Construction and characterization of a mutant of Rhodobacter sphaeroides with the reaction center as the sole pigment-protein complex

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Table I: Bacterial Strains and Plasmids

strain/plasmid	relevant characteristics	source/reference
E. coli		
DH5	*supE*44, Δ*lac*U169, (*ϕ80 lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	Sambrook et al. (1989)
S17-1	*thi*, *pro*, *hsdR*$^-$, *hsdM*$^+$, *recA*, RP4-2 (Tc::Mu Km::Tn7)	Simon et al. (1983)
Rb. sphaeroides		
NCIB 8253	wild type	R. A. Niederman (Rutgers University)
DPF2	genomic deletion of *pufBALMX*; insertion of Km^R gene	Hunter et al. (1991)
DD13	strain DPF2 with genomic deletion of *pucBA*; insertion of Sm^R gene	Jones et al. (1991)
DD13/G1	spontaneous green mutant of strain DD13	Jones et al. (1991)
RCO1	strain DD13/G1 complemented in trans with pRKEH10D	this work
RCLH11	strain DD13/G1 complemented in trans with pRKEH10	this work
plasmids		
pRK415	Tc^R derivative of RK2; mobilizable	Keen et al. (1988)
pSUP202	Ap^{R}Tc^{R}Cm^R; Mob$^+$ Tra$^-$; ColE1 replicon	Simon et al. (1983)
pRKEH10	Tc^R; 6.8-kb *Eco*RI–*Hin*dIII fragment encompassing *pufQBALMX* with engineered *Xba*I and *Bam*HI sites and unique *Sal*I site (in pRK415)	this work
pRKEH10D	Tc^R; pRKEH10 with *Pvu*I–*Nae*I deletion in *pufBA* (in pRK415)	this work

for which the three-dimensional X-ray crystal structure was deduced (Deisenhofer et al., 1985; Deisenhofer & Michel, 1989). This was closely followed by the publication of the X-ray crystal structure of the *Rb. sphaeroides* reaction center (Allen et al., 1987a,b). In *Rb. sphaeroides*, the reaction center is composed of three subunits, H, L, and M, and contains four bacteriochlorophyll *a* molecules, two bacteriopheophytin *a* molecules, and two molecules of ubiquinone, one of which is tightly bound (Q_A) and the other (Q_B) is free to exchange with quinone in the intramembrane ubiquinone pool. The special pair consists of an exciton-coupled dimer of bacteriochlorophyll *a* molecules each of which is located on either side of an approximate C_2 axis of symmetry. The remaining two bacteriochlorophylls, two bacteriopheophytins, and two quinones are arranged in pairs on either side of the C_2 axis. Despite this symmetry, electron transfer is asymmetric, proceeding along the L branch which is primarily associated with the L subunit. The underlying reasons for this asymmetry remain unclear, as do the precise reasons for the high quantum yield, which is caused by back-reactions within the reaction center occurring at a much slower rate than forward reactions. Also, the question of how excitation energy is transmitted from LH1 to the special pair remains unanswered.

Over the last few years, most studies of electron transfer within wild-type and mutant reaction centers have been performed on reaction centers that have been purified from bacterial membranes and solubilized in detergent, rather than on membrane-bound centers (Shuvalov et al., 1986; Coleman & Youvan, 1990). This is because the presence of large amounts of the antenna pigment–protein complexes in natural membranes complicates the acquisition and interpretation of experimental data. We have therefore constructed a strain of *Rb. sphaeroides* in which the reaction center is the sole pigment–protein complex. This has been achieved by deleting the *pufBALM* genes (coding for the LH1 β and α and reaction center L and M subunits, respectively) and *pucBA* genes (coding for the LH2 β and α subunits) and reintroducing the *pufLM* genes into this double-deletion strain in trans. An initial characterization of this strain is presented, which demonstrates that the reaction center complex in this strain is assembled, and possesses pigments in their normal orientations, despite the absence of light harvesting complexes. This strain provides an ideal experimental background for mutagenesis experiments designed to probe the mechanism of the reaction center.

Materials and Methods

Media, Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids are listed in Table I. *Escherichia coli* strains were grown in Luria broth with appropriate antibiotics. *Rb. sphaeroides* strains were grown under aerobic/dark or photoheterotrophic/light conditions in M22+ medium (Hunter & Turner, 1988) or in M22MAL medium (supplemented with 0.1% Casamino acids for growth in liquid culture). M22MAL medium was based on M22+ medium, but succinate, lactate, and glutamate were replaced by 30 mM DL-malate. Antibiotics were added where appropriate. Cells were grown photoheterotrophically at an approximate light intensity of 10 W m^{-2} or, where indicated, 150 W m^{-2}. Antibiotic concentrations (μg mL^{-1}) were *E. coli* ampicillin (200), tetracycline (10), streptomycin (25), kanamycin (30), *Rb. sphaeroides* tetracycline (1), streptomycin (5), and kanamycin (20).

Genetic Procedures, Site-Directed Mutagenesis, and DNA Nucleotide Sequencing. Recombinant DNA techniques were performed as described in Sambrook et al. (1989). Novel restriction sites were introduced into *Rb. sphaeroides* DNA using mutagenic oligonucleotides according to the method of Kunkel (1985), with templates based upon bacteriophage M13 or pBluescript II (Stratagene). Mutagenized sequences and deletion constructs were sequenced using Sequenase according to the method recommended by United States Biochemical Corp.

Conjugative Crosses. Plasmids to be introduced into *Rb. sphaeroides* were first transformed into *E. coli* strain 17-1. Matings were then performed as described in Hunter and Turner (1988).

Electron Microscopy. Cell samples were fixed overnight in 3% glutaraldehyde/0.1 M potassium phosphate (pH 7.4). After being washed in 0.1 M potassium phosphate (pH 7.4), the material was fixed for a second time in 2% aqueous osmium tetroxide for 4 h and then progressively dehydrated using 75% ethanol, 95% ethanol, 100% ethanol, ethanol/propylene oxide (50:50 v/v), and 100% propylene oxide. The sample was then infiltrated with Spurr resin (Agar Scientific) and polymerized at 60 °C for 17 h. Thin sections cut with a microtome were stained with saturated alcoholic uranyl acetate and Reynolds lead citrate and examined in a Philips CM10 transmission electron microscope.

Preparation of Membranes. Intracytoplasmic membranes were prepared using a French press as described in Clark et al. (1983).

Spectroscopy. Following matings, antibiotic-resistant colonies grown aerobically in the dark were screened for the presence or absence of light-harvesting complexes and reaction centers using a Guided Wave Model 260 fiber optic spectrophotometer (Guided Wave Inc., El Dorado Hills, CA). Modification of the fiber optic housings allowed spectra of the

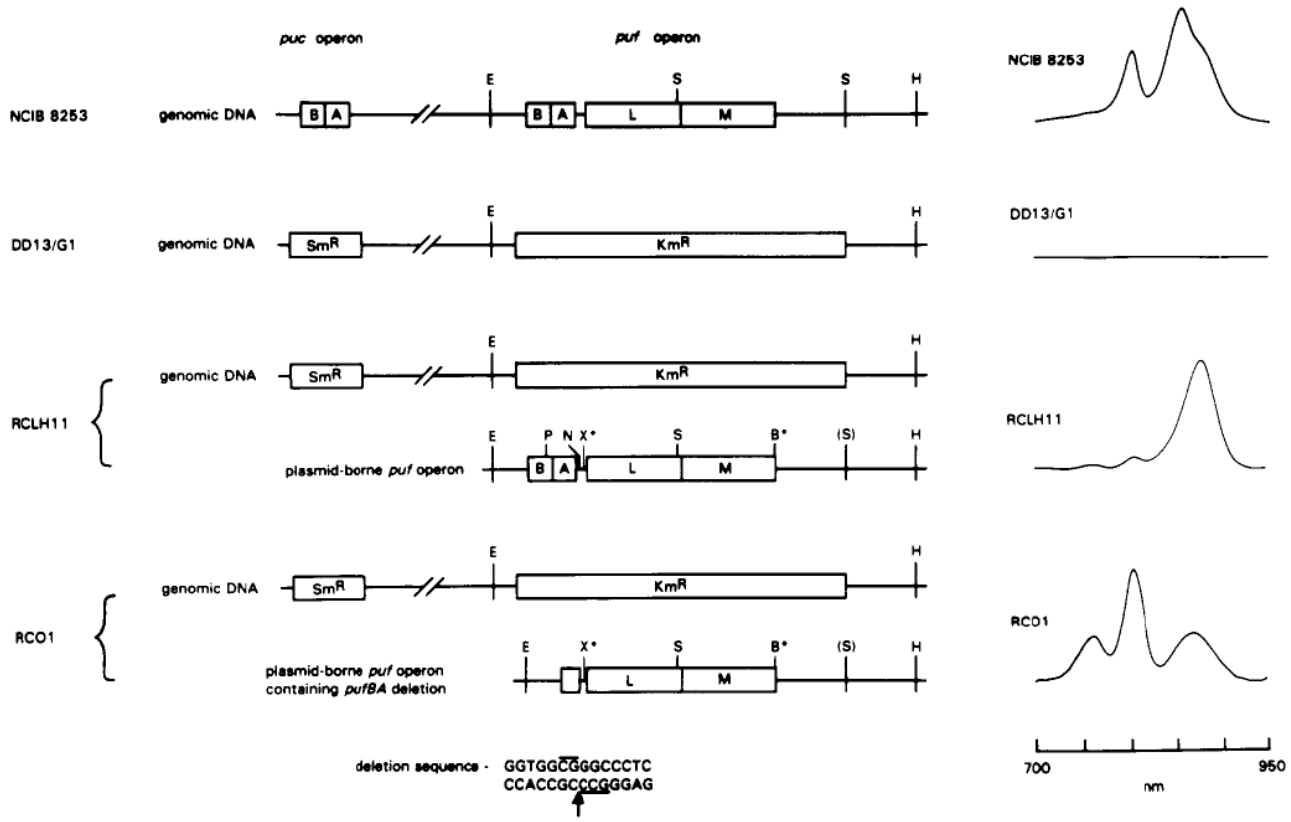

FIGURE 1: Diagrammatic genetic maps and near-infrared spectra of membranes from wild type, DD13/G1, and complemented strains. Strain RCLH11 is strain DD13/G1 complemented in trans with the *puf* operon. Strain RCO1 is strain DD13/G1 complemented in trans with a *puf* operon bearing a deletion in *pufBA*. The DNA sequence in the immediate region of this deletion is shown. Maps of the *puc* and *puf* operons and antibiotic-resistance cartridges are not drawn to scale. Brackets denote sites that have been removed by digestion, filling-in, and blunt-end ligation; asterisks denote sites that have been introduced by site-directed mutagenesis. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nae*I; P, *Pvu*I; S, *Sal*I; X, *Xba*I.

type shown in Figure 1 to be directly acquired from colonies of approximately 1 mm in diameter or greater. The same spectrophotometer was used to obtain cytochrome difference spectra from suspensions of membranes in a standard 1-cm path-length cuvette. Flash-induced absorbance changes due to cytochrome *c* and reaction centers were recorded in the single-beam spectrophotometer described in Jones and Jackson (1989). Membrane samples were preincubated in the dark under a continuous stream of argon for 15 min prior to data acquisition. The concentration of reaction centers in membrane samples was determined from an absorbance spectrum using $\epsilon_{802} = 2.88 \times 10^{5}$ M^{-1} cm^{-1} for isolated reaction centers (Feher & Okamura, 1978). Extents of reaction center and cytochrome (c_1 and c_2) oxidation were calculated as described in Jones and Jackson (1989).

Room temperature LD and CD spectra and low-temperature absorbance spectra were recorded using a laboratory-built spectrophotometer which was interfaced to a SUN3/SUN4 computer system for data analysis. Membrane samples were suspended in 50% (w/v) glycerol, and where appropriate were photooxidized by illumination with a 150-W halogen–tungsten light source which provided an incident light intensity of approximately 0.5 W m^{-2}. For LD spectra, membranes of strain RCO1 were prepared in biaxially squeezed 14.5% polyacrylamide/0.5% bis(acrylamide) gels inserted in 1-cm path-length cuvettes (van Amerongen et al., 1988).

RESULTS

Construction of a Green Double-Deletion Mutant. A mutant of *Rb. sphaeroides* devoid of LH1 and LH2 complexes and reaction centers was constructed by replacing the structural genes of the *puf* and *puc* operons with antibiotic-resistance cartridges. Precise details of the construction of these deletion/insertion mutations have been given elsewhere (Jones et al., 1992). Briefly, a 3.2-kb region of the *puf* operon extending from just upstream of ORFK to the *Sal*I site downstream of the 3′ end of *pufM* was replaced by a kanamycin cartridge and the resulting construct cloned into the suicide vector pSUP202. This was then transferred into wild-type *Rb. sphaeroides* NCIB 8253 by conjugative transfer from *E. coli* strain S17-1. Colonies were selected that were Km^R, nonphotosynthetic, and devoid of LH1 and reaction center complexes (using a fiber optic spectrophotometer; see Materials and Methods); all of the selected colonies possessed LH2 as the sole pigment–protein complex.

The chosen mutant strain, named DPF2, was then made LH2 through a second genetic manipulation. A 1-kb region of the *puc* operon encompassing the *pucBA* genes was replaced by a streptomycin-resistance cartridge and the resulting construct cloned into pSUP202 and transferred into strain DPF2 as described above. Colonies were then selected that were Km^R Sm^R, nonphotosynthetic, and devoid of all three pigment–protein complexes. The strain selected by this procedure was named DD13. A spontaneous green derivative of DD13 was then isolated (DD13/G1; Figure 1). DD13/G1 was used as a recipient for wild-type and altered *puf* operons as cytochrome *c* and reaction center absorbance changes in the 540–570-nm region of the spectrum are more easily measured in strains which possess green carotenoids.

Construction of a Reaction Center-Only Mutant. Several modifications were made to the wild-type *puf* operon to delete

the LH1 structural genes and to facilitate subcloning of the *pufL* and *pufM* genes. In order to make the *Sal*I site close to the 3′ end of *pufL* unique within the 6.8-kb *Eco*RI–*Hin*dIII fragment encompassing the *puf* operon, the second *Sal*I site downstream of *pufM* was removed by partial digestion with *Sal*I followed by filling-in of the resulting recessed 3′ termini and religation of the blunted ends. In addition, two new unique restriction sites were introduced by oligonucleotide-mediated site-directed mutagenesis. An *Xba*I site was created by changes (T→C and G→T) at positions 61 and 60 bp upstream of the first base of the start codon for *pufL*, and a *Bam*HI site was created by changes (C→G and C→T) at positions 5 and 10 bp downstream of the last base of the termination codon marking the 3′ end of *pufM*. In the resulting construct (pRKEH10), *pufL* can be isolated as an *Xba*I–*Sal*I fragment and *pufM* as a *Sal*I–*Bam*HI fragment.

In order to delete the structural genes for LH1, a 1.07-kb *Pst*I–*Xba*I fragment encompassing *pufBA* was subcloned into pUC18 and digested with *Pvu*I and *Nae*I. The resulting free ends of the major fragment were blunted and ligated, and the *Pst*I–*Xba*I fragment (now 0.83 kb) was cloned back into pRKEH10. The resulting construct, pRKEH10D, lacks the last 44 bp of *pufB* (150 bp total) and the whole of *pufA*. The portion of *pufB* deleted includes the C-terminal region which extends into the periplasm and approximately one-third of the membrane-spanning helix including the putative histidine ligand to B875 (J. Olsen, personal communication). The DNA sequence in the region of the *Pvu*I/*Nae*I deletion in pRKEH10D is shown in Figure 1, with the position of the ligation indicated by the arrow and the vestiges of the restriction sites indicated by the lines above (for *Pvu*I) and below (for *Nae*I) the sequence. The coding sequence for the residual part of *pufB* was out-of-frame with the coding sequence for *pufL*, excluding the possibility of the formation of a hybrid LH1β–RCL polypeptide. The two strong stem–loop structures located immediately downstream of the 3′ end of the residual of *pufB* (DeHoff et al., 1988) were retained in pRKEH10D.

Plasmids pRKEH10 and pRKEH10D were transferred to strain DD13/G1 by conjugative transfer from *E. coli* strain S17-1. Colonies were then selected under dark/anaerobic conditions that were Tc^R Sm^R Km^R and that exhibited either an LH1 absorbance peak at 875 nm (with pRKEH10, Figure 1) or reaction center absorbance peaks at 760 and 800 nm (with pRKEH10D, Figure 1). The transconjugants selected for further study were named RCO1 ($RC^+LH1^-LH2^-$) and RCLH11 ($RC^+LH1^+LH2^-$).

Photosynthetic Competence. Strains RCLH11 and RCO1 were both capable of photoheterotrophic growth on M22+ medium at a light intensity of 10 W m^{-2}, but strain RCO1 grew very slowly under these conditions even in the absence of kanamycin (doubling time >24 h). Growth of this strain could be accelerated by replacing the carbon sources in M22+ medium (succinate, glutamate, and lactate) with 30 mM malate. In this medium (M22MAL), strains RCLH11 and RCO1 grew with approximate doubling times of 10 and 13 h, respectively, at a light intensity of 10 W m^{-2} and in the presence of kanamycin. The wild-type NCIB 8253 grew with a doubling time of 4 h under the same conditions (no kanamycin). When exposed to an incident light intensity of approximately 150 W m^{-2}, strain RCO1 grew on M22(MAL) medium (plus kanamycin) with a doubling time of 4 h.

Confirmation that the cyclic electron-transfer chain was intact in strain RCO1 was obtained by measuring the flash-induced absorbance changes attributable to cytochrome (c_1 and c_2) and reaction centers in membranes in the absence and

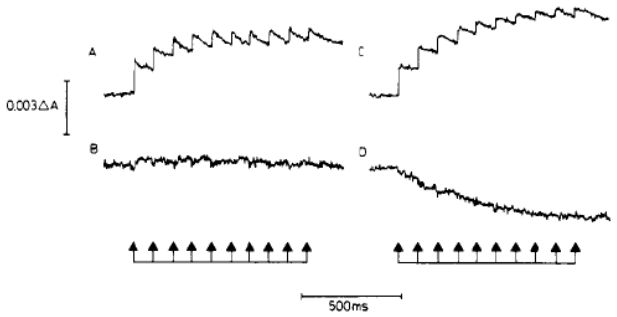

FIGURE 2: Flash-induced absorbance changes associated with cytochrome (c_1 and c_2) (B and D) and reaction center P870 (A and C) oxidation and reduction in membranes from strain RCO1. In all cases, the sample was exposed to 10 flashes fired at a frequency of 10 Hz. In (C) and (D), myxothiazol was added to 5 μM. Data in (A) and (C) are at 542 nm and are the average of 8 sweeps; data in (B) and (D) are at 552–542 nm and are the average of 2 × 8 sweeps, all at a sweep frequency of 0.0167 Hz.

presence of myxothiazol (Figure 2). Exposing the sample to 10 short flashes at 100-ms intervals progressively oxidized all the reaction centers in the sample (Figure 2A,C). The concentration of reaction centers in the sample was determined independently as described under Materials and Methods. The fact that eight or nine flashes were required to fully oxidize the reaction center pool could be explained if a proportion of photooxidized reaction centers was being reduced by cytochrome c_2 on a submillisecond time scale. However, no cycles of rapid cytochrome oxidation followed by slower re-reduction were observed following the first few flashes (Figure 2B,D), demonstrating that this was not the case. The gradual photooxidation of the reaction center pool during the flash train probably arose as a result of a combination of two factors. First, the results shown in Figure 2B,D indicate a lack of rapid donor to the reaction center (i.e., cytochrome c_2) in these membranes, and hence reaction centers accumulate in the oxidized state. Second, it is likely that the actinic flash was not saturating in this antenna-deficient mutant and so several flashes were required to fully oxidize the reaction center pool. Previous experiments with the same apparatus have established that this actinic source is approximately 96% saturating when measured using a sample of whole cells with an intact light-harvesting system (Jones & Jackson, 1989). The question of flash saturation is currently under more detailed investigation.

Despite the apparent lack of appreciable amounts of cytochrome c_2 in these membranes, the effect of myxothiazol in promoting a net oxidation of the cytochrome (c_1 and c_2) pool (Figure 2D) clearly showed that the reaction centers were coupled to the pool of cytochrome bc_1 complexes in these membranes. The relatively slow accumulation of oxidized cytochrome (c_1 and c_2) is consistent with the view that the vesicles lack appreciable levels of cytochrome c_2, which if present would be expected to promote rapid redox equilibration. Furthermore, it was noticeable that the total amount of photooxidizable cytochrome (c_1 and c_2) was low in these membranes. The maximum extent of cytochrome (c_1 and c_2) oxidation in Figure 2D corresponded to a concentration of 0.13 μM compared with a reaction center concentration of 0.46 μM (see Materials and Methods).

To investigate the size of the cytochrome *c* pool further, a ferricyanide-oxidized minus dithionite-reduced difference spectrum was recorded for the RCO1 membranes at the same dilution as in Figure 2. This spectrum revealed absorbance peaks due to *c*- and *b*-type cytochromes as expected (Figure 3a). To resolve the *c*-type cytochrome peak further, a ferricyanide-oxidized minus ascorbate-reduced spectrum was

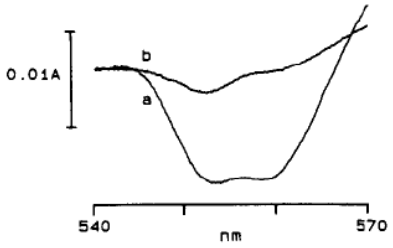

FIGURE 3: Reduced minus oxidized difference spectra of membranes of RCO1. (a) Dithionite-reduced minus ferricyanide-oxidized. (b) Ascorbate-reduced minus dithionite-oxidized.

recorded (Figure 3b). The *c*-type cytochrome peak was centered at 552 nm and yielded a concentration for total *c*-type cytochrome (measured at 552–542 nm) of 0.13 μM. This corresponded exactly with the value obtained for the total photooxidizable *c*-type cytochrome in these membranes (from Figure 2D).

An interesting feature of strain RCO1 is that because it lacks both LH1 and LH2 it is possible to observe the 760- and 800-nm reaction center peaks in samples of intact cells taken directly from growing cultures, allowing a semiquantitative estimate of the number of reaction centers per cell at various stages of growth. In mid-log-phase cells, we estimate that each cell contains approximately 3×10^4 reaction centers (based on the assumption that a culture with an $A^{600} = 1$ has 1×10^9 cells mL^{-1}).

Membrane Structure. A characteristic of wild-type strains of *Rb. sphaeroides* such as NCIB 8253 grown under photoheterotrophic conditions or chemoheterotrophically in the dark at low oxygen tension is a highly invaginated cytoplasmic membrane which contains the photosynthetic apparatus. Under the electron microscope, a thin section reveals that such cells are packed with circular membrane structures (Hunter et al., 1988). Electron microscopy of a thin section of *Rb. sphaeroides* RCO1 cells grown under photoheterotrophic conditions showed that in cells lacking both LH1 and LH2 the cytoplasmic membrane was not significantly invaginated (Figure 4A,B). Occasionally, isolated chromatophore-like structures were seen close to the cytoplasmic membrane, but these were insufficiently common to be described as a characteristic feature of these cells. In contrast, RCLH11 cells grown under these conditions clearly possessed tubular membrane structures within the cytoplasm (Figure 4C,D), confirming previous findings with *Rb. sphaeroides* strain M21 (Hunter et al., 1988) which has a chemically-induced mutation in the LH2 genes and hence displays the same phenotype as RCLH11 (Ashby et al., 1987).

77 K Absorbance Spectra. Figure 5 shows absorbance spectra of RCO1 membranes recorded under light-oxidized (Figure 5a) and dark-reduced (Figure 5b) conditions at 77 K, and the resulting difference spectrum. The peak at 860 nm in Figure 5a, attributed to the low-energy exciton component of the reaction center special pair, was almost completely bleached on illumination (Figure 5b), indicating photochemical charge separation. The other noticeable feature was an electrochromic bandshift of the 800- and 760-nm peaks attributed to the Q_y transition of the accessory bacteriochlorophylls and bacteriopheophytins, respectively, revealed in the difference spectrum (Figure 5c). This bandshift occurred in response to the presence of a net positive charge on the special pair in photooxidized reaction centers (Shuvalov et al., 1986).

Room Temperature Linear and Circular Dichroism Spectra. A room temperature LD spectrum of reaction centers in membrane vesicles is shown in Figure 6. The features of this spectrum agree with the LD spectrum of *Rb. sphaeroides* reaction centers reported by Rafferty and Clayton (1978). The broad band at 860 nm attributed to the special pair of bacteriochlorophylls had the highest overall LD/A, indicating that this transition was oriented approximately parallel to the membrane plane. On the blue side of this feature, the LD/A decreased steeply to a minimum at 812 nm, which may be attributed to the high-energy exciton component of the special pair oriented perpendicular to the membrane plane (Breton et al., 1989). The LD/A maximum in the 800-nm region attributed to the Q_y transition of the monomeric accessory bacteriochlorophylls corresponded to an angle of 72° to the normal of the membrane plane. The overall negative LD/A in the bacteriopheophytin region of the spectrum around 740 nm indicated an angle of approximately 40° relative to the normal of the plane of the membrane. All of the deduced angles are consistent with the findings of Breton (1988) for the reaction center of *Rb. sphaeroides* wild-type 2.4.1.

A room temperature CD spectrum of reaction centers in membrane vesicles is shown in Figure 7. The general shape of the spectrum confirmed that shown in Breton et al. (1989) for isolated reaction centers of *Rb. capsulatus* at room temperature and in Sauer and Austin (1978) for isolated reaction centers of *Rb. sphaeroides* R26 at 77 K, with positive bands at 800 and 865 nm and a negative band at 815 nm.

Discussion

The combination of a high-resolution X-ray crystal structure and a well-characterized genetic system has made the reaction center of *Rb. sphaeroides* and related species an ideal system for studying the phenomenon of photochemical charge separation, and the principles underlying protein structure in general. A number of site-directed mutations have been introduced into the reaction centers of *Rb. sphaeroides* and *Rb. capsulatus* in an attempt to unravel the mechanism of fast electron transfer from the special pair of bacteriochlorophylls to Q_A (Coleman & Youvan, 1990; Kirmaier et al., 1991; Mattoli et al., 1991), and reduction and protonation of the quinone at the Q_B site (Paddock et al., 1990; Takahashi & Wraight, 1990). To date, these studies have largely concentrated on the properties of reaction centers in detergent solution. In this paper, we describe a strain of *Rb. sphaeroides* in which the reaction center is the sole pigment–protein complex. This strain, named RCO1, is photosynthetically competent and, following photooxidation, is reduced by electrons derived from the quinol oxidase (Q_Z) site of the cytochrome b/c_1 complex.

The slow rate at which the cytochrome *c* absorbing in the 551-nm region was oxidized by the flash train suggests that most of the cytochrome in RCO1 membranes was in fact cytochrome c_1 [for comparison, see Bowyer et al. (1979) and Garcia et al. (1987)]. This is consistent with the data shown in Figure 3b in which the ascorbate-oxidizable cytochrome *c* gave an absorbance peak centered at 552 nm (Wood, 1980; Meinhardt & Crofts, 1982), and indicates a reaction center: b/c_1 complex ratio of approximately 3:1 in these membranes. All of the cytochrome *c* appeared to be accessible to the reaction centers. The reason for the low level of cytochrome c_2 in these membranes may lie in the arrangement of the cytoplasmic membrane in strain RCO1. In wild-type cells, chromatophores are formed in the French press from near-spherical invaginations of the membrane and contain relatively high levels of trapped cytochrome c_2. In contrast, the cytoplasmic membrane in RCO1 possesses few if any significant invaginations, and therefore it is likely that relatively little cytochrome c_2 is trapped within the vesicles formed during

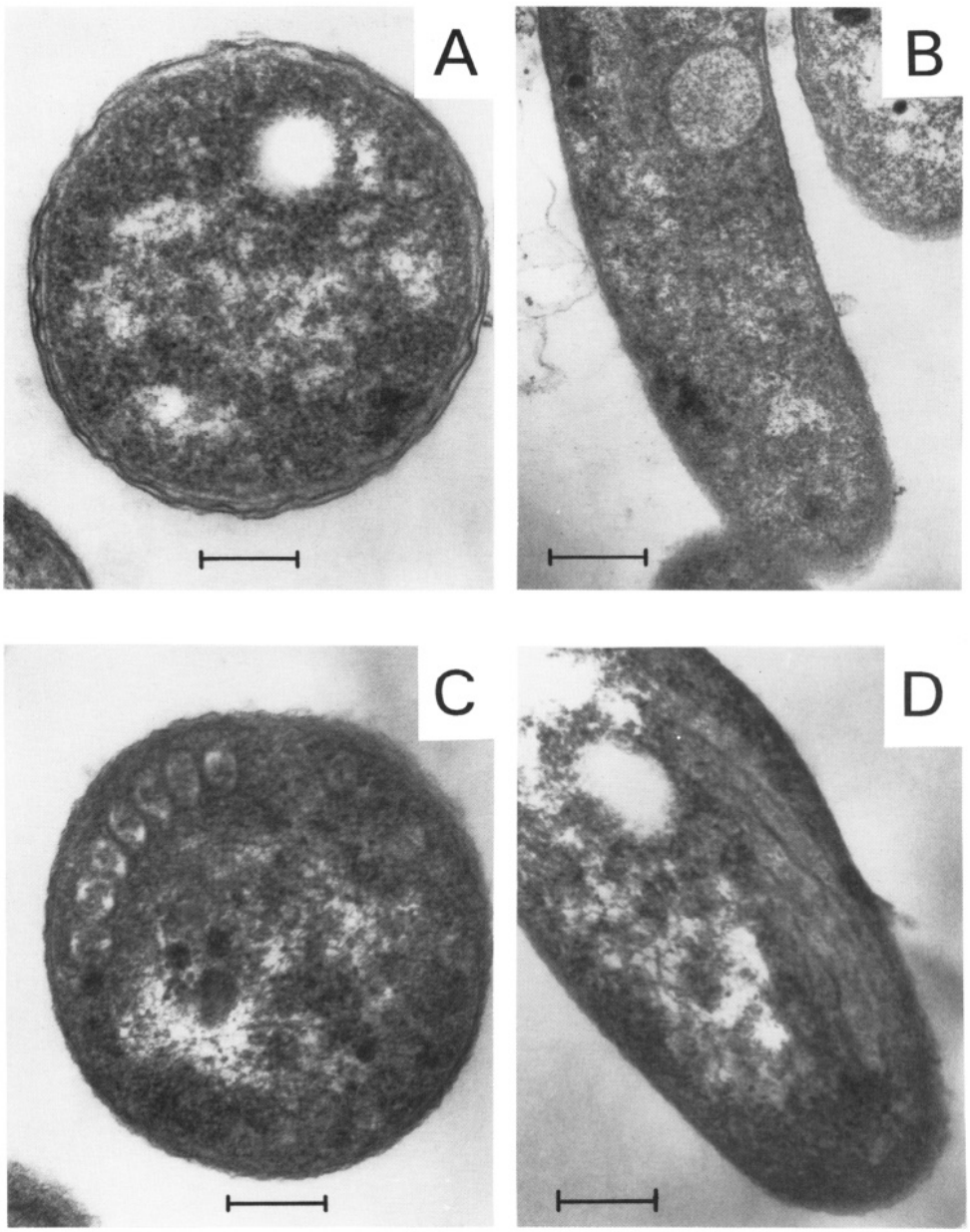

FIGURE 4: Electron micrographs of a thin section of photosynthetically grown cells of (A and B) RCO1 and (C and D) RCLH11. Scale bars represent 200 nm (A, C, and D) or 400 nm (B).

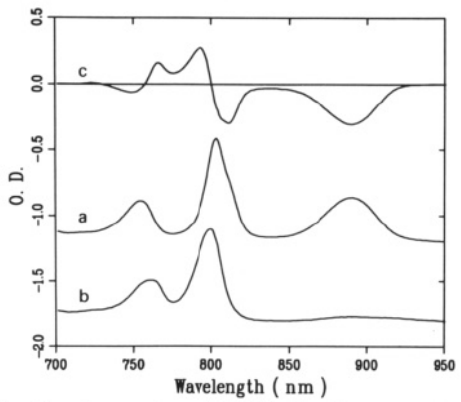

FIGURE 5: Low-temperature (77 K) absorbance spectra of (a) dark-reduced and (b) light-oxidized membranes of RCO1. The resulting difference spectrum is shown in (c).

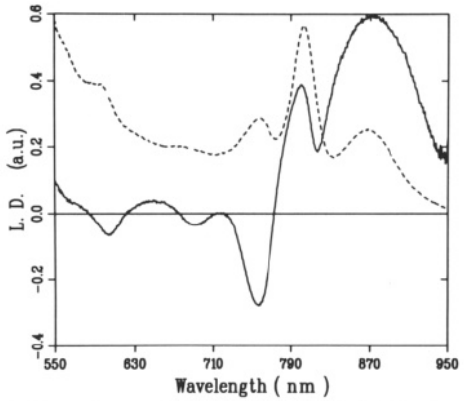

FIGURE 6: Room temperature absorbance (dashed line) and LD/A (solid line) spectra of membranes of RCO1.

passage through the French press. In this respect, membranes prepared from strain RCO1 bear some resemblance to the upper pigmented band isolated from the wild-type strain, which consists of membranes formed from areas of limited invagination at an early stage of development (Niederman et al.,

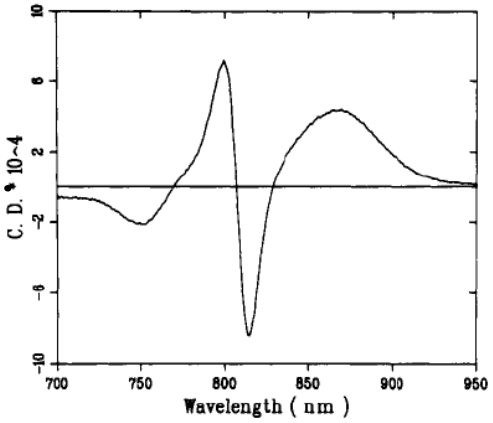

FIGURE 7: Room temperature CD spectrum of membranes of RCO1.

1979; Bowyer et al., 1985). It is further possible that a significant proportion of the membrane vesicles are in fact "wrong-side-out", with the cytochrome c_2 binding site exposed to the external medium, or exist as open sheets.

Given the lack of light-harvesting complexes in cells of RCO1, it was possible to obtain a semiquantitative estimate of the number of reaction centers in mid-log-phase cells growing under low-light photoheterotrophic conditions. The number arrived at, 3×10^4 reaction centers per cell, compares well with an earlier estimate of the number of reaction centers in cells of NCIB 8253 grown under conditions of low aeration in the dark, which yielded a figure of 2×10^4 reaction centers per cell (Hunter et al., 1985). These estimates suggest that the production of reaction centers in cells of RCO1 is not unduly inhibited by the absence of light-harvesting complexes, and approaches wild-type levels despite the lack of a highly invaginated cytoplasmic membrane.

Growth of strain RCO1 under photoheterotrophic conditions was greatly accelerated when the carbon sources of M22+ medium (succinate, glutamate, and lactate) were replaced by malate, a less reduced carbon source. This may reflect a need for the cyclic electron transport chain to maintain an optimal redox poise under light-limiting conditions (McEwan et al., 1985; Jones et al., 1990).

The relative growth rates of NCIB 8253, RCLH11, and RCO1 on M22MAL medium were consistent with the different abilities of these strains to harvest light. The rate of growth of RCO1 under these conditions was also broadly consistent with the rate of growth of a reaction center-only strain of *Rb. capsulatus* under low-light conditions (Garcia et al., 1991) which exhibited a doubling time of 11 h. Under high-light conditions, these authors observed a doubling time similar to that of their wild-type strain. Our findings support this result; at 150 W m^{-2}, strain RCO1 grows at approximately the same rate as NCIB 8253.

Strain RCLH11 forms tubular membranes when grown under photoheterotrophic conditions. This observation confirms earlier reports of tubular membrane structures in *Rb. sphaeroides* strains such as M21 (Hunter et al., 1988), which has a defect in the *puc* genes, RS104 (Kiley et al., 1988), which also has a defect in the *puc* genes and lacks colored carotenoids, and R26 (Lommen & Takemoto, 1978), which lacks carotenoids.

Strains of *Rb. capsulatus* exhibiting a reaction center-only phenotype have previously been described by Bylina et al. (1988) and Dorge et al. (1990). In both cases, these strains were constructed by complementing the *puf*-deletion strain U43 of Youvan et al. (1985) with a copy of the *puf* operon bearing one or more site-directed mutations in the LH1 genes which prevented correct assembly of the LH1 complex. The resulting transconjugants were deficient in LH1 and also deficient in LH2 by virtue of a point mutation in *pucC*, the function of which is unclear (Tichy et al., 1989). The ability of these strains to grow photoheterotrophically demonstrated that the reaction center can assemble in an active form in the absence of LH1 and LH2 spectral complexes. However, this did not exclude the possibility that the reaction center is stabilized in the membrane by association with LH1 and/or LH2 polypeptides, even if the latter are not part of an assembled complex. In the case of the strain of Dorge et al. (1990), pulse–chase experiments clearly showed that LH1 and LH2 polypeptides were synthesized and incorporated into the membrane but were turned over rapidly (Stiehle et al., 1990). The data presented in this report demonstrate unequivocally that reaction centers can assemble in an active form in the complete absence of light-harvesting α- and β-polypeptides. The possibility that reaction centers are stabilized in the membrane by association with the remaining portion of *pufB* can be discounted. Removal of the DNA sequence coding for the C-terminus region of the LH1 β-polypeptide (which in the wild type is located on the periplasmic side of the membrane) plus one-third of the transmembrane α-helical region should prevent the altered polypeptide from assembling in the membrane in a stable form. Even if this is not the case, previous experiments in which the entire coding sequence for *pufB* plus that for *pufA* was deleted produced transconjugant strains which also exhibited a reaction center-only phenotype and contained functional reaction centers (Jones et al., 1992). The plasmid (pRKEH10D) used in this paper to generate transconjugants with a reaction center-only phenotype is a significant improvement on that described in brief in Jones et al. (1992) as it can also be used to express *pufL* or *pufM* sequences that have been altered by site-directed mutagenesis.

Reaction centers in membrane vesicles from strain RCO1 display similar spectroscopic properties to reaction centers from *Rb. sphaeroides* in detergent solution. The spectroscopic properties of the pigments in reaction center and light-harvesting complexes serve as a useful indicator of the integrity of these complexes, and would reflect gross alterations in the pattern of reaction center assembly. It is known that the presence of some detergents may modify the absorbance properties of the chromophores and in some cases abolish them (Clayton & Clayton, 1981; Kramer et al., 1984; van Dorssen et al., 1988). The measurements conducted here on strain RCO1 demonstrate that the orientations of the reaction center pigments are consistent with those obtained for detergent-solubilized reaction centers and with crystallographic data (Breton, 1988). The spectra reported here are the first obtained for the *Rb. sphaeroides* reaction center in a bacterial membrane, demonstrating that previous data have provided an accurate reflection of the native complex. One valuable aspect of this strain is that it provides a useful system for the rapid assay of site-directed alterations to the *Rb. sphaeroides* reaction center complex without the need for any purification from attendant light harvesting complexes. Indeed, certain properties of mutated reaction centers can be evaluated in bacterial colonies using the fiber optic spectrophotometer. The ability to study altered complexes in the natural membrane is important as altered complexes are often unstable when extracted into detergent solution (Coleman & Youvan, 1990), leading to denaturation of the complex. It is also possible that altered complexes which do not denature on extraction undergo structural rearrangements which alter their biophysical properties. Such problems can be avoided by use of an ex-

pression system based on a double-deletion strain such as that described here.

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REFERENCES

Aagaard, G., & Sistrom, W. R. (1972) *Photochem. Photobiol. 15*, 209–225.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5730–5734.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6162–6166.

Ashby, M. K., Coomber, S. A., & Hunter, C. N. (1987) *J. Gen. Microbiol. 213*, 245–248.

Bowyer, J. R., Tierney, G. V., & Crofts, A. R. (1979) *FEBS Lett. 101*, 207–212.

Bowyer, J. R., Hunter, C. N., Ohnishi, T., & Niederman, R. A. (1985) *J. Biol. Chem. 260*, 3295–3304.

Breton, J. (1988) *NATO ASI Ser., Ser. A. 149*, 59–69.

Breton, J., Bylina, E. J., & Youvan, D. C. (1989) *Biochemistry 28*, 6423–6430.

Bylina, E. J., Robles, S. J., & Youvan, D. C. (1988) *Isr. J. Chem. 28*, 73–78.

Clark, A. J., Cotton, N. P. J., & Jackson, J. B. (1983) *Biochim. Biophys. Acta 723*, 440–453.

Clayton, R. K., & Clayton, B. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5583–5587.

Coleman, W. J., & Youvan, D. C. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 333–367.

DeHoff, B. S., Lee, J. K., Donohue, T. J., Gumport, R. I., & Kaplan, S. (1988) *J. Bacteriol. 170*, 4681–4692.

Deisenhofer, J., & Michel, H. (1989) *EMBO J. 8*, 2149–2170.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature 318*, 618–624.

Dorge, B., Klug, G., Gad'on, N., Cohen, S. N., & Drews, G. (1990) *Biochemistry 29*, 7754–7758.

Feher, G., & Okamura, M. Y. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 349–386, Plenum Press, New York.

Garcia, A. F., Venturoli, G., Gad'on, N., Fernandez-Velasco, J. G., Melandri, B. A., & Drews, G. (1987) *Biochim. Biophys. Acta 890*, 335–345.

Garcia, A. F., Mantele, W., Gad'on, N., Tadros, M. H., & Drews, G. (1991) *Arch. Microbiol. 155*, 205–209.

Hunter, C. N., & Turner, G. (1988) *J. Gen. Microbiol. 134*, 1471–1480.

Hunter, C. N., Kramer, H. J. M., & van Grondelle, R. (1985) *Biochim. Biophys. Acta 807*, 44–51.

Hunter, C. N., Pennoyer, J. D., Sturgis, J. N., Farrelly, D., & Niederman, R. A. (1988) *Biochemistry 27*, 3459–3467.

Hunter, C. N., McGlynn, P., Ashby, M. K., Burgess, J. G., & Olsen, J. D. (1991) *Mol. Microbiol. 5*, 2649–2661.

Jones, M. R., & Jackson, J. B. (1989) *Biochim. Biophys. Acta 975*, 34–43.

Jones, M. R., Richardson, D. J., McEwan, A. G., Ferguson, S. J., & Jackson, J. B. (1990) *Biochim. Biophys. Acta 1017*, 209–216.

Jones, M. R., Fowler, G. J. S., Gibson, L. C. D., Grief, G. G., Olsen, J. D., Crielaard, W., & Hunter, C. N. (1992) *Mol. Microbiol.* (in press).

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988) *Gene 70*, 191–197.

Kiley, P. J., Varga, A., & Kaplan, S. (1988) *J. Bacteriol. 170*, 1103–1115.

Kirmaier, C., Gaul, D., DeBey, R., Holten, D., & Schenck, C. C. (1991) *Science 251*, 922–927.

Kramer, H. J. M., van Grondelle, R., Hunter, C. N., Westerhuis, W. H. J., & Amesz, J. (1984) *Biochim. Biophys. Acta 765*, 156–165.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Lommen, M. A. J., & Takemoto, J. (1978) *Arch. Microbiol. 118*, 305–308.

Mattoli, T. A., Gray, K. A., Lutz, M., Oesterhelt, D., & Robert, B. (1991) *Biochemistry 30*, 1715–1722.

McEwan, A. G., Cotton, N. P. J., Ferguson, S. J., & Jackson, J. B. (1985) *Biochim. Biophys. Acta 810*, 140–147.

Meinhardt, S. W., & Crofts, A. R. (1982) *FEBS Lett. 149*, 223–227.

Niederman, R. A., Mallon, D. E., & Parks, L. C. (1979) *Biochim. Biophys. Acta 555*, 210–220.

Paddock, M. L., McPherson, P. H., Feher, G., & Okamura, M. Y. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6803–6807.

Rafferty, C. N., & Clayton, R. K. (1978) *Biochim. Biophys. Acta 502*, 51–60.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular cloning—a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sauer, K., & Austin, L. A. (1978) *Biochemistry 17*, 2011–2019.

Shuvalov, V. A., Shkuropatov, A. Y., Kulakova, S. M., Ismailov, M. A., & Shkuropatova, V. A. (1986) *Biochim. Biophys. Acta 849*, 337–346.

Simon, R., Priefer, U., & Puhler, A. (1983) *Biotechnology 1*, 784–791.

Stiehle, H., Cortez, N., Klug, G., & Drews, G. (1990) *J. Bacteriol. 172*, 7131–7137.

Takahashi, E., & Wraight, C. A. (1990) *Biochim. Biphys. Acta 1020*, 107–111.

Tichy, H.-V., Albien, K.-U., Gad'on, N., & Drews, G. (1991) *EMBO J. 10*, 2949–2955.

van Amerongen, H., Vasmel, H., & van Grondelle, R. (1988) *Biophys. J. 39*, 65–76.

van Dorssen, R. J., Hunter, C. N., van Grondelle, R., Korenhof, A. H., & Amesz, J. (1988) *Biochim. Biophys. Acta 932*, 179–188.

Wood, P. M. (1980) *Biochem. J. 189*, 385–391.

Youvan, D. C., Ismail, S., & Bylina, E. J. (1985) *Gene 38*, 19–30.