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Isolation and spectral characterization of photochemical reaction centers from the thermophilic green bacterium *Chloroflexus aurantiacus* strain J-10-f1

(photosynthesis/bacteriochlorophyll/pigment protein/evolution)

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ABSTRACT **A rapid procedure has been devised to extract photochemically active reaction centers from the green bacterium *Chloroflexus aurantiacus* strain J-10-f1 and a mutant lacking colored carotenoids (73-3). The isolated material was completely free of antenna bacteriochlorophyll and cytochromes and nearly free of carotenoids and had near IR absorption maxima at 865, 815, and 756 nm. On oxidation, the peak at 865 nm was bleached and the remaining two peaks were shifted to 806 and 757 nm. Although these spectral characteristics show general similarities to those in reaction center preparations from purple bacteria, there are some distinct differences. Comparison of the spectra of reaction centers of *Chloroflexus* and *Rhodopseudomonas sphaeroides*, adjusted to the same absorbance at 865 nm, showed that the absorbance at 815 nm in *Chloroflexus* was much less and that at 757 nm was much greater than the equivalent absorbances in *R. sphaeroides*. Unlike reaction centers from *R. sphaeroides* and other photosynthetic bacteria that have two molecules of bacteriopheophytin and four molecules of bacteriochlorophyll per unit, reaction centers from *Chloroflexus* appear to have three molecules of each pigment per unit. A prominent shoulder at 793 nm disappears concomitantly with the bleaching at 865 nm on oxidation of *Chloroflexus* reaction centers; this spectral component may represent the higher energy transition in the near IR of the two bacteriochlorophylls forming P865. While *Chloroflexus* has a light-harvesting pigment system very similar to that of the Chlorobiaceae, its reaction center has optical absorption characteristics similar to those of the Rhodospirillaceae.**

The photosynthetic apparatus of *Chloroflexus aurantiacus* is located in the plasma membrane and in light-harvesting structures called chlorosomes attached to the membrane (1). The chlorosomes contain all the bacteriochlorophyll (BChl) *c*, which absorbs near 740 nm, and some of the BChl *a* in a complex absorbing maximally at 790 nm (1–3). The plasma membrane contains the other BChl *a* molecules in antenna complexes absorbing near 800 and 865 nm (1–3) and in the photochemical reaction centers, which show reversible photobleaching at 865 nm due to P865, the presumed primary donor in the photochemical event (1, 2, 4). The chlorosomes are structurally and functionally similar to those of the green sulfur bacteria (Chlorobiaceae) (5–7), while the wavelength maxima of the membrane-bound BChl *a* complexes are similar to those of some species of purple bacteria (Rhodospirillaceae) and different from those of green sulfur bacteria (7). The photosynthetic apparatus of *Chloroflexus* therefore appears to be a unique combination of components normally found separately in different taxa (Rhodospirillaceae and Chlorobiaceae).

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Phylogenetically, *Chloroflexus* appears to be only distantly related to all other groups of photosynthetic bacteria, based on the cataloguing of 16S rRNA (8). *Chloroflexus* shows the deepest or most ancient division in all the true bacteria (8) and may be the closest extant photosynthetic organism to the common ancestor of all photosynthetic bacteria. Comparison of the characteristics of the photochemical reaction center of *Chloroflexus* with those of reaction centers purified from other bacteria could therefore enhance our understanding of the evolution of the photosynthetic apparatus. Furthermore, *Chloroflexus* is a thermophile and its reaction center could exhibit interesting thermal stability; little is known about the properties of any intrinsic membrane protein from thermophiles (9).

Recent attempts to isolate photochemically active reaction centers from *Chloroflexus* have been only partially successful (10). Reaction center complexes completely free of antenna BChl have not yet been isolated from any of the other green bacteria (7). A successful procedure is reported here that yields reaction centers from *C. aurantiacus* strain J-10-f1 and from a mutant lacking colored carotenoids, mutant 73-3.

MATERIALS AND METHODS

C. aurantiacus strain J-10-f1 was grown photoheterotrophically in D medium (11) containing yeast extract (1.0 g/liter; Difco), Casamino acids (2.0 g/liter; Difco), and glycylglycine (1.0 g/liter; free base; Sigma) and adjusted to pH 8.2 with 5 M NaOH before autoclaving. Cultures were grown at 55°C in 750 ml of medium in Roux bottles continuously sparged with 99.5% N_2/0.5% CO_2. Bottles were illuminated with 75-W reflector flood lamps at a distance of 17 cm. The medium was inoculated with 5 ml of an exponentially growing culture. The carotenoid-lacking mutant was obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (‡). It was grown the same as the wild-type parent strain with the following modification: the culture medium was sparged with N_2/CO_2 for 2 hr before adding a sterile aqueous solution of 1% (wt/vol) $Na_2S{\cdot}9H_2O$ to give a final concentration of 0.01%. The medium was then sparged for 1 hr before inoculation with the mutant. Cells were harvested 20–24 hr later, during the mid-to-late exponential phase of growth, by centrifugation at 10,400 × *g* for 8 min, washed twice with buffer (50 mM Tris·HCl, pH 8.0), and stored frozen. Tris·HCl buffer (50 mM) was used throughout all preparations. Frozen cells (wet weight 1.0–1.5 g) were suspended in 5 ml of buffer (pH 9.0) and broken by sonication (sonifier model W140 with

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Me_2DodNO, dimethyldodecylamine oxide; NIR, near infrared.

‡ Pierson, B. K. (1982) Fourth International Symposium on Photosynthetic Prokaryotes, Bombannes, France, September 1982, p. C26 (abstr.).

standard tip; Heat Systems/Ultrasonics, Plainview, NY) in an ice bath using four 30-sec pulses with 30-sec cooling/mixing periods between them. Disruption was complete as determined by microscopic examination. The suspension of disrupted cells was centrifuged for 10 min at 17,300 × *g* to remove debris. The supernatant is designated the crude extract.

For preparing reaction centers from strain J-10-fl, a total of 12 ml of crude extract ($A_{865} \approx 3$) was diluted to a total volume of 20 ml with buffer (pH 9.0). This diluted extract contained 50 mM NaCl and 1.0% (wt/vol) dimethyldodecylamine oxide (Me_2DodNO) (Onyx Chemical, Jersey City, NJ). The solution was incubated for 1 hr at 40°C and then chilled and diluted to 40 ml with buffer, pH 9.0/1% Me_2DodNO. The diluted extract was applied directly to a 2 × 4.5 cm DEAE-cellulose column (Whatman DE53) previously equilibrated with buffer, pH 9.0/0.1% Me_2DodNO. The column was washed with 4 to 5 column vol of buffer, pH 9.0/1% Me_2DodNO followed by 4 to 5 vol of buffer, pH 8.0/0.2% Me_2DodNO. The reaction centers were eluted with buffer, pH 8.0/30 mM NaCl/0.2% Me_2DodNO. Reaction centers were concentrated under N_2 by ultrafiltration using type PM30 ultrafiltration membranes (Amicon Systems, Danvers, MA). Reaction center activity was determined by measuring the total change in A_{865} due to reversible oxidation with potassium ferricyanide as oxidant and sodium ascorbate as reductant.

Visible and near IR (NIR) absorption spectra were recorded with an Aminco DW-2 spectrophotometer attached to a Midan microprocessor. A band-pass of 3.0 nm was used. Samples were reduced by adding a few grains of solid sodium ascorbate or sodium dithionite to the cuvette. Samples were oxidized with potassium ferricyanide. Cross-illumination for photooxidation of P865 was provided with a high-intensity monochromator using a xenon light source (Bausch and Lomb). UV absorbance was determined on a Beckman model 25 spectrophotometer.

Absorption spectra at 77 K were recorded using an optical Dewar flask for liquid N_2 and sample cells with an optical light path of 2 mm. Reaction centers were in 55–60% (vol/vol) glycerol for low-temperature spectra. The zero point for all quantitative absorbance determinations in the NIR was at 940 nm.

RESULTS

Isolation of Reaction Centers. After incubation of the crude extract with Me_2DodNO for 1 hr at 40°C, 80% of the initial P865 activity was still present. When the diluted detergent extract (pH 9.0, 25 mM NaCl, 1% Me_2DodNO) was applied to the column, all cytochromes and P865 bound to the column. The free BChl *c* (A_{670}) and free BChl *a* ($A_{770-780}$) passed through the column. The carotenoids, with broad absorption maxima at 440, 460, and 480 nm, were washed from the column with several volumes of buffer, pH 9.0/1.0% Me_2DodNO. Although such carotenoids were missing from the mutant, the column was also washed with the buffer. When the pH and the Me_2DodNO were decreased to 8.0–8.2 and 0.2%, respectively, 30 mM NaCl eluted the reaction centers completely free of cytochromes. At higher concentrations of salt, the cytochromes coeluted with the reaction centers. The purification procedure resulted in the recovery of 80% of the P865 activity loaded on the column. The final yield was at least 45% and was as high as 60–75% of the P865 activity in the crude extract. The isolated reaction centers were photochemically active and spectrally free from all other pigments. Hence, they were not noticeably colored until they were concentrated, when they had a gray-yellow appearance. The A_{280}/A_{865} ratio was 10.5 in strain J-10-fl as compared with approximately 3 in purified reaction centers from *Rhodopseudomonas sphaeroides* R-26. The A_{280}/A_{865} ratio rather than the A_{280}/A_{800} ratio was used for comparison because of the large difference in A_{800} in reaction centers from the two organisms. When the reaction centers were rechromatographed on DEAE-cellulose, the A_{280}/A_{865} ratio was decreased to 7 and the concentrated reaction centers were gray.

Spectral Characterization of the Isolated Reaction Centers. The absorption spectra at room temperature of oxidized and reduced isolated reaction centers from *C. aurantiacus* strain J-10-fl are shown in Fig. 1*A* (the spectrum of the reaction center from mutant 73-3 is not shown). The reaction-center preparations from both sources had essentially identical spectra. The NIR absorption maxima of the reduced preparation are at 865, 815, and 756 nm. The absorbances at 756 and 815 nm are of equal intensity and 1.3 times that at 865 nm. When the reaction centers were oxidized, the band at 865 nm was bleached, the 756-nm band shifted to 757 nm and, instead of a band at 815 nm, one was seen at 806 nm. In addition, a prominent shoulder at 793 nm disappeared on oxidation, which is seen as a loss of absorbance at 780–790 nm in the difference spectrum (Fig. 1*B*).

The absorbance at 687 nm is probably due to a chlorin formed by degradation of BChl *a* (12). The peaks at 605 and 534 nm were identified as the Q_x bands of BChl *a* and bacteriopheophytin (BPh *a*), respectively (12). The band at 605 nm greatly decreases on oxidation of P865 and the maximum shifts to 604 nm while the band at 534 nm shows no significant change. There is some absorption at 498 nm and some broad absorption at 400–500 nm that is probably due to carotenoid but is reduced when the reaction centers are rechromatographed. The Soret bands at 364 and 392 nm are due to BChl *a* and BPh *a* (12). The amount of carotenoid present per reaction center is less than one molecule per P865. The increased absorbance between 370 and 460 nm in the spectrum of the oxidized reaction centers (Fig. 1*A*) is due to the absorbance of $K_3Fe(CN)_6$, which is not seen in the spec-

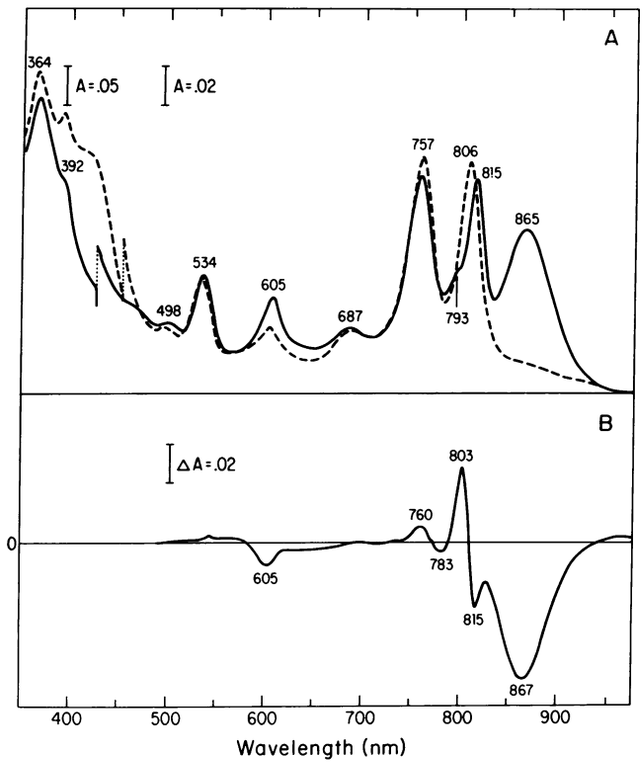

FIG. 1. Room temperature absorption spectra of oxidized (---) and reduced (——) reaction centers from *C. aurantiacus* J-10-fl. Reaction centers were oxidized by adding $K_3Fe(CN)_6$ and reduced by adding Na ascorbate. (*B*) Oxidized-minus-reduced difference spectrum of reaction centers from strain J-10-fl.

trum of the photooxidized material. Otherwise, the spectral changes (Fig. 1*B*) were identical when P865 was oxidized with light or with $K_3Fe(CN)_6$. Reduction in the dark of photooxidized P^+865 in the absence of added reductant took 10–15 min.

The 77 K absorption spectra of reduced and oxidized reaction centers from *C. aurantiacus* are shown in Fig. 2*A*. The NIR absorption maxima of reduced reaction centers are at 887, 813, and 757 nm, with a prominent shoulder at 791 nm. In the oxidized reaction centers, the band at 887 nm and the shoulder at 791 nm were lost, leaving bands at 806 and 757 nm. The difference spectrum (Fig. 2 *B*) shows the loss of absorbance at 887 nm, which is associated with oxidation of P865, and the associated decreases at 816 and 787 nm and an increase at 805 nm.

The absorption spectrum of reduced reaction centers isolated from *Chloroflexus* is superimposed on that from *R. sphaeroides* R-26 in Fig. 3*A*. The concentrations of the two preparations were adjusted so that the absorbances at 865 nm were equal. The difference spectrum (Fig. 3*B*) is flat through the 865 nm maximum, showing how similar the optical absorption properties are around 865 nm. A large difference in absorbance occurs at 803 nm, with an inflection at 780–790 nm probably due to the 790-nm shoulder in the *Chloroflexus* reaction center. There is an increased absorbance at 756 nm in *Chloroflexus* reaction centers. In the visible region, differences are seen in the Q_x band of BChl *a* (605 nm), which in *Chloroflexus* is 5 nm farther to the red than in *R. sphaeroides*, and the Q_x band of BPh *a* (534 nm), which is 2 nm farther to the blue in *Chloroflexus*. The BPh Q_x band is also sharper in *Chloroflexus*. Reaction centers isolated from *Chloroflexus* have slightly greater absorbance between 400 and 500 nm (carotenoid?) than do reaction centers from the carotenoid-lacking mutant of *R. sphaeroides*.

Attempts were made to estimate the bacteriopheophytin (BPh)/BChl ratio; however, extraction of these pigments from reaction centers by using acetone, methanol, or acetone/methanol, 7:2 (vol/vol) (13, 14) gave inconsistent quantitative

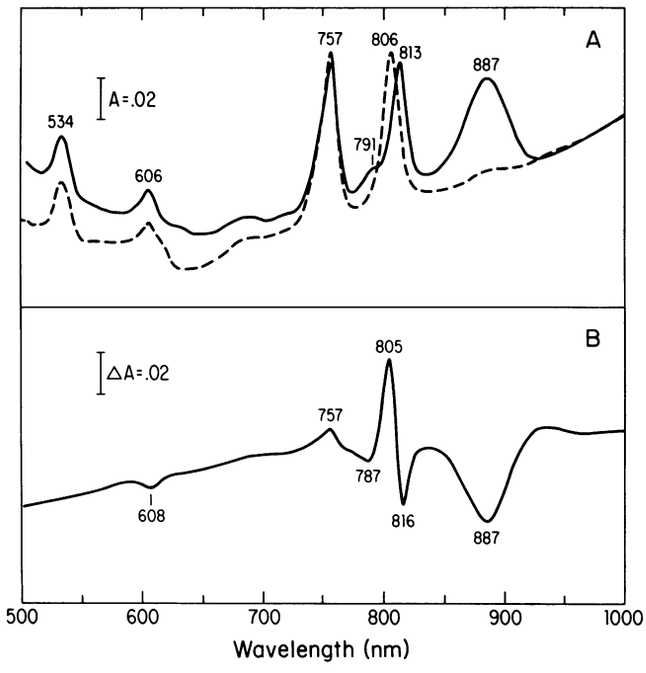

FIG. 2. (*A*) Absorption spectra at 77 K of oxidized (---) and reduced (——) reaction centers from *C. aurantiacus*. Reaction centers in 60% (vol/vol) glycerol were oxidized with $K_3Fe(CN)_6$ and reduced with $Na_2S_2O_4$. (*B*) Oxidized-minus-reduced difference spectrum at 77 K of reaction centers from *C. aurantiacus*.

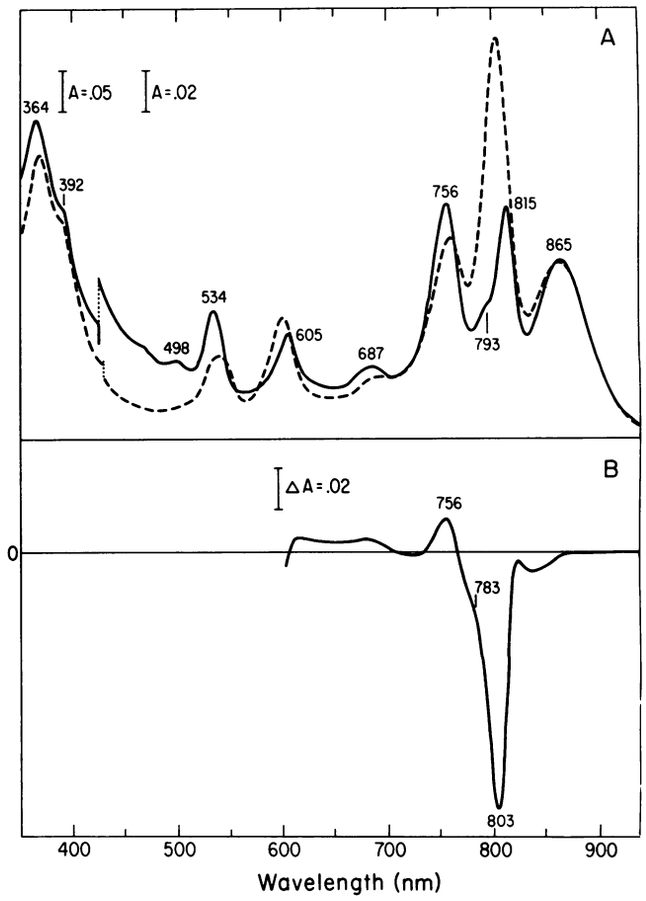

FIG. 3. (*A*) Room temperature absorption spectra of reaction centers from *C. aurantiacus* J-10-fl (——) and from *R. sphaeroides* R-26 (---) at equal A_{865} values. (*B*) Difference spectrum: reduced reaction centers from *C. aurantiacus* minus reduced reaction centers from *R. sphaeroides* (reduced with Na ascorbate).

results for the concentrations of reaction centers currently obtained. The ratio was therefore estimated by comparing the absorbance of the Q_x bands of BChl and BPh at 605 and 534 nm with their relative absorbancies in reaction centers from *R. sphaeroides*, in which the BChl *a*/BPh *a* (mol/mol) ratio is 2:1 (13, 14). The reaction center preparations from *Chloroflexus* were normalized at A_{865} to the reaction centers from *R. sphaeroides*. The areas under the 536- and 600-nm peaks in the spectra of reduced *R. sphaeroides* reaction centers were calculated and the area per mole of BPh *a* or BChl *a* was calculated by dividing by 2 or 4, respectively. These values were then applied to the areas under the 534- and 605-nm peaks in reaction centers of *Chloroflexus*. This approach is based on the assumption that the absorption properties of BChl and BPh in *Chloroflexus* are similar to those in *R. sphaeroides*, which seems to be a reasonable assumption useful for an estimate of pigment ratios. The means of five different determinations were 2.9 mol of BChl *a* (range, 2.7–3.0) and 2.8 mol of BPh *a* (range, 2.5–3.0). The best estimate of pigment content in a reaction center from *Chloroflexus* thus appears to be three molecules of BChl *a* and three molecules of BPh *a* for a ratio of 1:1. Assuming that two BChl *a* molecules per reaction center account for the 865-nm band, as in *R. sphaeroides*, then the A_{815} in *Chloroflexus* must be due to only one BChl *a* molecule. This seems reasonable because the A_{804} is due to two BChl *a* molecules per reaction center from *R. sphaeroides* and is 1.8 times the A_{815} in reaction centers from *Chloroflexus*.

DISCUSSION

Despite the large complement of carotenoids in chlorosomes and membranes of *Chloroflexus* (3), the large amount of cytochrome *c* in membranes (3, 15, §), and the large amount of accessory BChl *c* in chlorosomes (3), it is possible to obtain photochemically active reaction centers rapidly and in reasonably high yield from wild-type J-10-fl and its carotenoid-lacking mutant completely free of BChl *c*, antenna BChl *a*, and cytochromes and nearly free of carotenoid. The total preparation time starting with whole cells is less than 2 hr, including the 1-hr incubation with detergent. Although improvements in yield and elimination of extraneous protein are desirable, in its present form, the method permits the rapid preparation with minimal manipulation of reaction centers suitable for spectral analysis.

Even at the relatively high levels of detergent used here, several hours at 20°C or 30–60 min at 40°C were necessary to free the reaction centers completely from other membrane-bound BChl *a* complexes. The photochemical activity was quite stable under these extraction conditions. It is perhaps not surprising that solubilization of the membranes of the thermophilic *Chloroflexus* differs from that of mesophilic bacteria. *Chloroflexus* is known to have a different array of membrane lipids and proteins than found in other photosynthetic bacteria (3, 16, 17).

The reaction centers from the carotenoid-lacking mutant showed no significant spectral differences from the reaction centers isolated from the wild type. There was slightly less absorbance in the 450-to-500-nm region in reaction centers from the mutant. Either the mutant contains very small amounts of carotenoid associated with the reaction center or the absorbance in this region in both strains is due to something other than carotenoid.

The isolated reaction centers from the green bacterium *C. aurantiacus* bear no resemblance in their absorption properties to the reaction-center complexes isolated from other Chlorobiaceae (7) despite the similarities of the light-harvesting chlorosomes in these organisms. However, they exhibit considerable resemblance to those of purple bacteria although they do have some interesting and useful differences.

The absorption characteristics of the reversibly oxidizable pigment absorbing at 865 nm in *C. aurantiacus* are so similar to those of *R. sphaeroides* that difference spectra from reaction centers from both organisms at equal A_{865} show no differences from 850 nm to 950 nm (Fig. 3). The P865 of *Chloroflexus* and P870 of *R. sphaeroides* must then occupy very similar environments. Furthermore, the peak at 865 nm at room temperature is shifted to near 890 nm at 77 K (Fig. 2) as in *R. sphaeroides* (data not shown).

Chloroflexus apparently has one less BChl *a* and one more BPh *a* in each reaction-center unit than occurs in reaction centers isolated from *R. sphaeroides* and other members of the Rhodospirillaceae (18). While the ratio is different in *Chloroflexus*, it is interesting that the total number of chromophores in the reaction center is most likely six, as is the case in the reaction centers isolated from other photosynthetic bacteria (18).

The presence of BPh in this reaction-center preparation is not likely to be a preparative artifact. All preparations from all batches of cells including the mutant exhibited the same A_{756}/A_{815} ratio regardless of the age of the cells at time of harvest and of the stage of purification. The same ratio was observed even in the crudest preparations, heavily contaminated with cytochrome and carotenoid. BPh has recently been observed in purified membranes from *C. aurantiacus* (2). The light-minus-dark difference spectrum obtained from disrupted cells of *Chloroflexus* in buffer (4) is nearly identical with the difference spectrum obtained from isolated reaction centers (Fig. 1*B*). Comparison of the positions and relative intensities of the absorption maxima at 760 and 803 nm and the absorption minima at 815 and 867 nm in these two spectra indicates that the pigments in the reaction centers are not altered during the isolation procedure.

Of particular interest is that reaction centers from *Chloroflexus* contain only one BChl molecule in addition to those forming P865, rather than two, as in other organisms. Thus, the absorbance near 810 nm (803 nm in *R. sphaeroides* and 815 nm in *C. aurantiacus*) is contributed mainly by only one BChl molecule in *C. aurantiacus* and is about half as great as in *R. sphaeroides*. The equivalent peak is also 10 nm further in the IR in *Chloroflexus*, making it possible to discern the optical changes that occur between 780 and 815 nm on oxidation of P865 more closely than in other isolated reaction centers containing BChl *a*.

Oxidation of P865 is accompanied by a decrease in absorbance between 780 and 790 nm that is observable despite increases in absorbance that occur at 760 and 803 nm. A reasonable explanation of this change is that the 793-nm band represents the absorbance of the higher energy transition of the dimeric exciton forming P865, which would be lost on oxidation of P865. An alternative explanation is that the 793-nm band is a BPh molecule in a different environment than the others and that its absorption maximum is shifted on oxidation of P865. This seems unlikely because the 760-nm band, being relatively more intense in *C. aurantiacus* than in *R. sphaeroides* (Fig. 3*A*), would account for more BPh molecules (i.e., three) than the two it represents in *R. sphaeroides* reaction centers. The 793-nm band could be due to a BChl molecule only if P865 were monomeric and the second and third molecules absorbed at 815 and 793 nm. This seems unlikely because the Q_x band due to the three BChl molecules is so extensively bleached on oxidation, indicating that P865 is probably dimeric, as is believed for P870 in purple bacteria. Examination of the isolated reaction centers by biophysical techniques should reveal which possibility is correct.

The greater magnitude of the increase in absorbance at 803–806 nm compared with the decrease at 815 nm on oxidation of P865 (Fig. 1*B*) might be accounted for by the appearance of a band near 803 nm due to P^+865 in addition to a blue shift in the absorption maximum of the BChl molecule absorbing at 815 nm. In spectra from *R. sphaeroides* at room temperature (19), the spectral region from 780 to 802 nm is so dominated by the large absorbance from the two BChl molecules that it is difficult to know what specific changes accompany oxidation of P870. Linear dichroism studies of oriented chromatophores of *R. sphaeroides* suggest that P^+870 may add to the absorbance increase in that region and that the higher energy transition band of the dimeric P870 may be at 810 nm (20).

It is clear that the actual positions and relative intensities of some of the absorption bands in reaction centers from *C. aurantiacus* and *R. sphaeroides* are nearly identical while others are considerably different. The similarity of P865 in *C. aurantiacus* and P870 in *R. sphaeroides* is particularly striking. *Chloroflexus* thus appears to be very much like a purple bacterium that has a green bacterial light-harvesting system. If it is true that *Chloroflexus* diverged very early in the evolution of eubacteria (8), then the basic photochemical properties of the reaction center seen in the Rhodospirillaceae are likely to have been present in the earliest photosynthetic ancestors of both the Rhodospirillaceae and the Chloroflexaceae.

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