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Photochemistry and electron transfer in borohydride-treated photosynthetic reaction centers

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The photochemistry and electron-transfer activities of sodium-borohydride-treated reaction centers from the purple photosynthetic bacterium *Rhodopseudomonas sphaeroides* R26 have been investigated by both milli- and picosecond absorption techniques. Separation from the treated reaction center of the reduction product, apparently a reduced form of one of the two molecules of bacteriochlorophyll contributing to the 800 nm ground-state absorption band, is also reported. In the near-infrared region, differences between treated and untreated reaction centers are observed in both milli- and picosecond light-induced difference spectra. However, borohydride-treated reaction centers exhibit photochemistry and electron transfer which are indistinguishable from those observed in untreated reaction centers. These results indicate that normal activity occurs in reaction centers that contain both molecules of bacteriopheophytin, but only three of the usual four molecules of bacteriochlorophyll.

Introduction

Reaction centers from the purple photosynthetic bacterium *Rhodopseudomonas sphaeroides* contain six bacteriochlorin pigment molecules per reaction center, four of bacteriochlorophyll (BChl) and two of bacteriopheophytin (BPh) [1,2]. The primary charge separation process involves electron transfer in less than 10 ps from the excited singlet state of the primary electron donor (P), a complex of two of the BChls, to an electron acceptor (I), thought to involve one BPh and possibly one of the remaining BChls. This step is followed by electron transfer from I^- to the primary ubiquinone (Q_A) in approx. 200 ps at room temperature [3–5]. The functions of the BChl and BPh not implicated in P or I are completely unknown at present. That a BChl has a role in I, or as an intermediary electron carrier between P* and I, is also uncertain [6].

In the near-infrared ground-state absorption spectrum, the two BPh molecules give rise to a band near 760 nm, while the four BChl molecules contribute to two bands near 800 and 865 nm. The 865 nm band is due to P, while the 800 nm band has been associated mainly with the other two BChl molecules [2–5]. Recently, Ditson et al. [7] have shown that if a detergent-containing suspension of reaction centers from *Rps. sphaeroides* R26 is exposed to a high concentration of $NaBH_4$ at

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

moderately alkaline pH (approx. 10) for 8 h or so, the reaction center absorption spectrum is irreversibly altered: the 800 nm absorption diminishes by 40–50%, while the 760- and 865 nm bands are affected very little. At the same time, a new band grows in near 715 nm. With the aid of ancillary experiments on purified BChl and BPh in solution, it was concluded that one of the two 800 nm absorbing BChl molecules in the reaction center is converted nearly stoichiometrically by the borohydride reaction to a product absorbing near 715 nm. This product appears to be the Mg-free derivative of the affected BChl, in which only the acetyl group is reduced by $NaBH_4$ and not the ring-V keto group [7]. The remaining five pigments in the reaction center are apparently unaffected. Left unanswered were the questions of whether the BChl reduction product remains bound to the reaction center, and especially whether the borohydride-treated reaction centers retain photochemical activity.

We report here the results of investigations on borohydride-treated reaction centers from *Rps. sphaeroides* R26 which indicate that such reaction centers retain full photochemical activity. The BChl molecule in the reaction center that is reduced by $NaBH_4$ and subsequently removed thus appears to play no essential role in bacterial photosynthetic photochemistry or electron transfer.

Materials and Methods

Reaction centers from *Rps. sphaeroides* R26 were prepared as described [8]. For borohydride treatment, samples containing approx. 2 μM reaction centers in 220 ml 0.03% Triton X-100/10 mM Tris (pH 8.0) were placed in a degassed vessel and stirred under nitrogen atmosphere in the dark, while sufficient solid $NaBH_4$ was added to raise the pH to above 10; pH was monitored continuously. The progress of the reaction was followed by removing 1.0 ml aliquots from the reaction vessel, diluting 2-fold with 10 mM Tris (pH 8.0), and recording an absorption spectrum from 630 to 950 nm. Best results were obtained when the pH was held between 10.5 and 10.7 and the reaction allowed to proceed for 15 h or more. Under these conditions little overall sample degradation was noted, even for reaction times of 24 h or more, in contrast to earlier work in which rigorous protection of the sample from O_2 and light was not maintained during the borohydride reaction [7]. After completion of the reaction, samples were dialyzed overnight against 10 mM Tris buffer containing 0.03% Triton X-100. Separation of free pigment was achieved by loading the borohydride -treated samples onto a 2 × 5 cm DEAE-Sephacel column, pre-equilibrated with 0.1% LDAO/10 mM Tris (pH 8.0). The reaction centers were retained on the column while the straw colored eluant was collected. The column was then washed with 50 mM NaCl/0.1% LDAO/10 mM Tris (pH 8.0). Little or no additional color appeared in the eluant at this stage. The reaction centers were removed with 250 mM NaCl/0.1% LDAO/10 mM Tris (pH 8.0), dialyzed against 0.03% Triton X-100/10 mM Tris (pH 8.0)/20 μM EDTA and concentrated on an Amicon ultrafiltrator using an XM-100A filter.

Steady-state absorption and fluorescence spectra were recorded with commercial instruments (Cary 14 or 219, and an SLM 4000, respectively). The flash-induced kinetics in the milliseconds-to-seconds time range (P^+ kinetics and cytochrome *c* oxidation) were measured with a home-built single-beam spectrophotometer, with actinic flashes provided by a 10 μs xenon flashlamp.

The 10 Hz dual-beam picosecond transient absorption spectrometer used for the present studies has been described previously [9,10]. The 30 ps, 600 nm excitation flashes were attenuated to approx. 100 μJ, so that they were just saturating with respect to bleaching in the 865 nm band. For measurement of transient difference spectra and near-infrared kinetics, samples containing approx. 6.5 μM treated or untreated reaction centers were maintained at approx. 15°C, while flowing through a 4 mm pathlength optical cell. Kinetics in the visible were measured on approx. 35 μM samples in a 2 mm pathlength cell.

Results and Discussion

Absorption spectra

Fig. 1 shows a series of absorption spectra of reaction centers at various times after the start of the borohydride reaction. These spectra are similar to those reported by Ditson et al. [7], except that

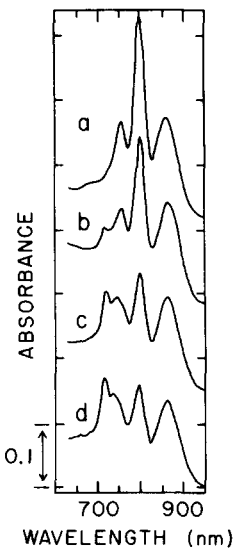

Fig. 1. Absorption spectra of reaction centers treated for varying times with $NaBH_4$: (a) 0 h; (b) 8 h; (c) 16 h; (d) 20.5 h. The baselines are shifted vertically by an arbitrary amount. In each case the absorbance at 950 nm is a true zero.

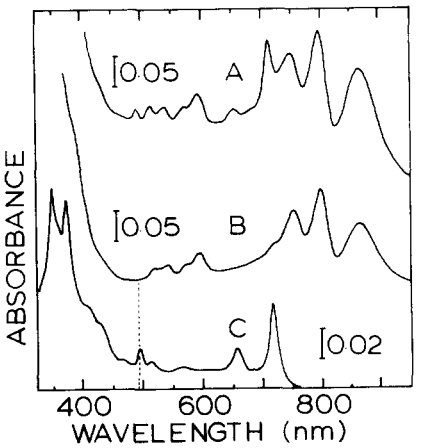

Fig. 2. Separation of the reduction products from reaction centers exposed to $NaBH_4$ for 15 h. (A) Absorption spectra of reaction centers after dialysis but before loading onto DEAE-Sephacel. (B) Absorption spectrum of reaction centers after purifying on DEAE-Sephacel. (C) Absorption spectrum of first eluant, containing only free pigment. Spectra are shifted vertically, as in Fig. 1.

we have been able to achieve a 50% reduction of absorption at 800 nm with substantially less overall degradation of the spectrum after prolonged borohydride exposure. The 865 nm peak declined by only 12% in the course of the treatment (21 h), during which the A_{800}/A_{865} ratio diminished from 2.2 to approx. 1.1. The precise ratio after the treatment is subject to some uncertainty due to a significant baseline arising from light scattering. The A_{800}/A_{865} ratio was found to have increased by 15–20% after final purification and concentration of the treated samples, so that the value for the preparations used in most of the measurements described below was about 1.4–1.5. If the endpoint of the borohydride treatment is taken as a ratio of 1.1, i.e., 50% of the native value, a ratio of 1.4 corresponds to approx. 70% modified reaction centers in the treated samples. The increase in A_{800}/A_{865} probably arises from an increased lability of the modified reaction centers, leading to a relative enrichment of native over modified reaction centers during chromatography.

Spectra of a partially treated sample (A_{800}/A_{865} = 1.4) before and after ion-exchange chromatography are shown in Figs. 2A and 2B, respectively. Note the substantial loss of the strong band near 715 nm after chromatography. Because this band is attributed to the BChl reduction product, we interpret this result to mean that the reduction product is not firmly bound to the reaction center. This suggests that the acetyl group of the borohydride-modifiable BChl, apparently the major point of attack of the borohydride [7], participates in the binding of this BChl to one of the reaction center's polypeptides. The long-wavelength absorption maxima at 655 and 715 nm in the pigment fraction (Fig. 2C) are consistent with the previous suggestion that the reduction product is 2a-deoxo-2a-hydroxy BPh *a* [7].

The washed reaction centers, after removal of free pigments, still retain several modified spectral features (Fig. 2B). These are apparent as distinct shoulders at 518, 574 and 718 nm, which roughly correspond to peaks in the spectrum of the eluted free pigments at 512, 564 and 716 nm. The free pigment peak at 495 nm is not seen in the treated reaction center spectrum.

Fluorescence spectra

The reduction products were further studied by fluorescence emission and excitation spectral analysis. The separated products, eluted from a DEAE-Sephacel column, showed two fluorescence

emission peaks at 660 and 717 nm, of similar intensity when excited at 370 nm. Almost identical peak positions were observed at room temperature and at 77 K. The excitation spectra of the two fluorescence bands were quite different. The 717 nm emission was effectively excited at 350, 375, 495 and 655 nm, in good agreement with the major features of the absorption spectrum of this sample (Fig. 2C). The 660 nm emission, however, showed major excitation peaks at 410, 430 and 615 nm, all of which are very minor features of the absorption spectrum. Similar but much weaker fluorescence was observed for the washed reaction centers, which retain several spectral features of the modified pigments in the absorption spectrum (Fig. 2B). However, the 717 nm emission exhibited an excitation peak at 515 nm, in agreement with the absorption spectrum, rather than at 495 nm as observed for the free pigment.

These results indicate the existence of (at least) two different reduction products of reaction centers exposed to prolonged borohydride treatment, or of one reduction product that is further modified during the subsequent work-up and chromatography. However, the 715 nm absorbing species is by far the dominant form in the free pigment state.

Photochemical and electron-transfer activity

Millisecond measurements. The treated reaction-center preparations are fully active photochemically and in regard to electron transfer. (This is also true before ion-exchange chromatography.) With no added ubiquinone or exogenous donors, flash illumination generated the state $P^+Q_A^-$ which recombined with a half-time (approx. 70 ms) indistinguishable from that of untreated reaction centers [11]. The percent bleaching of the 865 nm absorption band induced by a saturating flash was also the same as for untreated samples (approx. 88% [1]), indicating full activity in the treated preparation. Measurements of the relative quantum yield of cytochrome photooxidation in treated and untreated samples having the same 865 nm absorbance showed them to be the same within error ($\pm 10\%$), using either 600 or 860 nm excitation (Fig. 3). Cytochrome *c* oxidation is sufficiently tightly coupled to the rereduction of P^+ that this measurement represents a good measure of the photochemical yield [12,13].

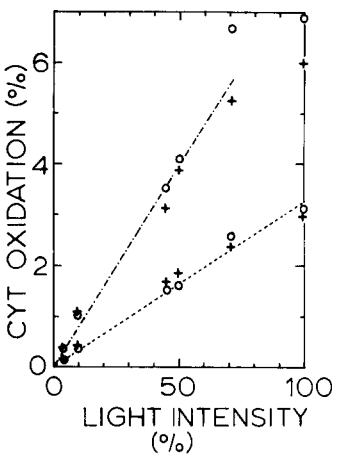

Fig. 3. Light-intensity dependence of cytochrome-*c* (CYT) oxidation measured at 550 nm on $NaBH_4$-treated (O) and untreated (+) reaction centers at two excitation wavelengths (600 nm, ------ and 860 nm, ·–·–·). 100% cytochrome-*c* oxidation, measured at 550 nm, refers to maximal turnover obtained with saturating (broad band) excitation. 100% light intensity refers to the unattenuated level of the two actinic wavelengths. Reaction centers (approx. 1 μM) were suspended in 10 mM Tris (pH 8.0)/100 mM NaCl/0.06% Triton X-100/20 μM ubiquinone-50 (Q-10).

Secondary quinone (Q_B) activity could be largely reconstituted by addition of ubiquinone. The P^+-rereduction kinetics in the absence of donors, but with excess ubiquinone, revealed fast ($t_{1/2} \approx 70$ ms) and slow ($t_{1/2} \approx 500$ ms at pH 8.0) components, which are associated with charge recombination of $P^+Q_A^-$ and $P^+Q_B^-$ respectively [11, 14,15]. These rates are not significantly different from untreated samples under similar conditions, i.e., in the presence of 0.06% Triton X-100 [14]. The relative amounts of fast (20%) and slow (80%) components in the treated samples indicate almost full reconstitution of Q_B activity, since even the controls showed 10% fast recombination.

Donation to P^+ by added cytochrome *c* is also unaffected by the borohydride treatment. Repetitive turnover in a series of flashes, with excess cytochrome *c* and ubiquinone, demonstrated that complete electron transport through the reaction center takes place (Table I). The magnitude of the cytochrome oxidation on the first flash yields the same amount of reaction center activity as was determined from the reaction center absorption spectrum and from the flash-induced P^+ signal.

TABLE I

CYTOCHROME TURNOVER BY $NaBH_4$-TREATED REACTION CENTERS.

Conditions as in Fig. 3.

Flash number	1	2	3	4	5	6
$\Delta A \times 10^3$ (500 nm)	−4.61	−3.87	−4.09	−3.70	−3.87	−3.78
ΔA (normalized)	1.0	0.84	0.89	0.80	0.84	0.82

The ratio of the second cytochrome turnover to that on the first flash (Table I) is in good agreement with the 80% reconstitution of Q_B activity determined from the P^+-rereduction kinetics. The small amplitude oscillations in the cytochrome turnover arise from the one-electron equilibrium: $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ and show that the two-electron gate function of the acceptor quinone complex is unperturbed by the borohydride treatment [8,15, 16]. The extent of cytochrome oxidation on a second flash relative to the first in a series remained constant (0.80 ± 0.05) as the flash repetition period was reduced from 1 s to 30 ms, showing that the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron-transfer

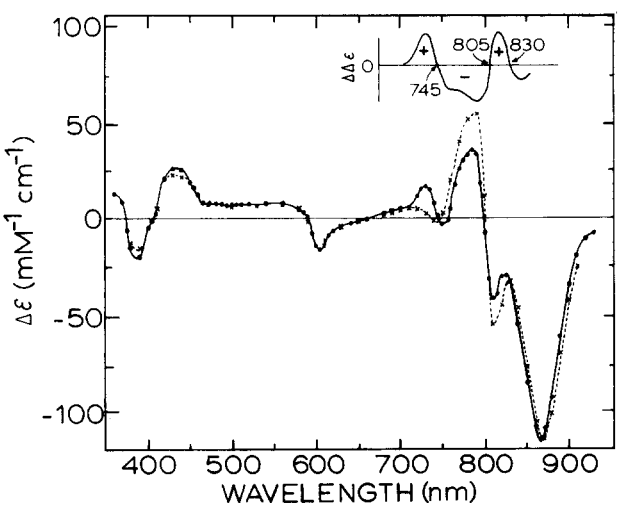

Fig. 4. Xenon flash-induced (ms) difference spectrum of $NaBH_4$-treated (●) and untreated (×) reaction centers, after dialysis in 10 mM Tris (pH 8.0)/0.03% Triton X-100. The samples contained 1–2 μM reaction centers. The difference spectra were normalized at 865 nm by a factor which also reflected the difference in the concentrations. Inset: difference between the near-infrared regions of the two spectra (treated minus untreated) showing three isosbestic points.

time is much shorter than 30 ms. In untreated reaction centers this process occurs with $t_{1/2} \approx 200$ μs at pH 8.0 [8,16].

Thus, there appears to be no significant difference between treated and untreated samples with regard to repetitive electron transfer into and out of the reaction center. However, differences are observed in the light-induced difference spectra of the charge separated states. Fig. 4 compares the millisecond light-induced difference spectra of $P^+ Q_A^-$ for treated reaction centers (solid) and untreated reaction centers (dashed). These spectra were normalized for ΔA at 865 nm; the ground-state spectra also differed by the same factor. The spectrum for the treated sample exhibits a new, band-shift-like feature centered at 745 nm with a peak at approx. 730 nm and a trough at approx. 755 nm. This is shown more clearly in the insert to Fig. 4, which shows the difference between the light-induced spectra of treated and untreated samples. The appearance of this feature is evidently a result of the modification of the reaction center by the borohydride treatment, and is present before and after ion-exchange chromatography. It is, therefore, not associated with the free, 715-nm absorbing reduction product. A possible interpretation of this light-induced absorbance change is that it represents a blue shift of a new absorption band centered at 745 nm and, indeed, the BPh absorption band centered at 760 nm in the ground-state spectrum acquires an asymmetry after the borohydride treatment indicative of a minor component on the short-wavelength side. In some preparations this was more marked and a definite blue shift of the ground-state absorbance maximum could be seen (Fig. 1d). Perhaps this new feature is associated with the Q_Y absorption band of one of the two BPh molecules, which has moved to shorter wavelengths as a result of disrup-

tion of interactions with the BChl molecule that has been modified by the borohydride treatment.

Picosecond measurements. The primary photochemistry of the borohydride-treated reaction centers was investigated with picosecond spectroscopy. For comparison purposes, transient-state spectra and kinetics were also measured with untreated reaction centers from the same initial preparation. Fig. 5A shows the absorption changes in the visible and near infrared for the untreated reaction centers excited with 30 ps flashes at 600 nm. These spectra are the same as those reported previously for *Rps. sphaeroides* reaction centers at room temperature [10,17–21]. The 33 ps spectrum (solid) can be assigned to P^+I^- (P^+BPh^-) and the 1.6 ns spectrum (dashed) to $P^+Q_A^-$. The complex absorption changes between 740 and 830 nm are not fully understood, even for these untreated reaction centers. For both transient states the absorption changes between 740 and approx. 770 nm are due mainly to the two BPhs, and the absorption changes between approx. 770 and 830 nm mainly to the two monomeric BChls that do not constitute P. In the visible region, electron transfer from I^- to Q_A causes relaxation of the broad absorption increase due to I^- (BPh^-) [22,23] centered at 665 nm as well as recovery of bleaching in the 545 nm Q_X band of a BPh (inset). (It appears that only the long-wavelength BPh absorbing near 545 and 765 nm in *Rps. sphaeroides* is an electron carrier between P and Q_A, whereas the short-wavelength BPh absorbing near 530 and 755 nm is not [23–26].)

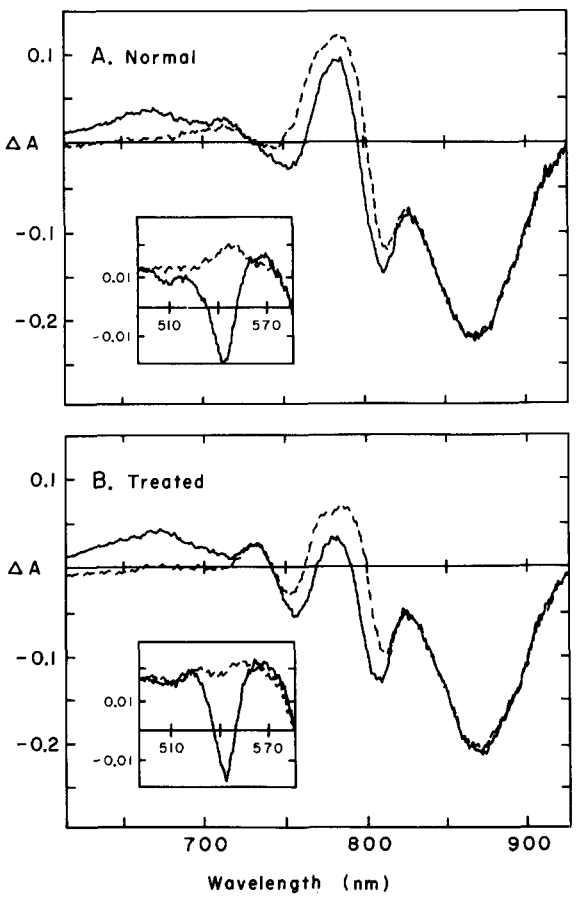

Fig. 5. Absorption difference spectra for approx. 6.5 μM *Rps. sphaeroides* R26 reaction centers at 33 ps (———) and 1.6 ns (- - - - - -) following the peak of a 30 ps, 600 nm excitation flash: (A) normal (untreated); (B) treated with $NaBH_4$ followed by ion-exchange chromatography so that 70% of the reaction centers had one 800 nm absorbing BChl removed. Each spectrum is the average of data acquired with approx. 500 excitation flashes and each was acquired in three 150 nm slices that agreed within experimental error ($\Delta A = \pm 0.005$) in the regions of overlap. The insets show the absorption changes in the region of the BPh Q_X ground-state absorption band, and were acquired using approx. 1000 excitation flashes.

Fig. 5B shows the absorption changes for a borohydride-treated sample that had the same absorbance in the 865 nm ground-state absorption band as the untreated sample used for Fig. 5A. All of the spectra of Fig. 5 show the measured absorption changes for the two samples; they have not been normalized. The 1.6 ns spectrum for $P^+Q_A^-$ in the treated reaction centers (dashed spectrum in Fig. 5B) is in very good agreement with the millisecond flash-induced spectrum shown in Fig. 3. The magnitude of the bleaching in the 865 nm band is the same in the normal (Fig. 5A) and borohydride-treated (Fig. 5B) reaction centers, and in both samples there is no recovery of this bleaching between 30 ps and 5 ns. The broad I^- (BPh^-) absorption increase centered near 665 nm and the BPh bleaching near 545 nm also have the same magnitudes in the 33 ps (solid) spectra in the treated and untreated reaction centers. Again, these observations reinforce the view that the treatment has not destroyed photochemical activity in the sample.

The difference between the near-infrared absorption changes for treated and untreated reaction centers at 33 ps (solid) and 1.6 ns (dashed) is

shown in Fig. 6A. These spectra are in excellent agreement with each other and with the one obtained from the millisecond flash-induced absorption changes (inset in Fig. 4).

The difference in the near-infrared region between the 33 ps and 1.6 ns spectra for the treated reaction centers (dashed spectrum in Fig. 6B) is essentially the same as that obtained in the untreated reaction centers (solid). These calculated difference–difference spectra emphasize the absorption changes associated with the reduction of I. (The reduction of Q_A causes some of the absorption changes in the 765 nm region.) Conversion of P^+I^- to $P^+Q_A^-$ results in the decay of net absorption decreases near 765 nm in the Q_Y region of the BPhs, and near 795 nm in the Q_Y region of the BChls.

The observed decay kinetics in these two regions, and also at 665 nm and 545 nm, are fit well by single exponential functions and the time constants are the same within experimental error in the borohydride-treated reaction centers and untreated samples. The measured decay times in the untreated sample are: 215 ± 20 ps in the 665 nm band of I^- (BPh^-), 263 ± 13 ps in the 545 nm Q_X and 266 ± 20 ps in the 765 nm Q_Y bands of BPh, and 127 ± 10 ps in the 795 nm Q_Y band of BChl. The time constants measured in the same four regions on the treated sample are, respectively, 206 ± 13, 250 ± 13, 271 ± 35 and 132 ± 19 ps. This detection-wavelength dependence of the observed kinetics has recently been well-documented in *Rps. sphaeroides* reaction centers at both room and low temperature [23]. It was proposed that the kinetics at certain wavelengths (for example, 665 nm) are a direct measure of the electron-transfer rate, whereas those at other wavelengths may contain contributions from nuclear relaxations involving the pigments and/or protein.

Considering that approx. 70% of the reaction centers in the treated samples were modified, we conclude that the rate of electron transfer from I^- to Q_A in the treated reaction centers is not appreciably different from that in untreated reaction centers. This is fully consistent with the unaltered quantum yield. The similarity in detection-wavelength dependence of the observed picosecond kinetics suggests that the additional relaxations are very similar in the treated and untreated samples.

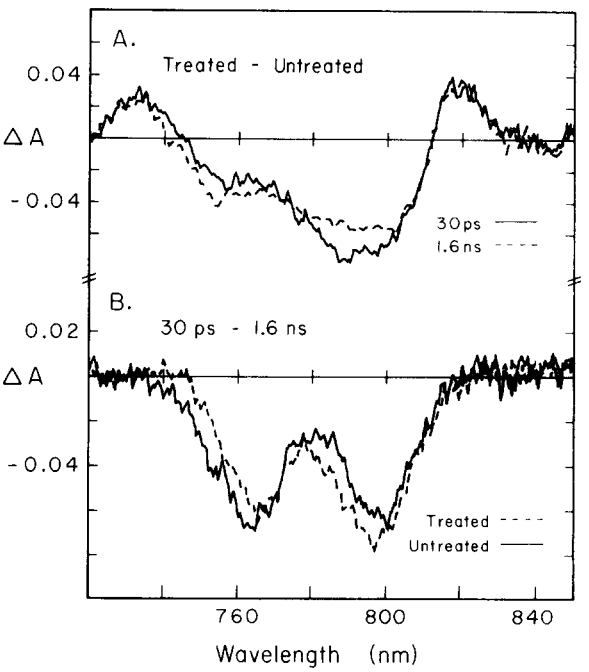

Fig. 6. (A) Calculated (treated minus untreated) near-infrared absorption changes from the spectra of Fig. 5 at 33 ps (solid) and 1.6 ns (dashed). (B) Difference between the absorption changes at 33 ps and 1.6 ns in the near-infrared region of Fig. 5 for untreated (———) and $NaBH_4$-treated (- - - - -) reaction centers.

The modifiable BChl cannot be the one responsible for the 795 nm kinetics and spectral feature of Fig. 6B, both of which are observed to be the same in treated and untreated samples.

The recent crystal structure of the *Rps. viridis* reaction center shows it to have a C_2 symmetry axis passing through the primary electron donor, P, with a BChl and a BPh molecule on each side [27]. If *Rps. sphaeroides* reaction centers have a similar structure, then our results, taken together with the previous measurements discussed above, suggest that the borohydride-modifiable BChl lies on the same side of the reaction center as the short-wavelength BPh, which does not appear to be an electron carrier.

In summary, on the basis of the foregoing results, we conclude that one specific molecule of BChl per reaction center is irreversibly modified upon treatment of the reaction center with $NaBH_4$, and that the modified pigment can be largely

removed from the protein by ion-exchange chromatography. The photochemical activity in the resulting modified reaction center, which contains only five bacteriochlorin pigments, is indistinguishable from that in the normal (untreated) reaction center that contains six bacteriochlorin pigments. It therefore seems that one of the four BChl molecules in the native reaction center plays no essential role in the photoconversion act itself. It may, of course, have some function regarding energy transfer or, possibly, could just be an evolutionary vestige. We conclude that the borohydride-modified reaction center is a new 'minimum' reaction center that is worthy of further study.

Acknowledgments

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