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Reaction centers of *Rhodobacter sphaeroides* R26 containing C-3 acetyl and vinyl (bacterio)pheophytins at sites $H_{A,B}$ *

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Abstract

The native bacteriopheophytin a in reaction centers of *Rb. sphaeroides* R26 has been exchanged with modified bacteriopheophytins (bacteriochlorins), as well as with plant-type pheophytins (chlorins). Emphasis is on four pigments, which differ by their C-3 substituents (vinyl or acetyl) or their state of oxidation (chlorin or bacteriochlorin). The native BPhe a, which is a member of this group, can be replaced by the other three at both binding sites, H_A and H_B. However, exchange at H_B proceeds more readily. Optical spectra (absorption, cd) show characteristic shifts, and the cd spectra indicate induced interactions between $H_{A,B}$ and $B_{A,B}$ and possibly also with P. Upon flash illumination, all modified reaction centers show reversible electron transfer to Q_B with recombination times comparable to native reaction centers. Forward rates and electron-transfer yields are also reported for some of the pigments.

Abbreviations: $B_{A,B}$ – binding sites for monomeric BChl's at the active and inactive branch, rsp.; BChl a – bacteriochlorophyll a; BPhe a – bacteriopheophytin a; Chl a – chlorophyll a; DDQ – 2,3-dichlor-5,6-dicyano-p-benzoquinone; DEAE – diethylaminoethyl; $H_{A,B}$ – binding sites for BPhe's at the active and inactive branch, rsp.; cd – circular dichroism; HPLC – high pressure liquid chromatography; LDAO – N,N-dimethyldodecyl-amine-N-oxide; MeOH – methanol; NMR – nuclear magnetic resonance; P – primary donor; p or gg – subscript indicating the esterifying alcohol, phytol or geranylgeraniol, rsp.; Phe a – pheophytin a; Phe b – pheophytin b; *Rb.* – *Rhodobacter*; *Rs.* – *Rhodospirillum*; RC – reaction center; TL-buffer – 20 mM Tris, pH 8, containing 0.1% LDAO; Tris – Tris-hydroxymethylaminomethane

Introduction

Pigment modification is a relatively recent tool to study structure function relationships of purple bacterial reaction centers (RC). The replacement of bacteriochlorophylls a and b (BChl a,b) by bacteriopheophytins a and b, respectively, and *vice versa*, is possible by site-directed mutagenesis of the apoproteins (Coleman and Youvan 1990; Schenck et al. 1990; Woodbury et al. 1990). More recently, inhibition of the last biosynthetic enzyme reducing three of the four double-bonds of geranylgeraniol, has led to RC containing BChl a_{gg} instead of BChl a_p (Bollivar et al. 1994). More extensively modified pigments can be introduced by an exchange procedure, using chemically modified tetrapyrroles. Previous investigations of the latter method (Scheer and Struck 1993; Scheer and Hartwich 1995) showed that a variety of bacteriochlorophylls (BChl) modified at the periphery or the central metal, can be exchanged selectively at the sites $B_{A,B}$ ('monomeric' bacteriochlorophylls). Neither the BChl at sites $P_{A,B}$ (special pair) nor the BPhe at sites $H_{A,B}$ were affected by these exchanges. The P-sites are

* This manuscript is dedicated to J. P. Thornber on the occasion of his 60th birthday.

either inaccessible or the RC are irreversibly destroyed once one or both of their BChl leave the RC even for only a short time. The H-sites are accessible for an exchange, too, but only few such modified (bacterio)pheophytins ((B)Phe) have hitherto been tested. In all cases, selective exchange occurred at the sites $H_{A,B}$ containing BPhe a in native RC, without affecting the BChl binding sites P or B. Obviously, the presence or absence of the Mg-atom decides whether the pigment is accepted in the B- or H-site, which correlates with the presence ($B_{A,B}$) or absence ($H_{A,B}$) of histidine as a ligand to the central Mg (Allen et al. 1987; El-Kabbani et al. 1991; Ermler et al. 1994).

The exchange with the plant pigment, pheophytin a (Phe a, a chlorin derivative) into both the H_A- and H_B-site, has been of particular interest both structurally and functionally (Scheer et al. 1992; Shkuropatov and Shuvalov 1993; Schmidt et al. 1994). The corresponding Mg-complex, Chl a, was not accepted at the $B_{A,B}$-sites (Struck 1990), indicating a greater structural flexibility at the H-sites. Depending on the measurement conditions, the redox potential of Phe a is in solution 150–170 mV more negative than that of BPhe (Watanabe and Kobayashi 1991, C. Geskes, private communication, Table 1). If this is preserved in the binding sites, the energies of the states $P^{+\bullet}B^{-\bullet}$ and $P^{+\bullet}H^{-\bullet}$ become comparable, or Phe a could even act as a barrier for the light-induced electron transfer from P to Q_A and stop the electron at B_A. However, electron transfer to Q_B was still possible to >75% in RC with both BPhe a replaced by Phe a. More detailed time resolved studies showed, that the electron transfer to the quinone is slowed down (380 ps in Phe-containing RC *versus* 200 ps in native ones), while the kinetic constants for the preceding faster processes are similar to those in native RC. However, the increased energy for the state $P^{+\bullet}H^{-\bullet}$ leads to thermal equilibration with $P^{+\bullet}B^{-\bullet}$ and even P^*, in agreement with the idea that the former is a real intermediate in the transfer process (Schmidt et al. 1994). These results demonstrated that the relative order of redox potentials is preserved in the RC and that population dynamics during electron transfer can be influenced considerably. They prompted us to investigate the effects of other modifications of BPhe a in RC from *Rhodobacter (Rb.) sphaeroides* R26.1 in a more systematic fashion.

Materials and methods

RC were isolated from anaerobically cultured *Rb. sphaeroides* R26.1 by repeated solubilization of the chromatophores with LDAO in the presence of 100 mM NaCl (modified after Feher and Okamura 1978) and purified on DEAE-cellulose (Whatman DE52).

BChl a and Chl a were extracted from *Rb. sphaeroides* 2.4.1 and *Spirulina geitleri* (SOSA Texco-co), rsp., and purified on DEAE-cellulose (Satoh and Murata 1978). [3-acetyl]-Chl a was obtained by oxidation of BChl a with DDQ (Smith and Calvin 1966). [3-α-hydroxyethyl]-BChl a was prepared by reduction of BChl a with $NaBH_4$ (Ditson et al. 1984), [3-vinyl]-BChl a by subsequent elimination of water from the 3-α-hydroxyethyl-substituent in refluxing toluene (Struck et al. 1990). Demetalation to the respective (B)Phe's was done with acetic acid (Rosenbach-Belkin 1988). All pigments were purifed on silica gel (type H, Merck) using preparative thin layer-chromatography (Scheer 1988) with mixtures of toluene and acetone (9:1, v/v) as eluent. All structures were verified by VIS-NIR absorption, ^{1}H-NMR and mass spectroscopy. The exctinction coefficients of the (bacterio)pheophytins were determined relative to the known ones of the respective (bacterio)chlorophylls (Smith and Calvin 1966; Scheer 1988; Struck 1990). The precise concentration of a solution ($\approx$5 μM, 2 ml) of the respective BChl-(derivative) pigment in ether was determined spectrophotometrically, and the solvent was evaporated in a stream of Ar. Acetic acid (100 μl) was added and evaporated after incubation for 2 min under Ar, by a stream of this gas. The resulting (bacterio)pheophytin was dissolved in exactly the original solvent volume, and a spectrum recorded. All values given in the figures result from >5 parallel experiments using different pigment concentrations.

The pigment exchange protocol of Struck et al. (1990) was modified. The (B)Phe-derivatives were dissolved in acetone and added to the RC solution in TL-buffer (20 mM Tris/HCl, pH 8, containing 0.1% LDAO). The concentrations were adjusted such that the final acetone concentration was 10%, and the modified pigment was present in a 5- to 10-fold excess over the native BPhe a in the RC. This solution was incubated for 60 min at 43.5 °C. After incubation, the excess of free and released pigments were removed by repeated chromatography on DEAE-cellulose.

Quantitation of pigment replacement was made by HPLC-analysis. The RC (3–4 μM in TL-buffer) were adsorbed on a DEAE-cellulose-column (0.5×2 cm)

and washed with distilled water to remove the detergent. The column is then briefly dried with a stream of N_2, and the pigments extracted with $CHCl_3/CH_3OH$ (5:1, v/v). The $CHCl_3$ is necessary to elute the (B)Phe's completely because of their better solubility in this solvent than in the methanol generally used (Scheer and Struck 1993). The organic extract containing the pigments is dried under a stream of Ar, redissolved in toluene and subjected immediately to HPLC analysis (Maeda et al. 1992) using a diode array detector (HP model 8452 A).

Recombination kinetics after photobleaching of the RC after a white nearly saturating μs-flash (Mecablitz electronic photoflash model 45CT-A, maximum power) was measured with a Transputer Integrated Diode Array Spectrometer (TIDAS, J&M, Aalen). 500 single spectra were collected with an integration time of 5 ms each. For this measurements, the RC were reconstituted with ubiquinone (UQ_{10}, Fluka). The analysis of the recombination times was made with the program 'scientific peakfit' (V. 3.0, Jandel, AISN Software 1991).

Results

Exchange conditions

The conditions of the exchange experiments for bacteriochlorophylls (Struck et al. 1990) had to be modified as follows: 1) the incubation-time was shortened to 60 min to increase the yield of modified RC recovered, 2) the temperature was increased to 43.5 °C to obtain a higher percentage of pigment replacement, 3) the solvent for the pigments was acetone instead of MeOH/ether because of the better solubility of the demetalated pigments in the former. For maximum pigment replacement, the procedure was repeated twice, and the RC purified on each stage (see Table 2). The extraction conditions for pigment analysis needed modification as well. Early results using methanol for extraction gave poorly reproducible results, because part of the modified (B)Phe's were retained on the DEAE-cellulose column to which the RC were adsorbed. Complete extraction was possible only with a mixture of $CHCl_3$ and CH_3OH (5:1, v/v). This corroborates the importance of the solvent system for quantitative pigment extraction (Maeda et al. 1992).

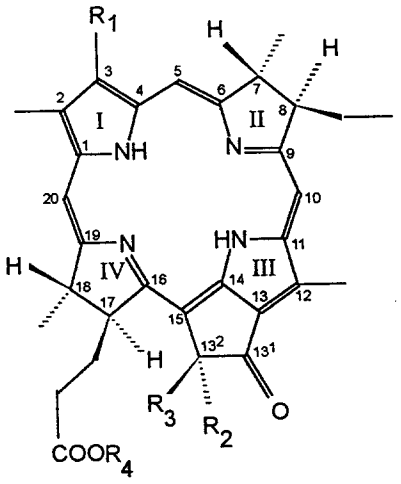
Modified pigments

The pigments tested in detail have modifications at the rings I and II (Fig. 1). A brief survey was made of pigments modified otherwise (see below). They were obtained by published procedures, and encompass the following structural features:

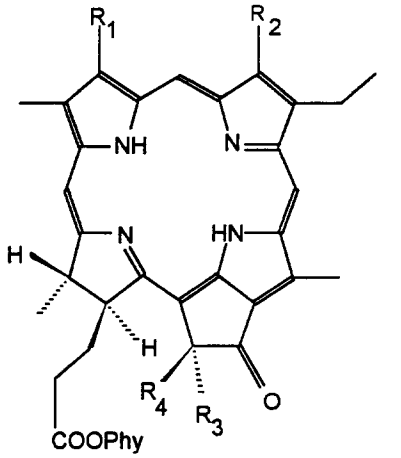
Phe a is the electron acceptor in the Photosystem II RC, which is believed to be related to those of purple bacteria. It differs from BPhe a in the hydrogenation state of ring II and in the substituent at C-3. [3-vinyl]-BPhe a and [3-acetyl]-Phe a (Fig. 2) bridge this gap stepwise: in the case of [3-vinyl]-BPhe a, ring II is saturated as in BPhe a, but C-3 carries the substituent of the plant pigment. Conversely, in [3-acetyl]-Phe a ring II is unsaturated as in Phe a, but C-3 carries the acetyl-group, characteristic of BPhe a. The absorption spectra of these pigments are dominated by the macrocyclic conjugation system (Fig. 2). Transformation of the bacteriochlorin to a chlorin results in a blue-shift of the Q_Y-band, a decreased Q_X-band, and a red-shift and stronger overlap of the Soret-bands (B_X, B_Y). The changes in the C-3 substituent cause only shifts in the spectra but do not change their type. The replacement of the [3-acetyl]-group by a [3-vinyl]-group causes a blue-shift of 287 cm^{-1} and 479 cm^{-1} in the Q_Y-band of the chlorins and the bacteriochlorins, rsp. The Q_X-band is also blue-shifted: 233 cm^{-1}/277 cm^{-1} for the chlorins, 447 cm^{-1} for the bacteriochlorins.

In the case of the plant-type pheophytins, the Q_X-band is always split into $Q_X{}^{0-0}$ and the vibrational side band $Q_X{}^{0-1}$ (Wolf and Scheer 1973). These bands shift with the structural change, but their separation remains fairly constant: 504 nm and 533 nm for Phe a, 510 nm and 541 nm for [3-acetyl]-Phe a. [3-acetyl]-Phe a has – as does BPhe a – two partly overlapping Soret-bands at $\approx$380 nm and 412 nm.

The structural changes of the related pigments (saturation of ring II *versus* unsaturation, acetyl-group *versus* vinyl-group) cause also differences in the redox potentials. Of particular interest with respect to the H_A-pigment as intermediate electron acceptor are the reduction potentials, which become 0.03–0.16 V more negative than for BPhe a in the modified pigments (Table 1).



Pigment	R_1	R_2	R_3	R_4
BPhe a_p	$COCH_3$	$COOCH_3$	H	$C_{20}H_{39}$
BPhe a_p'	$COCH_3$	H	$COOCH_3$	$C_{20}H_{39}$
BPhe a_{gg}	$COCH_3$	$COOCH_3$	H	$C_{20}H_{33}$
Pyro-BPhe a	$COCH_3$	H	H	$C_{20}H_{39}$
[3-vinyl]-BPhe a	$CHCH_2$	H	$COOCH_3$	$C_{20}H_{39}$



Pigment	R_1	R_2	R_3	R_4
Phe a	$CHCH_2$	CH_3	$COOCH_3$	H
Phe a'	$CHCH_2$	CH_3	H	$COOCH_3$
Pyro-Phe a	$CHCH_2$	CH_3	H	H
[3-acetyl]-Phe a	$COCH_3$	CH_3	$COOCH_3$	H
13^2-OH-Phe a	$CHCH_2$	CH_3	$COOCH_3$	OH
Phe b	$CHCH_2$	CHO	$COOCH_3$	H
[7^1-OH]-Phe b	$CHCH_2$	CH_2OH	$COOCH_3$	H

Fig. 1. Structures of the bacterial- (top) and plant-type pheophytins (bottom) tested (the absolute configuration at C-13^2 changes depending on the substituents present. We therefore prefer in the present context the 'natural' nomenclature).

Modified RC

Degree of pigment replacement

All pigments were used for two subsequent exchange cycles, and the percentage of pigment replacement determined at each stage by HPLC-analyses (Table 2). The exchanged pigments were always identified by their retention times relative to BPhe a on a silica gel column, and by their in-stream absorption spectra.

The pigments can be separated very well from BPhe a and possible by-products, which are mainly the C-13^2-epimeric 'prime' pigments, and the epimeric 13^2-hydroxylated products. As an example the HPLC-chromatogram of RC after double exchange of BPhe a against Phe a is shown in Fig. 3.

Because of the less polar 3-vinyl-group and the unsaturated ring II, Phe a has a shorter retention time than BPhe a on the polar SiO_2 column. One can see only traces of the former, and quantitation yields a pigment replacement of 95%. Nearly all pigments appear as 13^2-epimer mixtures, with the native configuration (13^2-$COOCH_3$ below the plane in Fig. 1) always in excess. The non-naturally configured ones, as well as the 13^2-OH–BPhe a, are products of chemical reactions during the exchange- or extraction-procedure. Storch (1993) has provided evidence that only the 'natural' epimer of BChl a and 13^2-OH–BChl a can bind to

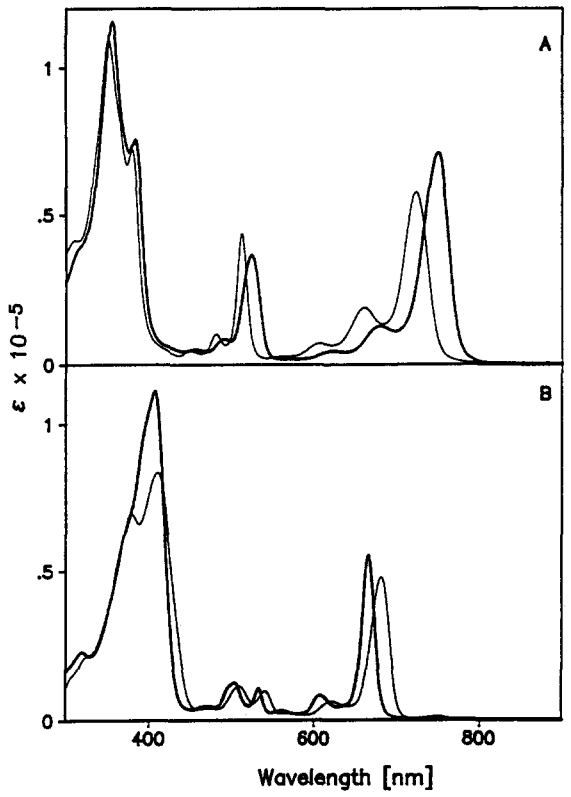


Fig. 2. (A) Absorption spectra of BPhe a (heavy line) and [3-vinyl]-BPhe a (thin line) in ether solution. (B) Absorption spectra of Phe a (heavy line) and [3-acetyl]-Phe a (thin line) in ether solution. Molar extinction coefficient (ε) given in [M^{-1} cm^{-1}].

Table 1. First reduction potentials of the four pheophytins differing in the C-3 substituent or ring II saturation. The cyclic voltammetry measurements were done by C. Geskes in the laboratory of G. Heinze (Freiburg) in tetrahydrofuran *versus* Ag/AgCl, with ferrocene used as internal standard reference. A comprehensive study on the redox properties of modified bacteriopheophytins shall be published separately

compound	E_{red}
BPhe a	−1.01
Phe a	−1.15
[3-vinyl]-BPhe a	−1.17
[3-acetyl]-Phe a	−1.04

sites $B_{A,B}$, and a similar stereoselectivity has been observed for chlorophyll synthetase (Helfrich et al. 1994) and chlorophyllase (Fiedor et al. 1992). While the 13^2-oxidation products were accepted well at $B_{A,B}$,

Table 2. Pigment replacement of modified pigments into RC. The percentage of pigment replacement was determined by HPLC-analyses, and all pigments identified by their retention times relative to BPhe a

Pigment	1. exchange	2. exchange
[3-vinyl]-BPhe a	50%	93%
Phe a	80%	95%
[3-acetyl]-Phe a	68%	80%

we have indications that 13^2-OH–Phe a can only be exchanged in the H_B-site of the RC (to be published elsewhere).

The exchange can be followed readily by spectrophotometry of the intact RC. Exchange with [3-vinyl]-BPhe a shows that the Q_Y-band of $H_{A,B}$ at 758 nm is decreasing while a new band at 730 nm appears. In the Q_X-region the broadened band at 536 nm for $H_{A,B}$ decreases, sharpens and shifts to 541 nm after the first exchange before it decreases with the second one. This indicates a preferential exchange of the 'inactive' site H_B, because we assign the 541 nm band after the first treatment to Q_X of the remaining BPhe a at H_A (Breton et al. 1989). Complementary changes are seen for the Q_X-band of the introduced [3-vinyl]-BPhe a. It absorbs at 521 nm after the first exchange (pigment replacement 50%), and shifts to 523 nm after the second exchange (pigment replacement of ≈93%, see below). The shift can be rationalized by differences in the binding sites, which (as with the native BPhe a) cause a red-shifted Q_X-band for the pigment in the H_A-site, as compared to H_B. The red-shift at high pigment replacement only, would then suggest a preferential exchange at H_B for low pigment replacement. Similar spectral shifts were seen with the other two pigments, suggesting always a preferential exchange at H_B.

Absorption spectra

The absorption- and cd-spectra of the maximally exchanged RC (Table 3) always show the characteristic features of the newly introduced pigments replacing the native BPhe bands, and only minor (but distinct, see below) changes in the BChl a-related bands (Figs. 4 and 5). The differences in the absorption spectra among the various pigments incorporated into the RC, are reminescent to those among the pigments in solution. The Q_Y-Band of $H_{A,B}$ at 758 nm is replaced by a strongly blue-shifted one at 674 nm (Phe a), 689 nm ([3-acetyl]-Phe a) or 730 nm ([3-vinyl]-BPhe a). The

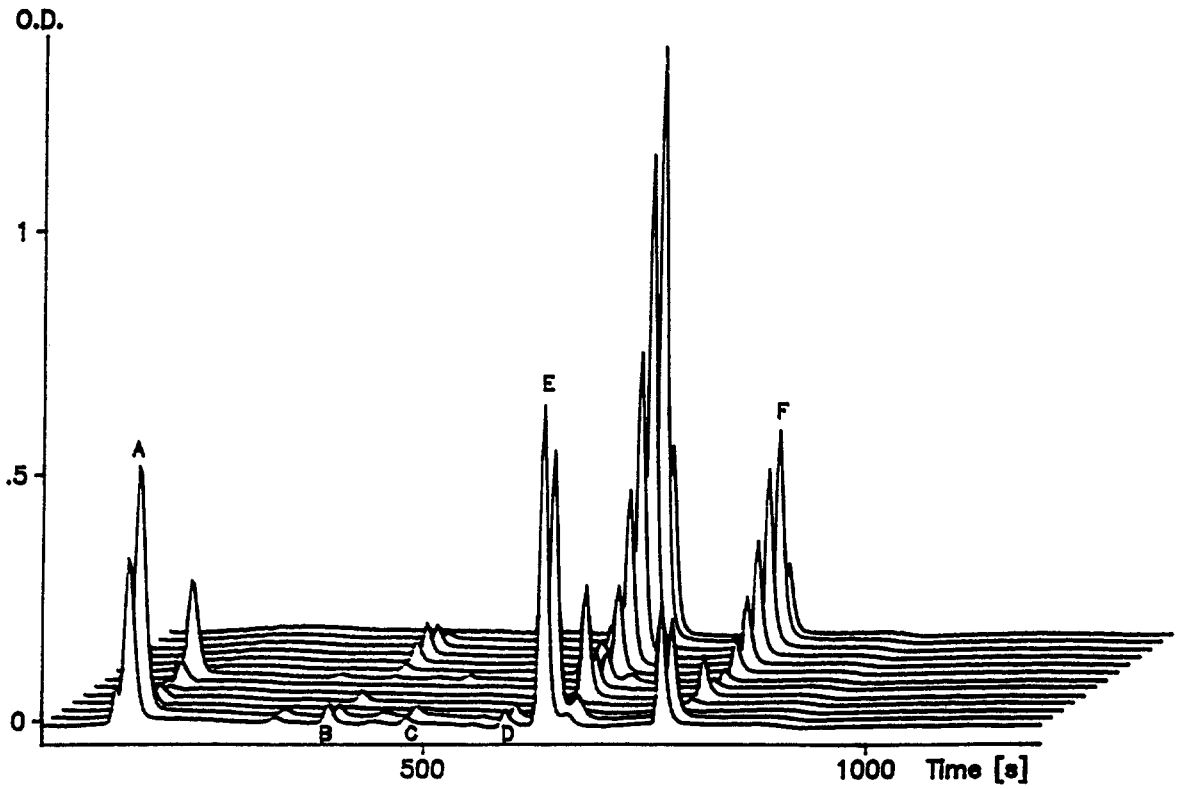


Fig. 3. HPLC of pigments extracted from RC which were exchanged twice with Phe a. The detection wavelengths from front to back were: 380, 400, 500, 530, 580, 620, 680, 730, 750, 760, 770, 780, 800 nm. Peak assignments: A: Phe a′/a; B: BPhe a; C: 13^2-OH–Phe a; D: BChl a′; E: BChl a; F: 13^2-OH–BChl a.

Table 3. Absorption maxima of the Q_X- and Q_Y-bands of the pigments in solution and in the RC, and the resulting EIRS (environment-induced-red-shift). In the case of the plant-type Phe's the Q_X-band is split into Q_X^{0-0} and the vibrational side band Q_X^{0-1} (Wolf and Scheer 1973)

Pigment	Ether [nm]		Protein [nm]		EIRS [cm^{-1}]	
	Q_X	Q_Y	Q_X	Q_Y	Q_X	Q_Y
BPhe a	524	750	537	758	462	141
[3-vinyl]-BPhe a	512	724	523	730	411	114
Phe a	504/533	667	509/542	674	195/312	156
[3-acetyl]-Phe a	510/541	680	516/544	689	228/102	192

characteristic split of the Q_X-band of the plant-type Phe's is also seen in the RC (Table 3). In the case of Phe a there is a significant peak in the Soret-region at ≈410 nm, which is assigned to the exchanged pigment.

All pigments tested show environment-reduced-red-shifts (EIRS) of their absorption bands (Table 3). These shifts show characteristic differences between the bacteriochlorins and chlorins, but appear to be rather constant within each group. In the bacteriochlorins the Q_Y-bands show small shifts (~150 cm^{-1}), while the shifts are larger for the Q_X-bands (~400 cm^{-1}). The Q_Y-EIRS is similar in the chlorins. The two Q_X-bands (see above) shift differently and alto-

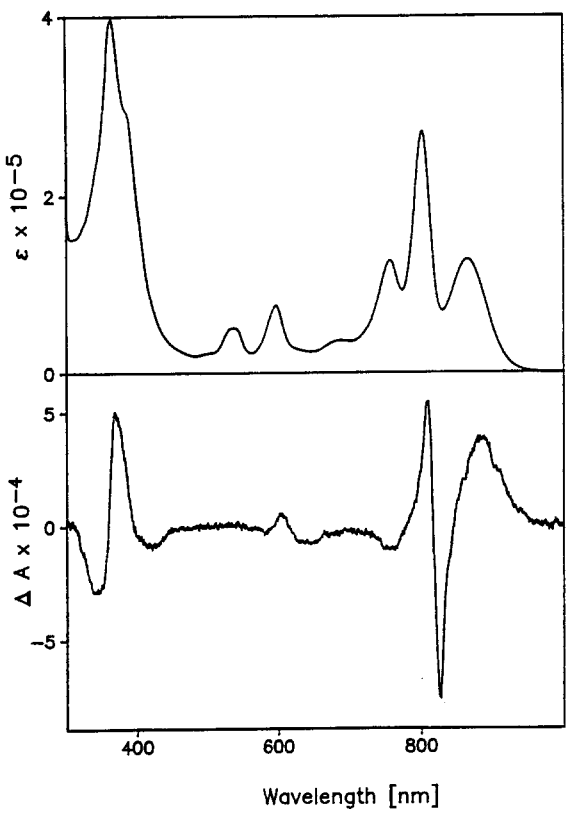


Fig. 4. Absorption spectrum (top) and cd spectrum (bottom) of native RC in TL-buffer. Molar extinction coefficient (ε) given in [M^{-1} cm^{-1}].

gether less than in the bacteriochlorins. The shifts in the Soret-bands ($B_{X,Y}$) were not evaluated due to the strong band overlap in this region.

The blue-shift (3–6 nm = 84–169 cm^{-1}) of the Q_X-band of $B_{A,B}$ and P in the modified RC as compared to native ones, indicates possibly an interaction between the pigments in the protein environment (Tables 3 and 4). The modified RC show also shifts of the Q_Y-bands of the dimer, P870, and the monomeric BChl's, and changes in the cd spectra. In our experience, the Q_Y-band of P870 is rather sensitive to environmental factors like type and concentration of detergent, temperature, etc. In view of this variability even in unmodified RC, the changes observed in modified ones (which are of the same size as in native RC) are probably insignificant. However, the shifts of the Q_Y-band of BChl a at sites $B_{A,B}$, which are neighbours to the modified pigments at $H_{A,B}$, are significant even if they are small. There is in particular always a distinct blue-shift of 3 nm if the acetyl-substituent is replaced by a

Table 4. Absorption maxima of the Q_X- and Q_Y-bands of $B_{A,B}$ and P in the modified RC

Pigment at $H_{A,B}$	Q_X ($B_{A,B}$; P)	Q_Y ($B_{A,B}$)	Q_Y (P)
BPhe a	598	803	865
[3-vinyl]-BPhe a	592	800	851
Phe a	595	800	862
[3-acetyl]-Phe a	593	803	865

vinyl-group, irrespective of the other structural modifications.

Cd spectra

The cd spectra of the modified RC all show moderate optical activities for the exchanged pigments, similar in magnitude to that of the native BPhe a. In all cases, the Q_Y-band of the native BPhe a at ≈755 nm disappears and a new blue-shifted one appears which is due to the modified pigment. Earlier experiments showed a distorted s-shaped signal in the Q_Y-region of $H_{A,B}$ after exchange with the modified pigments, indicating that there is an exciton coupling between the pigments at H_A and H_B. However, more recent studies with higher pigment replacement, showed that this signal appears only if the unspecifically bound pigment could not be removed completely. Only a single peak is seen, if all free pigment is removed. In the case of the plant-type Phe's (Phe a, [3-acetyl]-Phe a) the Q_Y-band has a positive sign, while in RC modified with [3-vinyl]-BPhe a the band is negative. These observations show, that there is no obvious exciton coupling between the two (B)Phe's. The increased optical activity of the modified pigments in the RC, as compared to monomeric solution, must be induced by the protein environment or by interactions with the BChl(s) (see above).

Furthermore, all cd spectra show the characteristic positive bands for the primary donor around 870 nm and for BChl–$B_{A,B}$ at ≈800 nm, but with different ratios. The ratio of the $B_{A,B}$-band to the upper exciton-band of P decreases after the exchange with a modified pigment.

Electron transfer

Electron transfer in the modified RC was evaluated by determining the recombination kinetics after flash-excitation. In these experiments, quinone (UQ_{10}) was added in excess to replenish both binding sites, because $Q_{A,B}$ is lost partly during exchange. Binding appears to

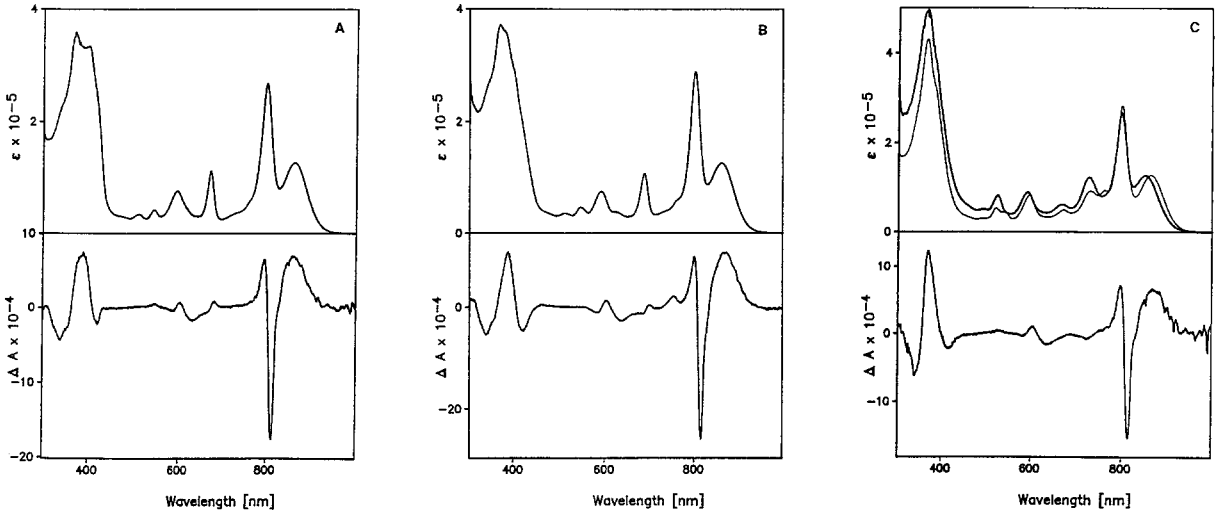


Fig. 5. (A) Absorption spectrum (top) and cd spectrum (bottom) of RC in which BPhe a is exchanged to 95% with Phe a in TL-buffer. (B) Absorption spectrum (top) and cd spectrum (bottom) of RC exchanged to 80% with [3-acetyl]-Phe a in TL-buffer. (C) Absorption spectrum (top) and cd spectrum (bottom) of RC exchanged with [3-vinyl]-BPhe a in TL-buffer. The absorption spectrum shows the RC exchanged once (thin line, 50% replacement) and twice (heavy line, 93% replacement). Molar extinction coefficients (ε) given in [M^{-1} cm^{-1}].

Table 5. Flash-induced electron transfer. Relative yields of electron transfer to $Q_{A,B}$ (bleaching at 870 nm), and the recombination kinetics of the modified RC. Complete spectra were taken at 5 ms intervals, and the kinetics evaluated at 870 nm and fit with biexponentials

Pigment at $H_{A,B}$	τ_1 [ms]	a_1	τ_2 [ms]	a_2	bleaching [%]
BPhe a	100 ± 2	0.3	730 ± 20	0.7	98
[3-vinyl]-BPhe a	110 ± 10	0.84	400 ± 50	0.16	60*
Phe a	120 ± 5	0.4	700 ± 35	0.6	77
[3-acetyl]-Phe a	100 ± 5	0.3	800 ± 34	0.7	64

*95% bleaching after first exchange, see text.

be less tight in the modified RC. Complete spectra were taken at 5 ms intervals, the data refer to the kinetics in the maximum of the Q_Y-band of P near 870 nm (Table 5).

In native RC the flash-induced bleaching is nearly quantitative (98%) under the measuring conditions. Bleaching was less with the exchanged samples (see Fig. 5). However, this should only be taken qualitatively since saturating flashes were used, which allow multiple excitation in case recombination to P occurs within the duration of the flash. The recombination kinetics can be fit by biexponentials in all four cases. The recombination times of the unmodified RC were 100 ms (0.3) and 730 ms (0.7, relative amplitudes in brackets). In the RC modified with Phe a or [3-acetyl]-Phe a recombination occurs with similar time constants. The recombination in RC modified with [3-vinyl]-BPhe can be fit monoexponentially (τ_1 = 110 ± 10 ms). In a forced two exponential fit, the major, fast component has again a rate constant $\tau_1 \approx 100$ ms similar to the one in native RC, with high amplitude, the second exponential has a shorter rate constant but this may be due to the decreased precision of this low-amplitude component. The major conclusion from these data is that electron transfer to the quinone is possible in all cases with good yields. A quantitative discussion is difficult in view of the flash conditions, which do not exclude double hits.

Only RC exchanged with Phe a were hitherto investigated in detail for the forward electron transfer (Shkuropatov and Shuvalov 1993; Schmidt et al. 1994). The time constants for the first steps (forma-

tion of $P^{+\bullet}B^{-\bullet}$ and $P^{+\bullet}H^{-\bullet}$) are similar to those in native RC: 2.3 ps and 0.9 ps for R26 and 3.5 ps and 1.5 ps for the modified RC. Formation of $P^{+\bullet}Q_A^{-\bullet}$ is slowed down from 200 ps to 380 ps. Details of these kinetics have been discussed in a previous publication (Schmidt et al. 1994).

Other pigments

Several other (bacterio)pheophytins have been tested for exchange. They are mentioned here only for completeness and have not yet been characterized in comparable detail; the full results will be published in due course. Two of these pigments are of the bacterial-type: BPhe a_{gg} contains geranylgeraniol instead of phytol at C-17[4]. BChl a_{gg} is the native pigment in RC from *Rhodospirillum (Rs.) rubrum*, but the BPhe's in the same RC are esterified with phytol (Walter et al. 1979). Pyro-BPhe a lacks the 13^2-$COOCH_3$-group and enolization at C-13^2 is therefore impeded. The four plant-type pheophytins were pyro-Phe a (lacking the $COOCH_3$-group at C-13^2), 13^2-hydroxy-pheophytin a (13^2-OH–Phe a, 13^2-H replaced by a hydroxy-group), pheophytin b (Phe b, formyl- instead of a CH_3-group at C-7) and [7-hydroxymethyl]-pheophytin b (7^1-OH–Phe b, hydroxymethyl-group at C-7). The preliminary results indicate that all pigments can be introduced into the RC, but some of them (BPhe a_{gg}, 13^2-OH–Phe a and Phe b) exchange into H_B only.

Discussion

Several binding sites of chlorophylls and bacteriochlorophylls have been studied by the technique of pigment exchange. These include sites in photosynthetically active complexes like that of the monomeric BChl's, $B_{A,B}$ (Scheer and Struck 1993), the core antenna complex B875 (Parkes-Loach et al. 1990) and the peripheral antenna complex B800-850 (Bandilla 1995) from purple bacteria and the green plant antenna complex LHCII (Paulsen et al. 1990), but also complexes involved in pigment biosynthesis and degradation (Griffiths 1991; Helfrich 1991; Fiedor et al. 1992; Helfrich et al. 1994). These studies have revealed functional groups of the pigment important for exchange, which are different for the different sites. Although this is only an operational criterion, it is likely that these differences reflect also binding properties of the various sites.

By comparison to the structural variations allowed for the $B_{A,B}$-sites of the bacterial reaction centers, the results presented here show that the BPhe binding sites $H_{A,B}$ allow for rather extensive structural changes, in particular the H_B-site. The pigments tested at the $B_{A,B}$-sites had modifications at ring I, II, the isocyclic ring and C-17[4] (Scheer and Struck 1993). Like the B-sites, the $H_{A,B}$-sites allow considerable modification of the C-3 substituent. The distance of the neighbouring amino acids (Ile, Ala) to the acetyl-group of H_A is at least 7 Å, i.e. there is no interaction possible between the groups (Ermler et al. 1994). It is noteworthy that pigments containing a vinyl-group at C-3 (Phe a, [3-vinyl]-BPhe a) give higher degrees of pigment replacement than pigments containing the (natural) 3-acetyl-group.

Chlorins, such as Chl a or [3-acetyl]-Chl a, could not be exchanged at the B-sites. By contrast, the BPhe-binding sites $H_{A,B}$ accept all plant-type Phe's tested. There is, however, a differentiation among the two sites: H_B appears to accept more extensively modified pigments than H_A. In particular, the integrity of the enolizable β-ketoester system is more important for the latter (13^2-OH–Phe a is e.g. only accepted at site H_B), and there is even a clear discrimination of the esterifying alcohol (phytol *versus* geranylgeraniol). A detailed study of these pigments shall be published separately.

There is also a kinetic discrimination of the two H-sites, which parallels that of the B-sites. In all cases studied, an exchange at B_B seems easier than at B_A, and the same is true for the exchange experiments of (B)Phe's ($H_B > H_A$). The more facile exchange of the inactive site H_B was originally suggested by the absorption spectra observed over several stages of the exchange (Fig. 5C). This preference is supported, too, by the efficiency of photobleaching. The best example is the exchange with [3-vinyl]-BPhe a. After the first exchange (50%) the quantum efficiency is 95%, similar to native RC, but decreases to 60% after the second exchange when H_A is nearly completely occupied (pigment replacement 93%). Assuming that the electron transfer proceeds *via* the A-branch only, this can be rationalized by an occupation of (mainly) the H_B-site after the first treatment (50% exchange), which leaves (mainly) the native BPhe a at H_A, and an exchange of the latter only after the second treatment, which then shows up in the electron transfer kinetics. Similar, albeit less pronounced differences at the different exchange states, were also found with the other two modified pigments.

Studies on the electron transfer in RC of *Rb. sphaeroides* (Shinkarev and Wright 1993) showed that there are two major pathways for recombination of $P^{+\bullet}$: direct electron transfer from $Q_A^{-\bullet}$ ($\approx$100 ms) or transfer from $Q_B^{-\bullet}$ via Q_A ($\approx$1 s), whereas direct transfer from $Q_B^{-\bullet}$ to P is an order of magnitude slower. In the most simple analysis, the latter is ignored, as well as any transfer *via* the pigment at sites H or B. In this case, the occupation of the Q_B-site can be estimated from the amplitude of the slow component. Such an analysis would indicate, that the occupation decreases with substitution of the pigments at sites $H_{A,B}$ in the order BPhe a $\approx$ [3-acetyl]-Phe a > Phe a $\gg$ [3-vinyl]-BPhe a. The small variations of τ_1 and τ_2 for the first three pigments, indicate that this analysis may be justified. These results support the idea that the intervening pigments at sites $H_{A,B}$ and $B_{A,B}$ are also in the modified RC not directly involved in the backtransfer, e.g. *viz.* a thermal population process, and that direct transfer from Q_B is negligible as well (Shinkarev and Wright 1993). RC modified with [3-vinyl]-BPhe a recombine mainly with the short time constant only, and even in a forced biexponential is τ_2 decreased. Using the above model this would indicate a different equilibrium constant between $P^{+\bullet}Q_A^{-\bullet}$ and $P^{+\bullet}Q_B^{-\bullet}$, or alternative routes of charge recombination (Shinkarev and Wraight 1993).

Little is so far known about the excited state properties of the modified pigments. We have recently begun to study in some detail the same four pigments which were emphasized in this work (BPhe a, [3-vinyl]-BPhe a, Phe a, [3-acetyl]-Phe a). This series may also shed some light on the relationship between the photosynthesis of higher plants and bacteria. Measurements of the fluorescence of Chl a, [3-acetyl]-Chl a, [3-vinyl]-BChl a and BChl a showed that the lifetime depends mainly on the unsaturation of ring II and much less on the C-3 substituent (Teuchner et al. 1994). The fluorescence lifetime is decreased in bacteriochlorins, while the intersystem crossing (ISC) efficiency is increased. Assuming that these excited-state characteristics are maintained in the native environment they may have been relevant for the eventual selection among the two pigment types. The choice of chlorins by the higher plants and other oxygenic organisms could be advantageous: with an oversupply of light, Chl a relaxation to the ground state by fluorescence would be one means of avoiding triplet generation. Under the same conditions, BChl a would make more ISC and form more of the long-lived triplet state. This is, however, less likely due to the prevailing low light, and damage would furthermore be reduced on account of the low oxygen in the habitat of these organisms.

It is noteworthy that all RC exchanged with the different (B)Phe's are capable of light induced electron transfer, although with decreased quantum yield. The detailed kinetic analysis of RC with Phe a at sites $H_{A,B}$ (Shkuropatov and Shuvalov 1993; Schmidt et al. 1994) indicates that the relative order of redox potentials measured in solution is maintained in the H_A binding site. However, in no case does the redox potential decrease sufficiently that the charge-separated state $P^{+\bullet}H^{-\bullet}$ lies energetically above that of P*, or even $P^{+\bullet}B^{-\bullet}$. Clearly, this aspect needs further studies of the kinetics and – if possible – the reduction potentials in the native RC, which are in progress.

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