

# CHEMICALLY MODIFIED PHOTOSYNTHETIC BACTERIAL REACTION CENTERS: CIRCULAR DICHROISM, RAMAN RESONANCE, LOW TEMPERATURE ABSORPTION, FLUORESCENCE AND ODMR SPECTRA AND POLYPEPTIDE COMPOSITION OF BOROHYDRIDE TREATED REACTION CENTERS FROM *Rhodobacter sphaeroides* R26

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**Abstract**—Reaction centers from *Rhodobacter sphaeroides* have been modified by treatment with sodium borohydride similar to the original procedure [Ditson *et al.*, *Biochim. Biophys. Acta* **766**, 623 (1984)], and investigated spectroscopically and by gel electrophoresis.

(1) Low temperature (1.2 K) absorption, fluorescence, absorption- and fluorescence-detected ODMR, and microwave-induced singlet-triplet absorption difference spectra (MIA) suggest that the treatment produces a spectroscopically homogeneous preparation with one of the 'additional' bacteriochlorophylls being removed. The modification does not alter the zero field splitting parameters of the primary donor triplet (<sup>1</sup>P870).

(2) From the circular dichroism and Raman resonance spectra in the 1500–1800 cm<sup>-1</sup> region, the removed pigment is assigned to Bchl<sub>M</sub>, e.g. the "extra" Bchl on the "inactive" M-branch.

(3) A strong coupling among all pigment molecules is deduced from the circular dichroism spectra, because pronounced band-shifts and/or intensity changes occur in the spectral components assigned to all pigments. This is supported by distinct differences among the MIA spectra of untreated and modified reaction centers, as well as by Raman resonance.

(4) The modification is accompanied by partial proteolytic cleavage of the M-subunit. The preparation is thus spectroscopically homogeneous, but biochemically heterogeneous.

## INTRODUCTION

The reaction center (RC)† of bacteriochlorophyll *a* (Bchl *a*) containing photosynthetic purple bacteria consists of three polypeptides, designated subunits H, M and L according to their electrophoretic mobilities. It contains six chromophores, i.e. 4 molecules Bchl *a* and 2 molecules bacteriopheophytin *a* (Bphe *a*), two quinones and one iron (Feher and Okamura, 1978; Parson, 1982). Two of the Bchl *a* molecules (Norris *et al.*, 1975) form the special pair serving as the primary electron donor designated P870 according to its long wavelength absorption around 870 nm. For one of the two Bphe *a* — molecules absorbing at 760 nm in the near infrared region, an intermediate acceptor function for the

electron during the charge separation has been established (see Parson, 1982 for leading references).

Crystallographic data from the RC of a Bchl *b*-containing purple photosynthetic bacterium, *Rp. viridis* (Michel, 1982; Deisenhofer *et al.*, 1984) and from *Rb. sphaeroides* (Chang *et al.*, 1986) show a C<sub>2</sub>-symmetry axis which divides the reaction center into two sets of pigments situated mainly on the L and M-subunit, respectively. The primary charge separation takes place most probably from the special pair situated on the symmetry axis, via Bphe<sub>L</sub> to Q<sub>L</sub> (formerly Q<sub>A</sub>) situated on the L- (or 'active') branch of the complex (Deisenhofer *et al.*, 1984; Vermeglio and Paillotin, 1982; Zinth *et al.*, 1985).

Much less is known on the function of the remaining two monomeric Bchl *a* molecules absorbing around 800 nm (Bchl<sub>L</sub>, Bchl<sub>M</sub>), and of the second pheophytin, Bphe<sub>M</sub>. If judged from its location between the primary donor and Bphe<sub>L</sub> (Deisenhofer *et al.*, 1985; Chang *et al.*, 1986), Bchl<sub>L</sub> should be functional in electron transport between the two. It is still unclear, however, if it acts as the very first acceptor in this process (Shuvalov and Duysens, 1986), or if some other mechanism like super-

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†Abbreviations: Bchl, bacteriochlorophyll; Bphe, bacteriopheophytin; the location on the L- or M subunit of the RC, is indicated by the suffix L and M, respectively; C., *Chloroflexus*; CD, circular dichroism; LDAO, dodecyl dimethylaminoxide; *Rb.*, *Rhodobacter*; RC, reaction center; *Rp.*, *Rhodospseudomonas*; RR, Raman resonance spectroscopy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TX-100, Triton X-100.

exchange, rapid hopping between a close and a distant site or yet another process is operative (Kirmaier *et al.*, 1985; Ogrodnik *et al.*, 1982; Breton, 1986; Zinth *et al.*, 1985; Knapp *et al.*, 1985; Wasielewski, 1986; Parson, 1982; Michel-Beyerle *et al.*, 1987; Fischer and Scherer, 1987). The participation of the second Bchl (Bchl<sub>M</sub>) and Bphe<sub>M</sub> situated on the M-branch is unclear. The latter can also accept an electron (Robert *et al.*, 1985; Michel-Beyerle *et al.*, 1987), but it is questionable if this also takes place under physiological conditions.

In view of the tight packaging of the pigments and the highly optimized charge separation, modified RC are of considerable interest both for the experimentalist and theoretician. The possibility to remove one of the six tetrapyrrolic pigments in bacterial reaction centers (Ditson *et al.*, 1984) provided for the first time an experimental tool to modify the tetrapyrroles in this complex. By treatment with sodium borohydride, about 50% of the bacteriochlorophyll (Bchl) absorbing at 800 nm can be removed. From kinetic (Maroti *et al.*, 1985; Holten, 1986; Shuvalov and Duysens, 1986; Breton 1986) and spectroscopic evidence (Robert *et al.*, 1986) it has been suggested that the 'inactive' Bchl<sub>M</sub> on the M-branch (Deisenhofer *et al.*, 1984; Chang *et al.*, 1986), is reduced at the 3-acetyl group and can then be dissociated from the remaining complex (Maroti *et al.*, 1985).

Selective modifications of this type are expected to be very useful in order to better understand the involvement of the different pigments in electron transfer. A basic requirement for evaluating the results is a thorough characterization of the modified preparations with respect to chemical composition, homogeneity, spectroscopy and kinetics. This is of particular importance because the homogeneity of the sample has been questioned more recently by Shuvalov *et al.* (1986), who discuss a product mixture in which the Bphe<sub>M</sub> is partly reduced. Here we want to present further data characterizing the "missing" Bchl *a* in NaBH<sub>4</sub> treated RC's, as Bchl<sub>M</sub>, which is strongly coupled to the other pigments, together with spectroscopic data supporting the homogeneity of the sample, but also results showing the treatment is prone to produce changes in the polypeptide(s) in addition to the pigment removal.

## MATERIALS AND METHODS

**Preparations.** *Rb. sphaeroides* R26 (DSM) was grown in 3001 batch culture at the Gesellschaft für Biotechnologie. (D-3301 Stöckheim). RC's were prepared as described earlier (Feher and Okamura, 1978). Bchl removal was performed according to the original method of Ditson *et al.* (1985) in the presence of LDAO as detergent. Solid sodium borohydride (5 mg = 0.13 mmol, can be replaced by the potassium salt) was added to the RC's (3 ml with  $A_{870} = 0.4 \text{ cm}^{-1} \cdot 9.4 \text{ nmol}$ ) in tris buffer (10 mM, pH=8.0)

containing LDAO (0.04% = 1.7  $\mu\text{M}$ ). The reaction was followed spectrophotometrically in the 300–900 nm range. To stop the reaction in kinetic experiments, aliquots were diluted 1 : 1 with a glucose solution (0.2 g/ml) which destroys excess reagent without affecting the RC. For polypeptide analysis, acetone was added alternatively which also precipitated the protein. For the low temperature spectra, the detergent was exchanged against Triton X-100 (TX-100). RC (0.1  $\mu\text{mol}$ ) were dialysed extensively against tris buffer (20 mM, pH 8.0) containing TX-100 (0.1%), loaded on a DEAE cellulose (DE 52, Whatman) column (2 × 8 cm) and subsequently washed with 10 column volumes of buffer. They were then eluted with NaCl (190 mM) in the same buffer. The typical absorption ratio  $A_{800}/A_{870}$  is 1.3. Chemical oxidation of reaction centers was done by titration with potassium ferricyanide.

**Chemicals.** Dodecyl dimethylaminoxide was from Fluka (Buchs), tris-HCl and TX-100 from Serva (Heidelberg), NaBH<sub>4</sub> and other chemicals from Merck (Darmstadt).

**Spectra.** Absorption spectra were recorded on a DMR 22 (Zeiss, Oberkochen). Absorption-difference spectra were measured with a ZWS II dual wavelength spectrometer in split-beam mode (Sigma, Berlin) equipped with a red-sensitive photomultiplier and home-built computer system. Extinction coefficients were determined with respect to the 870 nm transition of RC ( $\epsilon = 1.28 \times 10^5 \text{ cm}^{-2} \text{ M}^{-1}$ , Clayton and Wang, 1971). Since the intensity of this band remained unchanged during treatment with borohydride, the same value was used for modified RC. Circular dichroism spectra were obtained on a dichrograph V (ISA, Unterhaching) equipped with a silex data-handling system (Leanord, Lille) with a modified software. Wavelength calibration was done with the 360 nm Hg line in the near UV and with the 806 nm band of RC's (Reed and Ke, 1975) in the near infrared. Spectra of the relaxed state (fully reduced P870) were recorded in the presence of sodium ascorbate (1 mM) to prevent photooxidation by the measuring light. For the determination of molar ellipticities, the reaction was followed in the CD cuvette, and the decrease at the 870 nm band then used to calibrate all other bands in purified, modified RC.

Low temperature absorption spectra were taken by a homebuilt single-beam spectrometer, using a tungsten iodine lamp (150 W) for excitation, a Jobin-Yvon HRS.1 monochromator for dispersion and either a S1- or a S20-type photomultiplier for detection. The transmittance of the optical path without sample was divided by the transmittance with the sample and logarithmized by the computer to yield the optical density of the sample.

Fluorescence emission and excitation spectra were recorded with the same apparatus as described by Angerhofer *et al.* (1986). Fluorescence-ODMR spectra were measured with the ODMR set-up described earlier (Angerhofer *et al.*, 1985). The same microwave set-up in combination with the above described absorption photometer was used to record absorption-ODMR and microwave induced absorbance (MIA) spectra, the theory of which is described by den Blanken and Hoff (1983) and den Blanken (1983). Correction of the microwave induced transmission with the sample transmission was performed by the computer instead of an analog divider as used by den Blanken (1983).

Resonance Raman spectra were obtained with a setup described earlier in detail (Lutz, 1984; Lutz and Robert, 1985). They were obtained at about 30 K, using the 363.8 nm line of an Argon-ion laser which insures resonance at the top of the Soret bands of the bacteriochlorin pigments.

**SDS-PAGE** was done after a modified method of Laemmli (1970) using either a 11.5 to 16.5% acrylamid gradient gel or a constant 11.5% gel. Coomassie brilliant blue stained gels were scanned in Vitatron model UR400 scanner. The apparent molecular weights were determined with a set of hydrophilic markers.

## RESULTS

## Absorption spectroscopy

The titration with  $\text{NaBH}_4$  was followed by absorption (Figs. 1 and 2) and difference spectroscopy (Fig. 3) in a scattering insensitive system. The reaction mixture becomes slightly turbid during the reaction, and the 800 nm absorption decreases by about 50% as compared to its starting value (Fig. 1, traces A,B). A new absorption appears instead at 715 nm, whose intensity varies considerably among different experiments (see shoulder of the short-wavelength side of the 760 nm absorption band, Fig. 1, trace B). A second pronounced change occurs in the  $Q_X$  spectral region between 500 and 630 nm. The Bchl  $a$  absorption decreases, and both the  $Q_X$ -bands from Bchl and Bphe develop well resolved shoulders at their short-wavelength sides. Frequently, there is also a small absorption band around 480 nm present in the reaction mixture.

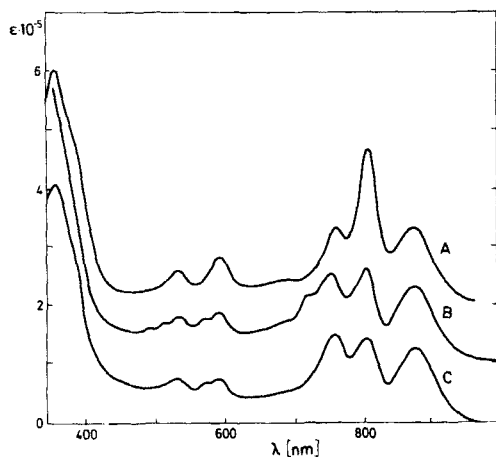


Figure 1. Absorption spectra at 298 K of reaction centers ( $5 \mu\text{M}$  in tris-LDAO buffer) before (A) and 16 h after (B) the addition of sodium borohydride ( $5\text{mg/ml}$  solution), and after purification on DEAE cellulose (C). Traces B and C are raised by 1 and  $2 \times 10^5 \text{ cm}^{-2}$ , respectively, to avoid extensive overlap.

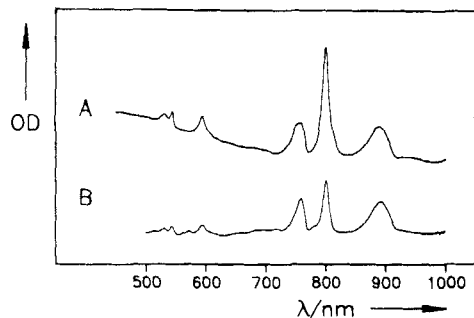


Figure 2. Low temperature absorption spectra (1.2 K) of modified, purified reaction centers (B) and of the original sample (A).

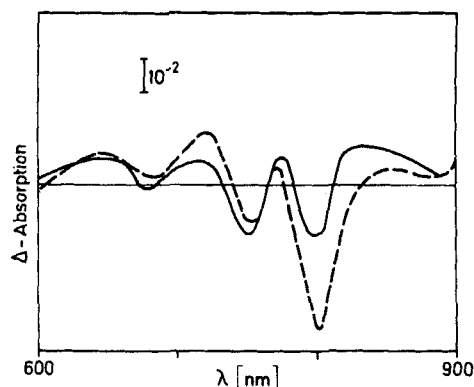


Figure 3. Absorption difference spectra at 298 K of the reaction of RCs with sodium borohydride. The spectra were taken 15 min (—) and 60 min (---) after addition of reagent, with untreated RCs as reference. Concentrations as in Fig. 1. Absorption of the original solution was 0.64 at 870 nm.

During purification of the modified RCs on DEAE-cellulose (Maroti *et al.*, 1985), the modified (reduced at C-3') pigment(s) are removed as indicated by the disappearance of the respective bands (715, 482, shoulder 508 nm). At the same time, the previously turbid solutions become clear again, and the ratio  $A_{800}/A_{870}$  is slightly increased. Typical preparations (Fig. 1, trace C) have an absorption ratio of 1.3.

The spectral changes in the tris-LDAO buffer system are principally similar to the ones described by Ditson *et al.* (1984) in the original procedure. In particular is the decrease of the 800 nm band more pronounced than in subsequent preparations using a tris/TX-100 buffer system (Maroti *et al.*, 1985; Shuvalov *et al.*, 1986). We found that the decrease of the 800 nm band is generally faster and more complete in the presence of LDAO. The 800/860 ratio changes from 2.1 to 1.4 within 6 hr and to 1.2 overnight, whereas a value of 1.6–1.8 was obtained after 24 h with TX-100.

The most pronounced feature in the low temperature absorption spectra (Fig. 2) is (like in the high temperature spectra) a decrease of the 800 nm band due to the loss of the Bchl<sub>M</sub> pigment. In addition, there are some differences resolved which were not discernible in the room temperature spectra (Maroti *et al.*, 1985). Two minor bands become apparent at 780 and 572 nm, and the shape of the Bphe band at 757 nm is different. Instead of consisting of two poorly resolved bands of equal intensity, it now appears to be a main band shifted to longer wavelengths with a short-wavelength shoulder.

The absorption difference spectra taken at later stages of the reaction ( $\geq 60$  min after borohydride addition, Fig. 3) reflect the absorption changes described above, but show as additional features a distinct positive maximum at 717 nm and a smaller one at 770 nm, together with negative bands at 800 and 750 nm. The  $Q_X$  region (data not shown) is

difficult to evaluate quantitatively because of the turbidity. Qualitatively, negative peaks are indicated at 580 and 530 nm and positive ones at 570 and 525 nm. In samples originally contaminated with a 2-deethyl-2-acetyl-chlorophyll *a* (Smith and Calvin, 1966), an additional negative peak around 680 nm appears corresponding to the reduction product(s) of the latter. The broad features in the 620–680 nm region are probably related to the products of this reaction (Steiner *et al.*, unpublished). Similar features are also seen in the published literature (see e.g. Fig. 2c of Maroti *et al.*, 1985).

The difference spectra at early stages of the reaction with NaBH<sub>4</sub> show a somewhat different pattern (Fig. 3). The two negative bands around 800 and 755 nm are of equal intensity. There is also a broad positive band above 800 nm. Its maximum is at shorter wavelengths than the P870: minus P870 difference spectrum (Clayton, 1980), which indicates that it does not arise from some oxidized P870 being present and then becoming reduced at the beginning of the experiment.

Since the reaction is accompanied by an increase of the pH to 10–10.5, the effect of high pH alone was tested in an independent experiment. Up to pH 10.5, the spectrum remains unchanged. At pH  $\geq$  11, the absorption around 860 nm decreases first, accompanied by an increasing absorption at 760 to 770 nm. At even higher pH values (pH 12.0) both the 860 and 800 nm bands diminish completely.

### Fluorescence

The excitation spectra of the P870 fluorescence at low temperatures (Fig. 4) are similar to the absorption spectra. The decrease of the 803 nm band is rather distinct, and a new band at 574 nm is clearly resolved. There is also a change in the 870

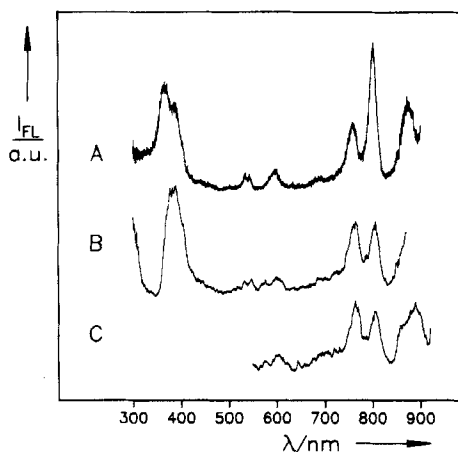


Figure 4. Fluorescence excitation spectra (1.2 K) of modified reaction centers (B,C) and of the original sample before treatment with sodium borohydride (A). Emission wavelengths 920 (A,B) and 933 nm (C). Spectra are not corrected for instrument response.

nm band, which becomes structured and broadened in the modified RC.

The emission spectra in the near infrared show two bands (Fig. 5). One at 917 nm is due to the reaction center, which is only weakly fluorescent. The one around 776 nm arises from contamination with free (= non-aggregated) pigments, Bchl and Bphe, as evidenced by the excitation spectra (not shown). They are strongly fluorescent and thus picked up with high sensitivity in the RC, which is only weakly fluorescent itself. The only significant change of the sample after modification with sodium borohydride, is an increase of this free pigment emission by approximately 150%. This is still at the lower limit of detection by absorption spectroscopy, and is probably responsible for the increase of absorption at 770 as compared to 870 nm in the modified sample (Fig. 1).

### Optically detected magnetic resonance

The fluorescence detected ODMR spectra (Fig. 6) support this interpretation. At short detection wavelengths, the spectra are related to a triplet with transitions at 500(+) and 903 MHz (–) characteristic of free Bchl (Beck *et al.*, 1983a). In the long-wavelength band, the triplet transitions are negative (474, 665 MHz) below 900 nm and change sign about 910 nm (474, 670 MHz). These two triplet signals with slightly different zero-field splittings and the sign reversal between 900 and 910 nm are typical for RC (Beck *et al.*, 1983b; Angerhofer *et al.*, 1984). The spectra of unmodified and modified RC are identical within the experimental error.

The absorption detected ODMR spectra (not shown) of *Rb. sphaeroides* RC show only two lines which are identical over the long wave-length ( $\lambda_{\max}$  = 870 nm) absorption band, and whose intensity follows the profile of this band. The transitions are both positive and have the same zero field splitting parameters as the triplet detected by fluorescence

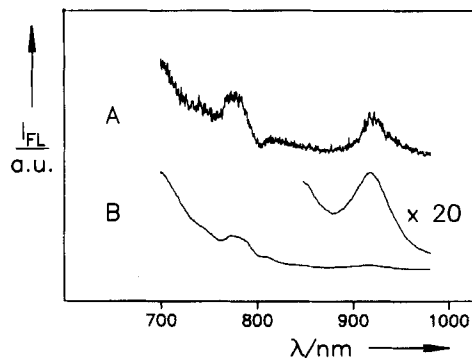


Figure 5. Fluorescence emission spectra (1.2 K) of modified reaction centers (B) and of the original sample before treatment with sodium borohydride (A). Excitation wavelength 390 nm. The inset is an amplification (20 fold) of trace (B).

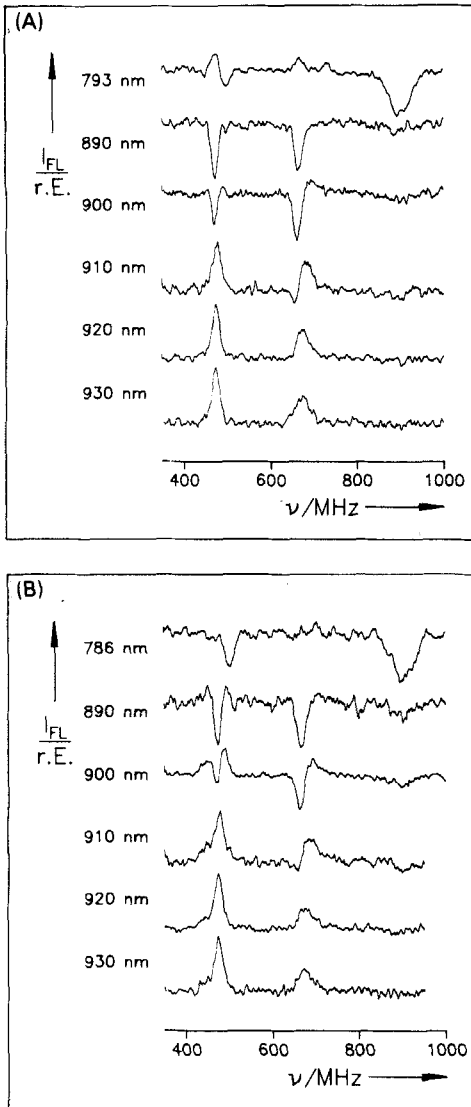


Figure 6. Fluorescence detected magnetic resonance spectra (1.2 K) at zero field of modified reaction centers (B) and of the sample before treatment with sodium borohydride (A). The detection wavelengths for the different traces are given in the insets. See materials and methods for experimental details.

ODMR at detection wavelengths  $\leq 900$  nm. Here again, the spectra of modified and unmodified RC are identical within the experimental error.

#### Microwave induced absorption difference

Significant differences between the two species occur in the microwave induced absorption difference spectra (MIA, also referred to as singlet triplet absorption difference by other authors, see e.g. Hoff 1986). Besides the intense band at 890 nm corresponding to the absorption of the primary donor, it shows a series of narrow bands in the 800 nm region. The absorption changes are identical for the  $|D+E\rangle$  and the  $|D-E\rangle$  transitions, although the

signal-to-noise ratio is better in the latter shown in Fig. 7, trace C. In modified RC, this region shows distinct changes. There is in particular a new positive band at 805 nm and a decrease around 795 nm. These changes become more clearly discernible in the difference spectrum (Fig. 7c). In addition to a dispersive signal at 797 and 802 nm there is a small positive signal at 763 and a negative signal (corresponding to an increase in singlet absorption in the modified RC) at 815 nm.

#### Circular dichroism

The untreated sample (Fig. 8) shows the well-known dichroic absorption maxima at 860 (+), 811 (-) and 795 (+) and a structured negative signal at 748 nm [Philipson and Sauer, 1973; Reed and Ke, 1973]. In the modified RC the S-shaped signal is shifted to shorter wavelengths 809 (-) and 779 nm (+)]. A distinct shoulder (-) becomes visible at 830 nm, and a new positive band appears at a position where the original sample had only a negative shoulder (762 nm). Less pronounced differences between treated and untreated RCs were found in the  $Q_x$ -region. Here the untreated sample shows extrema at 601 (+) and 631 nm (-). They are slightly blue shifted in the modified sample, and the minor one at 574 nm (-) is increased in intensity (Fig. 8). The minor positive band in the Bphe  $Q_x$ -region of untreated RC's has disappeared in the treated ones. The changes in the Soret region are even less pronounced. A minor shift becomes apparent only in the difference spectrum (not shown).

Quantitatively, the intensity of most bands is diminished. In the near infrared region, the positive peak at 860 nm commonly assigned to the special pair is decreased to 42% in treated RCs as compared to untreated RCs, the intensity of the S-shaped

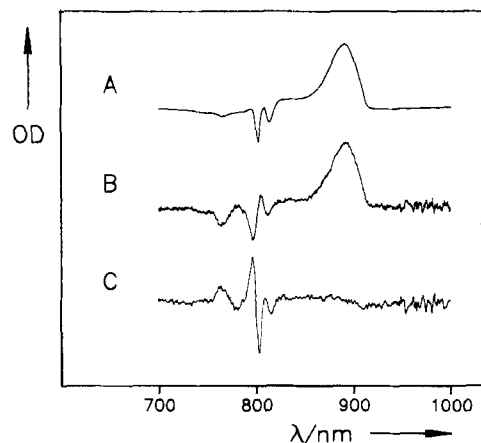


Figure 7. Microwave induced singlet triplet absorption difference spectra (MIA), at 1.2 K of modified RC (B) and of the original sample before treatment with sodium borohydride (A). Difference of the two spectra (C). All spectra were detected at the  $|D-E\rangle$  transition.

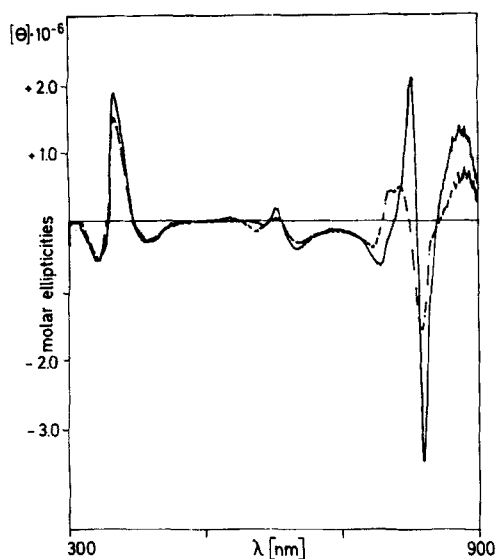


Figure 8. Circular dichroism at 298 K of RC in the relaxed state in the presence of sodium ascorbate (1mM). Before treatment with sodium borohydride (—), and after treatment and purification on DEAE cellulose (---). See Table 1 for wavelengths and molar ellipticities.

signal is even decreased to 37%. This reduced intensity of the strongest bands may be a major reason for the more structured CD spectra. Two bands in the  $Q_x$  region are decreased in intensity as well. The most significant change in this region is an increase of the negative extremum at 574 nm.

The CD spectra of chemically oxidized RC show the most pronounced changes in the 800 nm region (Fig. 9). The positive band at 799 nm which dominates the spectrum of the untreated RC, is decreased to 27%, and blue-shifted. Smaller changes include the shift of the negative band

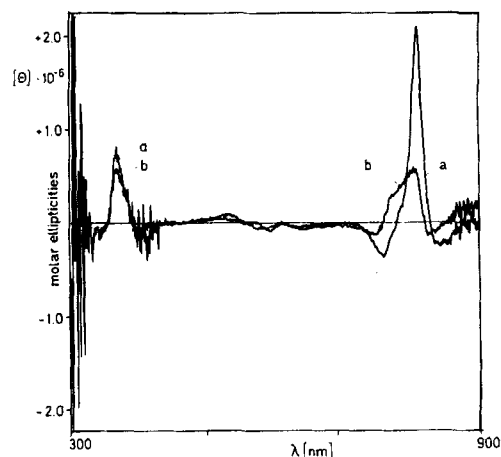


Figure 9. Circular dichroism at 298 K of RC in their oxidized state (P870<sup>+</sup> before (a) and after (b) treatment with sodium borohydride and purification on DEAE cellulose. See Table 1 for wavelengths and molar ellipticities.

around 762 to 738 nm, and the appearance of a short wave-length shoulder of the 800 nm band. Again, the changes in the other spectral regions are much less pronounced.

#### Raman-resonance spectroscopy

The Raman resonance (RR) spectra in the 1550–1750  $\text{cm}^{-1}$  spectral range of both untreated and borohydride treated RC are shown in Fig. 10. Treatment of the RC with borohydride results in a distinctive enhancement of the 1590  $\text{cm}^{-1}$  band relative to the 1615  $\text{cm}^{-1}$  one, indicating a decrease in Bchl concentration relatively to Bphe concentration. Similar quantitative changes are also observed in other regions of the RR spectra (not shown). The changes are more clearly seen in the

Table 1. Circular dichroism spectra [wavelength in nm (molar ellipticities  $\times 10^{-6}$  in  $\text{deg}\cdot\text{cm}^{-2}\cdot\text{mol}^{-1}$ )] of unmodified and modified reaction centers

Relaxed state		Oxidized state	
Unmodified	Modified	Unmodified	Modified
340 (-0.57)	342 (-0.56)	369 (+0.79)	341 (-0.13)
369 (+1.88)	368 (+1.52)	334 (-0.43)	369 (+5.65)
420 (-0.29)	420 (-0.26)	399 (-0.21)	405 (-0.15)
			398 (-7.52)
			400 (-0.07)
601 ( 0.17)	574 (-0.15)	591 (-0.11)	574 (-0.09)
631 (-0.41)	631 (-0.32)		586 (-0.09)
			611 (-0.034)
748 (-0.67)	737 (-0.40)	751 (-0.40)	738 (-0.15)
795 (+2.07)	762 (+0.41)	799 (+2.09)	792 (+0.56)
811 (-3.52)	779 (+0.48)	829 (-0.28)	812 (-0.17)
860 (+1.34)	809 (-1.60)		857 (+0.20)
	860 (+0.56)		

Relaxed state in the presence of sodium ascorbate (1mM), oxidized state in the presence of potassium ferricyanide (50 mM). Concentrations determined from A870 with molar absorptivity =  $1.28 \times 10^5 \text{ cm}^{-1}\text{M}^{-1}$  (Clayton and Wang, 1971).

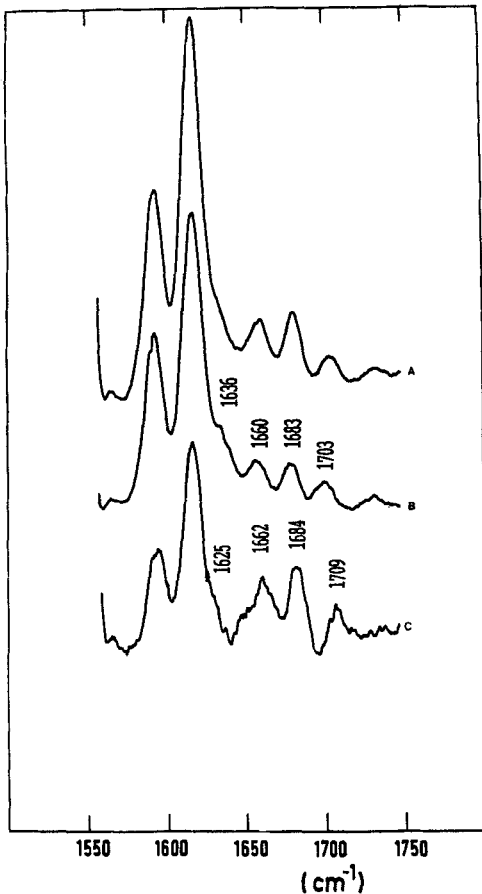


Figure 10. Raman resonance spectra of RCs before (a) and after treatment with sodium borohydride and purification on DEAE cellulose (b). Difference (a minus b) of the two spectra (c).

difference spectrum between untreated and borohydride treated RC (Fig. 10c). For this computation spectra have been properly weighted, by taking into account that the  $1700\text{ cm}^{-1}$  band arises purely from

the  $535\text{ nm}$  absorbing Bphe (Lutz and Robert, 1985). This difference spectrum displays two bands at  $1663$  and  $1680\text{ cm}^{-1}$  and two small S-shaped signals corresponding to shifts of bands occurring in untreated RC at  $1700$  and  $1625\text{ cm}^{-1}$ .

#### SDS-polyacrylamide gel electrophoresis

The studies pertaining to a change in the protein moiety were initiated by a chance observation. During routine tests for the polypeptide composition of RC after the borohydride treatment, we noticed an unusual band in SDS-polyacrylamide electrophoresis. The untreated samples show three peptides (H, M and L with apparent MW 28, 24 and 21 kDa, respectively), whereas in the treated ones an additional peptide fragment appears with an apparent MW of about 15.0 kDa (Fig. 11). At the same time, the intensity of the M band diminished, which suggest the latter as the origin of the new peptide with an apparent MW of 15 kDa. In view of the high pH (10–10.5) attained during the reaction with  $\text{NaBH}_4$ , control samples were subjected to pH 11 without addition of the reagent. Under these conditions, the additional polypeptide did not appear (Fig. 11). This indicates that only the  $\text{NaBH}_4$ -reduction but not the accompanying pH change leads to the cleavage of the peptide.

#### DISCUSSION

##### Homogeneity of the preparation

It had originally been proposed that the modification with sodium borohydride produces a species which lacks one of the Bchl pigments (Ditson *et al.*, 1984). There are, however, some questions about this straightforward interpretation. One is, that a modified pheophytin rather than the respective chlorophyll has been extracted from the reaction

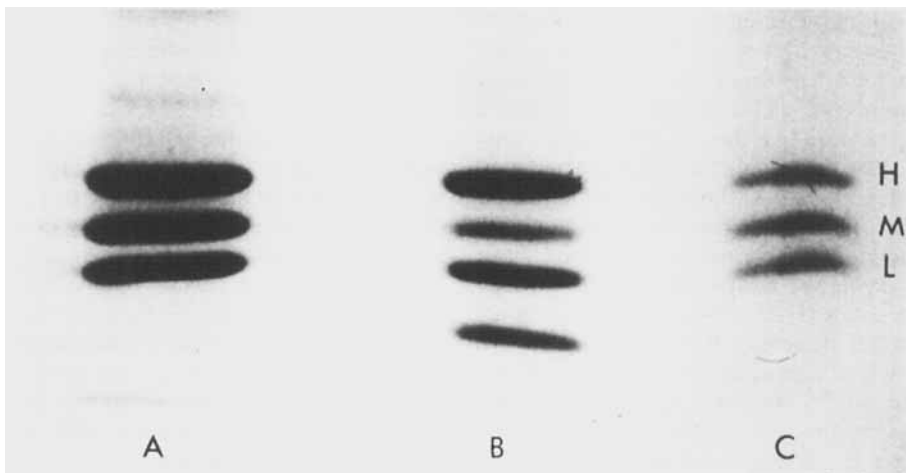


Figure 11. SDS polyacrylamide gel electrophoresis of RCs before (trace A) and after treatment with sodium borohydride for 16 h (trace B), and after incubation at pH11 for the same time (trace C). Gels stained with Coomassie brilliant blue.

mixture (Maroti *et al.*, 1985), although pheophytinization is unlikely at the high pH of the reaction. Shuvalov *et al.* (1986) have subsequently presented rather different conclusions on the identity of the preparation. Based on a comparison with RC from *Chloroflexus (C.) aurantiacus*, they suggested the presence of three species in the preparations: One (comprising about 40% of the mixture) containing a bacteriopheophytin of unspecified structure at the site of Bchl<sub>M</sub>, a second one (also about 40%) containing neither Bchl<sub>M</sub> nor Bphe<sub>M</sub>, and finally unmodified RC (20%). According to their argument, none of the species present would be the one lacking only Bchl<sub>M</sub>. The samples of these authors have been prepared in the same buffer system (LDAO as detergent) as used originally by Ditson *et al.* (1984), but the reaction has been stopped at an earlier stage, and the relative absorptions of the near infrared bands are different from ours. The situation is further complicated by our finding that the pigment modification is accompanied by a protein modification, more specifically by an apparent cleavage of a portion of the M-subunit (*vide infra*).

In view of the potential importance of the modified RC, it is then crucial to establish their homogeneity both in biochemical and in spectroscopic terms. Since Bchl<sub>M</sub> is apart less than 5 Å from the special pair, e.g. P870, it is expected that any change in its structure or its removal should lead to changes in the spectroscopic properties. We have therefore concentrated in the current work on methods which are sensitive to the state of P870 and to the homogeneity of the sample.

The most obvious contamination are free tetrapyrroles still adsorbed to the RC. If judged from the fluorescence emission in the near infrared, the same residual pigments, e.g. "free" Bchl and Bphe, are present both in modified and unmodified RC, although their amount is increased by a factor of 2.5 in the modified ones. In view of the high sensitivity of fluorescence spectroscopy for free pigments, we consider this impurity insignificant, however. By the same argument, fluorescence is then unsuited to detect any small contaminations having as low fluorescence yields as the intact complex. However, a mixture like the one discussed by Shuvalov *et al.* (1986) containing roughly equal amounts of three different species, should be visible if these species differ in their fluorescence spectra.

Magneto-optical double resonance methods have been used among the most sensitive tools to study the primary donor (Hoff, 1986). It has been shown by fluorescence detected ODMR, that there are (besides any free pigments) generally at least two triplets detected at long wavelengths in RC of two different photosynthetic bacteria, e.g. *Rb. sphaeroides* (Beck *et al.*, 1993b; den Blanken, 1983; den Blanken and Hoff, 1983) and *Rp. viridis* (Angerhofer *et al.*, 1984). The origin of these two slightly different signals with different signs is still under

discussion, but at least one of them visible also by absorption detection, is due to the triplet of the primary donors, P870 and P960, respectively. The modified sample shows identical fluorescence detected spectra with untreated RC. There is in particular no additional signal above 800 nm detection wavelength, and at shorter wavelengths there is only the signal which is typical for free pigments. In absorption mode, too, the modified sample is identical to the original one. If there were any mixture of different species present, the zero field splitting parameters and population-depopulation kinetics of their primary donors would then have to be very similar to each other. One of the species suggested by Shuvalov *et al.* (1986) has a pigment composition similar to RC from *C. aurantiacus*, whose zero-field splitting (zfs) values differ from those of *Rb. sphaeroides*. This species also shows a rather different MIA-spectrum (den Blanken *et al.*, 1983). Between 750 and 850 nm it shows a single strong absorption at 807 nm, a rather broad band around 770 nm with less intensity and additionally small bleaching at about 818 nm. From these characteristic features which were not reproduced in the modified RC from *Rh. sphaeroides*, it can be excluded that 40% of the RC-preparation have a structure similar to the ones of *C. aurantiacus*.

The finding of a protein modification in addition to the removal of one pigment molecule, makes the situation more complex again. Presently, we interpret this as a selective cleavage of one of the three RC subunits, e.g. M, since the 15 kDa band decreases the expense of the M-band. The possibility of proteolytic side reactions with sodium borohydride is documented (see e.g. Crestfield *et al.*, 1962). It should be noted, however, that the MW of hydrophobic polypeptides as estimated by SDS-PAGE is prone to considerable error (see e.g. the membrane polypeptides of photosynthetic bacterial membranes; Theiler *et al.*, 1984). Other modifications than cleavage can therefore presently not be excluded rigorously. Whatever the cause of the reaction, it is a result of the borohydride treatment rather than of the increasing pH that accompanies the reaction. Treatment of RC at high pH does not alter the polypeptide pattern even if the reaction center is already denatured (pH > 11). In view of the importance of the NaBH<sub>4</sub> reaction, the identity of the new polypeptide is currently explored.

In principle two different mechanisms could then be operative during reaction with borohydride. One is that all spectroscopic changes are essentially due to the removal of the pigment, and the spectroscopic changes induced by the protein modification are negligible. The other one is just the opposite. The protein modification induces such pronounced changes in the arrangement or environment of the remaining pigments, that these dominate the spectroscopy. The extreme of the second mechanism would not be unlikely in view of the tight packing



of the pigments in RC. Since the decrease of the intensity of M, and the concomitant increase of the 15 kDa band, amount to less than 40% at the end of the reaction, the final preparation should then be a mixture of two spectrally different products. This possibility is disfavored, however, by the spectroscopic results discussed above, which gave no indications of such a mixture being present. This leaves the first proposal, e.g. that spectroscopic changes induced by proteolysis (?) are insignificant at least as far as the methods applied are concerned. This result would be in line with earlier studies on Bchl proteins including RC from several bacteria, which showed remarkably little effects of proteolysis on their spectra and function (Wiemken and Bachofen, 1984; Steiner *et al.*, 1986).

#### Assignment of the pigment removed

The changes in the high (Fig. 1) and low temperature absorption spectra (Fig. 2) upon treatment with borohydride seem to be very simple (see below, however): there is a decrease of the 800 nm  $Q_y$  absorption band by approx. 40% and a decrease and split of the  $Q_x$  band around 580 nm. In untreated RC, the 800 nm band arises mainly from the two extra Bchl molecules, but other pigments, in particular P870, contribute to it. A decrease of this band by 40%, as well as the decrease of the  $Q_x$  band would then be compatible with the removal of one of the two Bchl.

An assignment of the pigment removed to Bchl<sub>M</sub>, can be made from several lines of arguments. The first is based on the CD spectra, which show much more pronounced changes than the absorption spectra (Figs. 8 and 9). Essentially all bands in the near infrared region are effected by the treatment, and the decrease in signal intensity is considerably more than expected for removal of a single, isolated pigment. Shuvalov *et al.* (1986) recently presented similar spectra of NaBH<sub>4</sub> treated RC. There are, however, distinct quantitative differences, the most significant one being that these authors found the intensity of the 870 nm band unchanged. This may reflect the different preparations. Since the relative band intensities in both preparations are rather similar, it is more likely, however, that the spectra of Shuvalov *et al.* are normalized to equal intensities of the 870 nm CD band. In the figure legend, this is only indicated for the low temperature spectra, however. (See note added in proof.)

The two pigments Bchl<sub>L</sub> and Bchl<sub>M</sub> are situated between P870, and the Bphe<sub>L</sub> and Bphe<sub>M</sub> molecules, respectively. The most pronounced effects upon removal of one of them are then expected on the immediate neighbors, e.g. P870 and Bphe<sub>M</sub> or Bphe<sub>L</sub>, respectively. The decrease of the 870 nm band then reflects the former interactions. The changes in the Bphe spectral region can be used to locate the removed pigment on the M-branch. The

CD spectrum of untreated RC show two poorly resolved negative bands in this region, which are present both in the reduced (P870) and the oxidized (P870<sup>+</sup>) complex. The major one is located at 757 nm and blue-shifted in the treated complex. The smaller one at longer wavelengths (shoulder at 773 nm) shows an apparent sign inversion, although it may be positive and superimposed on the stronger, negative band in the untreated RC. The absorptions of the two Bphe in the  $Q_y$ -region are only poorly separated, with Bphe<sub>L</sub> absorbing at 760 nm, and Bphe<sub>M</sub> at somewhat shorter wavelengths. The pronounced blue-shift of the latter would then place the removed Bchl on the M-branch. Unfortunately, the optical activity of the Bphe's is too small in the  $Q_x$  region to further evaluate this assignment.

The second line of arguments is based on the Raman resonance data. The frequency range presented in the results (Fig. 10) contains bands arising from double bond stretching modes, from which much information can be extracted: A methine bridge stretching mode of the Bchls occurs around 1615 cm<sup>-1</sup>, the frequency of which has been shown to be sensitive to the number (one or two) of axial ligands on the central Mg atom (Lutz and Robert, 1985). The stretching modes of the 2-acetyl and 9-keto carbonyls conjugated to the bacteriochlorin  $\pi$ -systems also occurs in this range (1620–1720 cm<sup>-1</sup>). These groups are present both in Bchl and Bphe and have been shown to play a key role in the intermolecular interactions of these pigments both *in vivo* and *in vitro* (Lutz and Robert, 1987). It was shown, that the 1590 cm<sup>-1</sup> band arises mainly from Bphe, and the relative intensity ratio between the 1590 and 1615 cm<sup>-1</sup> band can yield information about the Bchl/Bphe ratio.

The decrease of the 1615 cm<sup>-1</sup> band then reflects the removal of the Bchl (B800). This is further corroborated by bands in other spectral regions which are specific for either Bchl or Bphe. The former bands are always weakened relatively to the bands purely arising from Bphe (data not shown). The disappearance of two carbonyl groups in the difference spectrum corresponds to the removal of one and only one pigment in the RCs. Moreover, the possibility to compute this difference spectrum indicates that no important change in the Soret bands happens during this bleaching; otherwise no normalization between the spectra of Fig. 10 resulting in a coherent difference spectrum could have been done.

Based on this bleaching, it is now possible to assign the CO bands in the removed Bchl and at the same time infer their bonding states. The accessory Bchl that is removed upon borohydride treatment has one carbonyl band at 1662, and one at 1684 cm<sup>-1</sup>. The former one is assigned to the 3-acetyl group, because *in vitro* studies with Bchl *a* have shown this group between 1620 and 1660 cm<sup>-1</sup>, depending on its binding state. Its occurrence at

1662  $\text{cm}^{-1}$  then indicates that the 3-acetyl group is free in the Bchl removed. The band at 1684  $\text{cm}^{-1}$  is assigned to the 13<sup>1</sup>-keto group, which is at a frequency indicative of its being engaged in weak (or no) intermolecular interactions.

A more detailed inspection of the difference spectrum makes it clear, that borohydride treatment induces structural changes in the local environments of other pigments in addition to the bleaching of one accessory Bchl. Indeed, the 1625 and 1700  $\text{cm}^{-1}$  bands, both corresponding to the carbonyl stretching modes of the 535 nm absorbing Bphe<sub>M</sub> (Lutz and Robert, 1985) shift by a few  $\text{cm}^{-1}$  (less than 2  $\text{cm}^{-1}$  upshift for the 1625  $\text{cm}^{-1}$ , and 4  $\text{cm}^{-1}$  downshift for the 1700  $\text{cm}^{-1}$  band). From this fact, it can first be concluded, that the missing Bchl in borohydride treated RCs is most probably the one which is bound to the M branch, as the signals which appear the most influenced by the treatment are both due to Bphe<sub>M</sub>. The shifts of the Bphe<sub>M</sub> related bands are too small to correspond to any qualitative change in the liganding of the carbonyl groups; in particular, the keto group, which is free from bonding in untreated RC (1708  $\text{cm}^{-1}$ ) remains free upon borohydride treatment (1703  $\text{cm}^{-1}$ ). These changes may be induced directly by the removal of the Bchl<sub>M</sub> molecule, and its possible "replacement" by solvent molecules. Alternatively, they may as well be induced indirectly by this removal and result from local rearrangement of the protein around the molecule, involving e.g. displacement of a charged or aromatic residue. Such a rearrangement may be related to the same protein modification which gives rise to the new polypeptide of apparent MW 15 kDa.

The assignment of the removed Bchl to the M-branch (Bchl<sub>M</sub>) supports earlier kinetic results. The primary charge separation is not influenced by removal of one Bchl by treating RCs of *Rb. sphaeroides* with borohydride (Maroti *et al.*, 1985). It has been argued on this basis, that the Bchl removed is the one located on the 'inactive' or M side of the RC. In view of the yet undefined function of both Bchl pigments, this conclusion was not unambiguous, however, and has now been further substantiated.

#### *Differences in pigment interactions between modified and unmodified RC*

As discussed above, circular dichroism and light-induced absorption changes (Maroti *et al.*, 1985; Shuvalov *et al.*, 1986) of modified and unmodified RC show distinct differences which indicate rather complex couplings of the related bands. Corresponding, albeit less pronounced, effects are also observed in Raman resonance spectra.

Knapp *et al.* (1986) were able to reproduce a variety of optical spectra in the NIR spectral region of the RC from *Rp. viridis* based on the x-ray data

of Deisenhofer *et al.* (1984). More recently, this analysis has been extended to the RC of *Rb. sphaeroides* and *C. aurantiacus* (Scherer and Fischer, 1986). Scherz and Parson (1984) have used a different approach which emphasizes the strong hyperchromism of the Q<sub>y</sub> at the expense of the shorter wavelength bands upon aggregation of chlorophylls, which is even more pronounced in the CD spectra. Both treatments point to the strong couplings existing among the pigments in RC, such that most bands cannot be assigned to individual pigments but rather are of complex origin. The qualitative and quantitative changes observed upon removal of one of the six pigments support these couplings. The large signal decrease in the NIR and a much smaller decrease in the Soret can also be accounted for by hyperchromism. A full analysis of the spectral data is currently under way, which should also be a test case for the theoretical models on which the calculations are based.

The difference in the MIA spectra are another reflection of a change induced by the modification on the primary donor. At low excitation power, the observed spectrum is the difference between singlet and triplet absorption spectra of the primary donor and has been introduced under this name to photosynthetic systems (den Blanken and Hoff, 1983). It is thus selective to the primary donor, and it is also very sensitive due to the presence of a series of narrow bands in the 800 nm region. The spectral changes in the modified RC (Fig. 7) are very pronounced and indicate considerable differences in the coupling of the primary donor. The MIA spectra have recently been modelled for RC from three different bacteria (Knapp *et al.*, 1986; Scherer and Fischer, 1987) on the basis of the x-ray data of the RC from *Rp. viridis* (Deisenhofer *et al.*, 1984). A similar analysis assuming that Bchl<sub>M</sub> is removed in the modified RC, gave a reasonably good fit of the experimental data (Scherer *et al.*, to be published). Together with the Raman resonance data, we take this as further evidence that the major change induced by treatment with sodim borohydride, is indeed the removal of Bchl<sub>M</sub> as suggested originally by Ditson *et al.* (1984).

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*Note added in proof.* Drs Shuvalov and Shkuropatov have communicated to us that the room temperature CD spectra published in Shuvalov *et al.* (1986) were normalized at 870 nm. The decrease in modified RC was less than 20%. In view of the lesser degree of modification (judged from the ratio of the 800 and 870 nm absorption bands), this value is compatible with our data.