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THE PHOTOCHEMICAL REACTION CENTER OF THE BACTERIOCHLOROPHYLL *b*-CONTAINING ORGANISM *THIOCAPSA PFENNIGII*

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A photochemical reaction-center preparation has been made from a second bacteriochlorophyll *b*-containing organism, *Thiocapsa pfennigii*. The reaction-center unit is thought to be composed of one P-960, four bacteriochlorophyll, two bacteriopheophytin, one carotenoid molecules and polypeptides of M_r 40000, 37000, 34000, 27000 and 26000 probably plus quinones and metal atoms. The preparation also contains a low-potential cytochrome *c*-555 and a high-potential cytochrome *c*-557 bound to the reaction center in a 3–4:2–3:1 molar ratio with respect to P-960. The 40 kDa subunit is associated with the cytochromes, while the 37, 34 and 27 + 26 kDa subunits are proposed to be equivalent to the H, M and L polypeptides of bacteriochlorophyll *a*-containing reaction centers. The cytochromes are oxidized by P-960$^+$. The three near-infrared absorption bands at 788, 840 and 968 nm are assigned to bacteriopheophytin, bacteriochlorophyll and the primary donor (P-960), respectively. The 778 nm peak resolves into two at 77 K; no further resolution of the other two peaks occurs. Illumination of the sodium dithionite-reduced reaction centers at 77 K by 960 nm-light results in P-960, transferring one electron from cytochrome *c*-555 mainly to a bacteriopheophytin molecule, absorbing at 781 nm. A similar treatment at room temperatures reduces most of the two bacteriopheophytin molecules. It is argued that both bacteriopheophytin molecules, possibly with some contribution from bacteriochlorophyll, form an intermediary electron-carrier complex between P-960 and a quinone in *T. pfennigii*. We could not substantiate that a bacteriochlorophyll molecule precedes the bacteriopheophytins in the electron transfer sequence. Although the biochemical characteristics of the reaction center are very similar to those of the other known bacterioclorophyll *b*-containing reaction center, that from *Rhodopseudomonas viridis*, their spectral characteristics are not. This has helped elucidate more about the function of each spectral form and led us to conclude that the 850 nm form in *Rps. viridis* is not the higher energy transition of the special pair of bacteriochlorophyll molecules forming P-960. Laser-flash-induced absorbance changes in *T. pfennigii* reaction-center preparation should now lead to a more complete understanding of the mechanism of the primary photochemical event.

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; LDAO, lauryldimethylamine *N*-oxide; TLC, thin-layer chromatography.

Introduction

Much of our knowledge of the mechanism of the primary event of photosynthesis has been derived from studies on bacteriochlorophyll (BChl)

a-containing photochemical reaction centers [1–4], particularly that isolated from *Rhodopseudomonas sphaeroides* [5]. However, study of reaction centers purified from *Rhodopseudomonas viridis,* a BChl *b*-containing bacterium, has provided useful further insight into the process [6–11]. The former reaction-center preparation contains four BChl *a* and two bacteriopheophytin (BPh) *a* molecules, generally one carotenoid, 1–2 quinone molecules, an iron atom and equal stoichiometric amounts of three polypeptides of apparent sizes 21, 24 and 28 kDa [5]. While the function of each of the BChl and BPh molecules in the reaction center has not been unequivocally determined, it is believed that two BChl (but perhaps only one, see Pearlstein [12]) function as the primary electron donor (P-870) and some others, in particular one of the BPh molecules, in conjunction with one of the BChl molecules, act as an intermediary electron-carrier complex between P-870 and the first stable electron acceptor, a quinone (Q) [2–4,13,14]. A complete understanding of the primary event requires that the function and relative arrangement of all reaction-center constituents be known with certainty. Studies of the *Rps. viridis* reaction center proved advantageous in this respect, because it contains different pigments, BChl *b* and BPh *b*; cytochromes also form an integral part of the isolated complex. Furthermore, the *Rps. viridis* reaction center is the first such complex to be crystallized [15]. BChl *b* and BPh *b* absorb further into the near-infrared than all other photosynthetic pigments. This results in better separation of the spectral forms of both pigments than occurs in BChl *a*-containing reaction centers. Therefore, the spectrophotometric monitoring of each chromophore molecule's possible involvement in the electron-transfer pathway, among other this, is facilitated. Also, the cytochromes in the *Rps. viridis* reaction center feed electrons with a $t_{1/2}$ of approx. 1 μs to P^+960 (oxidized form of the primary electron donor), which leads to a trapping of electrons photo-ejected from P-960 on the intermediary carrier(s) in reaction centers in which Q has been previously reduced. Such carriers can then be spectroscopically identified (e.g., Refs. 7, 9–11, 16–19).

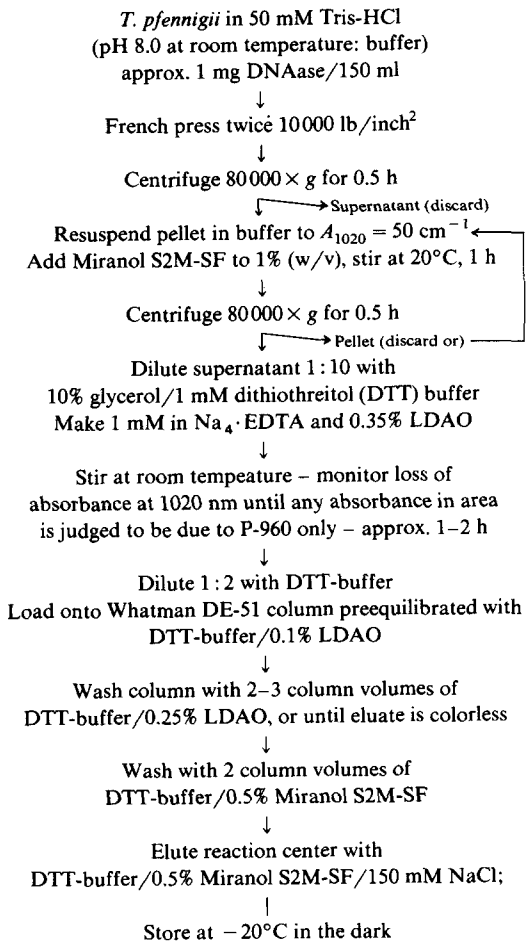
The *Rps. viridis* component has been used for all studies on BChl *b*-containing reaction centers, so far; however, investigations on broken membranes of another BChl *b*-containing organism, *Thiocapsa pfennigii,* revealed that its reaction center had absorption bands [7] and photochemical properties [20] somewhat different from those of *Rps. viridis.* Consequently, characterization of the *T. pfennigii* reaction center should extend our understanding of photosynthesis by increasing our knowledge of the structure and spectral composition of reaction centers and, by comparing such data with those for reaction centers from other bacteria, indicate which reaction-center features remain constant and are of general significance [21]. Furthermore, the spectral differences should be useful for solving the nature of the primary donor, the role of the BPh molecules and the identity of the intermediary electron carriers in the primary event [7].

Initial attempts to purify the *T. pfennigii* reaction center using LDAO as the surfactant gave a product enriched in the reaction center, but which also contained large amounts of a chlorin degradation product. The reaction-center preparation denatured rapidly above 4°C [7]. These undesirable features might be due to the use of LDAO [22]. We have, therefore, surveyed the effectiveness of other detergents for obtaining more stable reaction centers from *T. pfennigii.* We were successful in this aim and now describe a rapid method for the purification of the reaction center as well as some of its spectral, photochemical and biochemical properties.

Materials and Methods

Culture of bacterium. Cells of *T. pfennigii* (str. 9111) were grown photosynthetically in 10-liter carboys on a sulfide-medium [23] as modified by Pfennig [24] with 10 ml/l of the following heavy-metal solution used instead of the 'Schermatallosung' (in mg/l): EDTA · Na_2, 500 mg; $FeSO_4 \cdot 7H_2O$, 200 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2 \cdot 6H_2O$, 20 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; $CuCl_2 \cdot 2H_2O$, 1 mg and $Na_2MoO_4 \cdot 2H_2O$, 3 mg. The cells were harvested by centrifugation ($18000 \times g$) and washed in 50 mM Tris-HCl, (pH 8.0) at 20°C (buffer).

Preparation of the reaction center (Scheme I). The pellet was resuspended in buffer plus 1 mg



Scheme I. Purification procedure for photochemical reaction centers of *T. pfennigii*; DTT, dithiothreitol.

DNAase/150 ml of sample, passed twice through a French pressure cell at 10000 lb/inch^2, then centrifuged at 80000 × *g* for 0.5 h. Broken membranes were resuspended in buffer to give an A_{1020} = 50 cm^{-1}, and 40% (w/v) Miranol S2M-SF, which had been treated with catalase (10 μg/ml) to remove H_2O_2, was added to a final concentration of 1% (w/v). The sample was stirred at 20°C for 1 h, then centrifuged at 80000 × *g* for 0.5 h. The colored supernatant was diluted 1 : 10 with 10% glycerol/1 mM dithiothreitol buffer, then Na_4 · EDTA was added to give 1 mM followed by LDAO (pretreated with catalase, 10 μg/ml) to a final concentration of 0.35% (w/v). Thereafter, the mixture was stirred at room temperature. The absorbance at 1020 nm, which is almost entirely due to antenna BChl *b*, was monitored until the absorption remaining in the 950–1050 nm region was judged to be due only to P-960 which absorbs at 965 nm. The sample was then diluted 1 : 2 with 10% glycerol/1 mM dithiothreitol buffer and loaded onto a Whatman DE-51 column which had been preequilibrated with 10% glycerol/1 mM dithiothreitol buffer containing 0.1% LDAO. The column was washed with about three column volumes of 10% glycerol/1 mM dithiothreitol/0.25% LDAO buffer, or until the eluate was colorless. Following a wash with two column volumes of 10% glycerol/1 mM dithiothreitol/0.5% Miranol S2M-SF buffer, the reaction center was eluted with 10% glycerol/1 mM dithiothreitol/0.5% buffer Miranol S2M-SF/150 mM NaCl buffer, then stored at −20°C in the dark.

Spectrophotometry. Spectrophotometry was done on an Aminco DW-2 spectrophotometer coupled with a Midan microprocessor. An Aminco low-temperature attachment was used for spectrophotometric measurements at 77 K. An extinction coefficient of 100 mM^{-1} · cm^{-1} was used to determine the concentration of P-960 at 968 nm [6,7].

Light-induced oxidation of reaction-center cytochromes was measured at wavelengths of 500–560 nm following illumination of the preparation with 968 nm light from a Bausch and Lomb high-intensity monochromator.

Reduction of the intermediary electron carrier(s) was done as described in Refs. 7, 10 and 18. The reaction center, to which a few crystals of sodium dithionite had been added, was illuminated with a Bausch and Lomb high-intensity monochromator set at 1003 nm for illumination at 77 K or 968 nm for illumination at 293 K for periods of time given in the text. After illumination at 293 K, the sample was illuminated while cooling to 77 K. On some occasions, white light from a 500 W slide projector was used to illuminate the sample at 77 K.

Pigment analysis. Carotenoid analysis of the broken membranes and the isolated reaction center was performed as per Thornber et al. [6], except that the Silica Gel 1B plates were chromatographed in petroleum ether (35°–60°)/acetone (90 : 10, v/v). The colored bands were scraped

from the plate and eluted into ethanol for spectral identification. Saponification of the carotenoids in petroleum ether prior to chromatography was by addition of 30% (w/v) KOH in methanol, followed 20 min later by the addition of water. An extinction coefficient for 3,4,3′,4′-tetrahydrospirilloxanthin in organic solvent of 161 $mM^{-1} \cdot cm^{-1}$ was calculated using data in Ref. 25.

Cytochrome content. Cytochrome content was determined from oxidized-reduced difference spectra [6] using an assumed differential extinction (absorbance of α-peak minus absorbance at 540 nm) coefficient of 20 $mM^{-1} \cdot cm^{-1}$ for both cytochromes.

Polyacrylamide-gel electrophoresis. This was done according to Laemmli [26] with a 12.5% acrylamide running gel. All samples were incubated in a buffer (Tris-HCl) containing 3% (w/v) SDS ± 5% mercaptoethanol at 20°C for 20 min, then either loaded directly onto the gel, or boiled for 2 min and immediately cooled on ice to 20°C before loading. Gels were stained with 0.1% Coomassie Brilliant Blue R/10% methanol/7% acetic acid. Marker proteins were: phosphorylase *a*, bovine serum albumin, ovalbumin, catalase, soybean trypsin inhibitor and myoglobin. Staining to locate the heme of *c*-type cytochromes was by the method of Thomas et al. [27].

Chemicals. The following surfactants were used: lauryldimethylamine *N*-oxide (LDAO, Onyx, Jersey City, NJ); sodium dodecyl sulfate (Pierce); Nonidet P40 (Particle Data laboratories, Elmhurst, IL); sodium decyl sulfate (Sigma); Deriphat 160 (Henkel, Minneapolis, MN) and Miranol S2M-SF (Miranol, Irvington, NJ). Whatman DE-51 was from Pierce, the Silica Gel 1B thin-layer chromatography plates were from Baker and all other chemicals were from Sigma.

Results

Isolation of the reaction center

Six detergents were tested for their ability to solubilize the broken membranes of *T. pfennigii*: LDAO, SDS, sodium decyl sulfate, Nonidet P40, Deriphat 160C and Miranol S2M-SF. Miranol S2M-SF yielded an extract in which the color did not sediment during centrifugtion at 80 000 × *g* for 30 min. Such extracts were essentially spectrally identical to that of broken membranes and furthermore, retained P-960 activity. Deriphat 160C did not solubilize the membranes, while the other four detergents did so, but the absorbances at 790 and 840 nm, attributed to the reaction-center pigments [6,7] slowly decayed in such extracts. We thus selected the Miranol extract for further purification (see Scheme I) of the reaction center. It proved necessary to use LDAO to dissociate the antenna from the reaction-center complex and it was therefore added to the Miranol extract, but only for the minimum time necessary for it to achieve its effect and only after decreasing the Miranol concentration by diluting the extract with a buffer containing glycerol, dithiothreitol and Na_4EDTA. Glycerol and dithiothreitol seemed to mitigate the deleterious effects of LDAO (cf. [22]), while Na_4 EDTA appeared to accelerate the dissociation of the membrane proteins (cf. [7]). On addition of buffer, Na_4 EDTA and LDAO, the 1020 nm antenna absorption shifted, via a 930 nm form, to 800 nm. The 800 nm peak was further converted within minutes to a 685 nm-absorbing chlorin. Once the absorbance remaining in the 950–1050 nm region was judged to be due only to P-960, the extract was further diluted to stop the destructive effect of LDAO on the reaction center. This also permitted better binding of the reaction center to a DEAE-cellulose column in the next step. For chromatography, a DE-51 column was used, the more strongly binding ion-exchanger, DE-52, used for the preparation of *Rps. viridis* reaction centers [17], would not release the *T. pfennigii* reaction center after it was absorbed. Even so, with DE-51 the detergent-solubilized antenna BChl and chlorin derivative could not be washed from the column without again adding LDAO to the eluant. As soon as it was judged that all colored impurities had been eluted, LDAO was removed from the column by a Miranol wash (Scheme I). The reaction center was later eluted by a solution of 50 mM Tris-HCl/150 mM NaCl/0.5% Miranol S2M-SF/10% glycerol/1 mM dithiothreitol.

Spectral properties of the isolated reaction center

The *T. pfennigii* reaction center probably contains four BChl *b* and two BPh *b* molecules (see below). Its near-infrared absorption spectra in its

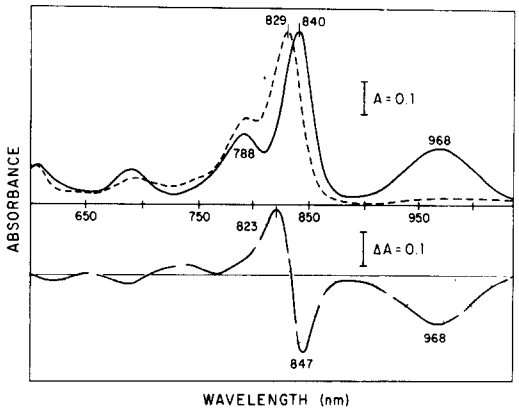


Fig. 1. Room-temperature absorption spectra of isolated (———) and ferricyanide-oxidized (- - - - -) reaction centers of *T. pfennigii*. The oxidized-minus-reduced difference spectrum is shown below (— — —).

isolated (reduced) and chemically oxidized form, are given in Fig. 1. As expected from preliminary work [6,7], there are three distinct peaks, compared to two in the *Rps. viridis* reaction center (Fig. 2). The three peaks behave similarly to those in BChl *a* reaction centers [1] upon oxidation. Thus, the 968 nm peak almost totally disappears, that at 840 nm shifts to higher energy and that at 788 nm shows little change. Therefore, a reasonable assignment of spectra forms to chromophores present in the *T. pfennigii* reaction center would be: the 968 nm peak due to the two BChl *b*

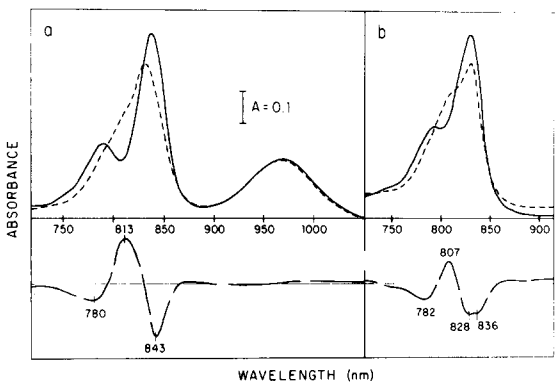


Fig. 2. Comparison of the room-temperature absorption spectra of (a) reduced and (b) $K_3(Fe(CN)_6^-$ oxidized reaction centers of *Rps. viridis* (- - - - -) with those of *T. pfennigii* (———). The concentrations of the two preparations were adjusted so that they had equal A_{965}. The difference between the two upper spectra is shown in the lower portion of the figure (— — —).

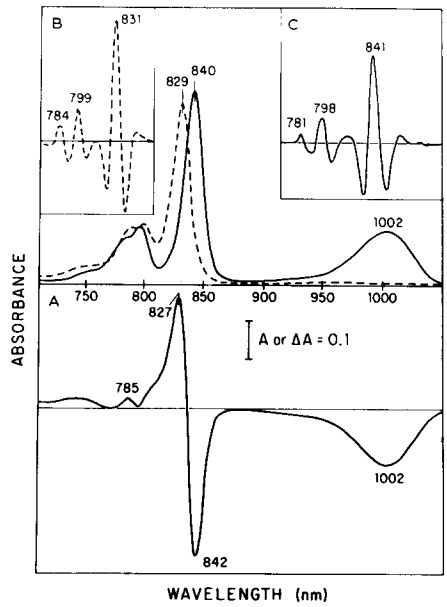


Fig. 3. (A) Spectra of isolated (———) and chemically oxidized (- - - - -) reaction centers of *T. pfennigii* at liquid N_2 temperature. The oxidized-reduced difference spectrum is shown in the lower half (———). Fourth derivatives of the 750–950 nm region of (B) oxidized (— — —) and (C) reduced (———) reaction centers from *T. pfennigii*. Wavelength interval for the fourth derivative is 4.8 nm.

molecules forming P-960; the 840 nm peak to the other two BChl *b* molecules; the 788 nm peak to the two BPh *b* molecules. The peak at 688 nm varies in height among preparations and, as previously [7,28], is regarded as being due to a chlorin degradation product of the antenna BChl *b*. The $\hat{Q}_x$ band(s) of BChl *b* occurs at 607 nm. The oxidized vs. reduced difference spectrum (Fig. 2, see also Fig. 3) differs from that of the *Rps. viridis* reaction center (see Fig. 6 in Ref. 6) in that the absorbance changes between 800–900 nm are due to the shifting of one rather than two or more spectral forms.

The *T. pfennigii* reaction-center spectra were compared in Fig. 2 with those of *Rps. viridis* by adjusting their concentrations so that their 965 nm absorbances were the same. Interestingly, the widths of the longest wavelength bands were then also equal. Spectra of *T. pfennigii* reaction centers have a central peak located at slightly lower energies (840 vs. 832 nm), and a tentatively assigned BPh peak at higher energies than their *Rps. viridis*

equivalents (Fig. 2). Spectral comparison (lower portion of Fig. 2) also shows that the troughs at 780 and 843 nm are more pronounced in the *T. pfennigii* reaction center, while the 813 and perhaps the 828 nm peaks, assigned to BPh *b* and BChl *b*, respectively (Refs. 8 and 29; see, however, Refs. 6 and 9), are stronger in the *Rps. viridis* component. Oxidized *T. pfennigii* reaction centers have greater absorbance at 782 and 828 and 836 nm and less at 807 nm. An attempt will be made in the Discussion section to rationalize these differences on the basis that the spectral forms of each of the BChl and BPh *b* molecules differ between the two preparations.

Low-temperature (77 K) spectra of reduced and oxidized *T. pfennigii* reaction centers, and fourth derivatives thereof, are given in Fig. 3. The equivalent spectra for the *Rps. viridis* complex can be seen in Refs. 6 and 28. In both cases, lowering the temperature shifts the absorbance of P-960 to longer wavelengths (968 → 1001 nm). The 788 nm band, assigned above to the two BPh molecules in the *T. pfennigii* reaction center, splits into two components at 77 K in both oxidized and reduced reaction centers. Fourth derivatives of this band and oxidized vs. reduced difference spectra show that the absorbance of both BPh molecules shifts slightly to longer wavelengths on oxidation of P-960, while absolute spectra show little change in their intensity during the shifts. Unlike *Rps. viridis* reaction centers, the 840 nm band in the *T. pfennigii* complex does not show multiple spectral forms on lowering the temperature. Thus, there is no visible equivalent of the 850 nm shoulder seen in 77 K spectra of *Rps. viridis* [6–9,28]. The sharpening of the 840 nm-peak that occurs at 77 K in *T. pfennigii* reaction centers is much more intense than that in the *Rps. viridis* component (cf. Fig. 3 with data in Ref. 28).

Biochemical properties of T. pfennigii reaction centers

Pigment content. Thin layer chromatography (TLC) of pigments extracted from the reaction center preparation showed that BChl *b* and BPh *b* were both present as were carotenoids. However, because of the instability in vitro of BChl *b* and BPh *b* in the presence of oxygen and/or light, we could not quantitate them accurately by TLC and/or extraction into organic solvents. The best that we have been able to do, until a convenient way is found to measure BChl and BPh *b* contents of material, is to adjust the heights (and widths) of the 960 nm bands of reaction center preparations of *T. pfennigii* and *Rps. viridis* to be equal, and then measure the areas under the 788 and 840 nm peaks in *T. pfennigii* and under the 830 nm band in *Rps. viridis* reaction centers (Figs. 1 and 2). Within 2%, the areas are the same; thus the ratio of BChl/BPh/P-960 in *T. pfennigii* reaction centers is probably the same as the 4 : 2 : 1 ratio previously determined [6,7] for the *Rps. viridis* component.

The two major carotenoids present in this bacterium have been identified as 3,4,3′,4′-tetrahydrospirilloxanthin and its aldehyde derivative, tetrahydrospirilloxanthinal [25]. We observed five carotenoid bands after TLC of organic solvent extracts of whole membranes [25], of which the two most prominent were confirmed as those given above by spectral characterization of bands eluted from the TLC plate. The saponified extract of the reaction center also contained these two major carotenoids which were present in the same proportion as occurred in whole membranes. A ratio

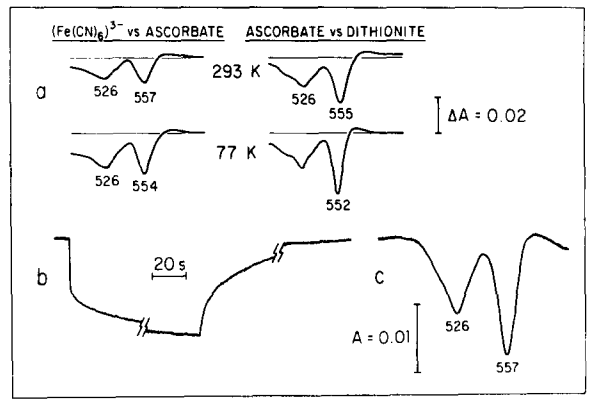


Fig. 4. (a) Chemical difference spectrum of *T. pfennigii* reaction centers in the 500–600 nm region. The upper traces were recorded at room temperature (293 K), the lower ones at liquid N_2 temperatures (77 K). The left pair of traces are the difference of $K_3Fe(CN)_6$ and sodium ascorbate-treated samples, the right-hand pair are the difference of sodium ascorbate- and dithionite-treated samples. (b) Absorbance change at 558 nm when isolated reaction centers were exposed to 960 nm light. (c) Light-minus-dark difference spectrum in the 500–600 nm region under the same conditions used to record (b).

of 0.8 mol carotenoid per mol P-960 was measured for the reaction center.

Cytochrome content. Two *c*-type cytochromes, a high potential cytochrome *c*-557 and low potential cyt *c*-555 [20] were identified and quantitated from ferricyanide vs. ascorbate and ascorbate vs. dithionite difference spectra, respectively (Fig. 4). A molar ratio of 2.5/3.5/1 for cyt *c*-557 : cytochrome *c*-555 : P-960 was measured.

Protein content. Electrophoresis of the dissociated center showed five major bands of M_r = 40000, 37000, 34000, 27000 and 26000 (Fig. 5) when the sample was not heated above 20°C in denaturing buffer. The 40 and 100 kDa bands stained intensely with tetramethylbenzidine/H_2O_2 and are probably cytochrome *c* bands (Fig. 5). Boiling the reaction center's preparation buffer, either in the presence or absence of mercaptoethanol, greatly reduced the intensity of the M_r 34000, 27000 and 26000 bands, and increased the amount of material at the top of the gel. Several fainter bands were present (Fig. 5) and are thought to be contaminants; this includes the 100 kDa band, which could not be dissociated into smaller components. A slight increase in purity of the reaction center was achieved by addition of either ammonium sulfate or poly(ethylene glycol) (PEG 6000), which precipitated some cytochrome material but not the reaction-center complex. Furthermore, the reaction center was spectrally less stable in the presence of the precipitants.

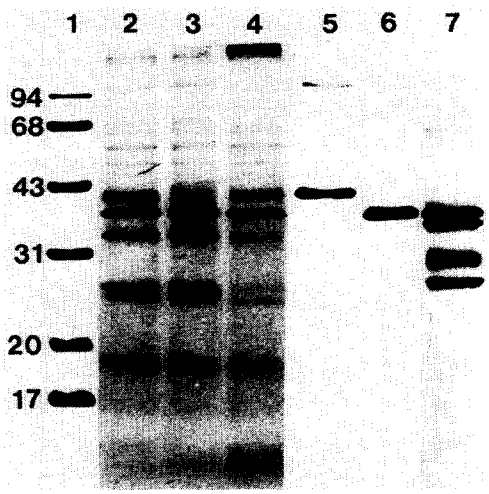


Fig. 5. Stained polyacrylamide gels of: lane 1, protein standards (see Methods); lanes 2–5: Reaction centers of *T. pfennigii*; lane 2, treated with denaturing buffer for 20 min prior to loading; lane 3, treated with denaturing buffer minus 2-mercaptoethanol for 20 min; lane 4, treated with denaturing buffer and heated for 2 min at 100°C prior to loading; lane 5, same sample as used for lane 2 but stained for heme. Lanes 6 and 7, reaction centers of *Rps. viridis* treated with denaturing buffer for 20 min prior to loading, lane 6 stained for heme and lane 7 for protein.

Photochemical properties of the isolated reaction center

The preparation is photochemically active. Exposure of the isolated material to 960 nm light resulted (a) in oxidation of the high-potential cytochrome *c*-557 by P^+-960 (Fig. 4), and (b) in the reduction of BPh by P*-960 (Fig. 6). In Fig. 4b, the time-course of the reversible, light-induced bleaching of the 558 nm absorbance is shown. A light-minus-dark differences spectrum (Fig. 4c) revealed that this absorbance change was due to oxidation of cytochrome *c*-557. One of the two hemes of cytochrome *c*-557 is rapidly photooxidized, while the second was oxidized much more slowly (Fig. 4b). Continuous strong white light (e.g., IR2 mode of the Cary 14R spectrophotometer) is requird to keep not only both cytochromes but also P-960 in a fully oxidized state (see Ref. 2).

Spectra before and after exposure of dithionite-reduced preparations to either 45 min illumination by white light at 77 K (Fig. 6A), or 3 min of 960 nm light at room temperature (Fig. 6B) were recorded at 77 K (see Fig. 6 legend for details). A 30 min illumination of a sample at 77 K was generally sufficient to complete all the absorbance changes that were to occur [20]. However, 3 min illumination by monochromatic 960 nm light completed most of the rapid changes at room temperature; thereafter further, much slower bleaching of all bands occurred (Seftor and Thornber, unpublished data). It has been established for other cytochrome-containing reaction centers [16] that low temperature illumination transfers one, and only one, electron from a low-potential cytochrome via the primary donor to I, an electron carrier intermediary between P-960 and Q. Illumination at room temperature causes more than one electron to be transferred when there is more than one I molecule preceding Q [10].

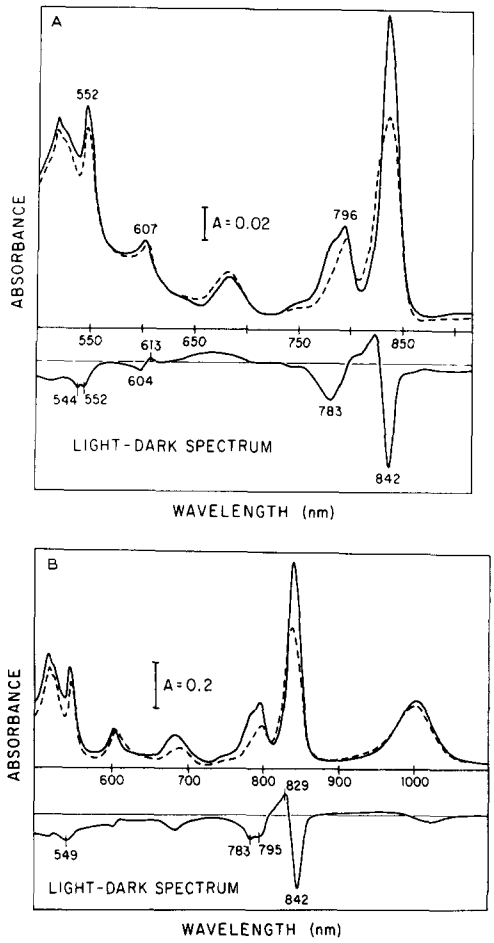


Fig. 6. (A) Liquid N_2 temperature spectra of sodium dithionite-treated reaction centers of *T. pfennigii* before (———) and after illumination with white light from a 500 W projector at 77 K for 45 min (— — —). The light-minus dark difference spectrum is shown in the lower portion of the figure. (B) Same as (A), but illumination was by 960 nm light for 3 min at room temperature (293 K). Note spectra were recorded at liquid N_2 temperatures in both cases.

Illumination of *T. pfennigii* reaction centers at 77 K (Fig. 6A) caused loss of the BPh band at 781 nm; the extent of an absorbance loss at 546 nm ($\hat{Q}_x$ band of BPh) and a broad absorbance increase aroung 685 nm (BPh^- band, [18]) are concomitant with reduction of one BPh molecule per P-960. The following absorbance changes in Fig. 6A are also noteworthy and will be explained later: a loss of some absorbance of the α-band of cytochrome *c*-555 (551 nm at 77 K, see below), a large decrease in absorbance and broadening of the 840 nm BChl peak and a shift of the 607 nm peak to longer wavelengths.

In order to use the absorbance of P-960 and the absorbance change at 551 nm (Fig. 6A) to measure how many electrons each P-960 transferred from cytochrome *c*-555 to an intermediary carrier(s) at 77 K, we needed to know the extinction coefficients for P-960 and cytochrome *c*-555 at 77 K. Thus, the spectrum of the preparation in buffered 55% glycerol/1 mM sodium ascorbate was measured at 293 K in the 2 mm cuvette used for low-temperature spectrophotometry. The cuvette was then cooled to 77 K and the spectrum rerecorded. Solid sodium dithionite was then added to the thawed sample to reduce cytochrome *c*-555, and the spectrum again recorded at 293 K and then at 77 K. From the 293 K spectra, P-960 and cytochrome *c*-555 concentrations could be estimated using the millimolar extinction coefficient given in the Materials and Methods section. Extinction coefficients at 77 K were 1.3-times that at 293 K for P-960 and 1.4-times that at 293 K for the cytochrome. Using these values we confirmed that each P-960 molecule transferred only one electron from cytochrome *c*-555 to a component(s) between P-960 and Q.

Illumination of reduced reaction centers at 293 K required only 1–3 min of exposure to 960 nm light to cause absorbance changes more extensive than did white light in 30 min at 77 K (cf. Fig. 6A and B). Low-temperature spectra before and after 3 min exposure to light at 293 K (Fig. 6B) revealed that both bands (at 781 and 799 nm) ascribed to the two BPh molecules have greatly decreased absorbance. The loss of $\hat{Q}_x$-band absorbance of BPh (approx. 545 nm) is larger than caused by illumination at 77 K, as would be concomitant with the reduction of more than one BPh molecule per P-960. No loss of cytochrome absorbance occurs. The absorbance changes of BChl are, quantitatively and qualitatively, very similar under both conditions of illumination (cf. Fig. 6A and B): there is a considerable decrease and broadening of the absorbance at approx. 840 nm, while the 1000 nm band, due to P-960, shifts to shorter wavelengths (data for the 1000 nm band shown only for illumination at 293 K). Furthermore, the changes in the region of the BChl $\hat{Q}_x$ are essentially identical in two cases.

Discussion

The reaction center of *T. pfennigii* was isolated by a procedure similar, but not identical, to that used to obtain the *Rps. viridis* complex [17]. Miranol was used to solubilize the photosynthetic membranes; thereafter, a short exposure of the extract to LDAO and EDTA separated the reaction center from extraneous membrane material. Glycerol and dithiothreitol seemed to stabilize the reaction center during exposure to LDAO. Unlike earlier isolation methods for this component [7], the new procedure yields reaction centers which are spectrally more stable; they undergo no spectral deterioration during weeks at −20°C, and only degrade slowly over a few hours at room temperatures.

The biochemical characteristics of the T. pfennigii and Rps. viridis reaction centers

These are remarkably similar, considering the spectral and nutritional differences between the organisms. They probably have the same carotenoid content (about 1 mol per mol P-960) and molar ratio of BChl/BPh/P-960 (4 : 2 : 1), but confirmation of the ratio requires the development of more rigorous procedures for the estimation of BChl and BPh *b*. There is no specificity of carotenoid type associated with the *T. pfennigii reaction center.*

The similarity of their cytochrome contents is striking. Two *c*-type cytochromes are bound to the *T. pfennigii* component, cytochromes *c*-555 and *c*-557, and they have approximately the same redox potentials as their counterparts in the *Rps. viridis* complex (Fig. 4, see also Ref. 20). There are 3–4 moles of heme of cytochrome *c*-555 and 2–3 moles of heme of cytochrome *c*-557 per mol of P-960; the latitude in these values indicates the uncertainty in the extinction coefficients used to quantitate them. In the *Rps. viridis* complex, the ratios are slightly smaller: cytochrome *c*-553/cytochrome *c*-558/P-960 = (2–3) : 2 : 1 [7]. The quantitative difference may be accounted for by the *T. pfennigii* preparations containing a contaminating cytochrome(s) of M_r 100000 (Fig. 5) and by the differential extinction coefficients varying for the cytochromes between and within the two organisms; a value of 20 $mM^{-1} \cdot cm^{-1}$ was assumed in all cases.

The dissociated reaction-center preparation shows several major polypeptides (Fig. 5) of which those of $M_r = 40000$, 37000, 34000, 27000 and 26000 are within 1000–2000 of their counterparts in the *Rps. viridis* complex and are thought to be subunits of the reaction center. The 40 kDa polypeptide band is brown and stains for heme, and may contain the hemes of both cytochromes *c*-557 and *c*-555 similar to the 38 kDa band in *Rps. viridis* component (Ref. 6 and Fig. 5). The band at 37 kDa is probably equivalent to the H-band in other purple bacterial reaction centers. The 34, 27 and 26 kDa bands are probably equivalent to the M- and L-bands of BChl *a*-containing reaction centers [5] because of their sizes and their aggregation on boiling (cf. [30]). Specific antibody studies and/or amino acid sequence determination will confirm or disprove these conclusions, and are also needed to decide whether or not the doublet at 27 and 26 kDa represents two forms of the L-band. Although proteolysis may explain the presence of the doublet, we found proteolytic inhibitors (EDTA, phenylmethylsulfonyl fluoride) did not change their relative proportions when added during isolation or when the reaction center would have had increased exposure to proteases during preparative procedures that took much longer. If the proposed equivalences are correct, the ratio of the amount of each polypeptide would be about the same in both *T. pfennigii* and *Rps. viridis* complexes (see Fig. 5).

We have not yet determined which quinones occur in the *T. pfennigii* reaction center; it is known that the *Rps. viridis* component contains 1 mol each of menaquinone and ubiquinone [31,32]. Further purification of the *T. pfennigii* complex is desirable to establish unequivocally its subunit composition, however, increased purity may be difficult to attain because the complex does not precipitate with standard, non-denaturing agents (e.g., ammonium sulfate) and because it has limited stability above 4°C.

The photochemical reactions in the isolated T. pfennigii reaction center

The preparation is photochemically active (Figs. 4 and 6). Illumination of dithionite-reduced reaction centers at 77 K bleached all of the 781 nm band and caused other absorbance changes at 546

and 551 nm, which we interpret as being due to the transfer of one electron from cytochrome *c*-555 via P-960 to reduce most, perhaps all, of one of the two BPh molecules, i.e., BPh_{781}. Most of the added electron density resides on the BPh molecule (cf. [13]) under the above conditions of illumination, and therefore, the changes in the 820–850 nm region must be mainly due to a peak shift of the BChl with the unpaired electron on the BPh, causing a large loss of oscillator strength of the shifting BChl(s) [19] and, to a lesser extent, due to reduction of BChl if it forms an acceptor complex with BPh as proposed by Shuvalov and Parson [13]. In addition, the change around 607 nm ($\hat{Q}_x$ band of BChl) must also be considered largely as a peak shift.

We believe that illumination of reduced reaction centers at room temperature (Fig. 6B) caused reduction of more than one BPh molecule. The 788 nm peak ($\hat{Q}_y$ bands of BPh_{781} and BPh_{798}) was almost completely bleached, and the extent of the absorbance change at 549 nm is concomitant with reduction of more than one BPh molecule [7]. The absorbance remaining in the 788 nm peak after illumination may be due to absorbance of BPh^- ion and/or incomplete reduction of all the BPh present. If our interpretation of the changes in the 820–850 nm region (above) is correct, then similarly very little of the transferred electron density resides on the BChl after room-temperature illumination, since the absorbance changes in the 840 and 610 nm regions ($\hat{Q}_y$ and $\hat{Q}_x$ bands of BChl) are quantitatively very similar under both conditions. It should be noted that the primary reaction in *Rps. viridis* reaction centers at room temperature, brought about by a laser flash, shows identical absorbance changes [11] in the picosecond time-domain to those resulting from continuous 960 nm-illumination of reduced reaction centers at 293 K, but not to those produced by illumination at 77 K [10] or by white light [29]. Thus, 960 nm illumination at 293 K may be the only condition used that causes physiologically significant reactions. On the basis of the present study and the former one on *Rps. viridis* [10], we suggest that in the primary photochemical event, an electron is driven from P-960 via the two BPh molecules (we cannot detect the reduction of one BPh molecule prior to the other) to a quinone molecule; one BChl molecule may be associated with one of the BPh molecule to form an acceptor complex [13]. We cannot eliminate the possibility that a BChl molecule precedes the BPh (molecules) in the electron transfer sequence cf. [14] in *T. pfennigii* reaction centers, since it may not be possible to trap electrons on the BChl molecule after both BPh molecules are reduced, due to a very rapid back-reaction between $BChl^-$ and $P960^+$ [3,13]. The 840 nm band can indeed be made to bleach almost completely after the 788 nm band has been bleached by prolonged (0.5–1.5 h) exposure of the reduced reaction centers to strong white light at room temperatures (Seftor and Thornber, unpublished data), which could indicate that a BChl molecule precedes the BPh molecules in the reaction. However, the prolonged, intense polychromatic light may cause the electron from P-960, or from other pigments, to perform non-physiological reactions [7,10]. Measurement of absorbance changes in *T. pfennigii* reaction centers, brought about by a laser flash, must now prove valuable in delineating further the primary event in photosynthetic bacteria. The spectral changes associated with reduction of either BPh molecule are known (Fig. 6), and, therefore, if a BChl molecule receives an electron before the BPh molecule during charge separation, the absorbance changes at the earliest time after the flash will be quite different.

Comparison of the spectral forms in T. pfennigii and Rps. viridis reaction centers

The absorbance of the *T. pfennigii* reaction center differs substantially from the *Rps. viridis* component by having three – as is usual for a bacterial reaction center (cf. [1]) – rather than two near-infrared peaks (Fig. 2). The assignments of BChl or BPh molecules to each of the three bands in *T. pfennigii,* made in the early part of the Results section, were substantiated by the experiments that followed. Thus, firstly, the 968 nm peak bleaches on exposure of the preparation to light or to oxidants (Figs. 1 and 3), as expected if it is due to the primary donor; secondly, exposure of dithionite-reduced reaction centers to light (Fig. 6) confirmed that the 788 nm peak is due to the Q_y bands of BPh, and that its resolution into two at 77 K (Fig. 3) is due to two spectral forms of BPh.

TABLE I

ASSIGNMENTS OF SPECTRAL FORMS IN REDUCED AND OXIDIZED BChl *b*-CONTAINING REACTION CENTERS TO CHROMOPHORE MOLECULES PRESENT

Data, wavelengths in nanometers, are from Figs. 3 and 6, and Ref. 29.

	Rps. viridis		*T. pfennigii*	
	Reduced	Oxidized [a]	Reduced	Oxidized
P-960	965	–	968	–
	820 [a]	–	≈ 830 [a]	–
$BChl_1$	851	804	≈ 841	≈ 831
$BChl_2$	834	828	≈ 841	≈ 831
BPh_1 [b]	805	811	781	784
BPh_2	789	791	798	799
P^+-960	–	832	–	≈ 830 [a]

[a] Data from Seftor and Thornber [29].

[b] Equivalence of BPh_1 between the two reaction centers based on this form being the BPh molecule to receive the electron when reduced reaction centers are exposed at 77 K to light (Fig. 6).

Table I gives our view on the assignment of chromophores to the spectra forms observed at 77 K in oxidized and reduced *T. pfennigii* and *Rps. viridis* reaction centers [29]. These assignments adequately explain the absorbance or difference spectra for both components as well as the spectral differences between the two (Fig. 2). For example, the differences between the two spectra in Fig. 2A are due to *Rps. viridis*, having its two Bph molecules each absorb approx. 15 nm towards longer wavelengths and one of its BChl absorb at shorter wavelengths than they do in *T. pfennigii*. Similarly, the relatively much larger shift (851 → 804 nm) of $BChl_1$ in *Rps. viridis* on oxidation of P-960 [29] explains in part the difference spectrum in Fig. 2B, and why the oxidized vs. reduced difference spectrum of *Rps. viridis* reaction centers [6,8] had two bands appearing in the 800–830 nm region, while *T. pfennigii* reaction centers have one (Figs. 1 and 3). Thus, some leeway is permitted in the environment of the reaction-center chromophores without impairing function. The virtual absence of a positive band at 850 nm in the 293 K difference spectrum (Fig. 2A) is not explained by the assignments made in Table I. This is due to the wavelength maximum of some, but not all, spectral forms shifting between 293 and 77 K, and therefore describing room-temperature spectra, such as in Fig. 2A, with 77 K spectral forms will not always succeed. This and other points regarding the spectral properties of reaction centers, particularly those of P-960, will be discussed in a subsequent paper.

For the present, it is appropriate to comment further only on our assigning two spectral forms to P-960 in Table I. Data on *Rps. viridis* led some groups [6,8,9,11] to conclude that P-960 has a higher energy transition at 850 nm and a lower energy transition at 965 nm, as expected if an exciton pair of BChl forms P-960. However, there is some disagreement on this point [12,13,29]. The data in this paper contribute to the matter as follows: since the environment forming P-960 must be very similar in *T. pfennigii* and *Rps. viridis* as judged by their almost identical 965 nm bands (Fig. 2), then if the higher energy transition is at 850 nm it should be seen in 77 K spectra of *T. pfennigii* reaction centers (e.g., Fig. 3). Its absence indicates to us that the 850 nm band is not due to the higher energy transition of P-960 [13]. If that transition is of sufficient oscillator strength to contribute to the absorbance of the preparation, and if it occurs at the same wavelength in both BChl *b*-reaction centers, then we can only conclude that it occurs within the 840 nm band. We have presented some evidence [29] that in *Rps. viridis* reaction centers at 820 nm band is due to this transition.

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