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RELATIVE ENRICHMENT OF P-870 IN PHOTOSYNTHETIC REACTION CENTERS TREATED WITH SODIUM BOROHYDRIDE

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Reaction centers from *Rhodopseudomonas sphaeroides* R26 in 0.03% LDAO/0.1 M Tris (initial pH = 8.0) were treated with sodium borohydride. The pH of the reaction center solution was never allowed to exceed 10. Absorption spectra taken at various times show that for approx. 8 h after the first addition of $NaBH_4$, A_{865} (P-870) and A_{760} diminish very little (no more than 15% loss each), while A_{800} diminishes markedly (45% loss) and a new peak (at 715 nm) grows in at approximately the same rate that A_{800} decays. Separate experiments on the absorption and ^{1}H-NMR spectra of purified bacteriochlorophyll (BChl) and bacteriopheophytin (BPh), and their respective $NaBH_4$-reduction products, reveal that A_{715} in $NaBH_4$-treated reaction centers most likely results from 2a-deoxy-2a-(hydroxy)BPh *a*, a BPh reduction product. We conclude that at least part of the BChl contributing to A_{800} in the reaction center is reduced at the acetyl group by $NaBH_4$, apparently with concomitant pheophytinization; if two molecules of BChl contribute equally to that absorption, one of them is reduced. Thus, it is plausible that, of the 6 bacteriochlorin molecules in the reaction center, only one is so configured that its acetyl group is both accessible to the solvent and reactive. In addition to providing a new geometric marker for reaction centers, the $NaBH_4$-reduction technique should make it possible to decide whether any small spectral change in the 800-nm region can be ruled out as the higher-energy exciton transition of the putative BChl special pair. The technique should be interesting to apply to reaction centers from other organisms, especially *Rhodopseudomonas viridis*. Because $NaBH_4$ treatment is unlikely to significantly modify protein structure, investigations of the photochemical properties of the modified reaction centers should be highly informative, especially since reversible bleaching of P-870 in $NaBH_4$-treated reaction centers now has been observed (Maroti, P., Wraight, C.A. and Pearlstein, R.M., unpublished results).

Introduction

In purple photosynthetic bacteria, each reaction center consists of three (or possibly four) polypeptides, four molecules of BChl, two molecules of BPh, one or two quinone molecules, an iron atom, and in some cases a bound *c*-type cytochrome [1]. Although, very recently, reaction centers from *Rps. viridis* have been crystallized and some X-ray diffraction data obtained [2], their detailed three-dimensional structure has still to be determined. Also, some doubt remains as to the identities of the earliest electron-transport components, especially the monomer-versus-dimer nature of the primary electron donor itself [3,4].

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; P-870, primary electron donor bacteriochlorophyll; LDAO, lauryldimethylamine *N*-oxide.

We report here a technique that, by chemically modifying reaction centers in a fairly precise way, provides a new avenue for obtaining structural information, and possibly an additional probe for the identity of the primary donor.

The present study arose from an attempt to obtain evidence for an in situ Schiff base link between a BChl or a BPh molecule and a lysine residue of a reaction center polypeptide [5]. The study was based on successful methods of demonstrating such a link between lysine and retinal in rhodopsin by reduction of the Schiff base with either sodium borohydride [6] or sodium cyanoborohydride [7]. Although results from the reaction center study were inconclusive with regard to the question of Schiff base links, they demonstrated interesting and unexpected phenomena.

We present evidence, first, that treatment of detergent-solubilized reaction centers with sodium borohydride causes a reduction reaction, preferentially involving a specific one of the BChl's contributing to the absorption at 800 nm. Second, we show from separate experiments on the borohydride reductions of BChl and BPh in a solvent, that the (irreversible) reduction product in the reaction center is most likely 2a-deoxy-2a-(hydroxy)BPh *a*. Thus, the borohydride reaction with the reaction center appears to affect only one of the six bacteriochlorin molecules present, and that one only at one of two possible reaction sites. Because A_{800} is partially 'bleached' by the borohydride reaction, while P-870 (and the BPh peak at 760 nm) is much less affected, the treated reaction centers are enriched in their P-870 content relative to other components. We discuss the application of this technique both as a structural probe and as a probe of the nature of the primary donor.

Materials and Methods

Rhodopseudomonas sphaeroides R-26 reaction centers were a gift of Colin Wraight. Trizma base was obtained from Sigma Chemical Co., St. Louis, MO; LDAO-30% (Ammonyx-10) from Onyx Chemical Co., Jersey City, NJ; sodium borohydride from Aldrich Chemical. Solvents were ACS Reagent grade from J.T. Baker Chemical, Phillipsburg, NJ. BChl was purified from *Rhodospirillum rubrum* according to the method of Strain and Svec [8].

Absorption spectra were recorded with a Cary 17 spectrophotometer (only monochromatic light incident on the sample). pH measurements were made with a Radiometer model 26 pH meter with combination electrode. Proton NMR spectra were taken on a Bruker model WP-200 (200 MHz) spectrometer. All measurements were done at ambient temperature.

Reaction centers were obtained and stored (at 4°C in the dark) as a suspension in 10 mM Tris-HCl (pH 8.0) containing 0.03% LDAO [9]. All pigmented samples were handled under green safelights. For the borohydride experiments, reaction centers were diluted approx. 100-fold with 0.1 M Tris (pH 8.0) containing 0.03% LDAO, to give a final reaction center concentration of approx. 3 μM. Various amounts of $NaBH_4$ were added as solid on the tip of a spatula. Solutions were mixed by inversion and spectra were recorded at various time intervals. Additional $NaBH_4$ was added as necessary; the pH of a solution was not allowed to exceed 10.

Borohydride reduction of BChl was done by addition of $NaBH_4$ to a solution of BChl in methanol. After a 30-min reaction time, a sample was divided in half. One portion was diluted with water then extracted with low-boiling-point petroleum ether. The second half was acidified with 6 M HCl in methanol, diluted with water, and then extracted with low-boiling-point petroleum ether. The solvents were evaporated, the samples redissolved in diethyl ether and their spectra were obtained. For NMR spectroscopy, samples were redissolved in acetone-d_6 at pigment concentrations of approx. 20 mM.

Results

Reaction centers

Absorption spectra of reaction centers exposed to $NaBH_4$ for various durations are shown in Fig. 1. With increasing exposure time, the spectra display both changes of shape and, especially after long exposure (27 h), significant overall loss of absorbance. Notable among the shape changes are the diminishing ratio of the 800 nm peak to that at 865 nm (both peaks due to BChl), the rise of a new peak at approx. 715 nm, a proliferation of peaks in

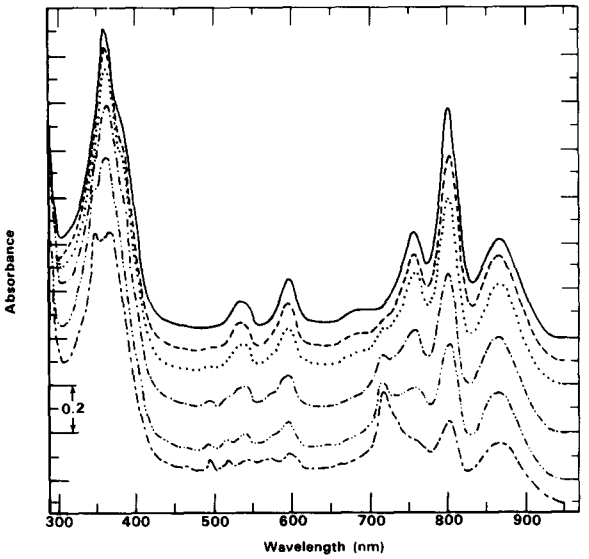

Fig. 1. Absorption spectra showing effect of $NaBH_4$ on reaction centers at various times. ————, Initial, — — —, 1.5 h; · · · · · ·, 2.5 h; ·—·—·, 5.5 h; —··—··, 7.8 h; ——·——·, 27 h. See text for further details.

the Q_x-region of the spectrum (approx. 500–600 nm), and substantial changes in the Soret (approx. 300–400 nm).

Table I shows the absorbance at 865 nm (A_{865}, also denoted P-870), and the absorbance ratios of other near-infrared peaks, as a function of time of exposure of the reaction centers to $NaBH_4$. Of particular interest is the constancy of the ratio of A_{760} (due to BPh) to A_{865} (due to BChl). For the first 8 h, the loss of absorbance at 865 nm is less than 20%, although after a day, the loss is nearly 50%. Note that during the early period, there is even a slight increase of 865 nm absorbance, presumably due to re-reduction of some oxidized P-870 present.

This effect is also evident in Fig. 2, in which the time dependences of the four infrared absorption peaks (not ratios) are plotted. One observes as well not only the close correlation of A_{760} with A_{865}, but also an apparent concomitance of the rise of A_{715} with the decay of A_{800}. These last two peaks both appear to display an initial phase (approx. 8 h or less) of relatively rapid change followed by a period of slower change.

Isolated pigments

To aid in the interpretation of the absorption

TABLE I

ABSORBANCE AT 865 NM AND ABSORBANCE RATIOS OF OTHER PEAKS FOR REACTION CENTERS TREATED FOR VARIOUS LENGTHS OF TIME WITH $NaBH_4$

Reaction time (h)	A_{865}	A_{800}/A_{865}	A_{760}/A_{865}	A_{715}/A_{865}
0 (initial)	0.400	2.38	1.05	0.26
1.5	0.420	2.02	1.01	0.33
2.5	0.410	1.85	1.06	0.49
5.5	0.370	1.73	1.03	0.57
7.8	0.340	1.53	0.97	1.00
27	0.220	1.28	0.91	1.77

spectra of BH_4-treated reaction centers, we observed absorption and ^{1}H-NMR spectra of BChl and BPh in organic solvents. For this purpose, we found it convenient to use BChl *a* purified from *R. rubrum*, in which the esterifying alcohol is geranylgeraniol [10], rather than phytol, as is the case for BChl *a* from *Rps. sphaeroides* (Fig. 3). However, this difference has negligible effect on the spectral properties of interest here.

Absorption spectra of the borohydride-reduction products of BChl *a* and BPh *a* in diethyl ether are shown in Fig. 4. Of particular interest are the longest-wavelength maxima (Q_y bands), which appear at 728 and 715 nm for the BChl and BPh reduction products, respectively. These are quite blue-shifted from the corresponding maxima for BChl *a* and BPh *a* in the same solvent, which occur at 770 and 749 nm, respectively [11]. Also noteworthy are the prominent and highly resolved

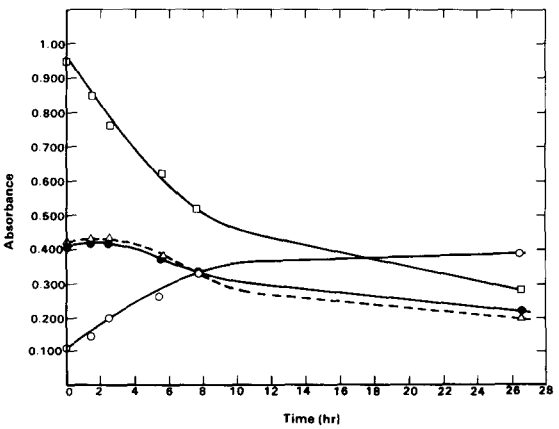

Fig. 2. Dependence of four absorbance maxima on time of exposure to $NaBH_4$. ●, A_{865}; □, A_{800}, △, A_{760}; ○, A_{715}.

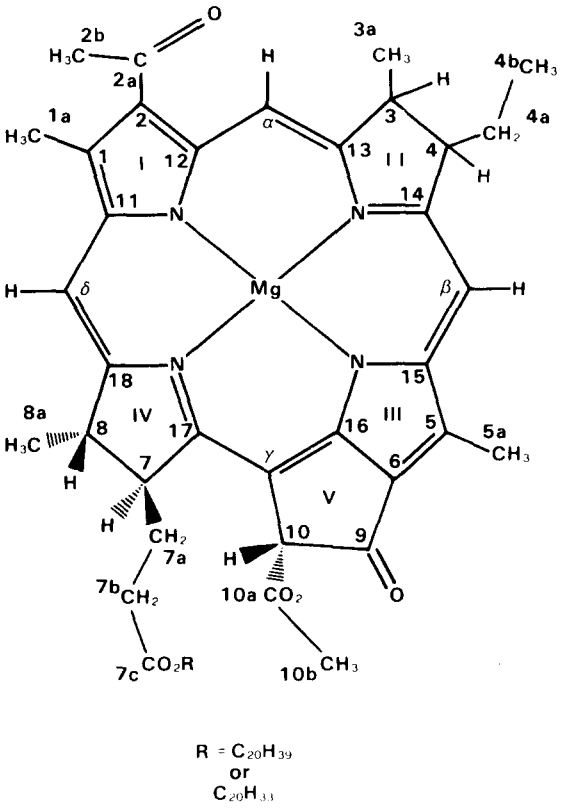

Fig. 3. Structure of bacteriochlorophyll *a*. R = $C_{20}H_{39}$ (phytyl) for BChl from *Rps. sphaeroides*, = $C_{20}H_{33}$ (geranylgeranyl) for BChl from *R. rubrum*.

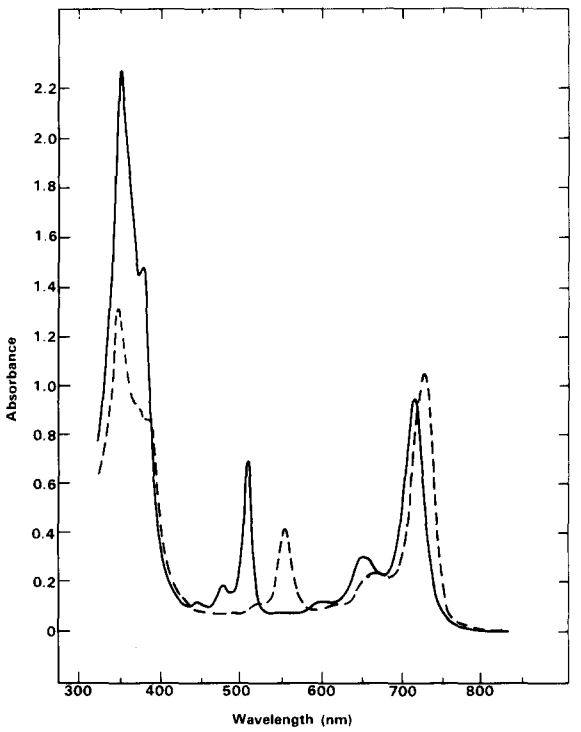

Fig. 4. Absorption spectra of $NaBH_4$-reduced bacteriochlorophyll (— — —) and bacteriopheophytin (————) in diethyl ether.

Q_x bands (approx. 500–560 nm), especially for the BPh product, in which the second vibronic overtone is clearly evident and the Q_x vibronic series is a good match to that of the Q_y. The absorbance ratio of Soret and Q_y peaks is almost a factor of 2 greater in the BPh product than in the BChl product.

The ^{1}H-NMR spectra of BChl *a* and its borohydride reduction product are shown in Fig. 5. With regard to the identification of the reduction product, there are several salient features. A one-proton multiplet appears at 5.7 ppm in the product spectrum (labelled '2a'; see Fig. 3 for numbering system) that is altogether absent in the BChl *a* spectrum. Decoupling experiments show that this resonance is closely coupled to a three-proton doublet at 1.6 ppm. The latter is apparently due to the 2b protons, but split by interaction with the 2a proton and shifted upfield nearly 1.2 ppm. Other protons relatively near C-2a, the 1a methyl and the α-methine, also display resonances that are split and appreciably shifted upfield. The remaining resonances are shifted to a much lesser extent. In particular, the proton resonance at C-10 is shifted not much more than 0.2 ppm and clearly retains its singlet character. Taken together, these results strongly imply that, under our experimental conditions, $NaBH_4$ reduces the (acetyl) carbonyl oxygen at C-2a, but leaves unaffected the carbonyl oxygen at C-9. We conclude that the BChl *a* reduction product is most likely 2a-deoxo-2a-(hydroxy)BChl *a*. Other NMR data (not shown) similarly confirm that the BPh *a* reduction product is most likely 2a-deoxo-2a-(hydroxy)BPh *a*.

Discussion

The interpretation of our principal findings is reasonably straightforward. From Figs. 1 and 2 and Table I, it is clear that treatment of reaction centers with $NaBH_4$ for periods of approx. 8 h or

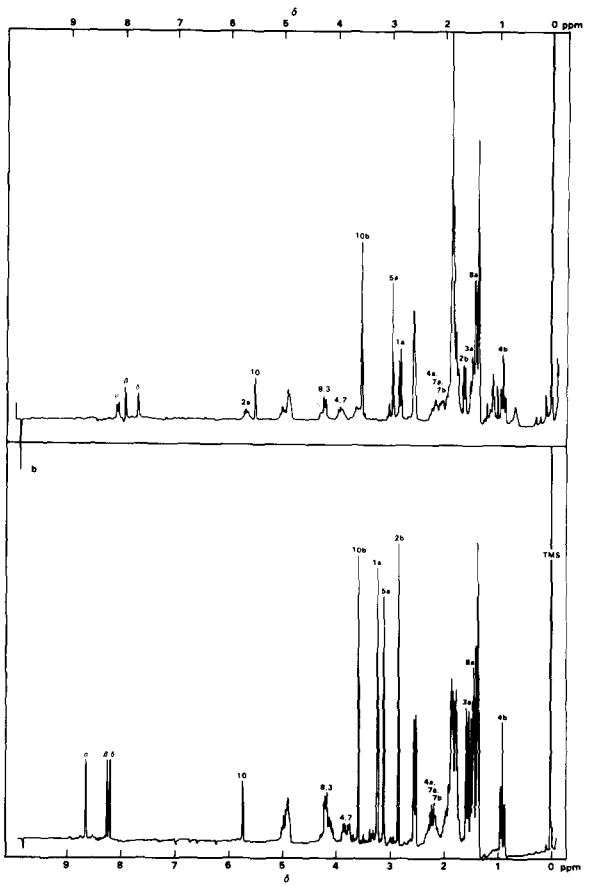

Fig. 5. ^{1}H-NMR spectra of BChl *a* (b) and its borohydride reduction product (a) in acetone-d_6. Chemical shift (δ) is in ppm relative to tetramethylsilane (TMS). Pigment concentrations approx. 20 mM. Assignments of BChl *a* resonances according to Sanders et al. [12].

less, induces a preferential loss of A_{800}, while A_{865} and A_{760} are relatively unaffected. The same data show that new spectral peaks grow in at the same rate that A_{800} is lost. The new peaks appear at about 715, 520, 490 and 347 nm. With the exception of the 490 nm peak, these compare well with the positions of the Q_y, Q_x, and Soret band maxima in 2a-deoxo-2a-(hydroxy)BPh *a* (Fig. 4), which occur at 715, 515, and 350 nm, respectively. We conclude that at least part of the reaction center BChl contributing to A_{800} is reduced at the acetyl group by $NaBH_4$. Indeed, Fig. 2 shows that if two molecules of BChl contribute equally to the absorption at 800 nm, one of them is reduced by $NaBH_4$. Apparently, pheophytinization occurs concomitantly. It is not yet established whether, in the detergent-containing solution, the 2a-deoxo-2a-(hydroxy)BPh remains associated with the reaction center protein complex.

This interpretation has implications for reaction center structure. Of the six bacteriochlorin molecules in the reaction center, apparently only one, i.e., one of the two BChl's contributing to A_{800} is located in such a way that its acetyl group is both reactive and relatively exposed to the solvent. Thus, the borohydride reduction potentially provides information on the orientation of this BChl, and possibly on its relative proximity to the surface of the protein complex. It is noteworthy that the borohydride reaction with this BChl in the reaction center proceeds approx. 20-times more slowly than with BChl isolated in methanol. This may be a consequence of the BChl acetyl group's binding to the protein.

The borohydride reduction method also provides a new means to investigate the dimeric exciton model of primary donor BChl in these reaction centers. According to the special pair hypothesis, which for purple photosynthetic bacteria seems to be firmly based on magnetic resonance data, two molecules of BChl in the reaction center constitute a nonbonded dimer that share the positive charge in the oxidized reaction center [3,4,13]. If such a dimer has certain geometric properties, e.g., Q_y oscillators are roughly parallel, there are then consequences for optical spectra as a result of exciton coupling between the two oscillators. This dimeric exciton model, some of the difficulties with which have recently been reviewed [3,4], interprets the 865 nm absorption of the reaction center as the lower energy transition of an exciton-split pair, and predicts the upper energy transition to be a weak one lying within the 800 nm absorption band. The earliest observation that was interpreted as evidence of such an upper transition was based on second derivative cryogenic absorption spectroscopy of these reaction centers [14]. Later observations have involved oriented linear dichroism or photoselection techniques [15]. In all cases, it is a small spectral feature that is inferred to be the upper transition. Borohydride-treated reaction centers would be useful in further studies of this sort. A small spectral feature near 800 nm, observed by derivative spectroscopy or photoselec-

tion techniques, should follow the P-870 borohydride-reduction kinetics, i.e., should be enhanced relative to A_{800}, if it is the upper exciton transition (although this would not prove it is such). If, on the other hand, a feature diminishes with the A_{800} kinetics, it can be inferred that this particular spectral feature does not correspond to an exciton transition of a dimeric primary donor.

Because, quite recently, reversible bleaching of P-870 in borohydride-treated reaction centers has been observed (Maroti, P., Wraight, C.A. and Pearlstein, R.M., unpublished results), photochemical investigations of these reaction centers also should be informative toward interpreting the complex absorption changes near 800 nm that occur when P-870 is oxidized to P-870$^+$ [4]. In the difference spectrum for the formation of P-870$^+$, if the absorption increase observed near 790 nm, along with the decrease near 815 nm, reflects a blue shift of the 800 nm-absorbing BChl's, then these changes should be reduced in amplitude in the borohydride-treated reaction centers. On the other hand, if the absorption increase at 790 nm is a new band in the absorption spectrum of P-870$^+$ [16], and the absorption decrease at 815 nm is a bleaching of the higher-energy excitonic component of P-870, then the difference spectrum (P-870$^+$–P-870) should be largely the same in normal and treated reaction centers.

Photochemical investigations on the intermediary electron acceptor, I [4,17], also might be informative in this regard. The acceptor I appears to involve a BPh absorbing at 760 nm interacting strongly with a BChl absorbing near 800 nm. If the BChl in this acceptor complex is the one that has been affected by the $NaBH_4$ treatment, then the difference spectrum for the reduction of I should be greatly perturbed in the treated reaction centers. The rate of electron transfer from I$^-$ to the quinone (normally 200 ps [4]) also might be perturbed.

The borohydride treatment should be tried with reaction centers isolated from other species of photosynthetic bacteria, especially *Rps. viridis*. Interest in the latter organism has intensified recently because of significant advances in structural studies related to its reaction centers [2]. However, *Rps. viridis* reaction centers have been studied attentively for a long time because of their unusual spectral properties [18]. Borohydride treatment may be able to resolve recent conflicting claims regarding whether the 820 nm [19] or the 850 nm [20] band (or neither) corresponds to a higher-energy exciton transition of P-960, the primary donor in *Rps. viridis*. The fact that *Rps. viridis* reaction centers, unlike *Rps. sphaeroides* reaction centers, have a tightly bound cytochrome *c*, lends additional interest [1].

As the absorption spectra of Fig. 1 attest, borohydride reduction is relatively gentle and unlikely to modify the protein structure of the reaction center complex in a significant way. The results presented here suggest that it may be worthwhile to investigate other gentle techniques for in situ chemical modification of Chl or BChl in reaction centers.

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