ISOLATED PHOTOCHEMICAL REACTION CENTERS FROM BACTERIOCHLOROPHYLL b - CONTAINING ORGANISMS

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Synopsis: Optical, ESR and pulsed laser spectroscopic examination of isolated bacteriochlorophyll (BChl) b - containing reaction centers (RCs) demonstrates that their composition (1 P960: 2 bacteriopheophytin (BPh) b: 2 cyt c 558: 2 cyt c 553; BChl/BPh = 2) and their mechanism for

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charge separation are very similar to those in BChl a-containing RCs; however, $P960^+$, the oxidized form of the primary electron donor, is apparently not a symmetrical dimer of BChl b. The 'primary' electron acceptor (X) is a quinone-iron complex, and I, the intermediary electron carrier between P960 and X, is BPh which interact(s) strongly with one or more of the other components in the RC. Comparison of the optical spectra of isolated RCs of the BChl b-containing bacteria, Rhodopseudomonas viridis and Thiocapsa pfennigii, with those of their intact cells shows that the BPh in the RC is NOT generated during the isolation procedure.

This paper summarizes the collaborative studies performed during 1976-77 by the laboratories listed above on the mechanisms of the primary photochemical event and the composition of the photochemical reaction center in BChl b-containing organisms.

Rhodopseudomonas viridis Reaction Center

Isolation. Although useful information had been obtained during 1968-72 from examination of SDS-RC preparations (1), further studies were hindered by the presence of a chlorin impurity absorbing at 685 nm. This contaminant which adhered tightly to the RC, arose from photo-oxidation of antenna BChl b during the preparative procedure. Inclusion of $Na_2S_2O_4$ in buffers used in RC isolation eliminated most of the chlorin (2). Recently Pucheu et al (3) have developed a better isolation procedure which uses the detergent, lauryldimethylamine oxide (LDAO), as the membrane-solubilizing agent and yields a chlorin-free product.

We (4) have shortened their procedure as follows: A 1% LDAO extract is made in which the absorption of the antenna BChl b has been shifted from 1015nm to 810nm. The extract is diluted to, or dialyzed against, 0.1% LDAO and then chromatographed on DEAE-cellulose; a RC-enriched fraction is eluted by 135mM NaCl. This eluate is adsorbed onto hydroxylapatite from which the RC is removed by 0.2M sodium phosphate, pH 7.0. Addition of ammonium sulfate to 22% (w/v) precipitates the RC which is redissolved in 50mM Tris, pH 8.0.

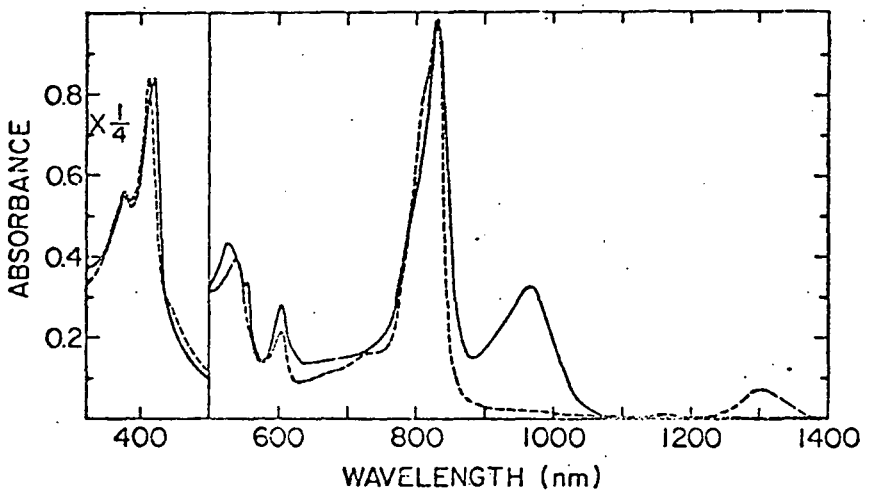

Fig. 1. Room temperature absorption spectra of R. viridis, LDAO-RCs in the reduced (——) and in the oxidized (- - - -) state recorded on a Cary 14R spectrophotometer.

Optical spectra and composition of the LDAO-reaction center.

Absorption spectra of the RC preparation are shown in Fig.1. On photo- or chemical oxidation P960 loses most of its absorbance at 958nm; the decrease at 605nm and increase at 1310nm are correlated with the formation of $P960^+$. The major near infrared (NIR) peak at 832nm is assigned to some BChl *b* molecules closely associated with P960; this peak shifts to 828nm when P960 is oxidised. BChl *a*-containing RCs exhibit three NIR bands: at ~870nm (due to P870, a special BChl *a* pair (BChl *a*)$_2$), ~800nm (due to BChl *a* closely associated with P870), and at ~760nm (due to BPh *a*). The presence of three corresponding bands in *R. viridis* RCs is not immediately apparent. The 960- and 832nm-peaks are homologous to the 870- and 800nm peaks, but only careful study of the spectrum reveals that a shoulder exists at ~790nm; further studies indicate that this is due to BPh *b* and therefore is homologous to the 760nm band in BChl *a*-containing RCs.

Treatment of LDAO-RCs with 0.1N acetic acid and/or 0.5% SDS converts all of the 960 and 832nm absorbance into that of a 685nm-absorbing chlorin, whereas the absorbance at 790nm characteristic of BPh *b* appears to be unaffected. Using $\varepsilon_M = 41{,}000 cm^{-1}$ for the Q_y band of BPh *b* *in vitro* (5) and an assumed $\varepsilon_M = 100{,}000 cm^{-1}$ at 958nm for P960 (1), two BPh molecules are found per P960. The ratio of BChl *b* to BPh *b* or P960 was also obtained from this experiment. The increase in 685nm absorbance that occurred when the 960 and 832nm peaks disappeared was known, and an approximate $\varepsilon_M = 34{,}000 cm^{-1}$ at 685nm for the chlorin was measured by converting LDAO-solubilized antenna BChl *b* absorbing at 796nm (ε_M = $91{,}000 cm^{-1}$) into the 685nm form by SDS/acetic acid treatment.

A probable molar ratio of 4BChl/2BPh/1P960 was thereby obtained.

The absorption bands in the visible region are due to cytochromes as well as BChl and BPh b and possibly carotenoids (cf.1). Two cytochromes are present - cyt c 553 (E_m = -12mV) and cyt c 558 (E_m = +330mV) (6,7). There are 2 hemes of c558 and 2-3 (probably 2) hemes of c553 per P960. Both can reduce $P960^+$ at room temperature (photooxidation of c558 can be seen in Fig.1; it has a $t_{\frac{1}{2}}$ of 200ns). At lower temperatures only c553 operates ($t_{\frac{1}{2}}$ = 1ms at 77°K).

Resolution of the 830nm band by 77°K optical spectrophotometry. The 832nm band is better resolved at 77°K, as was reported for the SDS-RC (2). The 790nm shoulder is now a peak and the 832nm band splits into a major component absorbing at 836nm, a smaller peak at 817nm, and a shoulder at 850nm. The 817nm component was not seen in SDS-RCs (2), which may explain the greater (3.2vs2.5) 830/960nm ratio in LDAO-RCs. On oxidation of P960, the 850nm shoulder disappears, the 836nm peak shifts to 830nm and a band appears at 808nm which obscures the 790 and the 817nm bands, if indeed the latter is still present. A difference spectrum (2) further substantiates that at least two bands disappear while two appear. The most probable assignment of the bands to the pigments in the RC is: 790nm to BPh, 817 and 836nm to two of the four BChls, and the 850nm shoulder and the 987nm peak to P960. $P960^+$ would account for the 808nm peak, for a 1315nm peak and possibly for part of the 830nm band. These assignments agree with Vermeglio and Clayton's (8) notion that P870 in R. sphaeroides absorbs at 870 and 810nm while $P870^+$ absorbs at 790nm.

ESR studies on $P960^+$. Chemical or photo-oxidation of P960 in cells of R. viridis or in SDS or LDAO-RCs yields ESR spectra of $P960^+$ with a g value of 2.0026. The line width of the signal is 11.8 ± 0.2 gauss at 130° and 230°K, whereas that of monomeric BChl $b^{\cdot+}$ in vitro is 14.0 gauss (5). ENDOR transitions occur at 1.6, 1.9, 3.3 and 4.0 gauss for $P960^+$ and at 1.7, 3.2 and 4.5 gauss for BChl $b^{\cdot+}$ in vitro. These data are incompatible with the symmetrical dimer model proposed for P870 and P700 (9,10). $P870^+$ and $P700^+$ exhibit line widths that are $1/\sqrt{2}$, and ENDOR transitions that are half, the values for monomeric BChl $a^{\cdot+}$ or Chl a^+ in vitro, and this has led to the conclusion that the unpaired electron in $P870^+$ or $P700^+$ is shared equally between a "special pair" of BChl a or Chl a molecules. Nevertheless P960 must be an oligomeric (probably dimeric) form of BChl b since the linewidth and ENDOR transitions are smaller than those of monomeric BChl b. ESR data on the triplet of P960 (7) support this notion; furthermore, monomeric BChl $b^{\cdot+}$ could not account for the absorption band of P960 at 850nm, nor that of $P960^+$ at 1310nm (Fig.1).

The "primary" electron acceptor (X) in R. viridis. The traditionally-defined "primary" electron acceptor in purple bacteria is now generally agreed to be a quinone-iron complex (QFe) which captures the electron photoejected from $(BChl)_2$. Q^-Fe gives rise to a well characterised ESR signal at g = 1.82 or at g = 2.0045 (due to the semiquinone radical) if the Fe has been removed (11,12). R. viridis LDAO-RCs exhibit the g = 1.82 signal whereas SDS-RCs show the g = 2.0045 signal. Thus in R. viridis, as in R. sphaeroides (12), SDS treatment interferes with the iron-quinone interaction either by extracting the iron, moving

it or changing its spin state (12). The quinone in R. viridis appears to be a menaquinone (cf.3).

The intermediary electron carrier (I) in R. viridis. Fig. 2 shows the absorbance changes that occur on a picosecond time scale following excitation of R. viridis RCs with short flashes. Similar measurements in R. sphaeroides have shown that an intermediary electron carrier (I) functions between $(BChl)_2$ and QFe (e.g. 13,14).

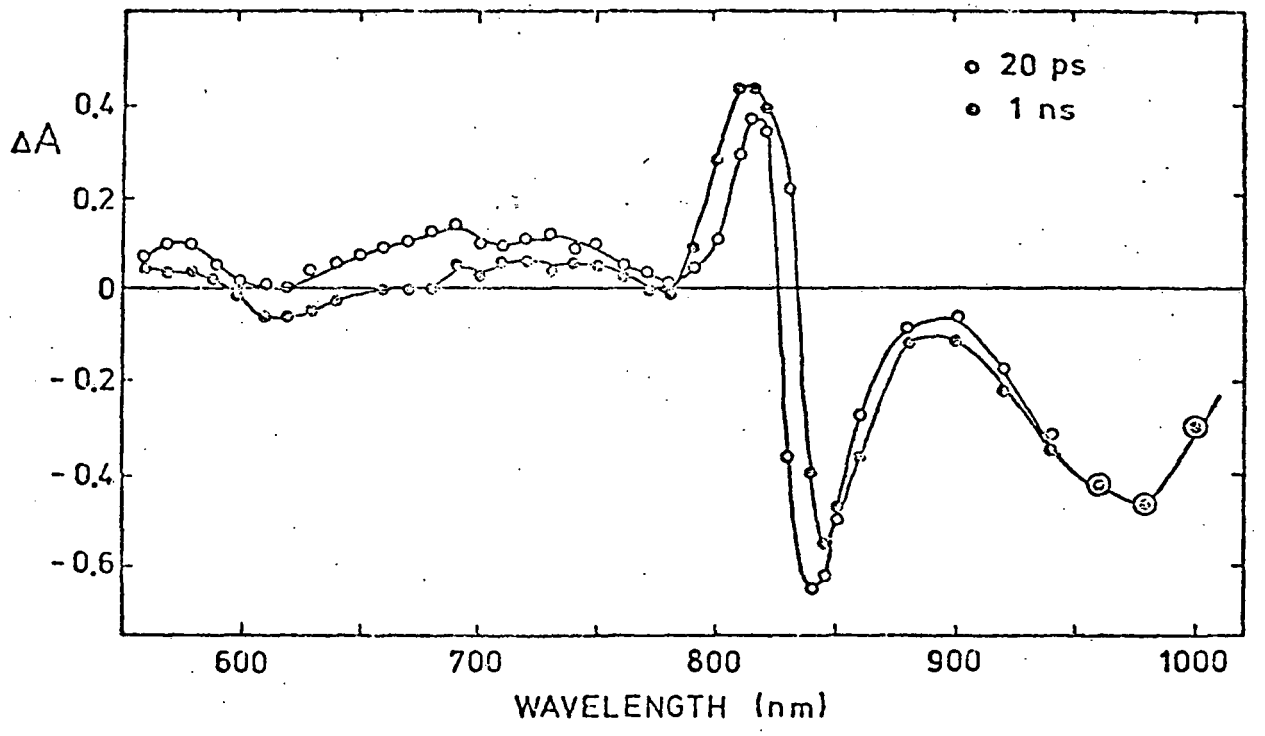

Fig. 2. Difference spectra of the absorbance changes resulting from excitation with an 8 picosecond flash measured after time delays of 20 picosec (open circles) and 1 nanosec (closed circles). Measurements employed approx. 52μM reaction centers of R. viridis in 50mM Tris-HCl-0.1% LDAO, pH 8.0; the path length was 1mm. Each point reflects the average of at least three measurements and typically has a standard deviation of ± 0.03.

Similar measurements in R. sphaeroides have shown that an intermediary electron carrier (I) functions between $(BChl)_2$ and QFe (e.g. 13,14). I has been proposed to be a BPh molecule (5,15). In such experiments the $(BChl)_2^{+\cdot}\ I^{-\cdot}$ state is only observed as a short-lived transient ($t_{\frac{1}{2}}$ = 200ps) and then only in the presence of $(BChl)_2^{+\cdot}$. Several groups (2,4,7,14,16-18) have recently devised a method for trapping RCs in the $(BChl)_2\ I^{-\cdot}$ state for periods of seconds to hours, permitting examination of $I^{-\cdot}$ by more conventional techniques. This is best accomplished in bacteria (e.g. Chr. vinosum, R. viridis) that possess cytochromes capable of reducing $(BChl)_2^{+\cdot}$ within a few μsec. Prolonged illumination (15sec-3min) of such bacteria or their isolated RCs in the presence of $Na_2S_2O_4$ is required to reduce I fully (QFe is directly reduced by the $S_2O_4^{=}$) because the rate of the reverse reaction $I^{-\cdot} \rightarrow P^{+\cdot}$ in 2-3 orders of magnitude faster than the $cyt \rightarrow P^{+\cdot}$ reaction.

The difference spectrum resulting from prolonged illumination of R. viridis LDAO-RCs in $Na_2S_2O_4$ is shown in Fig. 3B. The same spectrum is generated by illumination with light absorbed by BPh (790nm), by BChl (837nm) or by P960 (938 or 1027nm), indicating that it probably does not reflect a non-physiological reaction such as $BPh + S_2O_4^{=} \xrightarrow{h\bar{\nu}} BPh^{-\cdot}$. The spectrum agrees remarkably well with a spectrum of the absorbance changes that decay between 20ps and 1ns following excitation of LDAO-RCs poised at +200mV, i.e., the absorbance changes that decay when $I^{-\cdot} \rightarrow I$ (Fig.3A). We are confident therefore, that prolonged illumination of RCs treated with $Na_2S_2O_4$ yields the $(BChl\ \underline{b})_2\ I^{-\cdot}$ state. Comparison of the spectra in Fig.3 with those of BChl b, BPh b and their anions in vitro (5) show that a BPh molecule is almost certainly being reduced.

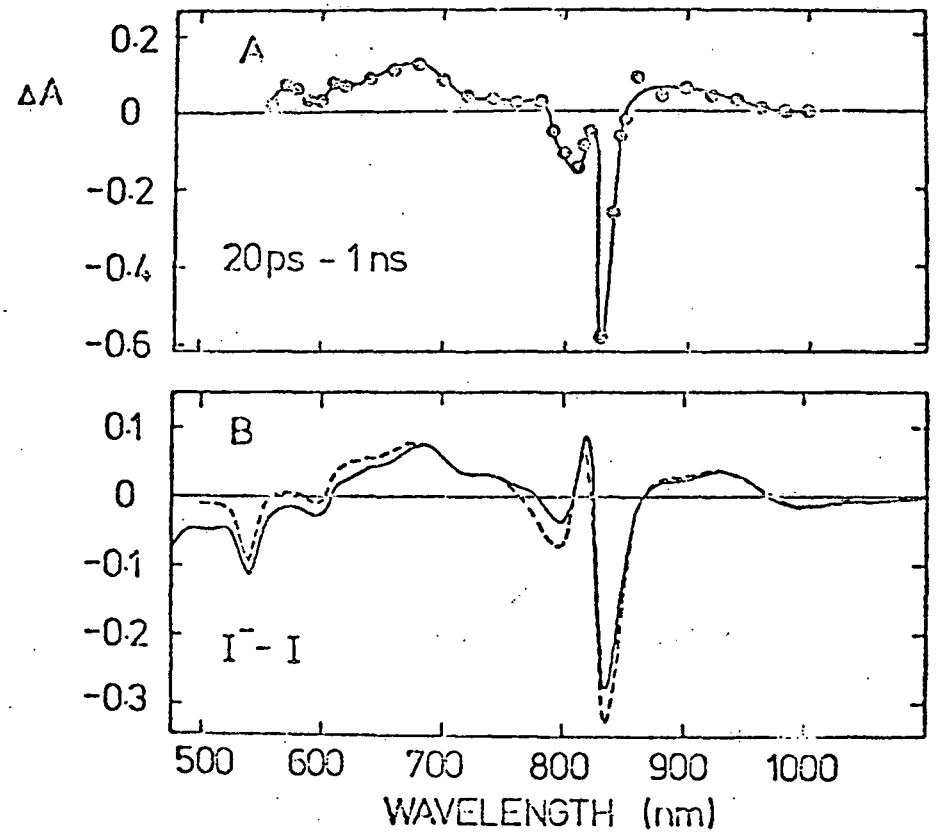

Fig. 3. Difference spectra of I^- - I in R. viridis.

(A) Difference spectrum calculated from the data in Fig.2 by subtracting the measurements made at 1ns from those made at 20ps.

(B) Difference spectra caused by illumination of RCs at low redox potentials. Solid curve: 3.4μM LDAO-RCs; dashed curve 3.6μM Triton X-100-RCs. Solid sodium dithionite was added to lower the potential in both cases and the samples were illuminated with white light. For the LDAO-RCs the difference spectrum was measured with the white light on, because the absorbance changes started to decay when the light was turned off. For the Triton-RCs the spectrum was measured with the light off since the absorbance changes lasted for many minutes after illumination ceased.

The loss of absorbance at ~545nm, the broad absorbance increase between 600-750nm and the increase around 900-950nm are characteristic of BPh $\rightarrow$ $BPh^{-\cdot}$ (5). However, the lack of an expected large absorbance loss at 790nm due to BPh $\rightarrow$ $BPh^{-\cdot}$, and the presence of one at 836nm, where BChl absorbs, are puzzling. The reduction of BPh could cause an electrochromic shift (i.e. from 836 $\rightarrow$ 800nm) of nearby BChl molecules, with the positive-going peak obscurring the absorbance loss at 790nm due to BPh — $BPh^{-\cdot}$. Other groups (13,16,17) have come to a similar conclusion. Alternatively, the 836nm band could result from an interaction between BPh and BChl, and the interaction could be broken by the reduction of BPh.

The ESR spectrum of $I^{-\cdot}$ at 130°K reveaks a free radical signal with g = 2.0035 $\pm$ 0.0002, 13 gauss linewidth and with ENDOR transitions at ~3.2 and ~2.7 gauss. Since these parameters are characteristic of both monomeric BPh $\underline{b}^{-\cdot}$ or BChl $\underline{b}^{-\cdot}$ *in vitro* (5), one can conclude only that $I^{-\cdot}$ is a monomeric anion radical of BPh or BChl *b*. At temperatures below 15°K, in membrane fragments of *R. viridis* $I^{-\cdot}$ exhibits a broad signal split by ~140G and centered at g = 2.003 (4,7); the g = 1.82 signal of $Q^{-}Fe$ is no longer seen. This could result from interaction between $I^{-\cdot}$ and $Q^{-}Fe$ (4). This notion is supported by data on the $I^{-\cdot}$ signal in SDS- and LDAO-RCs at <15°K: SDS-RCs which exhibit a g = 2.0045 of Q^{-} rather than the g = 1.82 signal of $Q^{-}Fe$ for $X^{-\cdot}$, lack the broad split signal of $I^{-\cdot}$ when illuminated but instead display a narrow (13G) signal at g = 2.003; whereas LDAO-RCs, which retain most of the g = 1.82 signal of $X^{-\cdot}$, exhibit a mixture of the broad and the narrow signals (4).

Some other points connected with I should be mentioned: At 300°K

the ΔA due to $I^{-\cdot}$ (Fig.3) begin to decay immediately illumination of LDAO-RCs + $Na_2S_2O_4$ is stopped. Alternatively, if the LDAO is exchanged for Triton X-100, or if SDS-RCs are used (2), hardly any decay has occurred 1 hr after illumination has ceased. Similarly, illumination of LDAO-RCs at 200°K results in irreversible ΔA for $I^{-\cdot}$ (13). LDAO may therefore act as an acceptor of electrons from $I^{-\cdot}$ in LDAO-RCs at room temperature (19). Secondly, both BPhs in the RC are apparently reduced as estimated from the ΔA at ~545nm ($\Delta\varepsilon_m$ = 13,000 (5), when LDAO- or Triton-RCs + dithionite are illuminated at 300°K, whereas at 200°K it has been precisely measured in Chr. vinosum that only one is reduced (13). The 300°K result merits further examination. Thirdly, in Triton-RCs with I pre-reduced, pulsed laser spectroscopy reveals that absorbance changes still occur; a bleaching is seen at 960nm with a shoulder at 850nm and with little change in the 760-830nm region. If these ΔAs reflect the excited singlet state of P960, a strong possibility when electron transfer from P960 to I is prevented, then they support the view that the "special pair", (BChl b)$_2$, absorbs at 850nm as well as 960nm (see above). Lastly, when X is reduced and I oxidized, illumination at a few °K produces a spin-polarized triplet (20). Under these circumstances, the electron leaves P960, goes to I and returns after spin rephasing, resulting in the formation of the triplet state of P960 (20). By using the triplet ESR signal to monitor the redox state of I, its midpoint potential was measured as -140mV (4,7). A more negative value of -620mV has also been reported (18,21). For comparison the value for BPh b in organic solvents is ~-530mV (5).

Thiocapsa pfennigii reaction center

Absorption spectra of intact cells of this BChl b-containing organism had indicated that the 790nm BPh b band is spectrally better separated from the 830nm-absorbing BChl than it is in R viridis (Fig.4). Attempts were therefore made to isolate the RC: an extract was made by treating broken cells with 0.5% LDAO in 50mM tris, pH 8.0, followed by centrifugation. The supernatant was made 33mM in EDTA and then incubated at 20°C until the 1024nm peak had completely shifted to 685nm. After the shift was complete (~1 hr), the solution was diluted to 0.1% LDAO and chromatographed at 4°C on DEAE-cellulose, from which a crude RC preparation was eluted with 50mM Tris-300mM NaCl - 0.05% LDAO, pH 7.8. Further necessary purification has not yet been achieved: Ammonium sulfate precipitation, warming or exposure to intense white light apparently destroy the RC.

The Th. pfennigii LDAO-RC spectrum (Fig.5) shows interesting differences from that of R. viridis (Fig.1): The presumed BPh b absorbance at 790nm is clearly distinguishable from the ~830nm peak which in Th. pfennigii is located at 837nm, and the far IR band of $P960^+$ is at 1270-nm. A chlorin contaminant causes the 685nm component (c.1). Two cytochromes with α-bands at 550 and 556nm are present in about the same ratio with respect to P960 as C553 and C558 are in R. viridis RCs.

ESR studies have been made on broken cells of Th. pfennigii. The spectra of $P960^+$, $Q_F e^-$ and the spin-polarized triplet are very similar to those in R. viridis. Likewise the ESR spectrum of $I^{\cdot -}$ at ~15°K suggests that $I^{\cdot -}$ is also magnetically coupled to $Q^- Fe$ in Th. pfennigii.

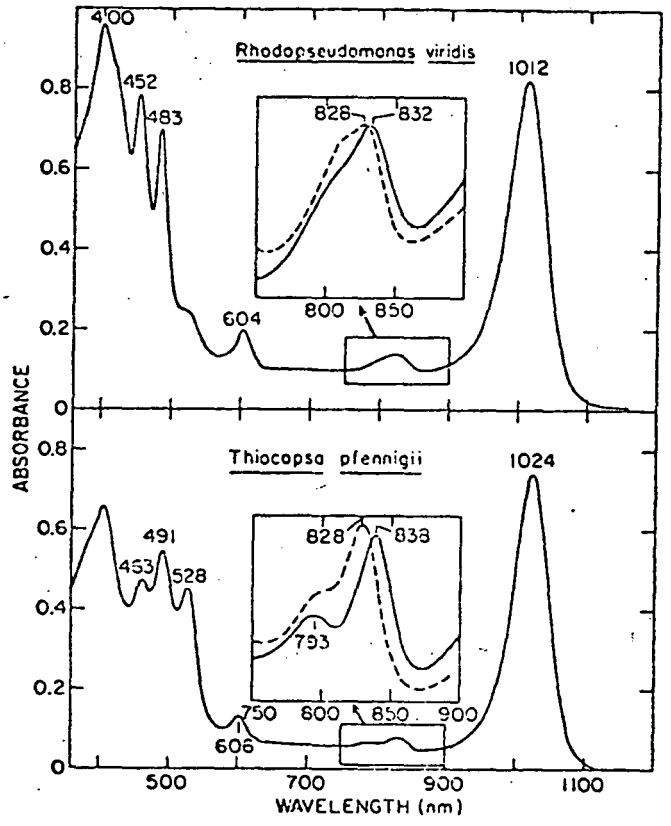

Fig.4. Absorption spectrum of intact cells of R. viridis and Th. pfennigii. In both cases the inset shows the spectrum of a more concentrated cell suspension around the 830nm peak. The dashed trace was recorded in the IR2 mode of the Cary 14R, i.e. in the presence of intense white light in which P960 is presumably in its oxidized form. Cells suspended in 50mM Tris-HCl, pH 8.0.

Is bacteriopheophytin a genuine constituent of the RC?

There have been suggestions that BPh in isolated RCs may be produced from BChl during the isolation procedure. Figs.1,4 and 5 provide evidence that this does not occur. The absorption spectra of intact cells of the two bacteria and of their 830nm bands in more concentrated cell suspensions are shown in Fig.4. Probably all the antenna BChl b

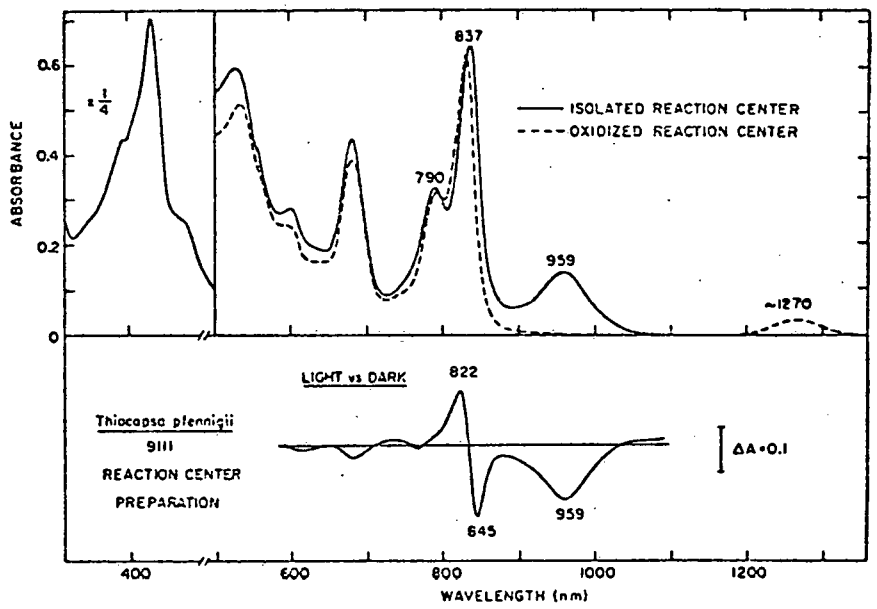

Fig.5 Absorption spectra of Th. pfennigii RC preparation in its reduced (———) and photooxidized (- - - -) state.

is accounted for in the 1012- and 1024nm peaks, and the peaks in the 750-850nm region are probably due to pigments in the RC. The shape and wavelength maxima of the latter peaks are replicated in the isolated RCs of the two organisms, both when P960 is reduced or oxidized. Thus the 793nm peak in Th. pfennigii whole cells and the 790nm shoulder in those of R. viridis apparently reflect the presence of BPh in the RC in intact cells, indicating that BPh is not manufactured during the isolation process.

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