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Modified reaction centers from *Rhodobacter sphaeroides* R26.

2: Bacteriochlorophylls with modified C-3 substituents at sites B_A and B_B

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Monomeric bacteriochlorophylls B_A and B_B in photosynthetic reaction centers from *Rhodobacter sphaeroides* R26 were exchanged with (13^2-hydroxy-)bacteriochlorophylls containing a 3-vinyl- or 3-(α-hydroxyethyl)-substituent instead of the 3-acetyl group. The corresponding binding sites must be tolerant to the introduction of the polar residue at C-13^2 *and* modifications of the 3-acetyl group. According to HPLC analysis, the exchange with both pigments amounts to ≦50% of the total BChl contained in the complex, corresponding to ≦100% of the monomeric BChl $aB_{A,B}$. The absorption spectra show significant changes in the Q_X and Q_Y-region of the monomeric bacteriochlorophylls. By contrast, the absorption of the primary donor (P870) and reversible photobleaching is retained. The circular dichroism is also unchanged in the 870 nm region. The positive cd band located at around 800 nm in native reaction centers, shifts with the (blue-shifted) Q_Y absorption(s) of B_A and/or B_B, whereas the position of the negative one remains nearly unaffected. The data indicate that the latter is the upper excitonic band of the primary donor, and that there is little interaction of the monomeric B_A/B_B with the primary donor.

Photosynthesis; *Rhodobacter sphaeroides*; Reaction center; Pigment modification; Pigment exchange; Chlorophyll; Bacteriochlorophyll *a*; [3-vinyl]-13^2-hydroxy-bacteriochlorophyll *a*; [3-(α-hydroxyethyl)]-13^2-hydroxy-bacteriochlorophyll *a*; Chromatography; Circular dichroism

1. INTRODUCTION

The recently demonstrated exchange of the 'monomeric' bacteriochlorophylls (BChl) B_A and B_B, has opened a new route to investigate structure-function relationships in photosynthetic reaction centers (RC) of *Rhodobacterspheroides R26* [1,2]. The function of these pigments is still controversial [3–17]. However, the finding of a new kinetic intermediate at ambient temperature between the initial excited state P^* of the primary donor, and the charge separated state $P^{\cdot+}$-$H_A^{\cdot-}$ in which the photoejected electron resides on the bacteriopheophytin (BPhe) of the A-branch, has provided strong evidence that one of them (B_A) is an intermediate acceptor in the primary charge separation process [16,17]. A similar role has been proposed for B_M in triplet energy transfer from P to the carotenoid in wild-type RCs [15]. To distinguish a stepwise electron transfer and other mechanisms like superexchange [7,8,11] it seemed desirable to introduce pigments with altered spectral and/or redox properties. As first pigments showing considerably blue-shifted absorption spectra, we have now incorporated bacteriochlorophylls containing 3-vinyl- or 3-(α-hydroxyethyl) substituents instead of the 3-acetyl group. Here, we wish to report the preparation, pigment analysis and some steady-state spectra of the modified RC.

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Abbreviations: BChl, bacteriochlorophyll *a*; BPhe, bacteriopheophytin *a*; RC, reaction centers; Rb., *Rhodobacter*; Cd, circular dichroism; P, primary donor; B, monomeric BChl; H, BPhe in RC. The subscripts 'A' and 'B' refer to the active ('L') and inactive branch ('M'), respectively, of the electron transport chain.

2. MATERIALS AND METHODS

Rb. sphaeroides R26 RCs were prepared by repeated solubilization of chromatophores and subsequent chromatography on DEAE-cellulose as described previously [18]. Final purification was achieved by density-gradient centrifugation (0.2 to 0.8 M sucrose in 10 mM Tris-HCl buffer, pH 7.6, containing 0.08% LDAO, 20 h, 240 000*g*). RCs were enriched in the 0.6 M region. They were withdrawn, dialyzed against Tris-HCl buffer (10 mM, pH 7.6, containing 0.08% LDAO) and stored at −20°C.

[3-(α-Hydroxyethyl)]-BChl (**4**) was prepared by reduction of Bchl (**2**) with potassium borohydride [19]. [3-vinyl]-BChl (**6**) was prepared from **4** by elimination of water from the 3-(α-hydroxyethyl)-group in refluxing toluene. Hydroxylation at C-13^2 was done as described in [20]. All products were characterized by absorption, fluorescence and ^{1}H-NMR spectroscopy. No attempts were yet made to separate stereoisomers. A detailed description of the syntheses will be published separately.

Exchange experiments were performed as previously [1,2] using a 20-fold excess of exogenous pigments over the BChl contained in the RCs. Excess pigments were removed from the incubation mixture by repeated chromatography on DEAE-cellulose.

The HPLC analysis was done on silica [1,21]. The detector for the system was a diode array photometer HP 8451A (Hewlett-Packard).

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It is controlled by a home-made program (available on request), which allows to detect simultaneously at selected wavelengths at rapid intervals. In the present case, we recorded at 8 wavelengths (360, 680, 700, 720, 740, 760, 780 and 800 nm) every 4 s. With this detection method, pigments can be discriminated in a rather fast and reliable manner. Data handling was done on a PC, using in part Supercalc (Computer Associates). Absorption spectra were recorded on a Perkin Elmer Lamda 2 spectrophotometer.

The extraction of pigments from RCs for HPLC analysis was done on small DEAE cellulose columns (5.20 mm). RCs in Tris-HCl buffer (10 mM, pH 7.6) containing 0.08% LDAO were adsorbed, then washed extensively with destilled water. Afterwards, most of the water was removed by flushing the column with argon, and the pigments extracted subsequently with methanol. The methanol solution was dried with a stream of nitrogen, and the pigments resolubilized in toluene for HPLC analysis. The entire extraction procedure was performed under dim safety light and completed within 30 min.

3. RESULTS

3.1. *Modified pigments*

The absorption spectra of 13^2-hydroxy-BChl (**3**), [3-(α-hydroxyethyl)]-13^2-hydroxy-BChl (**5**) and [3-vinyl]-13^2-hydroxy-BChl (**7**) are shown in Fig. 1. A comparison of the spectra with BChl (**2**) shows no shifts for **3**, and 26 (43) nm short wavelength shifts for compounds **7** and **5**, respectively, in the Q_Y region. Identical shifts are observed in the pigments **4** and **6** with the respective C-3 substituent, but lacking the 13^2-hydroxy-substituent (data not shown). The spectra in Fig.1 are normalized to the Q_Y-band. However, the relative intensities in the Q_X and Soret region indicate, that the Q_Y extinction coefficient is reduced in pigments **4–7**.

3.2. *Pigment composition of modified reaction centers*

Treatment [1,2] of RCs with the [3-vinyl]-pigment **7** results in a replacement of $\leq 50\%$ of total BChl (peak c) relative to Bphe (peak a), which is correlated with an increased content of **7** (peak d) (Fig. 2, see [1] for a chromatogram of an extract from native RCs). The exchanged pigment is identified by its identical mobility with the authentic pigment, and by its blue-shifted absorption resulting in maximum absorption in the 740 nm trace. The lower intensity in peak d at 740 nm, as compared to that of peak c at 780 nm, is due to differences in extinction coefficients, and off-center detection. Under the same conditions, the pigment **5** bearing a 3-(α-hydroxyethyl)-substituent, is exchanged as well, albeit with a lower rate (typically between 20% and 40%, HPLC data not shown). The corresponding pigments **4** and **6** lacking the 13^2-OH substituent, also yield exchange rates of this magnitude.

3.3 *Absorption of modified reaction centers*

The absorption spectra of the modified RCs (Fig. 3) show hardly any change (as compared to the native ones) in the 870 nm band of the primary donor but significant blue-shifts of the 800 and part of the 600 nm band assigned commonly to the Q_Y and Q_X bands, respectively, of the monomeric B_A and B_B. Together

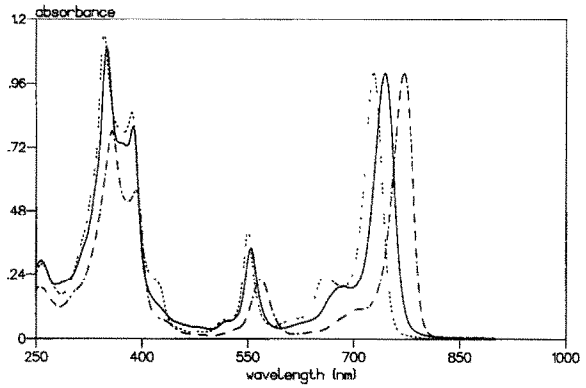
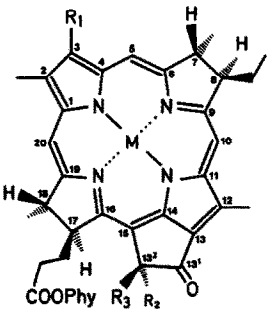


Fig. 1. Absorption spectra of free pigments in diethylether: 13^2-hydroxy-BChl (**3**) (-•-•-), and its 3-vinyl- (**7**) (——) and 3-(α-hydroxyethyl)-substitued analogue (**5**) (····). The spectra are normalized to 1.0 at their long-wavelength maxima.

with the analytical data we take this as direct proof that the monomeric pigments are the ones exchanged. This supports previous indirect ESR-evidence obtained with deuterated BChl [2]. There was no noticeable change in the BPhe content of the modified RCs, and the bleaching of the 870 nm band under strong light was normal and fully reversible, indicating no serious impairment of electron transfer to the quinone(s), and similar recombination kinetics.

The observed $\leq 50\%$ exchange of the total BChl with the [3-vinyl]-pigment **7**, corresponds to an exchange of $\leq 100\%$ of the monomeric pigments, e.g. an exchange of both B_A and B_B. This is similar to results obtained before with 13^2-hydroxy-Bchl [1]. For the other three pigments tested (**4,5,6**), the exchange is only incomplete. However, the maximum amounts of 40% are

Table I



Pigment	R_1	R_2	R_3	M
1	$COCH_3$	$COOCH_3$	H	H_2
2	$COCH_3$	$COOCH_3$	H	Mg
3	$COCH_3$	$(COOCH_3/OH)$*		Mg
4	$COHCH_3$*	$COOCH_3$	H	Mg
5	$COHCH_3$*	$(COOCH_3/OH)$*		Mg
6	$CHCH_2$	$COOCH_3$	H	Mg
7	$CHCH_2$	$(COOCH_3/OH)$*		Mg

*Stereochemistry unspecified

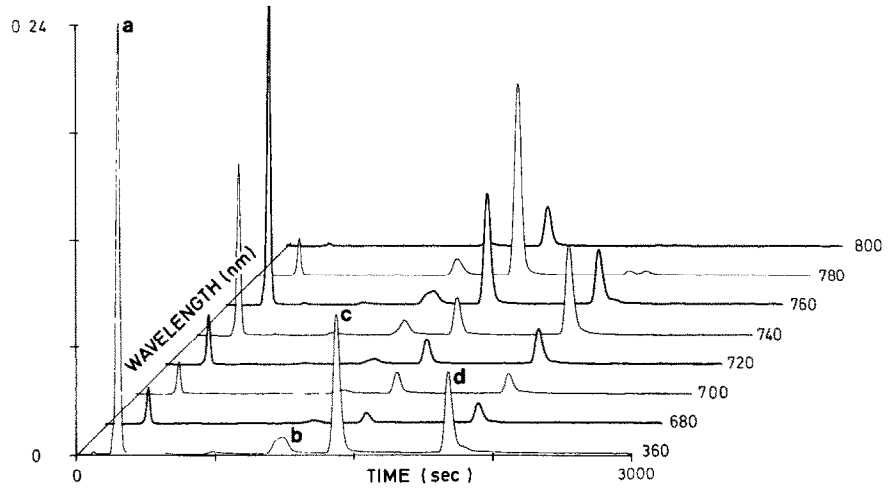


Fig. 2. HPLC chromatogram detected at different wavelength of pigments extracted from *Rb. sphaeroides* R26 RCs (on silica, see section 2) after exchange with [3-vinyl]-13²-hydroxy-BChl (**7**). BPhe (peak a), BChl (peak c), **7** (peak d). Peak b is a composite of BChl a′ and [3-vinyl]-BChl (**6**). The small peak eluting after (d) is due to 13²-hydroxy-BChl *a*. Detection wavelength for the different traces are given on the right side [nm].

well above the value of 25% expected for a selective exchange of only one of the monomeric pigments. We conclude, therefore, that for these pigments, too, both the B_A and the B_B sites are accessible and suitable.

As compared to the ether solution of **3**, its absorption bands in the RC at the $B_{A,B}$-sites show long-wavelength shifts of 25 nm (Q_X) and 31 nm (Q_Y) [1]. A similar comparison for the pigments **5** and **7** bearing modified C-3 substituents, yields for Q_Y shifts of a similar magnitude (30 nm) between the ether solutions and the respective modified RCs (note, the change of the extinction coefficients between **3** and **7** (vide supra) is also seen in the protein complex). The Q_X shifts are, by contrast, different among the pigments. With **5**, the shift is 16 nm (550 to 566 nm), with **7** it is 20 nm (554 to 574 nm). The constant protein-induced Q_Y shift for all pigments investigated here, indicates that the origin for the spectral changes between organic solvent and protein are neither disturbed by a modification at the position 3, nor at position 13², which lie both on or near the *y*-axis. The different Q_X shifts, on the other hand, may be the result of some indirect structural change along the *x*-axis, or variations in the magnesium ligation [22] which are induced by the modifications at position 3 and 13².

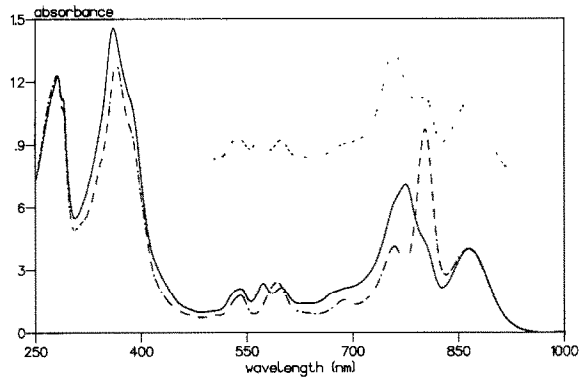


Fig. 3. Absorption spectra of RCs from *Rb. sphaeroides* R26 after exchange with 13²-hydroxy-BChl (**3**) (-•-•-), and with its 3-vinyl (**7**) (——) and 3-(α-hydroxyethyl) substituted analogue (**5**) (····). The spectra are adjusted to A_{865} = 0.4. The dotted spectrum is displaced vertically to avoid extensive overlap in the 750–800 nm region by 0.7 a.u.

3.4. *Circular dichroism of modified reaction centers*

Of particular interest are the circular dichroism spectra of the modified RCs (Fig. 4). The anisotropy of the lower band of the primary donor around 870 nm is the same within the spectral noise for all three modified RCs shown, which is also identical to that of native RC ($\Delta A/A = 23 \pm 1 \cdot 10^{-4}$). The spectra are, therefore, normalized to 1 at this position. Two other features are noteworthy in the comparison: there is (i) a blue-shift of the positive band occuring around 800 nm in native RC, concomitant to the blue-shift of the 800 nm absorption

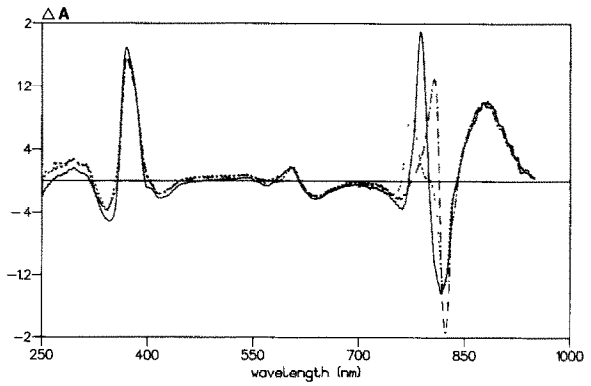


Fig. 4. Circular dichroism spectra of modified RCs from *Rb. spheroides* R26. Assignments as in Fig. 3. The spectra are normalized to 1.0 at the lower excitonic band of the primary donor.

band, and there is (ii) only little change in the intensity ($\leq 70\%$) and position of the main negative band. The extremum of the latter is slightly blue-shifted in RC containing the 3-vinyl-substituted **7**, but this appears to be a result of a partial separation of two previously overlapping bands, with one remaining at the original position. In RC containing the 3-(α-hydroxyethyl)-pigment **5**, this band is narrow again and of the same shape as in RC containing **2** or **3**. We take this as a strong indication, that this negative cd band has major contributions from the upper excitonic band of the primary donor.

The data indicate at the same time, that there is little spectral mixing among the primary donor and the monomeric Bchl. In view of the experimental (see [23–26]) and theoretical effort (see [27–29]) to understand these interactions, a more detailed analysis of the spectra including low-temperature linear and circular dichroism is in progress.

4. DISCUSSION

It has been shown that bacteriochlorophylls modified at the position 3 and 13^2 are 'accepted' selectively by the binding pockets for the monomeric pigments B_A and B_B, and that pigment exchange into RCs is a valuable new tool to investigate structure-function relationships. The [3-vinyl]-BChl was chosen, because it constitutes a link between BChl and the plant pigment, chlorophyll *a* (which does not exchange under these conditions [2]). Modifications close to the 2-methyl group of BChl also change the electron density at this group, which has been suggested to be involved in electron transfer [28].

There are only few (bacterio)chlorophyll-proteins in which modified pigments have been incorporated. Evolution [30–32] or site directed mutagenesis [33,34] has led to several RCs in which BPhe replaces BChl, and vice versa. These changes are always related to a replacement of a histidine residue ligated to a central Mg, with a non-polar one, or vice versa. The notion of a high degree of discrimination between the BChl and BPhe by the different binding sites, is also borne out by the lack of exchange of BPhe with bacteriochlorophylls in the experiments reported here.

Chemically modified pigments have been incorporated in the antenna complex B873 of *Rhodospirillum rubrum* [35]. The selection rules between this complex and the RC from *Rb. sphaeroides*, are different, however. The RC is tolerant to a hydroxylsubstituent at 13^2, whereas this pigment was not accepted by the B873 apoprotein. Information on the exchangeability of pigments **4–7** in the B873 complex is not yet available.

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