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FURTHER STUDIES ON THE COMPOSITION AND SPECTRAL PROPERTIES OF THE PHOTOCHEMICAL REACTION CENTERS OF BACTERIOCHLOROPHYLL *b*-CONTAINING BACTERIA

J.P. THORNBER [a], R.J. COGDELL [b], R.E.B. SEFTOR [a] and G.D. WEBSTER [b]

[a] *Department of Biology and Molecular Biology Institute, University of California Los Angeles, CA 90024 (U.S.A.), and* [b] *Department of Botany, University of Glasgow, Glasgow G12 8QQ, Scotland (U.K.)*

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Summary

1. There are no basic differences in the biochemical composition of photochemical reaction centers between bacteriochlorophyll *a*- and bacteriochlorophyll *b*-containing purple bacteria. Both types have the same stoichiometric ratios of bacteriochlorophyll/bacteriopheophytin/special pair (i.e., 4 : 2 : 1) and of carotenoid/bacteriochlorophyll (i.e., 1 : 4), and the molecular weight equivalencies of the polypeptide subunits of the reaction center are basically similar.

2. The isolated reaction center of *Rhodopseudomonas viridis* contains polypeptide subunits of 38, 35, 28 and 24 kilodaltons. The latter two are aggregated by heat. Of the two *c*-type cytochromes present in the isolated material, *c*-553 (and possibly *c*-558) is coincident with the 38 kilodalton component. It is concluded that the 35, 28 and 24 kilodalton polypeptides correspond to the so-called H, M and L subunits of bacteriochlorophyll *a*-containing reaction centers.

3. The isolated reaction center contains carotenoid which can apparently be removed from the reaction center by 4% (w/v) lauryldimethylamine oxide. 1′,2′-dihydroneurosporene (50—70%) and 1′,2′-dihydrolycopene (30—40%) constitute the bulk of the reaction center carotenoid. There is no specificity of carotenoid type inserted into the reaction center.

4. The spectral forms of the chromophores in the 830-nm band of room and 77 K absorption spectra of reaction centers of *Rps. viridis* have been studied via fourth derivatives of the spectra. Four forms with maxima at 787, 812—813, 834—835 and ~850 nm are present. We attribute the forms to bacteriopheophytin *b* (787 nm), two 'voyeur' bacteriochlorophyll *b* molecules (one at 812—

813 nm and one at 834—835 nm), and a spectral component of *P*-960 (~850 nm). Each spectral form in the 830-nm band occurs at the same wavelength maximum and in the same relative proportions in both intact photosynthetic membranes and isolated reaction centers. We thus conclude that each form, in particular the bacteriopheophytin and 812—813 nm bacteriochlorophyll components, is present in the intact photosynthetic membrane, and that they are not generated by treatment of the membranes with detergents; furthermore, we believe that the absorption bands between 780 and 860 nm in spectra of whole cells of *Rps. viridis* and *Thiocapsa pfennigii* are due to the absorbance of reaction center chromophores.

Introduction

The primary photochemical event of photosynthesis takes place within a membrane-bound pigment-protein complex termed the photochemical reaction center. Studies on purified reaction centers from which most or all extraneous material, in particular the antenna or light-harvesting chlorophylls, has been removed, enables the primary charge separation to be spectrally monitored more readily than in the intact membrane, and permits the data obtained to be interpreted less equivocally. From such studies we are learning more about the primary photochemical mechanism. Reaction centers with the smallest number of chromophore molecules per unit have been isolated from some purple bacteria (for reviews see Feher and Okamura [1], Olson and Thornber [2], and for a very recent treatment Vadeboncoeur et al. [3]. Ref. 2 summarizes the comparative biochemistry of reaction center preparations isolated from all types of photosynthetic organisms). Reaction centers purified from bacteriochlorophyll *a*-containing purple bacteria are composed of 6—7 pigment molecules (four bacteriochlorophylls, two bacteriopheophytins and generally one carotenoid), an Fe-quinone complex, and three polypeptide, labeled H, M and L, with respective molecular weight equivalences of 28, 24 and 21 kilodaltons [1—3] (however see Clayton and Clayton [4]) per reaction center unit. Spectral studies on isolated reaction centers have revealed that two of the four bacteriochlorophyll molecules form a 'special pair', the primary electron donor (*P*-870), and one of the bacteriopheophytin molecules acts as an intermediate electron carrier between *P*-870 and the iron-quinone complex in the primary photochemical event [5].

Rps. viridis and *Th. pfennigii* are two purple bacteria that contain bacteriochlorophyll *b* rather than bacteriochlorophyll *a*. Although these organisms have yielded reaction center preparations [4,6—8], the biochemical and spectral composition of the reaction center has not been sufficiently characterized to make optimum use of much of the biophysical data that have recently been accumulated for these organisms (see, for example, Refs. 8—11). The aim of the research described here was to advance such knowledge to the same state achieved for bacteriochlorophyll *a*-containing reaction centers. Bacteriochlorophyll *b*-containing reaction centers offer some advantages over their bacteriochlorophyll *a*-containing counterparts: for example, the Q_y transitions of the component pigments are spectrally more separated [7], permitting the molecu-

lar events occurring during primary photochemistry to be more easily described; secondly, reaction centers of *Rps. viridis* and *Th. pfennigii* are isolated with the two membrane-bound, *c*-type cytochromes attached to the reaction center polypeptides; these cytochromes can feed an electron very rapidly (within 1 μs) to *P*-960$^+$, the oxidized form of the primary electron donor ('special pair') in *Rps. viridis*. It is easier with the cytochromes present to photochemically trap electrons in the intermediate electron acceptor [8,11, 12], thereby permitting its chemical identification. As in the case of other reaction centers, a bacteriopheophytin molecule has been shown to participate in the early photochemical events by receiving an electron from the photo-excited state of *P*-960 [12,13]; Shuvalov and Asadov [11] propose that an 830-nm-absorbing pigment receives the photoejected electron from *P*-960 prior to its transfer to the pheophytin molecule.

Some of the questions about the mechanism of the primary event in all purple bacteria could be better resolved if bacteriochlorophyll *b*-containing reaction center preparations are further characterized spectrally and biochemically. Thus in this paper we sought to settle the following points about which the various groups who have studied *Rps. viridis* reaction centers have differences of opinion: its chlorophyll and pheophytin content and in particular the assignments of these pigments to the near-infrared spectral forms observed, its carotenoid and cytochrome content and its polypeptide composition. Finally we wished to established that the bacteriopheophytin present in the reaction center is not an artefact of the preparative procedure but a genuine component of the reaction center in the whole cell.

Materials and Methods

Growth of organisms

Cells of *Rhodopseudomonas viridis* were grown photosynthetically in 25-l flasks on a medium described by Trosper et al. [6]. Cells of *Thiocapsa pfennigii*, a culture of which was kindly supplied by Dr. N. Pfennig, University of Gottingen, F.R.G., were grown photosynthetically in 25-l flasks on the inorganic medium described by Pfennig [14] and containing Na_2CO_3 as the carbon source and Na_2S as reductant. Cells were harvested by centrifugation, and photosynthetic membranes were isolated following breakage of the cells in a French Pressure cell at 10 000 lb/inch^2 by differential centrifugation. A little DNAase was added to the cells prior to breakage.

Preparation of Rps. viridis reaction centers

These were obtained by the method described in Prince et al. [8] following treatment of the membranes with lauryldimethylamine oxide. Concentrated reaction centers (approx. 50 μM *P*-960) prepared by this method were further purified prior to polypeptide analysis. This was achieved by electrophoresing the reaction center solution in Deriphat 160-containing gels [15]. The reaction center complex migrated as a pigmented band which could then be excised from the gel for further analysis. The molecular weight equivalence of the complex in the Deriphat gels was measured by comparison with the following protein standards (figures in kilodaltons): mollusc hemocyanin (290), spectrin

(230 and 215), β-galactosidase (130). phosphorylase A (100), bovine serum albumin (68), ovalbumin (43), soybean trypsin inhibitor (21.5) and lysozyme (14).

Analysis of polypeptide composition of reaction centers

The brown band excised from the Deriphat 160 gel was incubated for 1 h in denaturing buffer [16] and then either applied directly or heated at 100°C for 2 min and then applied to a 10% acrylamide Laemmli gel [16]. The electrophoretic behavior of *Rps. viridis* reaction centers was compared to that of *Rps. sphaeroides* reaction centers. After electrophoresis the gels were stained overnight in 0.1% Coomassie Brilliant Blue R250/25% isopropanol/10% acetic acid and then destained for 24 h with 5% methanol/7% acetic acid. The following proteins were used as molecular weight markers (figures in kilodaltons): catalase (57.5), ovalbumin (43), carbonic anhydrase (29), myoglobin (17.2). The gels were scanned in a Joyce-Loebl microdensitometer to estimate the relative proportions of protein in each band of the reaction center. In some gels the presence of *c*-type cytochromes was detected by the H_2O_2-tetramethylbenzidene procedure of Thomas et al. [17].

Analysis of the carotenoid content of reaction centers and photosynthetic membranes

There are four main carotenoid types in *Rps. viridis* membranes: neurosporene, 1′,2′-dihydroneurosporene, lycopene and 1′,2′-dihydrolycopene [18]. Their separation on Silica gel TLC plates is difficult since they are all hydrocarbons of very similar chain lengths. Therefore, prior to analysis of isolated reaction centers, we first verified that we could obtain a similar carotenoid analysis of whole photosynthetic membranes as that reported by Malhorta et al. [18].

The material to be studied was extracted with 10 vols. of acetone and then 10 vols. of methanol. This procedure was repeated until no further pigment was removed. The extracts were pooled and 400 ml of 40—60°C petroleum ether added. Acetone and methanol were removed from the ether layer by washing with warm, salty water. The ethereal extract, containing all the pigments, was dried over anhydrous sodium carbonate. The sodium carbonate absorbed most of the bacteriochlorophyll and bacteriopheophytin, the remainder of which was removed by partitioning against 95% aqueous methanol. The volume and absorption spectrum of the ethereal layer was recorded and used to quantitate the total carotenoid content (see below).

This extract was rotary evaporated to dryness in the dark. A small volume of diethyl ether was added to dissolve the carotenoids, which were then spotted on a Silica gel TLC plate. The carotenoids were separated from each other by chromatography of the plates in 40—60°C petroleum ether/acetone (99 : 1; v/v). It was found that optimum separation occurred when the chromatography was repeated for one or more times. Each colored component was scraped off the plate, the carotenoid eluted from the Silica gel by petroleum ether and the absorption spectrum and volume of the extract recorded. This procedure permitted the relative amount of each carotenoid to be determined. These data then allowed a calculation of an average, combined extinction coefficient for

that particular carotenoid mixture at 468 nm, the maximum absorbance of the total extract, and, therefore, the total amount of carotenoid in the extract. We repeated each determination with a 5-fold different amount of starting material. In each case the difference between the two separate determinations was less than ±2%.

The following extinction coefficients were used: Neurosporene and its dihydro derivative 169 $mM^{-1} \cdot cm^{-1}$ at 436 nm, and lycopene and its dihydro derivative $185^{-1} \cdot cm^{-1}$ at 468 nm in 40—60°C petroleum ether [18].

Spectroscopy

All spectra were recorded on an Aminco DW-2 spectrophotometer with a Midan microprocessor attachment, except for the data shown in Fig. 3 which were recorded on a Cary 14R spectrophotometer. Room temperature spectra were measured in 1.0-cm cuvettes while liquid nitrogen temperature spectra were recorded in 0.2-cm cuvettes. Fourth derivatives of the absorption spectra were calculated by the Midan microprocessor; the derivative interval is given in the respective figure legends.

Results and Discussion

(a) Polypeptide composition of Rps. viridis reaction centers

Reaction centers prepared from *Rps. viridis* membranes by lauryldimethylamine oxide-solubilization followed by DEAE-cellulose chromatography are spectrally pure except for a chlorin contaminant absorbing at ~685 nm [7,8, 12]. They contain more polypeptides than expected even for a preparation that contains bound cytochromes. However, a purer preparation can be obtained by electrophoresis of the chromatographically prepared lauryldimethylamine oxide-reaction center in polyacrylamide gels containing Deriphat D-160 (see Methods). A brown band of apparent mol. wt. ~110 000 (cf. Ref. 19) is observed. The absorption spectrum of this band shows the presence of the reaction center pigments with very little contamination by the chlorin derivative (Fig. 1). This brown band was excised from the gel and soaked in denaturing buffer for one hour. It was then placed with or without prior heating at 100°C onto a Laemmli gel [16]. The denatured but not heated sample exhibited four protein bands of molecular weight equivalences 38, 35, 28 and 24 kilodaltons

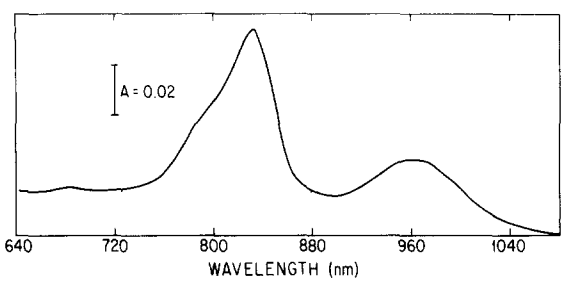

Fig. 1. Room temperature absorption spectrum in near-infrared of the photochemical reaction center zone excised from a Deriphat 160-containing polyacrylamide gel [15] on which a lauryldimethylamine oxide-prepared reaction center had been electrophoretically purified.

(Fig. 2). Variation in the molecular weight equivalence measured on gels of 10%, 12.5% and 15% acrylamide were within 2 kilodaltons for any band. Electrophoresis of heated, denatured samples revealed the almost complete absence of both the 28 and the 24 kilodaltons bands due to aggregation. This aggregation seems to be a phenomenon of M and L reaction center subunits. Figure 2, which compares the electrophoretic behavior of isolated reaction centers from *Rps. viridis* and *Rps. sphaeroides* demonstrates the loss of M and L subunits when reaction centers are boiled prior to electrophoresis. After electrophoresis of dissociated *Rps. viridis* reaction centers the largest polypeptide remained colored; however, the bulk of chlorophyll pigments electrophoresed well ahead of the polypeptides. The color of the 38 kilodalton band and its heavy staining for H_2O_2-peroxidase activity with tetramethylbenzidene indi-

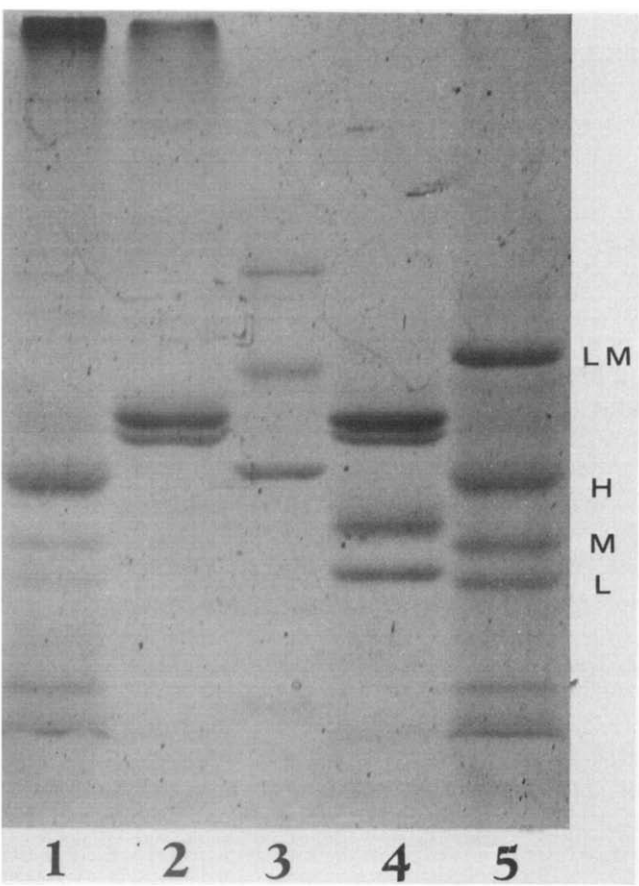

Fig. 2. Electrophoretic pattern on 12.5% polyacrylamide gels [16] of *Rps. viridis* and *Rps. sphaeroides* strain 2.4.1 reaction center preparations. Columns 1 and 2: preparations heated at 100°C for 2 min in denaturing buffer; Column 3: marker proteins; Columns 4 and 5: preparations incubated at room temperature in denaturing buffer. The *Rps. viridis* preparation used (columns 2 and 4) was a gel slice excised from a Deriphat 160-containing polyacrylamide gel; the slices were incubated in denaturing buffer for 1 h (columns 2 and 4) prior to heating at 100°C (column 2). The *Rps. sphaeroides* reaction center was only partially purified, note the presence of lower molecular weight polypeptides from the light-harvesting complexes.

cates the presence of heme. Spectral examination of this band revealed that cytochrome *c*-553 was present while the other cytochrome, *c*-558, known [12] to be present in the isolated reaction center preparations of *Rps. viridis*, cannot be observed anywhere on the gel either by spectroscopy or by staining for heme. Treatment with denaturing buffer apparently 'denatures' *c*-558 so that it cannot be detected.

On the basis of: (1) the observed size of the polypeptides in comparison with those of bacteriochlorophyll *a*-containing reaction centers; (2) the effects of heating the reaction center in denaturing buffer prior to electrophoresis, and (3) the results of staining for heme, we conclude that the four polypeptides correspond to: one or both *c*-type cytochromes (38 kilodaltons), the H subunit (35 kilodaltons), the M subunit (28 kilodaltons) and the L subunit (24 kilodaltons). Further work is necessary to locate unequivocally the polypeptide that contains the heme of cytochrome *c*-558. There are two possibilities, either the *c*-558 is removed from the cytochrome yielding an apoprotein that fails to enter the gel or the *c*-558 heme is attached to a 38 kilodalton polypeptide. The ratio of polypeptide staining after electrophoretic separation of a denatured and unheated sample indicates a probable ratio of 2 : 1 : 1 : 1 mol/mol polypeptide of the 38, 35, 28 and 24 kilodalton bands, respectively. This ratio favors the second possibility. However, it remains to be seen whether the two different hemes are bound to the same 38 kilodalton polypeptide or whether the 38 kilodalton polypeptide is composed of two dissimilar polypeptides.

Previous work by Clayton and Clayton [4] demonstrated that the isolated *Rps. viridis* reaction centers contained three subunit polypeptides of 41, 37 and 31 kilodaltons while Pucheu et al. [7] observed four polypeptides of somewhat similar sizes to those reported here, 45, 37, 29 and 23 kilodaltons in their preparation. Clayton and Clayton [4] heated their preparation prior to denaturing electrophoresis. This would result in the aggregation of the L and M polypeptides, which might account in part for the discrepancy between their work and that reported by Pucheu et al. [7] and in this paper. Thus we do not subscribe to the view [4] that there is any substantial difference between the polypeptide pattern of the reaction center of *Rps. viridis* and those of *Rps. sphaeroides*, *Rhodospirillum rubrum* and *Chromatium vinosum*.

(b) Carotenoid composition of the Rps. viridis reaction center

The first examination of this reaction center, prepared by hydroxyapatite chromatography of sodium dodecyl sulfate-solubilized membranes [6,19], revealed the presence of carotenoid; however, the carotenoids present were incorrectly identified (cf. Ref. 18). Subsequent studies showed the absence of carotenoids (see Fig. 1 in Ref. 4 and Trosper, T., personal communication) when relatively high concentrations (4%) of lauryldimethylamine oxide were used to prepare the reaction center whereas Lutz et al. [20], also studying lauryldimethylamine oxide-prepared material, noted the presence of carotenoid in a *cis*-conformation. In the present work we sought to determine the true situation with respect to the reaction center carotenoid(s), and to explain the apparently conflicting data.

We have identified and quantitated the carotenoids in chromatographically isolated lauryldimethylamine oxide-reaction centers to the best of our ability

TABLE I

PERCENTAGES OF CAROTENOIDS IN *RPS. VIRIDIS* CELLS AND IN THE ISOLATED REACTION CENTER

Carotenoid [a]	Wavelength maxima in petroleum ether (nm)	Whole cells [b]	Reaction center preparations	
			1	2
1′,2′-Dihydroneurosporene	412, 436, 446	65 ± 3.5%	66%	54%
Neurosporene	412, 436, 446	16 ± 2%	(30%)	(39%) [c]
1′,2′-Dihydrolycopene	442, 468, 500	14 ± 2%		
Lycopene	442, 468, 500	5 ± 0.5%	4%	8%
P-960/carotenoid (mol/mol)			1:0.92 [d]	1:0.88 [d]

[a] Traces of other carotenoids were present.

[b] Average of three determinations.

[c] Some neurosporene was present but insufficiently separated from 1′,2′-dihydrolycopene to permit its quantitation.

[d] These ratios were calculated using the extinction coefficient for *P*-960 described by Clayton and Clayton [4], and for simplicity we have not included the uncertainty factor inherent in their value.

considering the similar chromatographic behavior of four of the carotenoids present in the organism. The data are given in Table I. Four carotenoids and a trace of a fifth occurred in the isolated material. About one carotenoid molecule was present in each reaction center entity (Table I). The relative concentrations of the carotenoid types in the reaction center are quite similar to those in the intact membrane. We thus conclude that there is no specificity of the carotenoid inserted into the reaction center, and that higher concentrations of lauryldimethylamine oxide [4] than used in the current and previous studies [8,20] remove carotenoid from the reaction center. This specific removal of the carotenoid should facilitate further study of carotenoid function in the reaction center.

(c) Bacteriochlorophyll and bacteriopheophytin content of Rps. viridis reaction centers

Difficulties in measuring the content of these pigments are encountered if they are extracted from the isolated reaction center into organic solvents: firstly, it is difficult to extract totally these pigments from the reaction center; secondly, spectral or chromatographic separation of the chlorophyll from the pheophytin, which is required for the estimation of each, is virtually impossible to achieve quantitatively (i.e. without loss of some of either pigment (cf. Ref. 4)). We overcame these difficulties by making measurements on an aqueous solution of reaction centers before and after certain treatments.

One such procedure has been described briefly by one of us [12]: lauryldimethylamine oxide-prepared reaction centers were suspended in a solution of 0.5% sodium dodecyl sulfate-0.1 N acetic acid (Fig. 3). This solution immediately converted the 960- and 830-nm peaks (Fig. 1) into one absorbing at 784 nm. Since the disappearance of a band at 600 nm (Q_x transition of bacteriochlorophyll) and the existence of one at 540 nm (Q_x transition of bacteriopheophytin) were concomitant with the spectral shift, we conclude that bacteriochlorophyll in the reaction center was converted into detergent-solubilized bacteriopheophytin (cf. Ref. 4). The 784-nm peak (Q_y of bacteriopheophytin)

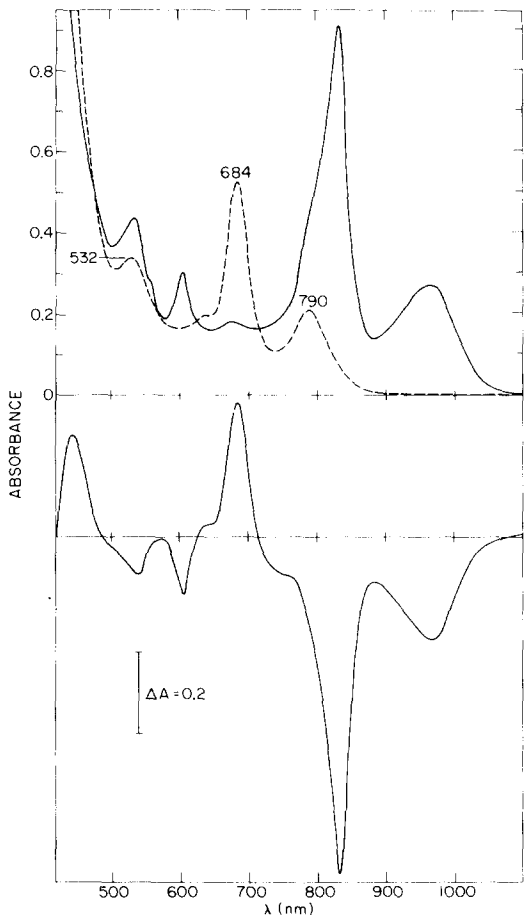

Fig. 3. Effect of suspension of *Rps. viridis* reaction centers in 0.5% sodium dodecyl sulfate/0.1 N acetic acid. Solid trace (top) spectrum of untreated reaction centers; dashed trace (top) spectrum 15 min after addition of detergent and acid. Lower trace; difference spectrum of two traces in top of figure.

shifted to 684 nm (a chlorin derivative [21]) during a 15-min period after addition of the acid while an absorbance peak at 790 nm was not so affected Fig. 3). It was surmized [12] that this peak was due to the bacteriopheophytin originally contained in the reaction center. Using the absorbance of the 960, 790 and 684 nm peaks and extinction coefficients of 100 [12], 41 [21] and 34 [21] $mM^{-1} \cdot cm^{-1}$, respectively, it was possible to quantitate the ratio of *P*-960/bacteriopheophytin/bacteriochlorophyll. A most probable molar ratio in isolated reaction centers of 1 : 2 : 4 was reported [12].

A slightly different procedure has now been used to further substantiate the ratio originally reported: lauryldimethylamine oxide-prepared reaction centers were suspended in 50 mM Tris-HCl/1% (w/v) sodium dodecyl sulfate/150 mM NaCl, pH 8.0, containing a pinch of sodium ascorbate. Heating this solution at 55°C for 1 min resulted in the loss of the 963-nm and 834-nm peaks (see Fig. 1) which were replaced by a single peak at 797 nm. Since no loss occurred in the absorbance of the 600-nm band (Q_x of bacteriochlorophyll), we deduced

that *P*-960 and the 'voyeur' bacteriochlorophyll molecules had been converted to detergent-complexed bacteriochlorophyll (rather than to pheophytin or chlorin derivatives) by heat. This view was supported by the observation that addition of acetic acid (to 0.1 M) to the heated solution changed the 797-nm and 600-nm peaks to those (784 and 540 nm) characteristic of bacteriopheophytin. We found that the addition of $K_3Fe(CN)_6$ to the heated sample changed much of the 797-nm peak into a chlorin peak at 684 nm while some absorbance, centered at 790 nm and presumed to be bacteriopheophytin originally present in the reaction center (cf. older procedure above), could not be similarily blue-shifted by the oxidant. A difference spectrum of the solution before and after addition of $K_3Fe(CN)_6$ revealed that a chromophore absorbing maximally at 812 nm (solubilized bacteriochlorophyll) in the 797-nm peak was converted to the 684-nm chlorin derivative. The spectra obtained during the above sequence of treatments enabled the ratio of reaction center chromophores to be estimated: *P*-960 was calculated from the absorbance at 963 nm in the reduced starting solution using an extinction coefficient of 123 ± 25 $mM^{-1} \cdot cm^{-1}$ [4]; bacteriopheophytin *b* from the absorbance at 790 nm in the heated and oxidized material using a coefficient of 41 $mM^{-1} \cdot cm^{-1}$ [21]; total bacteriochlorophyll from the loss of absorbance at 812 nm occurring on oxidation of the heated solution ($\epsilon = 91$ $mM^{-1} \cdot cm^{-1}$ [21]). Data from five measurements on two different preparations of reaction centers gave a molar ratio of 1 ± 0.2 *P*-960/1.9 ± 0.5 bacteriopheophytin/3.6 ± 0.8 total bacteriochlorophyll. The majority of the uncertainty in these values comes from the extinction coefficient used to estimate *P*-960. We conclude that the same pigment ratio occurs in the reaction centers of bacteriochlorophyll *a*- and *b*-containing organisms, and that each unit of reaction center has seven chromophore molecules: four bacteriochlorophyll, two pheophytin and one carotenoid.

It is interesting that bacteriopheophytin in the reaction center is not converted to a chlorin derivative. The difference between its absorption maximum (790 nm) and that of bacteriopheophytin (~782 nm) produced when acetic acid is added to detergent-solubilized bacteriochlorophyll may indicate that in the reaction center bacteriopheophytin is sequestered in a manner which prevents access of the oxidant even when the reaction center is denatured. This is obviously a point worthy of further investigation.

(d) Analysis of the absorption spectra of photosynthetic membranes and isolated reaction centers of Rps. viridis and Th. pfennigii

Shuvalov and Asadov [11] expressed doubt whether the ~812-nm spectral form of bacteriochlorophyll *b* observed in isolated reaction centers of *Rps. viridis* is actually present in the reaction center in situ; they suggested [11] that it probably represented a contaminating pigment molecule, presumably a consequence of the purification process. Furthermore, one can argue that the occurrence of bacteriopheophytin *b* in isolated reaction centers is also an artefact of the preparative procedure since chlorophylls can easily be altered to pheophytins upon solubilization. This latter point, if correct, would invalidate many recent studies (e.g. Refs. 8—13) on the mechanism of the primary photochemical event performed using isolated reaction centers. We consider it unlikely that the pheophytin is artefactual since the esterifying alcohol in bac-

teriopheophytin is chemically different from that in bacteriochlorophyll at least in *Rps. rubrum* [22]. Nevertheless, until these doubts are settled, an unequivocal elucidation of the photosynthetic mechanism is impossible. We therefore examined these uncertainties by comparing the spectrum of purified reaction centers with that of whole membranes in the 830-nm region where the two bacteriopheophytins, two 'voyeur' bacteriochlorophylls and *P*-960 molecules of the reaction center contribute all of the absorbance in isolated reaction centers [12] and much of the total absorbance of whole membranes [23—25]. In this manner we could see whether any new spectral species arise in the reaction center during the preparative procedure. We also sought to classify which spectral forms in the 830-nm band were due to the absorbance of which reaction center chromophores.

Both intact membranes and isolated reaction centers of *Rps. viridis* exhibited four spectral forms (787—790 nm; 812—813 nm; 834—835 nm and ~850 nm) in the 830-nm band at room and at liquid nitrogen temperatures when *P*-960

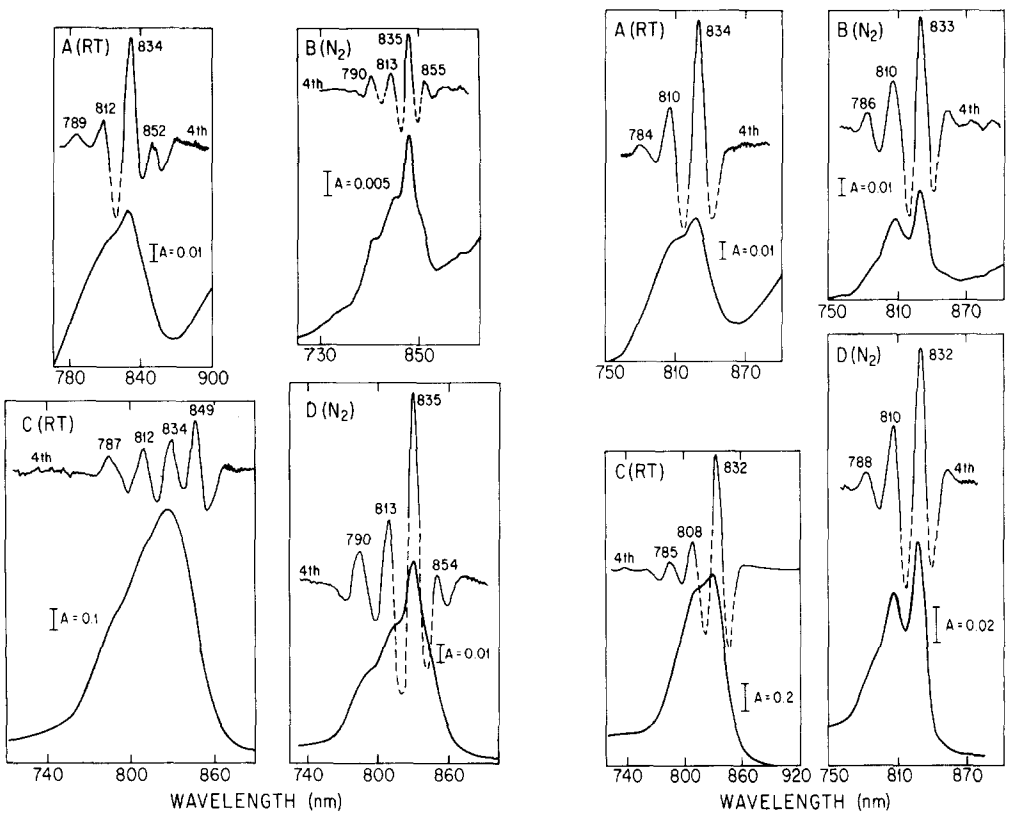

Fig. 4. A and C. Room temperature absorption spectra and fourth derivatives thereof of (A) isolated photosynthetic membranes and (C) isolated reaction centers of *Rps. viridis* having *P*-960 in the reduced form. B and D. Same as A and C, respectively, but measurements made at 77 K. Bandpass used to record absorption spectra = 5.0 nm. Wavelength interval used for obtaining derivatives = 4.8 nm.

Fig. 5. Same as Fig. 4 but measurements made after oxidation of *P*-960 with potassium ferricyanide crystals.

was reduced (Fig. 4). While the four forms can be readily observed in 77 K spectra, fourth derivatives of spectra were required to reveal them at room temperature. Spectra equivalent to those in Fig. 4 are presented in Fig. 5 but in this case *P*-960 has been chemically oxidized. Three rather than four spectral forms are now observed. The 784—788 nm form is still present whereas the ~850 nm form is missing; the 834—835 nm form is shifted slightly (2—3 nm) to shorter wavelengths as is the 812—813 nm peak which is now more prominent in the fourth derivative spectra. For both the reduced (Fig. 4) and oxidized (Fig. 5) cases the existence of the same spectral forms and the similar shape of the 830-nm band in the intact membrane and in the isolated complex demonstrate that virtually no change occurs in the nature, number or environment of the chromophores of the reaction center during isolation. Thus bacteriopheophytin and the 812-nm form are genuine components of the reaction center.

One should be able to correlate the information in Figs. 4 and 5 with oxidized-minus-reduced difference spectra of *Rps. viridis* reaction centers (Fig. 6, see also Refs. 6, 8, 12) in which it can be clearly seen in 77 K spectra that two forms at 808 and ~830 nm appear and two at ~838 and ~850 nm (as well as that at ~990 nm) disappear on oxidation of *P*-960. We deduce from such correlations that *P*-960 absorbs in the near-infrared region at 960 and ~850 nm (cf. Refs. 9, 12) while *P*-960$^+$ absorbs at 808 nm (cf. Refs. 9, 10), and that on oxidation of *P*-960 an electrochromic shift of the 834—835 nm form to a shorter wavelength (832 nm) occurs. We also surmize that the 810-nm spectral form in oxidized reaction centers represents two spectral forms: the 812-3 nm component, also observed in reduced reaction centers, and the 808-nm component due to *P*-960$^+$. These two forms are insufficiently separated spectrally that

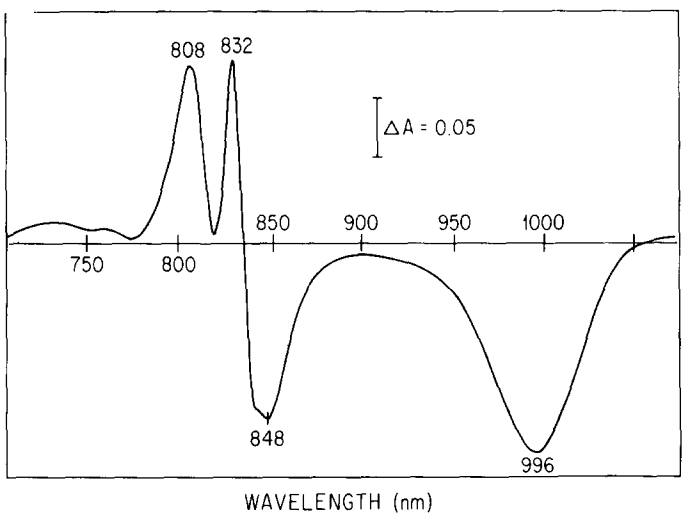

Fig. 6. Liquid nitrogen temperature oxidized-minus-reduced difference spectrum of lauryldimethylamine oxide-prepared reaction centers of *Rps. viridis*. Samples were frozen in 55% (v/v) glycerol/25 mM Tris-HCl, pH 8.0. A few crystals of sodium ascorbate or potassium ferricyanide were added to ensure reduction and oxidation of *P*-960, respectively. Bandpass of measuring light was 2.0 nm.

TABLE II

ASSIGNMENT OF SPECTRAL FORMS IN THE NEAR INFRARED REGION OF *RPS. VIRIDIS* REACTION CENTERS TO THE CHROMOPHORES PRESENT

Chromophore	Spectral form in reaction center	
	Reduced (nm)	Oxidized (nm)
Special pair (bacteriochlorophyll *b*)$_2$	965 (~990 at 77K) and ~850	1310 [6,8] and 808
'Voyeur' Bacteriochlorophyll *b*	834—835	832
'Voyeur' Bacteriochlorophyll *b*	812—813	812—813 *
Bacteriopheophytin *b*	787—790	784—788
Bacteriopheophytin *b*		

* Not separated from 808-nm component by direct spectral analysis.

neither absolute nor fourth derivative spectroscopy can resolve them and consequently they appear as a single form with a wavelength maximum intermediate between the two.

Our assignments of reaction center chromophores to the spectral forms observed in Figs. 4—6 are given in Table II. The data in section (c) above indicated that there are six molecules of porphyrin-containing chromophores (excluding cytochromes) in each unit (i.e. per one *P*-960 molecule) of the *Rps. viridis* reaction centers (four bacteriochlorophyll *b* and two bacteriopheophytin *b*) and that the 784—790 nm forms in reduced and oxidized reaction centers as well as in whole membranes are contributed by the bacteriopheophytin molecules, and that the bacteriopheophytins absorb close to 790 nm. We therefore concluded that the two 'voyeur' bacteriochlorophylls give rise to the 812-nm and 834 nm spectral forms, one or both of which undergo an electrochromic shift when *P*-960 is oxidized (cf. Ref. 10). The remaining near-infrared bands (~850 and 960 nm) we assigned (cf. Ref. 9, 12) to the absorbance of the special pair of bacteriochlorophyll *b* molecules that form the primary electron donor. Thus to account for all the spectral forms and the spectral changes observed upon oxidation of the reaction center requires only the use of the absorbance of the six porphyrin chromophore molecules measured to occur in each reaction center entity.

Our interpretations (Table II) are in complete agreement with the assignments made by Paillotin et al. [10] from linear dichroism studies of whole membranes of *Rps. viridis*, and with those made earlier by Philipson and Sauer [26]. We were perhaps able to pinpoint more accurately the wavelength maxima of the spectral forms. However, Paillotin et al. [10] were able to document a small shift of the 812 nm-spectral form to longer wavelengths on oxidation of *P*-960, which our experimental approach did not enable us to detect. Shuvalov and Asadov [11] made some different assignments: they proposed that an 814-nm transition (equivalent to our 812-nm transition) belongs to a contaminating bacteriochlorophyll *b* molecule, and that the 850-nm transition is contributed by a bacteriochlorophyll *b* monomer and not by the special pair of chlorophyll molecules which form *P*-960. In support of our view we emphasize the following points: (1) The 812-nm form is present in intact membranes (Fig. 4), and occurs in the same proportion with respect to other components of the 830-nm band in the reaction center fraction throughout the preparative

procedure. (2) If the 812-nm form were contaminating bacteriochlorophyll *b* it would almost certainly be oxidized with time to a 684-nm chlorin derivative (see section (c) above); this does not occur (Fig. 5). (3) Three spectral forms (812, 834 and 850 nm) have to be accounted for by the four bacteriochlorophyll *b* molecules (see (c) above) in the reaction center. Since two of them form *P*-960, the other two can only contribute two of the three 800-nm forms. Thus one of these forms must be a spectral component of one of the transitions already assigned. (4) In contrast to Shuvalov and Asadov [11] we find that the spectral form at ~850 nm does disappear when *P*-960 is oxidized. Therefore, in light of much existing data [9,10,27] the most likely assignment for the 850-nm component is that it represents the forbidden transition of the special pair.

The 830-nm band of another bacteriochlorophyll *b*-containing organism, *Th. pfennigii*, was examined (Fig. 7) in the same manner used above for *Rps. viridis*. Significant differences in the number and wavelength maxima of the spectral forms occur between the two organisms. Only two forms (798 and 838 nm) are observed in the room temperature spectrum of *Th. pfennigii* membranes (see also the spectrum in Fig. 5 of Ref. 12). On lowering the temperature to 77 K the 798-nm band splits to give two forms at 782 and 800 nm; however, there is no resolution of the 838-nm band. Thus there is no evidence

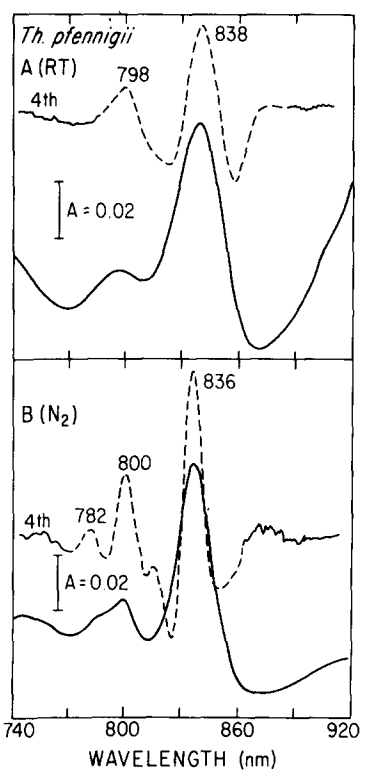

Fig. 7. Room temperature (a) and 77 K (b) absorption spectra (———) of 830-nm band of *Th. pfennigii* photosynthetic membranes. The fourth derivative of each spectrum is given above each absorption curve (- - - - -). The wavelength interval for obtaining derivatives was 4.8 nm. Bandpass for recording absorption spectra was 5.0 nm.

for an ~850 nm component of the special pair (cf. Table II) in *Th. pfennigii*, nor is there any form intermediate between 790 and 838 nm as occurs in *Rps. viridis*. We surmise that the 'voyeur' chromophore, equivalent to that giving rise to the 812—813-nm band in *Rps. viridis*, absorbs maximally very close to the maximum of the other 'voyeur' chlorophyll molecule in *Th. pfennigii* as occurs in the bacteriochlorophyll *a*-containing reaction centers. This would account for the distinct valley between the 790-nm and 838-nm peaks in spectra of *Th. pfennigii*, its absence in those of *Rps. viridis*, and for the greater ratio of the 838-nm absorbance to those at 790 and 960 nm in *Th. pfennigii* [12]. It is conceivable that there is an ~850 nm band due to *P*-960 in *Th. pfennigii*, but that the relatively greater absorbance at 838 nm may prevent its spectral visualization. Comparison of the shape of the 830-nm band of intact membranes with that of a purified reaction center of *Th. pfennigii* [12] reveals just as with *Rps. viridis*, that the 750—850-nm absorbance peaks in whole membranes are contributed by reaction center chromophores and that little or no change occurs in the spectral characteristics of the reaction center chromophores during solubilization and fractionation of the membrane. It is unclear from spectral data in the literature [28] whether the two other known bacteriochlorophyll *b*-containing organisms (*Rps. sulfoviridis* and *Ectothiorhodospira halochloris*) are spectrally like *Rps. viridis* or like *Th. pfennigii*; *E. halochloris* may even represent a third spectral type (cf. Ref. 28).

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