Selective photochemical reduction of either of the two bacteriopheophytins in reaction centers of *Rps. sphaeroides* R-26

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The bacterial photosynthetic reaction center (RC) contains two bacteriopheophytin (BPh) molecules, only one of which (characterized by $Q_{x,y}$ maxima at 540 and 760 nm) has been found to function in the normal photochemistry. We present here the formation of a new RC redox state, in which the apparently inactive BPh ($Q_{x,y}$ maxima at 530 and 752 nm) is selectively trapped in a reduced state by secondary photochemistry. This BPh is found to be associated to a bacteriochlorophyll (BChl), forming a BChl-BPh complex analogous to the photochemically active BChl-BPh acceptor complex. Electron transfer between the two BPhs is found not to occur.

Reaction center *Photosynthesis* *Bacteriopheophytin* *Bacteriochlorophyll* Rps. sphaeroides *Electron acceptor*

1. INTRODUCTION

The photochemistry of the bacterial photosynthetic reaction center (RC) appears to follow a single, specific sequence of one-electron transfers (reviews, [1-6]). Electron transfer occurs first from a light-generated excited state of a bacteriochlorophyll (BChl) dimer B_2, to an acceptor complex I_A, which primarily involves a bacteriopheophytin (BPh) H_A, in close association with another BChl, B_A. A subsequent transfer then occurs from I_A^- to the first of the RC quinone acceptors, Q_A [1-6]. However, the bacterial RC also contains an additional BChl and BPh [1-7], designated here, B_B and H_B. An involvement of B_B and H_B in the RC photochemistry has not yet been found (reviews [1-6]). The photochemically active H_A is readily distinguished spectrophotometrically from H_B in the Q_X band region, where these components have absorption maxima at 542 and 530 nm, respectively [6,8].

The recently determined X-ray structure for *Rhodopseudomonas viridis* RCs has shown that this apparent inactivity of the 'voyeur' B_B and H_B is all the more surprising since two BChl/BPh pairs are seen to be symmetrically arranged at nearly equivalent positions about the B_2 on the RC C_2 symmetry axis [9]. The fact that each of the BChl/BPh pairs share a similar structural relationship to the B_2 suggests that both could function as pathways for electron removal from the B_2 [10]. A schematic representation of the RC chromophore organization is shown in fig.1. The close cor-

Abbreviations: B_A, B_B, the reaction center bacteriochlorophylls, of which only B_A has been found to function as an early acceptor; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; H_A, H_B, the reaction center bacteriopheophytins, of which only H_A has been found to function as an early acceptor; I_A, a complex involving B_A and H_A; I_B, a complex involving B_B and H_B; MV, methyl viologen; Q_A, Q_B, reaction center primary and secondary quinone acceptors; RC, reaction center

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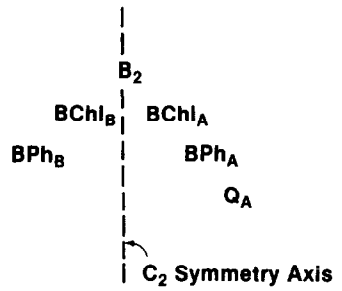

Fig.1. Schematic representation of the reaction center components.

respondence in dichroism properties for all of the bacterial RCs [11–13] shows that the general features of the *Rps. viridis* RC organization is likely to be common for all of the photosynthetic bacteria.

Here we describe the formation of a new RC redox state, the specific photochemical reduction of H_B, the BPh not normally associated with photochemical activity, in RCs of *Rps. sphaeroides* R-26. The light-dependent reduction of H_B appears to be a product of an incompletely understood secondary reaction involving the redox mediator, methyl viologen (MV). However, with the formation of the H_B^- state, we show that: (i) H_B reduction is accompanied by absorption changes of the BChl bands analogous to those produced by H_A reduction. (ii) There is no rapid electron transfer between H_A and H_B (on the 1–2 h time scale at room temperature). (iii) The specific H_B reduction provides a new RC redox state which can be used to test for the involvement of B_B and H_B in the normal, I_A photochemical pathway. (iv) Based upon these data, we suggest that difference spectra associated with Q_A and Q_B reduction in *Rps. sphaeroides* [14–16] may be re-interpreted to show that Q_A is coupled to I_A, while Q_B is coupled to the I_B complex.

2. MATERIALS AND METHODS

RCs of *Rps. sphaeroides* R-26 were isolated with the detergent lauryldimethylamine *N*-oxide (LDAO) essentially as described in [17]. The detergent LDAO was exchanged for Triton X-100 by washing the RCs fixed on a DEAE-cellulose column with 0.05% Triton, 10 mM Tris, pH 8.0.

The rationale for the photochemical trapping of reduced electron acceptors in RCs poised at low redox potentials in the presence of electron donors to B_2^+ has been previously described (e.g. [18–20]). Here, anaerobic RC solutions (0.05% Triton, 10 mM Tris, pH 8.0) containing either cytochrome *c* or MV (concentrations as noted in figure legends) were poised reduced with 2 mM $Na_2S_2O_4$, added from a 0.2 M stock in 1 M Tris, pH 8.0. The actinic light from an 800 W lamp was filtered through 10 cm of water and a Schott RG 714 filter. The RC solutions in sealed anaerobic curvettes were maintained at 22°C during the 50–150 min illuminations.

Spectra were recorded on a Cary 17 spectrophotometer, and differences were taken from spectra stored on a Tracor TN 1710 signal averager.

3. RESULTS

A series of absorption spectra of dithionite reduced *Rps. sphaeroides* R-26 RCs plus MV (4:1 molar ratio of MV:RC) is shown in fig.2. The absorption of the MV has been subtracted by a reference buffer sample. The bottom spectrum was taken before illumination, the middle after 50 min of illumination and the top after the curvette was opened to air, which reversed the absorption changes produced by illumination. The illuminat-

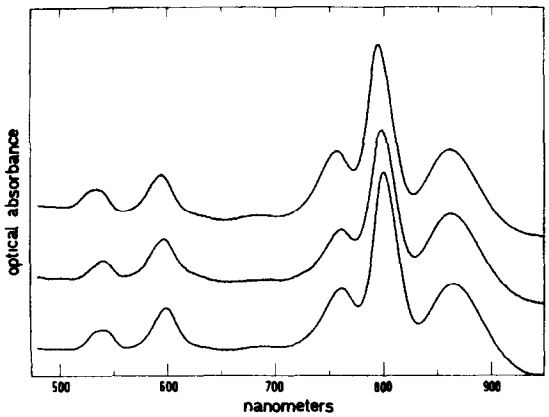

Fig.2. Absorption spectra of *Rps. sphaeroides* RCs at room temperature. Bottom, spectrum before illumination; middle, after 50 min of illumination ($\lambda > 750$ nm); top, after removal of the cuvette stopper, and left exposed to air overnight. RCs, 35 μM; MV, 140 μM; 0.05% Triton X-100, 10 mM Tris, pH 8; 1 mm path length.

ed-minus-before illumination difference spectrum is shown in the lower spectrum of fig.3. The specific reduction of the H_B BPh is clearly indicated by the selective bleaching of BPh Q_y and Q_x bands at 752 and 530 nm. In addition, an involvement of a BChl molecule is shown by the complex absorption changes near 800 and 590 nm, with a maximal absorption decrease at 806 nm.

This difference can be compared to the I_A^--I_A spectrum [18–20] produced by a similar illumination, except with cyt *c* instead of MV as an electron donor to the RC [20]. The spectrum (fig.3, top) shows bleaching at 760 and 540 nm, characteristic of H_A reduction, and complex absorption changes near 800 and 590 nm, with a maximal bleaching at 804 nm [18–20]. The similarity of the two spectra in fig.3 suggests that the illumination in the presence of MV leads to the reduction of a complex I_B which primarily involves the BPh, H_B.

The yield of I_B reduction is found to depend on MV concentration. At ratios of MV : RC below 2 : 1 (tested over a range of RC concentrations between 6 and 60 μM) a variable amount of I_B^- is found. Above this ratio, the I_B^- state is maximally formed. Illumination times were varied between 50 and 150 min, but only minimal further absorption changes occurred after the first 60 min. For example, with cyt *c* as the electron donor to the RC, additional illumination did not yield further bleachings of

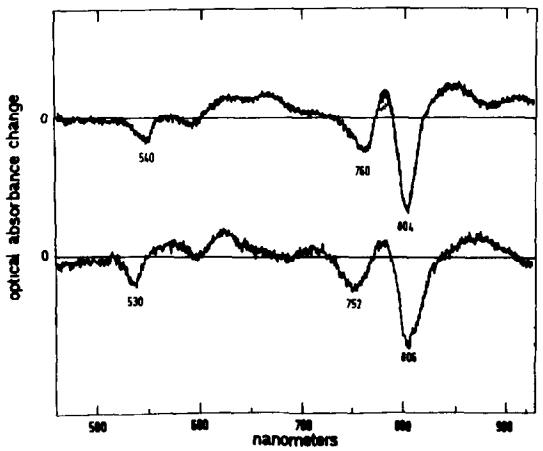

Fig.3. Room temperature I_A^--I_A (top) and I_B^--I_B (bottom) difference spectra. The I_B^--I_B spectrum was obtained from the subtraction of the after and before illumination spectra, as shown in fig.2. The I_A^--I_A spectrum was obtained as described in [20].

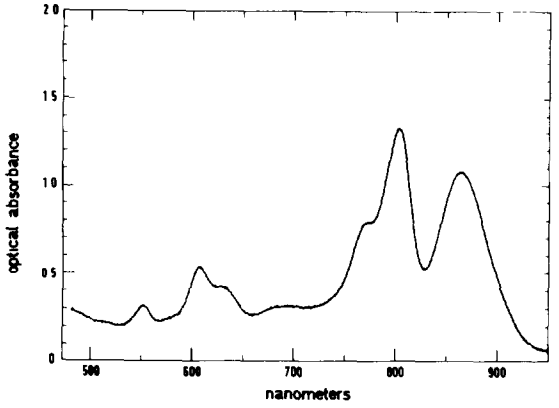

Fig.4. Absorption spectrum of *Rps. sphaeroides* RCs after 30 min of illumination at room temperature. 85 μM MV, 50 mM sodium borate, 0.05% Triton, pH 9.5.

BChl and BPh bands after I_A^- formation. Schenck et al. [21] with *Rps. sphaeroides* and Thornber et al. [22] with *Rps. viridis* have shown that additional BChl and BPh reduction occurs following I_A^- formation under similar conditions. The stability of I_A^- or I_B^- states were extremely sensitive to the presence of oxygen, and exposure to air relaxed the absorption changes produced by illumination.

The reduction potential of a buffered solution in the presence of excess dithionite is limited by the redox mid-point of the hydrogen couple (1/2 H_2/H^+), and lower potentials will be produced by raising the pH (e.g. [23]). Illumination of RC plus MV solutions poised at pH 9.5 with 2 mM $Na_2S_2O_4$ (ϵ_h = −570 mV) showed extensive absorption decreases for all bands except those of the B_2, suggestive of a dual formation of I_A^- with I_B^- (fig.4). However, a residual H_A absorption at 760 and 540 nm remained even after 150 min of illumination. The spectrum also shows the appearance of a new absorption near 630 nm which cannot be assigned to the MV buffer solution. These absorption changes were reversible by exposure of the sample to air.

4. DISCUSSION

Previous reports have shown that in combination with I_A reduction, the accessory B_B and H_B molecules can also be reduced by prolonged illumination of RCs at room temperature and poised at low redox potential [21,22]. Here, we

have shown more specifically that either acceptor complex I_A or I_B can be reduced selectively, characterized by the bleaching of the individual H_A and H_B BPh absorption bands at 760/540 nm and 752/530 nm, respectively. The dependence of I_B^- formation on the MV:RC ratio shows that it is most likely a product of a secondary photochemical pathway involving MV. The fact that H_B does not function as a primary acceptor of the B_2 with high yield has been shown by the absence of appreciable 530 nm absorption changes in the transient states formed by flash excitation [1-3,24], and by the absence of measurable flash-induced B_2 oxidation following the reduction of the I_A complex [21,25,26]. Although a single mechanism cannot explain all observations, we propose two possible mechanisms for the light-induced formation of the I_B^- state. First, that I_B^- could be formed by a secondary photochemical pathway of low yield, possible through the $B_2 \rightarrow B_B \rightarrow H_B$ sequence. In this case MV (ϵ_m −440 mV), which exists in a mixture of reduced and oxidized forms with excess dithionite at pH 8.0 (ϵ_h −480 mV), provides both a reductant to reduce the light-generated B_2^+ and an acceptor to oxidize I_A^-. After many cycles this permits the I_B^- state to accumulate at a lower yield. At high pH MV is more completely reduced and both I_A^- and I_B^- can accumulate following prolonged illumination. This scheme requires the additional assumption that MV can react with H_A^- but not H_B^-. An alternative possibility is that MV acts as a redox mediator between H_A and H_B, operating after H_A^- has been trapped by MV reduction to B_2^+ in the $[B_2^+H_A^-]$ state. In this case it must be assumed that H_B has a higher redox potential than H_A, and that in the absence of a redox mediator there is no direct equilibration between the two BPhs. This second hypothesis, however, is not supported by previous observations which showed that additional B_B or H_B reduction can occur after initial I_A^- accumulation, following prolonged illumination of *Rps. sphaeroides* [21] and *Rps. viridis* [22] RCs in the presence of excess dithionite and only *c*-cytochromes as donors to B_2^+.

Although the photochemistry leading to I_B^- accumulation is not fully understood, the ability to generate selectively either the I_A^- or I_B^- state does demonstrate new features about the RC. First, the similarity of the BChl absorption changes in the $I_B^- - I_B$ spectrum to those in the $I_A^- - I_A$ spectrum shows that H_B interacts with a BChl in the I_B complex in the same manner in which H_A is coupled to B_A in the I_A complex. This suggests that there are two nearly equivalent pairs of BChl/BPh complexes in the *Rps. sphaeroides* RC, which is consistent with the equivalent arrangement of two BChl/BPh pairs seen in the *Rps. viridis* RC X-ray structure [9].

Secondly, the fact that both the I_B^- and the I_A^- states are stable on the 1-2 h time scale shows that there is no rapid electron transfer between H_A and H_B.

Thirdly, the reduced I_B represents a new RC redox state, which can be used to look for perturbation of the predominate B_2-B_A-H_A electron transfer pathway. Preliminary results (unpublished) show that the reduction of I_B does not appreciably alter the yield of P^R, the B_2 triplet state (see [1-3] for description) at temperatures below 100 K, suggesting that the presence of I_B^- does not alter the light-induced $B_2^+I_A^-$ charge separation. This gives further evidence that I_B does not function as an acceptor for B_2 with high yield. The preference for I_A rather than I_B as an acceptor in spite of their equivalent positions about the B_2 as found in *Rps. viridis* [9], suggests that the choice of the photochemical pathway could be determined by specific interactions between BChl molecules, or by different protein environments.

Finally, a proposal for the localization of the ubiquinone acceptors, Q_A and Q_B, in the *Rps. sphaeroides* RC can be made. Previous work by Vermeglio and co-workers [14-16] characterized the BChl and BPh absorbance changes associated with Q_A and Q_B reduction. Q_A reduction was shown to cause a BPh red shift, with a zero crossing near 760 nm, and less well defined shifts in the BPh Q_x region [14,15] which are consistent with a blue shift centered at 540 nm. BChl absorbance changes are also seen near 800 nm, and a blue shift of the B_2 Q_y band centered at 860 nm [14,15]. With Q_B reduction the BPh red shift is seen to be smaller and centered at lower wavelengths near 750 nm, while a blue shift centered at 530 nm is seen in the Q_x region [14-16]. BChl shifts are also seen near 800 nm, but the B_2 absorptions are not appreciably altered [14-16].

The optical properties of H_A and H_B described here suggest that it is possible to interpret these ab-

sorption changes as arising from a specific interaction of Q_A with the I_A complex, and Q_B with the I_B complex. However, the amplitudes of the absorption shifts may suggest that Q_A and I_A are more closely associated than are Q_B and I_B.

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