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Modified bacterial reaction centers. 4. The borohydride treatment reinvestigated: comparison with selective exchange experiments at binding sites $B_{A,B}$ and $H_{A,B}$

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Pigments of borohydride-treated reaction centers of *Rhodobacter sphaeroides* R 26 and *Rhodopseudomonas viridis* were analyzed by HPLC with polychromatic detection. In both species, pigment composition and contents were unchanged. Reaction centers from *Rhodobacter sphaeroides* R26 were prepared in which bacteriochlorophylls ($B_{A,B}$) and bacteriopheophytins ($H_{A,B}$) were exchanged with their potential borohydride products reduced at C-3^1. [3-Hydroxyethyl]-BChl *a* exchanges selectively into the $B_{A,B}$ pockets, and 3^1-OH-BPh *a* to the $H_{A,B}$ pockets. Stable reaction centers are obtained in both cases. A comparison of the absorption and circular dichroism spectra of reaction centers after exchange with 3^1-OH pigments, and of borohydride-modified reaction centers, reveal distinct differences. It is concluded that during borohydride reduction none of the pigments is chemically modified or extracted from the reaction centers.

Introduction

Reaction centers (RC) from the purple photosynthetic bacteria *Rhodobacter* (*Rb.*) *sphaeroides* and *Rhodopseudomonas* (*Rp.*) *viridis* contain six bacteriochlorin molecules, viz. four bacteriochlorophylls (BChl) *a* or *b*, and two bacteriopheophytins (BPhe) *a* or *b*, respectively. The RC of *Rb. sphaeroides* consists of three polypeptides, designated subunits H, M, L according to their electrophoretic mobilities; and contains an additional cytochrome *c* in *Rp. viridis*. The crystal structure is known for both RCs [1–4]. The 'primary' charge separation process involves electron transfer in less than 4 ps from the excited singlet state of the primary donor, a complex of two of the bacteriochlorophylls, to BPhe (H_A) [5]. Recently, evidence was obtained for the direct involvement of BChl (B_A) as an intermediate electron carrier (Ref. 6, but see Ref. 7 for a controversial discussion). The functions of the BChl and BPhe not implicated in charge separation (BChl B_B and BPhe H_B) are unknown; an involvement of BChl B_B in triplet energy transfer is discussed [8,9].

In view of the tight packing of the pigments and the highly optimized charge separation, modified reaction centers are of considerable interest both for experimentalist and theoretician. Today there are several possibilities available to modify either the amino-acid [10–16] and/or the pigment composition [17–20]. Historically, however, treatment with borohydride was the first method by which reaction centers from *Rb. sphaeroides* R26 with modified spectral properties were accessible [21]. It was generally accepted (but see [22]), that treatment with borohydride removed about 50% of the monomeric bacteriochlorophylls absorbing at 802 nm. From kinetic and spectroscopic evidence it had been suggested that the 'inactive' BChl *a* (B_B), is reduced at the 3-acetyl group by this treatment, and then subsequently lost from the remaining complex [9, 21, 23–25]. As a side reaction, partial cleavage of the M-subunit has been observed in the case of *Rb. sphaeroides* R 26 reaction centers [23].

Abbreviations: *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*; RC, reaction center; BChl, bacteriochlorohyll; BPhe, bacteriopheophytin; $B_{A,B}$, BChl and $H_{A,B}$, BPhe binding sites, the subscripts refer to the location on the active (A) and inactive (B) branches, respectively; LDAO, dodecyldimethylamine oxide.

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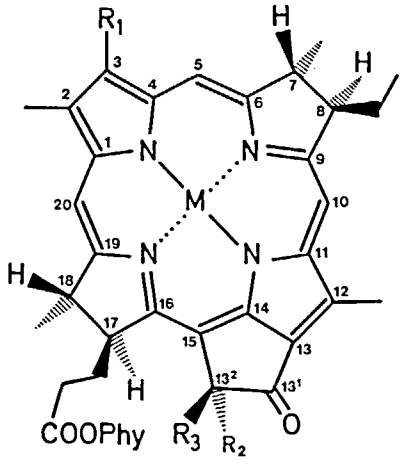

Scheme I.

Pigment	Compound			
	R_1	R_2	R_3	M
BChl *a*	$-COCH_3$	$-COOCH_3$	-H	Mg
BChl *a'*	$-COCH_3$	-H	$-COOCH_3$	Mg
BPhe *a*	$-COCH_3$	$-COOCH_3$	-H	H_2
3^1-OH-BChl *a*	$-CHOHCH_3$	$-COOCH_3$-H		Mg
3^1-OH-BPhe *a*	$-CHOHCH_3$	$-COOCH_3$-H		H_2
3-Vinyl-BChl *a*	$-C_2H_3$	$-COOCH_3$-H		Mg
13^2-OH-BChl *a*	$-COCH_3$	$-COOCH_3$-OH		Mg
13^2-OH-BPhe *a*	$-COCH_3$	$-COOCH_3$-OH		H_2

Recently, exchange of monomeric BChl at the $B_{A,B}$ binding sites of RC from *Rb. sphaeroides* and of monomeric BPhe at the $H_{A,B}$ binding sites of RC from *Rb. sphaeroides* and *Rp. viridis* with pigments modified at the 3-acetyl group was achieved [19,20]. In particular 3^1-OH-BChl *a* can be introduced into $B_{A,B}$ and 3^1-OH-BPhe *a* into $H_{A,B}$, which are the pigments discussed as reduction products obtained with borohydride. Contrary to what was expected from the postulated reaction mechanism of borohydride treatment, this exchange yielded stable RC, which do not lose the modified pigments during common work-up procedures. This conflicting results led us to re-investigate the pigment compostion, absorption and circular-dichroism (CD) spectra of RC after borohydride treatment. The data show that the major spectral changes induced by it are not caused by a partial loss of pigments.

Material and Methods

Reaction centers. Rb. sphaeroides R26 RC were prepared by repeated solubilization of chromatophores and subsequent chromatography on DEAE-cellulose as described previously [23]. *Rp. viridis* RC were isolated by dissociation of the chromatophores with LDAO (5%) [26], and subsequent chromatography on DEAE-cellulose. The final purification was achieved in both cases 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, 240000 × *g*). RC 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.

Borohydride treatment. Borohydride treatment of *Rb. sphaeroides* R26 RC was done as described in Ref. 24, that of *Rp. viridis* ones as described in Ref. 22. The treated RC were purified on DEAE-cellulose followed by density-gradient centrifugation, as above.

Exchange experiments. Exchange experiments for the $B_{A,B}$ binding sites were performed as published [19,20] by incubating the RC at 42.5 °C for 90 min in Tris-buffer (20 mM, 0.08% LDAO, 10% MeOH) containing the 3^1-OH modified pigments in a 20-fold excess. Afterwards, excess pigments were removed from the incubation mixture by repeated chromatography on DEAE-cellulose. The yield of recovered RC was $\leqq$ 30%. For the exchange of the 3^1-OH-BPhe *a* into the $H_{A,B}$ binding sites, the pigments were dissolved in a minor amount of diethyl ether, diluted with MeOH, and then added to the RC as in the case of $B_{A,B}$ exchange.

Pigments. BChl *a* was extracted from *Rb. sphaeroides* and reduced to [3-hydroxyethyl]-BChl *a* (3^1-OH-BChl) with $NaBH_4$ as published before [19]. Demetalation to the respective pheophytin, 3^1-OH-BPhe *a* was done with dilute HCl. The structures of the products, had the expected absorption, fluorescence, ^{1}H-NMR and mass spectra. No attempts were made to separate the $3^1,13^2$ diastereomers. A full account of the chemistry will be given separately.

Pigment analysis. Extraction of pigments from RC for HPLC analysis was done with MeOH under Ar on DEAE-cellulose as described in Ref. 19. The HPLC analysis was done on silica or C-18 reversed phase with a diode array detector (HP 8451) as reported [19]. Absorption spectra were measured on a Lambda 2 spectrophotometer (Perkin Elmer), CD spectra with a dichograph V (Jobin-Yvon).

Results

Rb. sphaeroides R26

Absorption after BH_4 treatment

The spectrum shows the typical changes reported previously [21] (Fig. 1). As compared to untreated RC, there is a decrease of the absorption at 802 nm and a concomitant small, broad increase at 765 nm. There are also some small shoulders in the Q_X region of the monomeric BPhe *a* at 542 nm and of the BChl *a* at 598 nm. There is no distinct increase in the 720nm region, if well purified RC are used for the reaction.

Absorption after exchange of $B_{A,B}$ with 3¹-OH-BChl a

3¹-OH-BChl *a* was obtained by borohydride reduction of BChl *a* in MeOH. Due to the removal of the conjugated carbonyl substituent at C-3, the absorption of this pigment is blue-shifted. In the Q_Y and Q_X region, this shift amounts to 43 nm (766 cm^{-1}) and 18 nm (566 cm^{-1}), respectively. No reduction of the 13¹-C = O group [27–29] was observed, even after prolonged standing and repeated addition of the reagent. Partial exchange with 3¹-OH-BChl *a* of monomeric BChl *a* at the $B_{A,B}$ binding sites [18,19] yields RC with the absorption spectrum shown in Fig. 1d [19]. There is a decreased absorption (compared to untreated reaction centers) at 802 nm which correlates to an increase at 756 nm. This shift of 46 nm (759 cm^{-1}) compares with the one observed in solution upon reduction (see above), but it should be kept in mind that this band also contains contributions from the BPhe Q_Y band and the maximum may not be exactly that of the 3¹-OH-BChl *a* introduced. The short-wavelength shift is more distinct in the Q_X region. There are no spectral changes at 865 nm and 756 nm. From this, it is concluded that the BChl *a* dimer (P870) and the BPhe in the H-binding sites are not exchanged under these conditions. The intactness of P870 was also demonstrated qualitatively and quantitatively by ENDOR spectroscopy (with W. Lubitz et al., not shown). The modified RC were stable during repeated chromatography on DEAE-cellulose and ultracentrifugation.

The spectral changes in the long-wavelength region are partially similar to those of borohydride-treated reaction centers. An interpretation in the Q_Y region is difficult, because there is an overlap of the BPhe *a* bands, and the one(s) of the newly introduced 3¹-OH-BChl, which are shifted to shorter wavelengths. However, there is a significant difference in the Q_X region of the bacteriochlorophylls. The '3¹-OH-BChl *a* reaction centers' show a new absorption at 562 nm, whereas the borohydride-treated reaction centers show only a short-wavelength shoulder ($\approx$ 568 nm). The stability and the spectra are evidence, that the main effect of borohydride treatment can not readily be explained by the presence of 3¹-OH-BChl *a*. A stereoselective reduction, coupled with a pronounced dependence of native absorption on the configuration at the two asymmentric centers, can presently not be ruled out, however, by the chromatographic data.

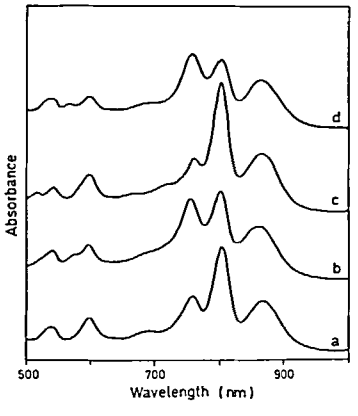

Fig. 1. Absorption spectra of modified reaction centers of *Rb. sphaeroides* R26 (absorption at 865 nm normalized to 1) of: (a) untreated RC; (b) RC after treatment with borohydride, and subsequent purification on DEAE-cellulose and density-gradient centrifugation; (c) RC with 3¹-OH-BPhe *a* in the H_B-binding site; (d) RC with a partial exchange of 3¹-OH-BChl *a* into the $B_{A,B}$ binding sites. The features at the long-wavelength side of the 870 nm band in traces a and d are an instrument artefact.

Absorption after exchange of $H_{A,B}$ with 3¹-OH-BPhe a

Borohydride-reduction of BPhe *a* in organic solvents yields 3¹-OH-BPhe *a* [19,21]. Treatment under exchange conditions with 3¹-OH-BPhe *a* yields RC which are stable during repeated chromatography on DEAE-cellulose. The purified RC have an absorption spectrum as shown in Fig. 1c. The absorption decrease at 756 nm (compared to untreated reaction centers) correlates with an increase at 723 nm. A short-wavelength shift is also obvious in the BPhe Q_X region. There are no spectral changes in the BChl a Q_Y region at 865 nm and at 802 nm, indicating that only BPhe, but neither the dimer P870, nor the monomeric BChl *a* at $B_{A,B}$ is exchanged under these conditions. A spectral comparison of these 3¹-OH-BPhe *a* reaction centers with the borohydride-treated reaction centers shows that in the latter the poorly resolved short-wavelength shoulder in the Q_X region of BPhe *a* (535 nm) could not be explained by presence of 3¹-OH-BPh *a* at the $H_{A,B}$ site(s), because this pigment absorbs in the exchanged RC at 515 nm.

CD spectra

A concomitant analysis of the CD spectra of modified reaction centers support the results of the absorption spectra analysis (see also [23]). Fig. 2 shows the CD spectra of untreated and modified reaction centers of *Rb. sphaeroides* R26. The anisotropy of the lower band of the primary donor around 870 nm is the same within the spectral noise for all reaction centers shown ($\Delta A/A = 23 \pm 1 \cdot 10^{-4}$). The spectra are therefore normalized at this position. The reaction centers after treatment with borohydride and purification (Fig. 1b) show a decrease and broadening of the band around 800 nm. So the absorption change upon borohydride treatment is accompanied by a change in CD activity, which is in conflict with the exchange results. Reaction centers after exchange of the monomeric BChl *a* at $B_{A,B}$ show a charactersitic decrease of the upper positive band around 800 nm (which is commonly assigned

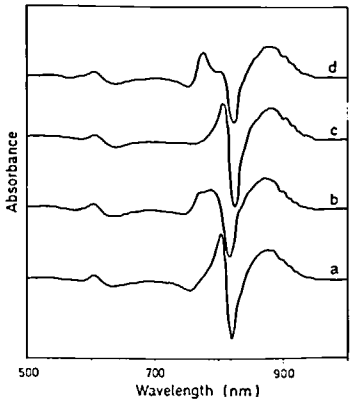

Fig. 2. Circular dichroism spectra of reaction centers of *Rb. sphaeroides* R26. The anisotropy of the lower band of the primary donor around 870 nm is the same within the spectral noise for all reaction centers shown ($\Delta A/A = 23 \pm 1 \cdot 10^{-4}$). The spectra are therefore normalized at this position. Samples a–d as in Fig. 1.

to the monomeric bacteriochlorophylls), and a concomitant increase around 780 nm. Similar results with [3-vinyl]-BChl *a* are discussed elsewhere [19]. After the exchange of BPhe at $H_{A,B}$ with 3^1-OH-BPhe *a*, there is an increase of the broad negative band around 760 nm. This change is also typical for exchanges of several modified bacteriopheophytins, e.g., [3-vinyl]-BPhe *a* into the $H_{A,B}$ binding site (data not shown, see Ref. 20 for a preliminary discussion), but does not correspond to the CD of borohydride-treated RC.

Taken together, the exchange experiments show that 3^1-OH-BChl *a* can replace selectively BChl *a* in the $B_{A,B}$ binding sites and 3^1-OH-BPhe *a* can replace selectively BPhe *a* in the $H_{A,B}$ binding sites. Also, the latter can replace BPhe *b* in $H_{A,B}$ of *Rp. viridis* (see below). All exchanges yield RC which are stable towards the common purification procedures, and in particular to the ones used to purify borohydride-treated RC [22–24]. The selection of the binding sites obviously depends mainly on the presence or absence of the central Mg. This corroborates results obtained with [3-vinyl]-analogues of BChl *a* and BPhe *a* [19,20] which also exchange selectively into the $B_{A,B}$ and $H_{A,B}$ binding sites, respectively [20]. It is also corroborated by mutagenetic studies [10–16] and by the unusual pigment composition of *Chloroflexus aurantiacus* RC [30–32]. The stability and the spectroscopic properties of the complexes into which the 3^1-hydroxy analogues are introduced are in contradiction to the common interpretation that borohydride treatment leads to chemical reduction of BChl *a* bound to B_B at C-3^1, and its subsequent loss from the reaction center. A loss may be expected, however, if BChl *a* at B_B were reduced and demetallated simultaneously, because 3^1-OH-BPhe *a* does not exchange into the $B_{A,B}$ binding

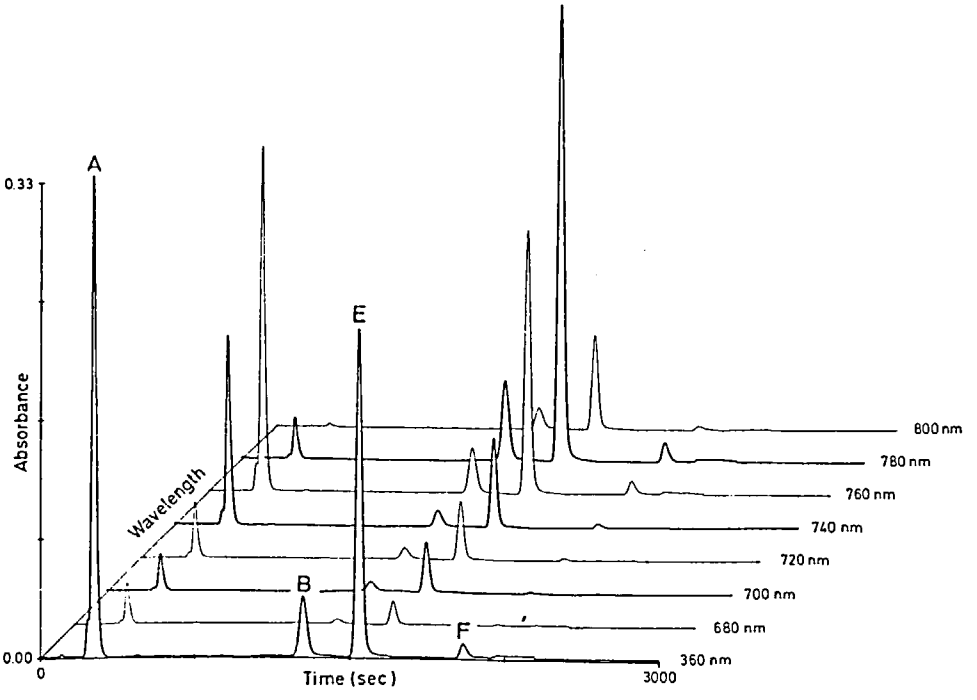

Fig. 3. Multiwavelength-detected HPLC-chromatogram of the pigments extracted from untreated reaction centers of *Rb. sphaeroides* R26 (extract from the preparation shown in Fig 1a). Peak assignments as in Fig. 4.

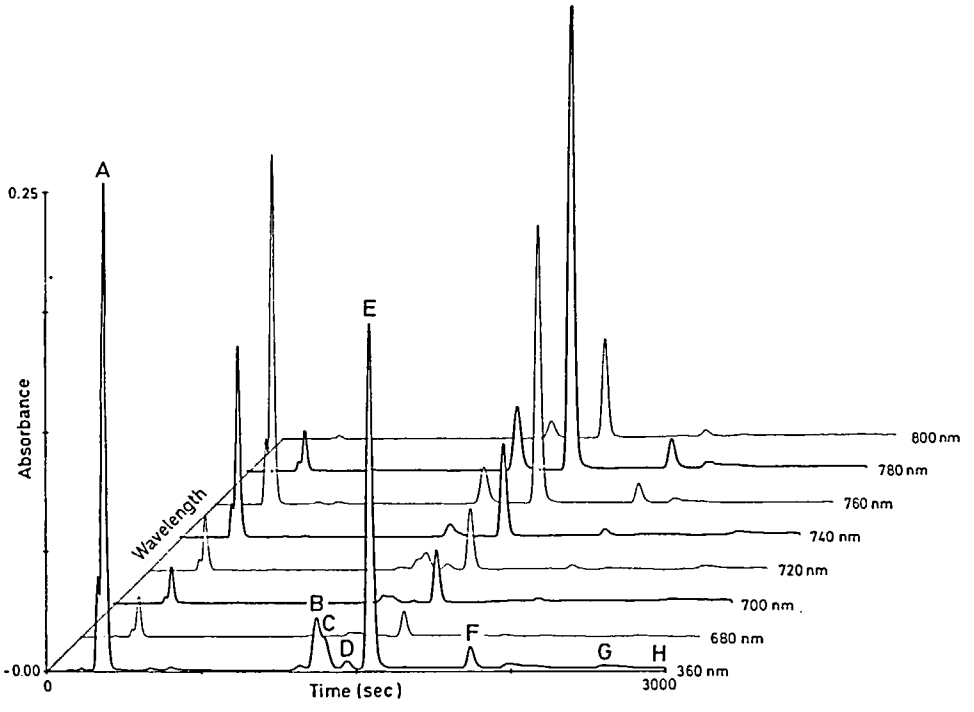

Fig. 4. Multiwavelength-detected HPLC-chromatogram of the pigments extracted from borohydride-treated reaction centers of *Rb. sphaeroides* R26 (extract from the preparation shown in Fig. 1b). (A) BPhe *a*; (B) BChl *a'* (C,D) 3^1-OH(*R,S*)-BPhe *a*; (E) BChl *a*; (F) 13^2(*R,S*)-OH-BChl *a*; (G,H) 3^1-OH(*R,S*)-BChl *a*.

site as shown above *. Indeed, from borohydride-treated reaction centers only 3^1-OH-BPhe *a* was isolated [24]. Although it is difficult to understand how demetallation may occur under the alkaline reaction conditions, it was tacitly assumed that this is the final product of the treatment. To test this possibility, a quantitative analysis of the tetrapyrroles in reaction centers was done.

HPLC analysis

Fig. 3 shows the chromatogram of a pigment extract of untreated reaction centers from *Rb. sphaeroides* R26. Two peaks corresponding to BPhe *a* (peak A) and BChl *a* (peak E) occur in a characteristic intensity ratio, that translates after correction for absorptivities into the 2:1 molar ratio typical for untreated reaction centers. The chromatogram in Fig. 4 shows a pigment extract of borohydride modified RC from the same organism. There is no significant change in the BPhe *a*/ BChl *a* ratio (compared to untreated reaction centers). If the integrated peak areas are normalized to the 870 nm absorption, the same amounts of both BChl *a* and BPhe *a* are present in untreated and in borohydride-treated reaction centers. The unchanged BPhe *a*/BChl *a*; BChl *a*/P870 and BPhe *a*/P870 ratios in the pigment extract of borohydride-treated RC contradict the common interpretation, that a decrease in the 802 nm absorption is due to a loss of BChl *a*. The identical quantitative amounts of BChl *a* and BPhe *a* in treated and untreated reaction centers also exclude the possibility of a simultaneous loss of BChl *a* and BPhe *a* in a $2/1 \pm 0.15$ ratio. The pronounced changes in the absorption spectrum can therefore not be explained by a bulk loss of monomeric BChl *a*.

There are several additional minor bands visible in the choromatograms of borohydride-treated RC, which represent minor amounts of 3^1-OH-BPhe *a* (peak C, D) and 3^1-OH-BChl *a* (peak G, H). The presence of traces of these 3^1-OH compounds in the pigment extract can be most readily explained by the presence of traces of free (or unspecifically bound) BChl *a* or BPhe *a*, respectively, in the original RC. Variable purity of RC used for the borohydride treatment, would then also explain the puzzling variations of the intensity of the 720 nm band seen in crude reaction mixtures, but never in RC purified thereafter [23]. As mentioned in the previous section, this band is absent if carefully

* Strictly speaking, the exchange experiments only give an 'exchange capacity'. In the case of BPhe vs. BChl, the exchange selectivity matches the selectivity observed after site directed mutagenesis of ligating histidine residues, but the relationship still has to be explored for more extensively modified pigments.

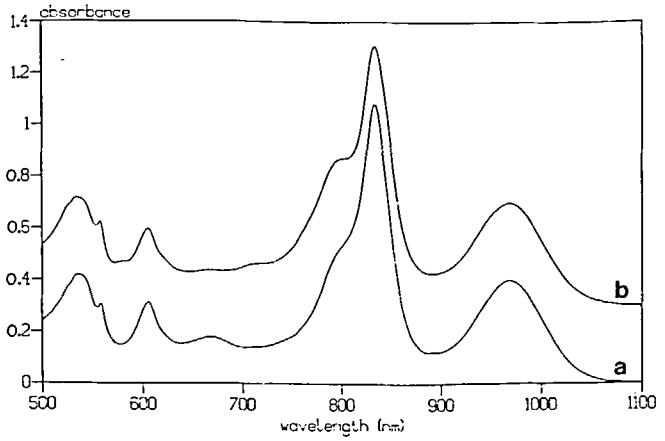

Fig. 5. Absorption spectra of modified reaction centers of *Rp. viridis* (absorption normalized at 965 nm to 1) of: (a) untreated RC; (b) RC after treatment with borohydride, and subsequent purification on DEAE-cellulose and density-gradient centrifugation.

purified RC are treated. Small amounts of BChl *a'* (peak B) in the pigment extract of borohydride-treated reaction centers are as observed in other treatments [18] and cannot explain the changes in the absorption spectrum, either.

Rp. viridis

Absorption after borohydride treatment

The absorption changes upon treatment of *Rp. viridis* RC with borohydride are less spectacular than in *Rb. sphaeroides*. After purification, they show a characteristic change of the shoulder at ≈ 790 nm and of the 830 nm band (Fig. 5). A decrease in absorption at 834 and 820 nm correlates to an increased absorption at 775 nm, as can be seen in the difference spectrum (data not shown). These changes correspond to the ones reported by Shuvalov et al. [33]. There are also small changes in the 650–750 nm region, which we consider insignificant, however. The band ≈ 680 nm corresponds to oxidation products of BChl *b* [34]. An absorption increase at 720–750 nm appears after borohydride treatment and indicates free pigments that can be removed by repeated chromatography.

HPLC analysis

The HPLC-chromatograms of untreated RC of *Rp. viridis* after careful extraction show two peaks corresponding to BPhe *b* (peak a) and to BChl *b* (peak c) (Fig. 6). There are also oxidation products [34] which absorb all around 680 nm, and which appear in peaks d and e in variable and minor amounts under the conditions used. Pigment extracts of borohydride-modified reaction centers (Fig. 7) turned out to have the same BPhe *b*/BChl *b* ratio as untreated ones. The quantitation with respect to P960 is less precise in this case, due to the aforementioned ready oxidation of BChl *b* and BPhe *b* to products of the chlorin spectral type, which amount to ≦ 15% of the total pigment. However, unless there is a transformation and release of BChl *b* and BPhe *b* in a 2:1 ratio in borohydride-treated *RC of Rp. viridis*, the data are only compatible with the

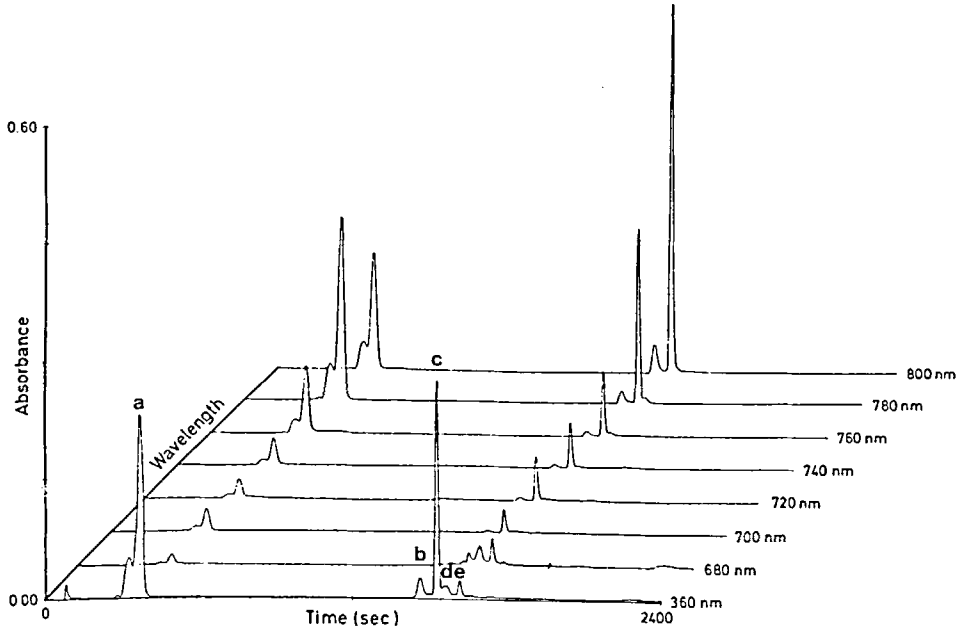

Fig. 6. Multiwavelength-detected HPLC-chromatogram of the pigments extracted from untreated reaction centers of *Rp. viridis* (extract from the preparation shown in Fig. 5a). (a) BPhe *b*; (b) BChl *b'*; (c) BChl *b*; (d,e) oxidation products of BChl *b*.

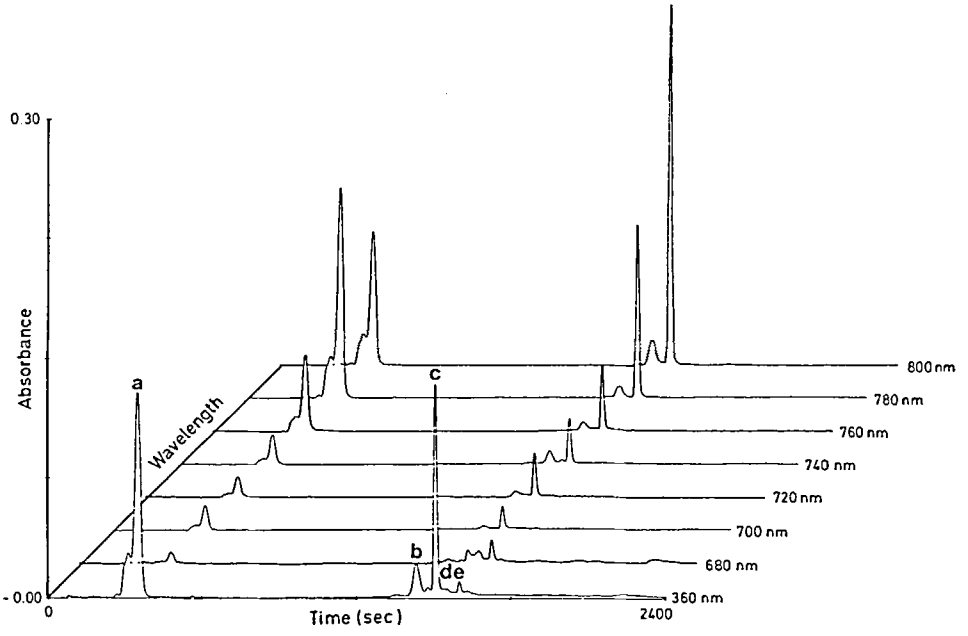

Fig. 7. Multiwavelength-detected HPLC-chromatogram of the pigments extracted from borohydride-treated reaction centers of *Rp. viridis* (extract from the preparation shown in Fig. 5b). Peak assignments as in Fig. 6.

fact that there is no change in the pigment content in this species, either.

Discussion

The notion that BChl at the B_M site is reduced and then removed from RC by borohydride treatment rested on several lines of evidence: (1) The 802 nm absorption is decreased by approx. 40%. If this band contains contributions of the Q_Y-band of both monomeric BChl, and of the upper excitonic band of the special pair, this corresponds to a substantial loss of the former [21]. In *Rp. viridis* RC, the spectral differences are less distinct, but it was assumed that the increase of the shoulder at 790 nm (Fig. 5b) might be caused by the loss of a pigment absorbing around 820 nm, probably B_B, too [22]. (2) 3^1-OH-BPhe was identified by ^{1}H-NMR and chemical correlation in the supernatant of borohydride-treated RC from *Rb. sphaeroides* [24]. (3) Electron transport is unimpaired in borohydride-treated RC [35–39]. (4) Borohydride modification is inhibited in wild-type RC, which contain one carotenoid per RC, bound in close proximity to and apparently protecting the BChl in B_M [2,4]. If the conclusion presented above is correct that there is no change in pigment composition, it should be able to explain the referenced results on this basis, too.

The following explanations are suggested: (1) The decrease of the 802 nm band is due to an environmental effect. A precedent is the apparent loss of BChl *a*-B800 in the B800–850 antenna from *Rb. sphaeroides*, which is induced by the detergent SDS, and reverted after addition of LDAO [40,41]. The Q_Y-bands in this complex show strong red-shift and hyperchromism. If both are reduced by some environmental effect, one would expect an overall decrease in absorption in the Q_Y-range, and a blue-shift. This is indeed observed in B800–850 upon SDS treatment. Similar, albeit irreversible, changes are seen in RC upon borohydride treatment. The broad increase in the 750 nm region is not as pronounced as the decrease around 802 nm, and it has hardly been discussed, but it is obvious in Fig.1b, and also noticeable in previous reports on the subject [21–25]. A considerable change in about 1/3 of the RC population, e.g., proteolysis of the M-subunit, has been found by Beese et al. [23]. Although this can hardly explain the homogeneous spectral features, it indicates at least that there is (are) specific reaction(s) of $NaBH_4$ with the protein. (2) The finding of 3^1-OH-BPhe instead of 3^1-OH-BChl has been puzzling from the beginning. Under the alkaline reaction conditions reaching pH 10, a demetallation of any Mg-bacteriochlorin is not a likely process, yet a Mg-free pigment was unambigously identified [24]. An explanation in the framework of the findings presented rests on the fact that the 3^1-OH-BPhe *a* in the reaction mixture of RC with borohydride had never been quantitated carefully. It could thus be a product not of an integral RC-bound pigment, but rather of a BPhe impurity in the RC. This could also explain previous observations that the intensity of the 725 nm band arising in the reaction mixture and assigned to a pigment reduced at C-3^1 shows considerable and previously unaccountable variations [23,25]. The pigment could also arise (together with 3^1-OH-BChl *a*) if some of the RC were irreversibly destroyed by the BH_4

treatment. (3) As long as the environmental effect acts only on the B_M site, this result is conceivable with the previous as well as the new interpretation. (4) The same is true for the protective effect of carotenoids.

In conclusion, the notion that there is no change in pigment composition, but rather a changed interaction of the protein with BChl in the B_M binding site by some as yet unaccounted mechanism, does not conflict with known facts. We are aware of unpublished X-ray results (Allen, J., personal communication), which also indicate that the B_M binding site of BH_4-treated RC from *Rb. sphaeroides* is still occupied by a tetrapyrrole. There are furthermore items of evidence which indicate that the pigment is even still bound rather tightly to a distinct site. These are (a) that the pigment composition is highly reproducible, (b) that it is the same after different purification procedures (here DEAE-cellulose and/or gradient centrifugation) and (c) that there is a characteristic CD activity in treated RC which implicates a strong interaction. It may be useful to reinvestigate the LD-spectra of BH_4-treated RC [42] with regard to this aspect.

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