Characterization of the Pigments in Reaction Center Preparations from *Rhodopseudomonas spheroides*

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SUMMARY

The pigments in reaction center preparations from *Rhodopseudomonas spheroides* strain R-26 have been characterized. The absorption spectra and magnesium content of both intact preparations and organic extracts as well as thin layer chromatography of the extracts indicate that the pigment complex of the reaction center contains both bacteriopheophytin *a* and bacteriochlorophyll *a*. The functional unit of the reaction center contains 2 bacteriochlorophyll *a* molecules and 4 bacteriochlorophyll *a* molecules. In dark-adapted preparations, the extinction coefficients for the three red and near infrared absorption maxima of the reaction center are: 1.6 × 10^5 M^-1 cm^-1 at 757 nm, 3.0 × 10^5 M^-1 cm^-1 at 803 nm, and 1.4 × 10^5 M^-1 cm^-1 at 865 nm.

Reaction center preparations enriched with respect to the components of the primary photochemical reaction and completely devoid of light-harvesting bacteriochlorophyll have been isolated from several photosynthetic bacteria by a variety of techniques (1-11). In studies on the blue-green mutant strain R-26 of *Rhodopseudomonas spheroides*, an electron transfer complex has been isolated by Triton X-100 fractionation (4). This complex contains the specialized reaction center bacteriochlorophyll, the associated primary electron acceptor, and components of a cyclic electron transfer system (12). More recently, reaction center preparations have been isolated by treating chromatophores of this bacterium with another nonionic detergent, Ammonyx LO, lauryldimethylamine-*N*-oxide (9, 10). These preparations are devoid of many of the secondary components of the photosynthetic electron transfer system and afford obvious advantages for studying the primary photochemical reaction.

A detailed understanding of the mechanism of the primary photochemical reaction of bacterial photosynthesis requires both the identification and quantification of those components which catalyze the transfer of an electron from bacteriochlorophyll to the primary electron acceptor, presumably a non-heme iron compound (14, 15). Clayton (16), on the basis of BChl

analyses on rather crude preparations obtained by iridic chloride oxidation of the light-harvesting BChl, indicated that each reaction center most likely contains 3 specialized BChl molecules, 2 P800 and 1 P870, although other possibilities have subsequently been discussed (17). Feher (9) concluded from magnesium analyses and second derivative spectra that each reaction center contains 5 BChl molecules. During the writing of this report, Mauzerall (18) has reported that he now believes the reaction center contains 4 bacteriochlorophyll and 2 bacteriopheophytin molecules.

Here we present a full report of our experimental results on the identification and quantification of the pigments in reaction center preparations. Some of these results have previously been communicated in preliminary form (19). In addition to the specialized bacteriochlorophyll *a* molecules, bacteriopheophytin *a* has been identified as a normal component of the reaction center. The stoichiometry and extinction coefficients of these components have been determined. These results are discussed with regard to the organization of the pigments in the reaction center.

EXPERIMENTAL PROCEDURES

Materials—Triton X-100 (octylphenylpolyoxyethanol) was a gift from Rohm and Haas Research Division, Philadelphia, Pa. Ammonyx LO (lauryldimethylamine-*N*-oxide) was a gift from Onyx Chemical Co., Jersey City, N.J. Thin layer chromatographic media I.T.L.C. type SA was obtained from Gelman Instrument Co., Ann Arbor, Mich. Acetone, methanol, petroleum ether (b.p. 30-60°) and NaSO₃ of reagent grade were purchased from Matheson. Bacteriochlorophyll *a* was a generous gift from Mr. Tom Meyer in the laboratory of Dr. Gilbert Seely and bacteriopheophytin *a* was prepared by the method of Smith and Benitez (19). All other chemicals were of reagent grade from commercial sources.

*Rhodopseudomonas spheroides* strain R-26 was grown on modified Hutner medium containing succinate as the carbon source, harvested by centrifugation, and stored as a frozen paste at -10°. The reaction center complex was isolated from chromatophores by Triton X-100 fractionation procedure described previously (12) and the reaction center P870 protein was isolated by treating the complex with Ammonyx LO using a procedure similar to those of Clayton and Wang (10) and Feher (9), which will be reported separately. The reaction center preparations were maintained at 4° in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1% Ammonyx LO.

Analytical Procedures—Absorption spectra were measured at
25° in a Cary model 14 recording spectrophotometer equipped with a scattered transmission attachment. Glass-stoppered quartz cuvettes of 1 cm path length were used. Bacteriopheophytin concentrations were calculated from the extinction coefficient of 28.4 mm⁻¹ cm⁻¹ at 524 nm in the petroleum ether extracts (20).

Magnesium ion concentrations were determined in a Perkin Elmer model 303 Atomic Absorption spectrophotometer. Samples were ashed to dryness in the presence of sulfuric and nitric acids, dissolved in 0.01 M H₂SO₄, and compared with MgSO₄ standards treated similarly.

Thin layer chromatography was carried out by the method of Sherma and Lippstone (21). Samples in anhydrous petroleum ether were applied to silica gel-impregnated glass fiber sheets (Gelman SA) which had been previously activated at 100° for 1 hour. The solvent was removed under a stream of argon in the dark and the chromatograms were developed in 2-propanol-petroleum ether (1:99, v/v). Spots were visualized by their absorbance under white light and fluorescence emission under ultraviolet light. For preparative isolation of BPheo, sheets were streaked and developed as above and the strongly fluorescent pink bands were cut out and eluted with diethyl ether.

The reaction center pigments were extracted into acetone-methanol and transferred into petroleum ether for spectral and chromatographic analysis. Samples were maintained in dim light at 4° and mixed vigorously after additions of 9 volumes of acetone-methanol (7:2 v/v), 10 volumes of petroleum ether (b.p. 30-60°), and 0.6 volume of 2 M KCl. In order to convert the bacteriochlorophyll to bacteriopheophytin, 0.6 volume of 5 N HCl was added to some samples. After 15 min the samples were mixed again and centrifuged for 5 min at 2000 × g. The upper organic layer was removed and the lower aqueous layer was extracted with an additional 10 volumes of petroleum ether. The first and second petroleum ether extracts were combined, washed with 0.3 volume of water, and dried over anhydrous sodium sulfate. The combined petroleum ether fractions were concentrated to the appropriate volume by evaporation under a stream of argon.

**RESULTS**

The absorption spectrum of the reaction center preparation from *R. spheroides* strain R-26 is shown plotted in a wave number scale in Fig. 1 and on a wave length scale in Fig. 2 (solid lines). The reaction centers exhibit absorption bands at 535 and 597 nm in the yellow and three absorption bands at 757, 803, and 865 nm in the red and near infrared. The absorbance of the bands at 597 and 865 nm are maximal when these preparations are exposed only to the monochromatic measuring beam. The large half width of the 535 nm band is interpreted to be the result of multiple BPheo components.

The three near infrared absorption bands of reaction centers are closely approximated by the sum of three Gaussian curves obtained by computer fitting as shown in Fig. 1 (dashed lines). The slight discrepancy between the sum of the calculated bands (open circles) and the measured absorption spectrum (solid line) most likely results from asymmetry on the short wave length sides of the absorption bands. Similar asymmetry has been observed in the absorption spectra of purified BCHl and BPheo solutions (22, 29).

The band widths and areas of the resolved red and near infrared absorption bands are listed in Table I. The areas or intensities of these three absorption bands are approximately equal with ratios of 0.9:1.2:1.0 relative to the band at 1156 cm⁻¹. Some variation in the band areas results from different amounts of overlap by the adjacent asymmetric absorption bands.

Attempts to quantify the BCHl and BPheo in reaction centers from spectra of intact preparations or from spectra of aneuous...
acetone-methanol extracts were not satisfactory. The BPheo band near 525 nm which is broad in the intact reaction centers is narrow in the extracts but the BChl band near 590 nm broadens in the polar organic solvent. To avoid these anomalies of absorption spectra measured in polar solvents, the pigments were transferred to dry petroleum ether.

Absorption spectra of intact reaction centers and of the pigments extracted into petroleum ether are shown in Fig. 2. The specialized environment in the reaction center is lost when the pigments are extracted into the organic solvent and the absorption maxima shift toward shorter wave lengths. The maxima at 535 and 597 nm in the intact reaction centers are replaced by absorption maxima of BPheo and BChl in the extract at 524 and 576 nm, respectively, in the yellow region and a broad asymmetric band resulting from the unresolved maxima in the red. The recovery of pigments in the extracts was essentially quantitative and did not change significantly when the time or number of extractions was increased. The ratio of BPheo to BChl in the extracts was not changed appreciably when either NaHCO₃ or sodium ascorbate was included during the extraction. Furthermore, the oxidative degradation product of BChl which absorbs maximally near 680 nm (24) was negligible in absorption spectra of the reaction center preparations or the organic extracts.

The identities of the reaction center pigments determined by thin layer chromatography are shown in Fig. 3. The two pigments present in the petroleum ether extracts were separated by this chromatographic system. The more rapidly migrating pink-colored and highly fluorescent pigment migrated with authentic bacteriopheophytin a and the migration of the blue-colored pigment was coincident with authentic bacteriochlorophyll a. After acidifying the extracts, only BPheo in an increased amount was present.

Absorption spectra of the neutral and acidified extracts are shown in Fig. 4. The 577 nm BChl maxima and the asymmetry of the far red band due to the presence of BChl in the neutral extract are absent in the acidified extract. The absorbance of the BPheo bands at 524 and 750 nm is also greater in the acidified extract. After correcting for the absorbance by the overlapping BChl band in the neutral extracts, comparison of the 524 nm absorbance in the neutral and acidified extracts indicate that the BPheo concentration is approximately 2.6 times higher in the acidified extracts.

Absorption spectra of the bacteriopheophytin purified by thin layer chromatography from neutral and acidified extracts are shown in Fig. 5. The samples of BPheo which had been separated from the BChl by this procedure exhibited absorption spectra which were essentially identical with the spectrum of authentic bacteriopheophytin a. After thin layer chromatography, the BPheo recovery from either the neutral or acidified extracts was near 70% and the removal of any absorbance by overlapping BChl bands allowed more accurate comparison of the BPheo concentrations in the two extracts. The BPheo recovered from the acidified extracts was 2.8 to 3.0 times greater than the BPheo recovered from neutral extracts prepared from the same amount of reaction center preparation. Conversion of the reaction center BChl to BPheo produced a 3-fold increase of the BPheo concentration in the acidified extract consistent with a molar ratio of 1 BPheo to 2 BChl in the neutral extract from reaction centers. However, the broad band width of the 535 nm absorption maximum of BChl at 577 nm is absent and the absorbance of the BPheo band at 524 nm is increased.

![Fig. 3. Thin layer chromatography of the pigments from reaction center preparations. Samples of neutral (A), and acidified organic extracts (B), bacteriopheophytin a (C) and bacteriochlorophyll a (D), were chromatographed on silicic acid impregnated glass fiber paper in petroleum ether containing 2-propanol (10%, v/v). The neutral extract contained BChl which migrated more slowly than the BPheo (see arrows) and the acidified extract contained only BPheo.](http://www.jbc.org/)

![Fig. 4. Absorption spectra of reaction center pigments in a neutral extract (---), and an acidified extract (--), at the same total pigment concentration in dry petroleum ether. In the acidified extract the yellow absorption maximum of BChl at 577 nm is absent and the absorbance of the BPheo band at 524 nm is increased.](http://www.jbc.org/)

![Fig. 5. Absorption spectra of bacteriopheophytin samples in diethyl ether purified from the same amount of reaction centers by preparative thin layer chromatography of neutral (---) and acidified extracts (--). The BPheo recovery from the acidified extract was approximately three times that from the neutral extract.](http://www.jbc.org/)
absorption band of intact reaction center preparation (Fig. 2) indicates that each reaction center contains 2 BPheo molecules or a total pigment composition of 2 BPheo and 4 BChl molecules.

Values of the extinction coefficient for the 865 nm absorption band in the reaction center preparations are listed in Table II. Based on a reaction center composition of 2 BPheo and 4 BChl, the extinction coefficients near 140 mm$^{-1}$ cm$^{-1}$ are calculated from the absorbance at 865 nm in dark-adapted preparations and the magnesium and BPheo concentrations in the intact preparations and organic extracts. A differential extinction coefficient of 120 mm$^{-1}$ cm$^{-1}$ is calculated from the data in Table II for the approximately 85% decrease in the 865 nm absorbance accompanying oxidation of the reaction center by actinic illumination or by a chemical oxidant (4, 9, 10, 25). This value is consistent with measurements of the absorbance changes accompanying the photochemical transfer by the reaction center of a single electron from cytochrome $c$ (13, 17, 26, 27).

### Table II

| Basis of determination | Extinction coefficient mm$^{-1}$ cm$^{-1}$ at 865 nm |
|------------------------|-----------------------------------------------|
| 4 Mg$^{2+}$ in intact reaction center preparation | 124 |
| 4 Mg$^{2+}$ in neutral organic extract | 154 |
| 2 BPheo in neutral organic extract | 122 |
| 6 BPheo in acidified organic extract | 142 |

In agreement with the fluorescence measurements, the quantum efficiency for P870 bleaching by excitation of the 757 nm band is lower, near 80% and exitation at either 803 or 865 nm has an efficiency near 100% (13, 31). These properties of the reaction center chromophores are consistent with the assignments of the 757 nm red maximum and the 535 nm yellow maximum as absorption bands due to electronic transitions associated primarily with bacteriopheophytin molecules and the 803 and 865 nm infrared maxima and the 597 nm yellow maximum as absorption bands due to transitions associated primarily with the bacteriochlorophyll molecules (P800 and P870).

The analyses of intact reaction center preparations and organic extracts indicate that this pigment complex contains both bacteriochlorophyll and bacteriopheophytin in a molar ratio of 2:1. However, the functional unit of the reaction center pigment complex contains twice the minimal number of pigments or 4 BChl and 2 BPheo molecules. The broadening of the BPheo absorption band at 535 nm in reaction centers (Fig. 2) indicates that 2 BPheo molecules are present in slightly different environments in the pigment complex and the extinction coefficient calculated on this basis is in good agreement with values previously obtained for the functional reaction center unit which transfers a single electron from cytochrome $c$ (13, 17, 26, 27). The extremely low yield of fluorescence emission from the P800 band (25, 31) suggests that a nonfluorescent BChl dimer remains even when the P870 band at 865 nm is bleached. Further details of the molecular organization of the pigments in the photosynthetic reaction center are currently being sought from circular dichroism measurements.

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