor (≤1–3 cm^{-1}) shifts in frequencies of the bands (Fig. 7). Although subpopulations of unchanged and/or $Pheo_{D2}$ only-exchanged RCs are expected to exhibit the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectra identical to that of the control RCs, the absence of large changes in the spectrum of modified preparations could indicate that an overall chlorin macrocycle geometry and possible hydrogen-bonding interactions of the pigment carbonyl groups with the $Pheo_{D1}$ protein pocket were comparable for Pheo *a* and 7^1-OH-Pheo *b*, both in neutral and anionic forms. The only clear difference between the difference spectra in Fig. 7 is that a positive band at 1561 cm^{-1} is decreased upon pigment exchange. A residual signal at 1560 cm^{-1} in modified preparation apparently belongs to the population of unchanged and/or $Pheo_{D2}$ only-exchanged RCs.

As a whole, the results obtained in both the visible/near-IR (Fig. 6) and mid-IR (Fig. 7) spectral regions are consistent with the functional integrity of the modified RCs, indicating that the inserted 7^1-OH-Pheo *b* can operate as an electron acceptor with accumulation of its radical anion in the presence of a reductant that is able to rapidly rereduce P_{680}^{+} in the photogenerated radical pair $P_{680}^{+}7^1$-OH-Pheo b^{-}.

3.5. $NaBH_4$-treatment

Previously, it has been shown that treatment of native PSII RCs with $NaBH_4$ led to the preferential irreversible chemical modification of $Pheo_{D2}$ to form 13^1-deoxo-13^1-hydroxy-Pheo *a* (13^1-OH-Pheo *a*) inside RCs, while $Pheo_{D1}$ remained largely unchanged [14]. In the present study, we used this reaction to obtain information on absorption properties of 7^1-OH-Pheo *b* at the $Pheo_{D2}$ site of 7^1-OH-Pheo *b*-modified RCs. The absorption difference spectrum for reactions of $NaBH_4$ with the pigments in the control preparations (Fig. 8, solid line) was found to be very similar to that previously described for native PSII RCs [14]. The spectrum shows absorbance decreases at 679.5 and 542 nm reflecting a loss of the Q_y and Q_x absorption bands of Pheo *a*, respectively, and an appearance of the bands at 655 and around 500 nm due to the formation of 13^1-OH-Pheo *a*, most probably at the $Pheo_{D2}$ site [14]. The absorption difference spectrum for $NaBH_4$-treatment of 7^1-OH-Pheo *b*-modified RCs (Fig. 8, dotted line) exhibits generally similar features, however, the Q_y band of a newly formed pheophytin hydroxy-derivative is significantly shifted in this case to 649 nm. The observed blue shift is consistent with a shorter wavelength position of the Q_y absorption band of 7-deformyl-7-hydroxymethyl-13^1-deoxo-13^1-hydroxy-Pheo *b* (7^1-OH-13^1-OH-Pheo *b*) (650.5 nm) with respect to that of 13^1-OH-Pheo *a* (653 nm) in diethyl ether solution (Fig. S2, Supplementary data). These facts indicate that 7^1-OH-Pheo *b* exchanged into PSII RCs was subjected to the reaction with $NaBH_4$, forming a hydroxy-derivative, most likely 7^1-OH-13^1-OH-Pheo *b*. The light-induced electronic (400–950 nm) $Pheo_{D1}^{-}/Pheo_{D1}$ difference spectra for untreated and $NaBH_4$-treated 7^1-OH-Pheo *b*-modified preparations measured in the presence of sodium dithionite and methyl viologen were very close to each other both in shape and amplitude after their normalization to comparable concentrations of RCs (Fig. S3, Supplementary data). This confirms that it is the inactive-branch 7^1-OH-Pheo *b* molecule that was selectively involved in the reaction with $NaBH_4$ in the modified preparations. Otherwise, a reduced yield of $Pheo_{D1}^{-}$ photoaccumulation would be observed for $NaBH_4$-treated preparations because, according to previous estimates for 13^1-OH-Pheo *a* [15], 7^1-OH-13^1-OH-Pheo *b* is expected to have too negative reduction potential to participate in charge separation. Due to the incomplete pheophytin replacement, the difference spectrum for $NaBH_4$-treatment of the 7^1-OH-Pheo *b*-modified RCs (Fig. 8, dotted line) should include also absorbance changes reflecting chemical modification of native Pheo *a* to 13^1-OH-Pheo *a* in a fraction of unchanged and/or $Pheo_{D1}$ only-exchanged RCs. However, such signals seem to be too small to be clearly discernible in the experimental difference spectrum. Based on these data, the negative feature at 677 nm dominating the red region of the difference spectrum for $NaBH_4$-treatment of 7^1-OH-Pheo *b*-modified RCs and the prominent bleaching observed in this spectrum at 543.5 nm (Fig. 8, dotted line) can be assigned to a loss of the Q_y and Q_x absorption bands of 7^1-OH-Pheo *b* at the $Pheo_{D2}$ binding site due to its conversion into 7^1-OH-13^1-OH-Pheo *b*. A weak negative shoulder at ~665 nm (Fig. 8, dotted line) is possibly due to the formation of 7^1-OH-13^1-OH-Pheo *b* from a minor amount of 7^1-OH-Pheo *b* molecules that are non-specifically bound to the periphery of the RC protein, absorbing near 665 nm.

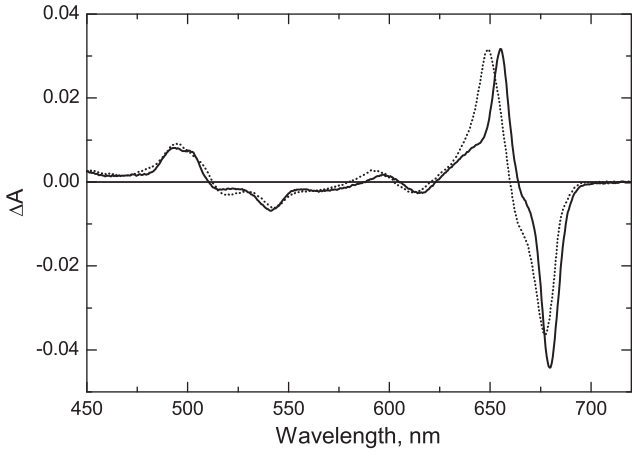


Fig. 8. Absorption difference spectra for reactions of $NaBH_4$ with the pigments in control (solid line) and 7^1-OH-Pheo *b*-modified (dotted line) PSII RC preparations. Spectra were obtained at room temperature and normalized at the maxima of their positive bands at 655 and 649 nm, respectively. The difference spectra were obtained under conditions when $NaBH_4$-treatment resulted in about 50% decrease of the dipole strength of the pheophytin Q_x absorption band (not shown).

4. Discussion

The data obtained show that 7^1-OH-Pheo *b*, a pigment that is not naturally present in PSII, could be incorporated into about 70% of the $Pheo_{D1,D2}$ binding sites in isolated PSII RC preparations under pigment exchange conditions. The resulting 7^1-OH-Pheo *b*-modified RCs remained essentially functional in primary charge separation, as evidenced by the photoaccumulation of 7^1-OH-Pheo b^- upon illumination in the presence of sodium dithionite and methyl viologen (Figs. 6 and 7). This implies that the P_{680}^+ 7^1-OH-Pheo b^- radical pair formed in the active cofactor branch of the modified RC is thermodynamically accessible from the excited primary electron donor state. It seems reasonable to expect that the selective pigment exchange at the pheophytin sites of PSII RCs should have little effect on the chlorophyll binding sites, and that the electronic structure and the oxidation potential of the primary electron donor remained unaltered. The function of the active Pheo *a* as an intermediate electron acceptor is largely determined by its reduction potential, which is modulated by the RC protein matrix. The photochemical activity of the 7^1-OH-Pheo *b*-modified RCs suggests that the protein environment of the $Pheo_{D1}$ pocket affects the redox properties of Pheo *a* and 7^1-OH-Pheo *b* in a similar manner, and that the close similarity between the $E_{1/2}^-$ potentials for the one electron reduction of the two pigments in solution (−1.15 and −1.17 V, respectively [39]) appears to be maintained in RCs. An analogous situation, namely, preserving a solution reduction potential difference between the pigments in the PSII RC protein matrix, seems to be also characteristic of the 13^1-OH-Pheo *a*/Pheo *a* pair. Indeed, exchange of Pheo *a* at the $Pheo_{D1}$ pocket with 13^1-OH-Pheo *a*, the reduction potential of which when in solution strongly shifted (~0.3 V) to negative values with respect to that of Pheo *a* [15], has resulted in preventing electron transfer in the 13^1-OH-Pheo *a*-modified RCs, most likely because the free energy level of the $P_{680}^+13^1$-OH-Pheo a^- radical pair was placed well above that of the excited primary electron donor state. Vavilin et al. [32] have presented arguments that the midpoint reduction potential of Pheo *b* in organic solvents should be close to the reduction potential values known for Pheo *a* in vitro. The authors relate this similarity in potentials with the ability of Pheo *b* to function as an electron acceptor in the chlorophyll *b*-containing mutant RCs of *Synechocystis* sp. PCC 6803 in lieu of Pheo *a*. Pigment exchange experiments performed earlier with bacterial RCs have also indicated that the relative order of redox potentials measured for (bacterio)pheophytins in solution was maintained in the active-branch H_A binding site [45]. Thus, in agreement with other data, the present work shows that a comparison of the in vitro redox potentials of pigments is a useful approach for a primary selection of extraneous pheophytin molecules as possible candidates for functioning in charge separation in site-exchanged PSII RCs.

According to recent models for the electronic structure of the PSII RC [24–26,73], the Q_y optical transitions of the RC core pigments are excitonically coupled, but their mean site energies are different. As a consequence of this difference in site energies, the RC exciton states are partially localized, and (except for the chlorophylls of the special pair P_{D1}/P_{D2}) each pigment dominantly contributes to only one of the states [24]. With this model, the bleaching at 677 nm that is observed in the absorption difference spectrum for $NaBH_4$-treatment of the 7^1-OH-Pheo *b*-modified RCs (Fig. 8, dotted line) due to the transformation of the 7^1-OH-Pheo *b* molecule at the $Pheo_{D2}$ site into 7^1-OH-13^1-OH-Pheo *b* indicates that the inactive-branch 7^1-OH-Pheo *b* mainly contributes to an exciton state at about 677 nm. Similarly, an exciton state which is dominated by Pheo *a* at the $Pheo_{D2}$ site in control RCs is localized near 680 nm, as it can be inferred from the peak position of the Q_y bleaching band at 679.5 nm seen upon $NaBH_4$-treatment of these RCs (Fig. 8, solid line, and Ref. [14]). The observed ~2.5 nm blue shift between the Q_y exciton bands of $Pheo_{D2}$ in the 7^1-OH-Pheo *b*-modified and control preparations is close to the difference between the Q_y absorption maxima of 7^1-OH-Pheo *b* and Pheo *a* in solution (3 nm in diethyl ether; Fig. 2), suggesting that pigment–pigment and/or pigment–protein interactions that are responsible for the modulation of absorption properties of the pheophytin molecule in the $Pheo_{D2}$ protein pocket are largely similar for Pheo *a* and 7^1-OH-Pheo *b*. It is possible that this blue shift of the dominant Q_y $Pheo_{D2}$ exciton state upon pigment replacement could qualitatively explain the appearance of the narrow positive peak at 674 nm in the 100 K absorption difference spectrum 'modified-minus-control RCs' (Fig. 5B). A negative lobe that is expected for such a shift might constitute a part of the absorption decrease at 680 nm (Fig. 5B), which is not resolved at 100 K. Thus, one can conclude that the Q_y absorption of the inactive-branch 7^1-OH-Pheo *b* is located at about 677 nm, being significantly shifted to red with respect to the Q_y absorption maximum of the pure pigment in solution (664 nm in diethyl ether) (Fig. 2). At present there is no consensus on the absorption properties of the inactive Pheo *a* in PSII RCs: the absorption at 670–672 nm [10,21,74–79] or at 676–680 nm [9,14,30,40,52,80] has been ascribed to the $Pheo_{D2}$ Q_y transition. The data presented in this work are consistent with the latter assignment, suggesting that $Pheo_{D2}$ mainly contributes to the low-energy side of the PSII RC Q_y absorption band at about 677–680 nm in both native and 7^1-OH-Pheo *b*-modified RCs. Recent $NaBH_4$-treatment experiments have shown that $Pheo_{D2}$ appears to absorb at ~680 nm in the RC of the functionally active spinach PSII core complexes as well [40].

A remarkable feature is that the spectral position of the Q_y absorption of the 7^1-OH-Pheo *b* molecule at the active $Pheo_{D1}$ site of modified RCs is apparently different from that at the $Pheo_{D2}$ site. As it follows from the $Pheo_{D1}^-$ photoaccumulation measurements in the visible/near-IR region, a new bleaching is observed near 665 nm in the double difference spectrum (Fig. 6B), which suggests that the photoreducible 7^1-OH-Pheo *b* molecule dominantly contributes to the absorption band centered in this wavelength region. The same band is apparently seen as the positive peak located at 664 nm in the difference spectra for pigment exchange (Figs. 4B and 5B). We conclude therefore that the Q_y absorption band of the active-branch 7^1-OH-Pheo *b* molecule at ~665 nm is significantly blue-shifted with respect to the 677 nm exciton band dominated by the inactive pigment. This means also that there is practically no difference between the positions of the Q_y optical transitions of 7^1-OH-Pheo *b* in the $Pheo_{D1}$ protein pocket and in diethyl ether (Fig. 3).

The spectral position of the $Pheo_{D1}$ Q_y absorption band in PSII RCs is not yet established unequivocally: different authors placed it either at ~670–672 nm [24,77] or in the range of 676–682 nm [7,9,14,15,21,30,74–76,78,80–83]. According to the model by Raszewski et al. [24], the main bleaching at 682 nm in the $Pheo_{D1}^-/Pheo_{D1}$ electronic difference spectrum of control RCs (Fig. 6A) could be explained by a blue shift of the low-energy exciton band dominated by the active branch chlorophyll Chl_{D1} due to the change in excitonic coupling to this molecule upon $Pheo_{D1}$ reduction. Electrochromic effects induced by formation of $Pheo_{D1}^-$ would compensate the bleaching of an exciton state dominated by $Pheo_{D1}$, which is proposed to be localized at about 671 nm [24]. With this model, a shorter wavelength Q_y absorption of the active 7^1-OH-Pheo *b* molecule in modified samples (~665 nm) as compared to that of the active Pheo *a* in control RCs (~671 nm) would be in line with a blue-shifted position of the Q_y absorption maximum of 7^1-OH-Pheo *b* with respect to that of Pheo *a* in solution (Fig. 3). However, in the framework of this model, it is difficult to rationalize an observation that the double-difference spectrum for photoaccumulation of $Pheo_{D1}$ (Fig. 6B) does not show a positive feature at ~671 nm due to replacement of the native Pheo *a* by 7^1-OH-Pheo *b*.

An alternative model by Ganago et al. [67] suggests that the main bleach at 682 nm in the $Pheo^-/Pheo$ difference spectrum of control RCs (Fig. 6) is composite, consisting of two component: the bleaching of the $Pheo_{D1}$ dominated Q_y exciton band at 680 nm upon reduction of Pheo *a* superimposed with the negative lobe of the differential signal due to a blue band shift of a Chl *a* pigment near 680 nm. A small positive peak at 672 nm (Fig. 6) is also attributed to this chlorophyll band shift [67]. According to this model, the pigment exchange at the $Pheo_{D1}$ site

would be accompanied by a decrease in the $Pheo_{D1}^{-}/Pheo_{D1}$ difference spectrum of the bleaching at 682 nm (due to a loss of Pheo *a*) and by a concomitant appearance of a bleaching of the newly introduced 7^1-OH-Pheo *b* molecules at about 665 nm. This expectation is well consistent with the differential signal at 682/665 nm in the double difference spectrum (Fig. 6B). Then, by analogy to bacterial RCs (see, for example, Ref. [84]) a differential signal at 672/682 nm remaining in the $Pheo_{D1}^{-}/Pheo_{D1}$ difference spectrum of modified preparations (Fig. 6A, solid line) might be attributed to an electrochromic shift and a decrease in the dipole strength of the nearby accessory Chl_{D1} Q_y band upon the $Pheo_{D1}^{-}$ formation. We note, however, that in the current literature there is some controversy as to whether Chl_{D1} dominantly absorbs at about 680 nm [25,85,86] or at ~670 nm [13,29,87]. It should also be taken into account that a fraction of unchanged and/or $Pheo_{D2}$ only-exchanged RCs in modified preparations are expected to have the $Pheo_{D1}^{-}/Pheo_{D1}$ difference spectrum indistinguishable from that of control RCs.

Summarizing, of the two models considered, the second one, placing the dominant Q_y excited state of the active Pheo *a* at about 680 nm, appears to provide a somewhat easier interpretation of the data presented here. We note that there is also another literature model [21] which also suggests that the Q_y-region site energy of $Pheo_{D1}$ in isolated PSII RCs is at ~680 nm. This assignment was based on recent 5 K absorption and hole-burning experiments performed with isolated RC preparations from spinach and wild-type *C. reinhardtii* (at different levels of intactness) as well as from the *C. reinhardtii* mutant D_2-L209H, in which Pheo *a* at the $Pheo_{D1}$ binding has been genetically replaced with Chl *a* [21]. However, this model proposes that the site energy of $Pheo_{D2}$ is at ~670 nm, an assignment that is not consistent with the results obtained for isolated spinach RCs (this work, [14,15,30,52]) and spinach PSII core complexes [40], which suggest that $Pheo_{D2}$ absorbs at ~680 nm in both types of preparations.

Concerning possible reasons for the shorter wavelength position of the Q_y absorption band of 7^1-OH-Pheo *b* at the $Pheo_{D1}$ site compared to the bands of 7^1-OH-Pheo *b* at the $Pheo_{D2}$ site and of Pheo *a* at both binding sites, we can only speculate that the nature of the C-7 substituent is an important factor to determine the absorption spectrum of Pheo *a* in the $Pheo_{D1}$ protein pocket of PSII RCs, while the role of this side group in the $Pheo_{D2}$ pocket is less specific. The finding that the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectrum of 7^1-OH-Pheo *b*-modified samples is close in shape to that of control RCs (Fig. 7) suggests a general similarity of structural arrangements and molecular interactions (i.e., hydrogen-bonding at C-13^1 and C-13^3 [53,69,70,88–90]) of the active Pheos in the two RC preparations. This seems to prevent explaining the Q_y absorption feature of the active 7^1-OH-Pheo *b* molecule by its totally 'wrong' position in the $Pheo_{D1}$ protein pocket as compared to the active Pheo *a*. However, in the absence of crystallographic data for modified RCs, one cannot exclude that the introduction at C-7 of the hydroxymethyl group instead of the methyl group causes subtle structural deformations of the chlorin macrocyclic system and/or induces non-native interactions between the pheophytin cofactor and its nearest environment that have a role in determining the absorption properties of the pigment, but not to the extent of strongly modifying the FTIR spectrum. We notice in this context that the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectra of the two RC preparations are not completely identical to each other, differing in the intensity of the positive band at 1561 cm^{-1} (Fig. 7). Although this band has not been assigned previously, it could be tentatively attributed to an absorption change of the ring CC mode of $Pheo_{D1}^{-}$ (see Supporting information in Ref. [70]). The decrease of this band in the $Pheo_{D1}^{-}/Pheo_{D1}$ FTIR difference spectrum of the modified preparation might reflect small changes in the ring geometry of 7^1-OH-Pheo *b* at the $Pheo_{D1}$ site as compared to Pheo *a* due to the difference between the pigments in the C-7 substituent. Recently Vavilin et al. [32] have shown that the maximum of the Q_y absorption band of the photoreducible Pheo *b* in RCs of the chlorophyll *b*-containing mutant of *Synechocystis* sp. PCC 6803 was observed at 650 nm, also being very close to the absorption peak of pure Pheo *b* in organic solvents. It is interesting that both the 7-formyl group of Pheo *b* and the 7-hydroxymethyl group of 7^1-OH-Pheo *b* differ from the hydrophobic 7-methyl group of Pheo *a* by their potential hydrogen-bonding ability: the formyl is a hydrogen-bond acceptor, and the hydroxymethyl can be both a hydrogen-bond acceptor and donor (see Ref. [47]). It is unclear, however, whether such ability is realized in the modified RCs.

In conclusion, we described the functionally active chemically modified D1/D2/Cyt *b*559 RC complex from spinach in which pheophytin binding sites $Pheo_{D1}$ and $Pheo_{D2}$ were partially occupied by the exogenous pigment, 7^1-OH-Pheo *b*. This preparation appears to represent the first example of the PSII RCs containing an externally introduced modified pheophytin molecule that is involved in the electron-transfer reaction. Upon subsequent treatment of the 7^1-OH-Pheo *b*-modified RCs with $NaBH_4$, the photochemically active complex was also prepared with $Pheo_{D1}$ and $Pheo_{D2}$ sites containing two different exogenous pigments, 7^1-OH-Pheo *b* and 7^1-OH-13^1-OH-Pheo *b*, respectively. One may hope that the ability of 7^1-OH-Pheo *b* to functionally replace the active-branch Pheo *a* will be useful to the study of the mechanism and dynamics of primary charge separation in PSII RCs by time-resolved optical spectroscopy. In particular, the blue-shifted spectral position of the Q_y absorption band of the 7^1-OH-Pheo *b* molecule at the $Pheo_{D1}$ site of modified RCs with respect to the absorption of other core pigments could help to define specific absorbance changes associated with the transient reduction of the pheophytin electron acceptor, whose changes are difficult to distinguish from other signals in this spectrally congested region when only native PSII RC preparations are employed.

Acknowledgements

This work was supported by the Russian Academy of Sciences (the programs "Molecular and Cell Biology" and "The fundamentals of technologies of nanostructures and nanomaterials"), by the Russian Foundation for Basic Research (grants 10-04-01308 and 13-04-40297-H), and by the program "Leading Scientific Schools" (SS-4771.2014.4). We thank Dr. M. G. Zvereva for help in preparing the pheophytins used in this study. We are grateful to Dr. R. A. Khatypov for his participation in the initial stage of the work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.08.004.

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