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The electron transfer chain of aerobically grown *Rhodopseudomonas viridis*

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Chemotrophically grown *Rhodopseudomonas viridis* contains little bacteriochlorophyll *b* and no detectable reaction center. Membranes isolated from chemotrophically grown *Rps. viridis* appear to contain cytochrome *d* and cytochrome *o* terminal oxidases. The membranes also appear to contain a cytochrome bc_1 complex, since cytochrome c_1 ($M_r = 31\,000$, detected by immunoblotting) and *b*-cytochromes are present. Chemotrophically grown *Rps. viridis* contains neither the membrane-bound, reaction center-associated cytochrome *c*-552/*c*-558 peptide nor any soluble cytochrome c_2. A previously undetected soluble *b*-type cytochrome, apparently absent in phototrophically grown *Rps. viridis*, was found in chemotrophically grown cells. Preliminary measurements indicate that cytochrome *b*-560 is probably a dimer of about 20 kDa subunits and has an oxidation–reduction midpoint potential of −20 mV.

Introduction

Light-dependent electron transfer reactions of phototrophically grown cells of the bacteriochlorophyll-*b*- (BChl-*b*)-containing, purple non-sulfur bacterium *Rhodopseudomonas viridis* have been widely studied. In particular, the recent elucidation of the detailed three-dimensional structure of the *Rps. viridis* reaction center [1–4] has stimulated kinetic studies of the early, light-driven electron transfer reactions in this bacterium [5–8]. The role of the soluble *Rps. viridis* cytochrome c_2 as the electron donor to the reaction center-associated cytochrome *c*-558 in phototrophically grown cells has been demonstrated [9]. Evidence has also been obtained for the presence of a cytochrome bc_1 complex in the membranes of phototrophically grown *Rps. viridis* [10,11], suggesting that the pattern of light-driven secondary electron transport reactions in *Rps. viridis* may be similar to that found in bacteriochlorophyll-*a*-containing, purple non-sulfur bacteria such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* [12]. In contrast to this substantial body of information available about photosynthetic electron transport in *Rps. viridis*, very little is known about respiratory electron flow in chemotrophically grown *Rps. viridis* cells, despite the fact that it has been known for some time that this bacterium is capable of chemotrophic growth in the dark in the presence of oxygen [13]. In this report we present evidence for both similarities and differences between the electron transfer components of the respiratory chain in chemotrophi-

Abbreviations: BChl, bacteriochlorophyll; EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate.

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cally grown *Rps. viridis* and those of the cyclic electron transfer system present in *Rps. viridis,* grown phototrophically under anaerobic conditions.

Materials and Methods

The original culture of *Rps. viridis* used in these studies came from the culture collection of Professor Norbert Pfenning (University of Konstanz, F.R.G.). Cells were grown at pH 6.8–7.0 on a malate-containing medium which was supplemented with 0.05% yeast extract and vitamins [14]. Phototrophic growth was carried out essentially as described previously [10]. For chemotrophic growth, 3 liter batch cultures started with a 5% (v/v) inoculum of phototrophically grown cells were incubated in the dark at 25 °C in 4 liter glass bottles and stirred gently. Semiaerobic growth conditions were provided by gassing the cultures with air at increasing flow rates as the cell density increased to a maximum ($A_{650}^{1cm} = 0.81$). Exponentially growing cells ($t_d = 16–20$ h) were transferred when A_{650}^{1cm} reached 0.6–0.8, using a 10% (v/v) inoculum. After the bacteriochlorophyll content of the cells reached a minimum, typically after three such transfers, the cells were harvested by centrifugation, washed once with 10 mM Tris buffer (pH 8.0) and stored at −20 °C.

Cells grown under either semiaerobic conditions in darkness or under anaerobic conditions in the light were broken by sonication in 10 mM potassium phosphate buffer (pH 7.6), containing 0.1 mM PMSF. Unbroken cells and cell debris were removed by centrifugation for 10 min at 30 000 × *g*. EDTA and potassium ferricyanide were added to the supernatant to yield final concentrations of 2 mM and 0.1 mM, respectively, after which the sample was centrifuged for 4 h at 255 000 × *g* in a Beckman Type 60 Ti ultracentrifuge rotor. The supernatant, after dialysis against 2 mM Mes buffer (pH 7.3), was utilized as the source of soluble electron transfer proteins. *Rps. viridis* cytochrome c_2 was purified from the supernatant of phototrophically grown cells according to [15]. The sedimented membranes were washed once in 10 mM potassium phosphate buffer (pH 7.6) containing 2 mM EDTA but no ferricyanide, prior to determination of protein, BChl *b*, cytochrome and iron-sulfur protein content.

Absorbance spectra were obtained using an Aminco DW-2a or a Perkin-Elmer Lambda 5 spectrophotometer. EPR spectra were recorded using a spectrometer system described previously [16]. BChl *b* was determined after extraction into 7 : 2 (v/v) acetone/methanol [17]. Protoheme and heme *c* were determined as described by Morrison and Stotz [18]. Oxidation-reduction titrations were carried out in a thin-layer electrochemical cell as described previously [19]. Protein was determined either according to a modification [20] of the method of Lowry et al. [21] or with the bicinchoninic acid method [22], using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis was carried out in the presence of 0.1% (w/v) SDS after solubilization with 4% (w/v) SDS on 1.5 mm thick 10–20% gradient slab gels [23]. Gels were stained for protein with Coomassie brilliant blue or for heme with 3,3′,5,5′-tetramethylbenzidine plus H_2O_2 [24]. Western blots, using an antibody against *Rb. sphaeroides* cytochrome c_1 (generously supplied by Professor Chang-An Yu), were carried out as described in Ref. 25 except that 2% (w/v) powdered milk dissolved in 10 mM Tris-HCl buffer (pH 7.5)/150 mM NaCl, and 0.1% (v/v) Tween-20 replaced 1% bovine serum albumin as the blocking reagent used followed the protein transfer to nitrocellulose. Molecular weight standards were obtained from Bio-Rad laboratories. An affinity-purified antibody against cytochrome c_2 from *Rb. sphaeroides* was a generous gift from Professor Samuel Kaplan.

Results

Fig. 1 shows the absorbance spectrum of membranes isolated from chemotrophically grown *Rps. viridis*. Although the membranes contain some carotenoid, they contain very little BChl *b* (see Table I) and the spectrum is dominated by the absorbance contributions of the membrane-bound cytochromes. If inocula of chemotrophically grown cells were transferred and grown under phototrophic conditions (i.e., anaerobically in the light) for three generations, BChl *b* was detected in amounts comparable to those found in cells grown continuously under phototrophic conditions and growth occurred at a rate ($t_d = 5–6$ h) typical of

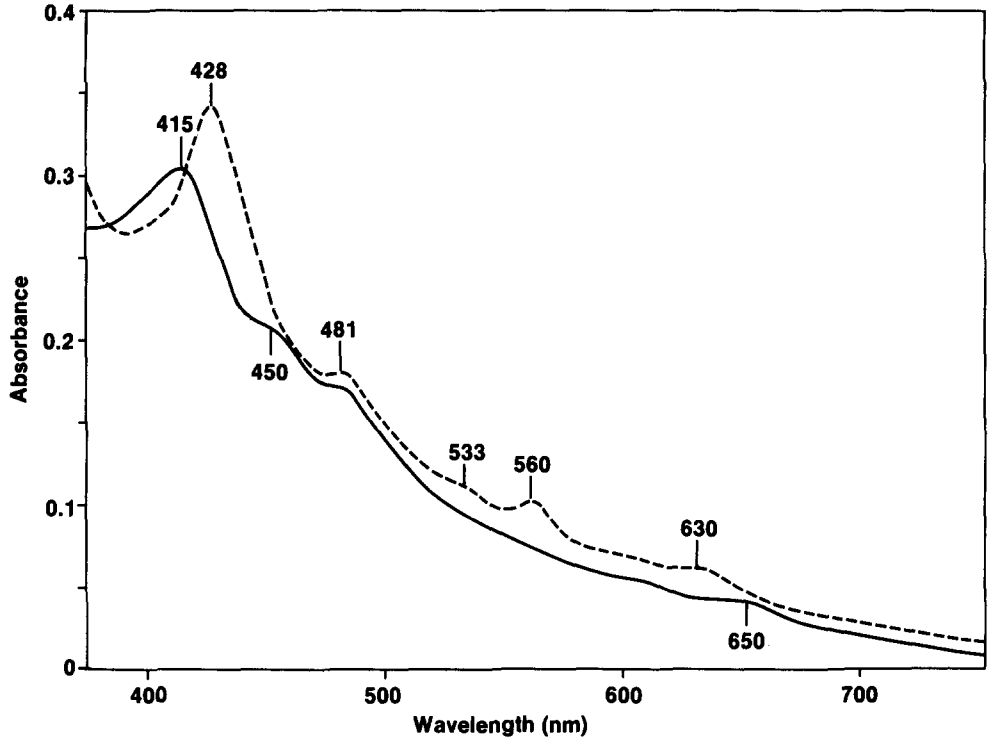

Fig. 1. Absorbance spectra of membranes from chemotrophically grown *Rps. viridis*. The spectrum of the membranes, isolated as described in Materials and Methods, was determined in a 1 cm optical pathlength cuvette at a spectral resolution of 1 nm (———). Small solid aliquots of sodium dithionite were then added until no further absorbance changes were detected and the spectrum was re-recorded (— — —).

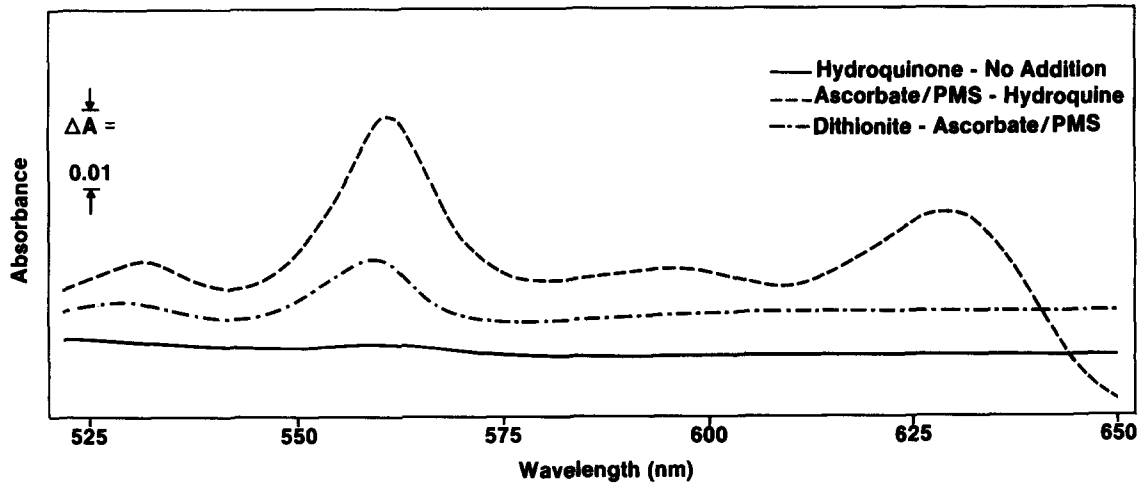

Fig. 2. Cytochrome difference spectra of membranes from chemotrophically grown *Rps. viridis*. Membranes, isolated as described in Materials and Methods, were placed in both sample and reference cuvettes to establish a baseline. Increasingly more electronegative reductants were then added sequentially as indicated, to obtain the difference spectra. Hydroquinone, sodium ascorbate and sodium dithionite were added as small solid aliquots until no further absorbance changes were detected. PMS was added from an aqueous stock solution to yield a final concentration of 1 μM. Optical pathlength, 1 cm; spectral resolution, 1 nm.

phototrophic growth. Adding sodium dithionite as a reductant to membranes of chemotrophically grown cells causes a series of spectral changes consistent with the reduction of *b*-type cytochromes and also appears to cause the absorbance band at 650 nm to shift to 630 nm. The cytochrome components can be more readily observed in reduced minus oxidized difference spectra. Fig. 2 shows that only a barely detectable absorbance change can be observed over the cytochrome α- and β-band region in a hydroquinone-reduced minus ferricyanide-oxidized difference spectrum of membranes isolated from chemotrophically grown *Rps. viridis*. Such a difference spectrum would be expected to reveal only relatively high potential components ($E_m \geqslant +230$ mV) because hydroquinone is a weak reductant ($E'_m = +290$ mV). The difference spectrum of Fig. 2 differs greatly from that obtained with membranes isolated from phototrophically grown *Rps. viridis*, which shows a prominent peak at 558 nm due to the reaction center-associated, high potential ($E_m = +300$ mV) cytochrome *c*-558 [10]. This observation, and others to be discussed below, suggest that cytochrome *c*-558 is absent in chemotrophically grown *Rps. viridis*.

An (ascorbate + PMS)-reduced minus hydroquinone-reduced difference spectrum (Fig. 2), which would be expected to reveal components with $E'_m \geqslant +70$ mV but $\leqslant -230$ mV [10], shows an absorbance maximum at 561–562 nm, most probably due to the presence of a *b*-type cytochrome. The asymmetry (detectable in expanded spectra) of this α-band – a shoulder is present at about 565 nm – suggests the possibility of multiple *b* cytochromes being present in the membranes. The ascorbate/PMS minus hydroquinone difference spectrum also exhibits a prominent feature at 630 nm that was not seen in similar difference spectra of membranes isolated from phototrophically grown cells [10]. This component can also be observed in the absolute spectra of Fig. 1. The identity of the component(s) absorbing at 630 nm in the reduced form and 650 nm in the oxidized form has not been definitely established, but the spectral data are consistent with the presence of a cytochrome *d* terminal oxidase in the membranes of chemotrophically grown *Rps. viridis* [26].

The dithionite-reduced minus ascorbate/PMS-reduced difference spectrum of membranes

TABLE I

BCHl AND HEME CONTENT OF *RPS. VIRIDIS* MEMBRANES

Protein concentrations were determined using the bicinchonic acid method.

Membrane source	Chromophore content (nmol/mg protein)		
	BChl *b* ($\times 10^{-3}$)	heme *c*	protoheme
Phototrophically grown cells	250	300	35
Chemotrophically grown cells	15	45	350

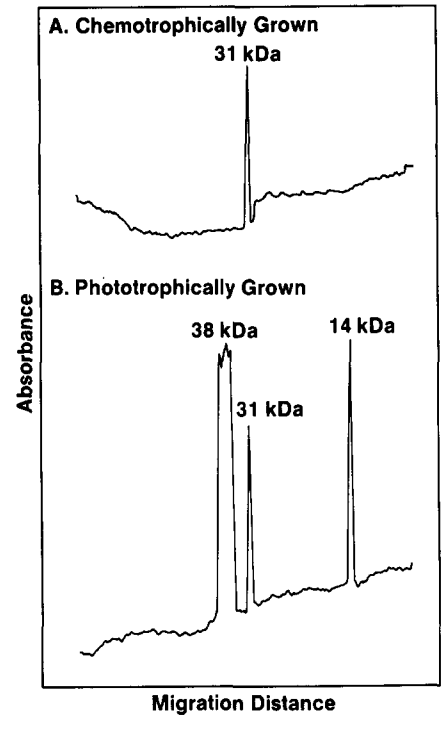

Fig. 3. Heme *c*-containing, membrane-bound peptides of *Rps. viridis*. Membranes isolated from phototrophically grown (A, 0.15 mg protein) or chemotrophically grown (B, 0.15 mg protein) were solubilized and, after polyacrylamide gel electrophoresis, stained from heme as described in Materials and Methods. The resulting gels were scanned at 580 nm with a densitometer. Molecular weights were calculated from a gel lane loaded with molecular weight standards and stained for protein as described in Materials and Methods.

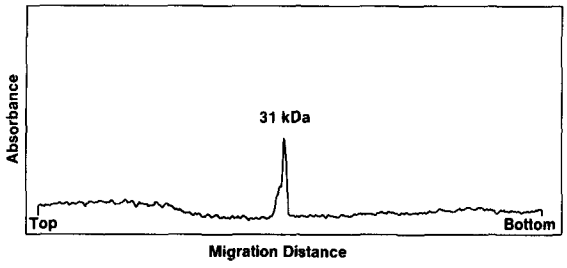

Fig. 4. Immunoblotting of membranes from chemotrophically grown *Rps. viridis* for cytochrome c_1. Membranes (0.1 mg protein) were subjected to polyacrylamide gel electrophoresis in the presence of SDS and the separated peptides were transferred to nitrocellulose as described in Materials and Methods and treated with an antibody against *Rb. sphaeroides* cytochrome c_1. After visualization using horseradish peroxidase-linked goat anti-rabbit IgG, the immunoblot was scanned at 580 nm with a densitometer. The molecular weight was determined from a lane of the same gel containing molecular weight standards and stained for protein.

isolated from chemotrophically grown *Rps. viridis* (Fig. 2), which would be expected to reveal low potential ($E'_m < +70$ mV) components, has a peak at 559 nm. This difference spectrum, which suggests the presence of a low-potential *b*-type cytochrome, is quite different from that observed with membranes isolated from phototrophically grown *Rps. viridis* [10] which is dominated by a peak at 552 nm due to the presence of the reaction center-associated, low-potential ($E'_m = 0$) cytochrome *c*-552. This observation suggests that cytochrome *c*-552 (like cytochrome *c*-558) is absent in chemotrophically grown *Rps. viridis*. In fact, none of the difference spectra of Fig. 2 provides unambiguous evidence for the presence of *c*-type cytochromes in these membranes. However, as shown in Table I, chemical analysis for heme *c* does indicate the presence of cytochrome *c* in these membranes. The difficulty of observing any *c*-type cytochromes in the difference spectra arises from the high ratio (7.8 : 1) of cytochrome *b* (protoheme prosthetic group) to cytochrome *c* in these membranes. No heme *a* was detected in these heme analyses, suggesting the absence of a cytochrome aa_3-type terminal oxidase.

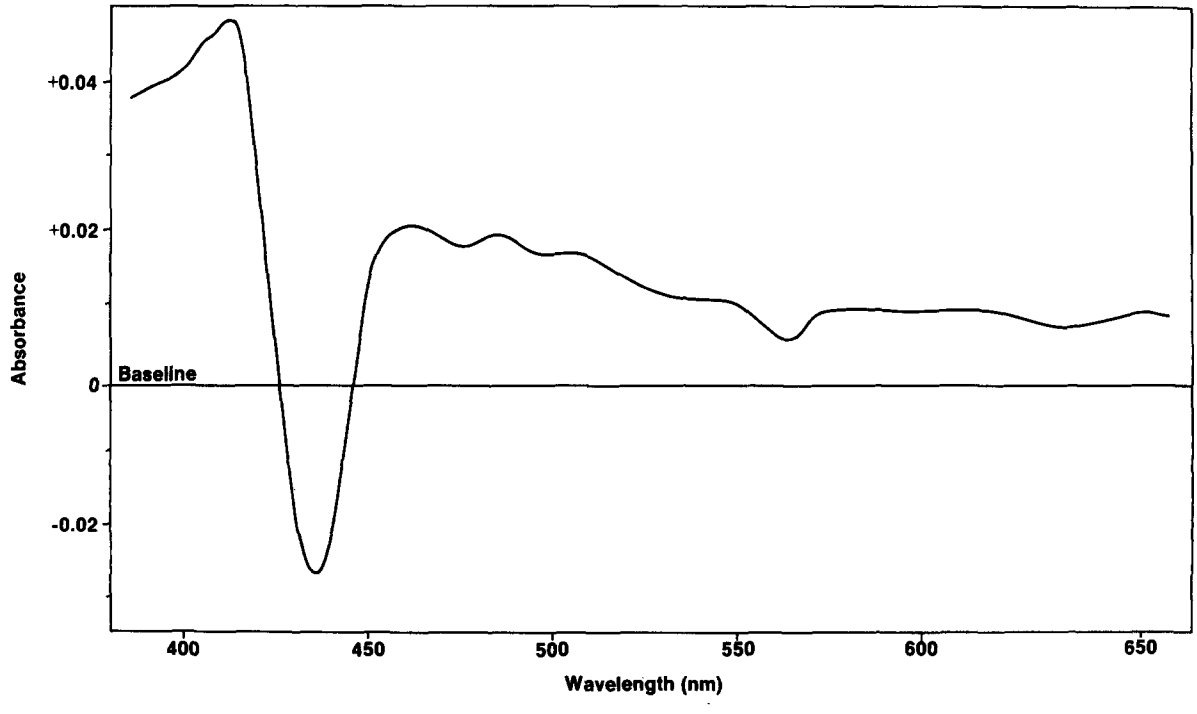

Fig. 5. CO-difference spectrum of membranes from chemotrophically grown *Rps. viridis*. The sample and reference cuvettes, containing identical membrane suspensions, were reduced with sodium dithionite under an N_2 atmosphere and a baseline was recorded. The sample cuvette was then exposed to CO in the dark for 2 min and the spectrum was recorded. The spectrum shown was corrected by subtracting the initial baseline. Spectral resolution = 1 nm.

Further evidence for the presence of *c*-type cytochrome in the membranes of chemotrophically grown *Rps. viridis* was obtained by staining polyacrylamide gels of the membranes, after electrophoresis in the presence of SDS, for heme *c* [18]. Fig 3A shows a densitometer trace of such a gel of membranes isolated from chemotrophically grown cells, with results obtained from phototrophically grown cells presented for comparison (Fig. 3B). The preparation from chemotrophically grown cells shows a single heme *c*-containing component of 31 kDa, while that from phototrophically grown cells contains components of 38 and 14 kDa in addition to that at 31 kDa. The two components present in phototrophically grown but not in chemotrophically grown cells can be assigned to the reaction-center-associated cytochrome *c*-558/*c*-552 peptide [1,2,4,10,27] and soluble cytochrome c_2 [15], respectively. We assume that some soluble cytochrome c_2 is associated with the membranes or is trapped in membrane vesicles formed during cell breakage and can thus be detected in the membrane fraction. Electrophoresis of isolated purified cytochrome c_2 from *Rps. viridis* under conditions identical to those of Fig. 3 gave a single heme staining component with an apparent M_r of 14000, the same value obtained for the low-molecular-weight component found in electrophoresis of membranes isolated from phototrophically grown cells (data not shown). We had hoped to confirm the identity of the 14 kDa component as cytochrome c_2 using immunological techniques, but no antibody against *Rps. viridis* cytochrome c_2 was available and, in dot-blot assays, an antibody against *Rb. sphaeroides* cytochrome c_2 failed to cross-react with isolated, purified *Rps. viridis* cytochrome c_2.

We had earlier suggested that a 31 kDa, heme-*c*-containing peptide detected in the membranes of phototrophically grown *Rps. viridis* was likely to by cytochrome c_1 [10,11]. In Western blot analyses, a 31 kDa component in the membranes of chemotrophically grown cells was observed to cross-react with an antibody against *Rb. sphaeroides* cytochrome c_1 (Fig. 4). Similar results were obtained with membranes isolated from phototrophically grown cells (data not shown). We have thus positively identified cytochrome c_1 as a component of the *Rps. viridis* electron transport system and shown it to be present during growth under both phototrophic and chemotrophic conditions.

Observations described above suggested the likely presence of a cytochrome *d* terminal oxidase in chemotrophically grown *Rps. viridis*. The possible presence of additional oxidase components was examined using CO-difference spectra. Fig. 5 shows such a spectrum (dithionite-reduced, CO-equilibrated minus dithionite-reduced) for membranes isolated from chemotrophically grown *Rps. viridis*. Maxima are present at 413, 461, 485, 507 and 542 nm and minima at 435 and 563 nm. Although more than one CO-binding component is likely to be present, the difference spectrum of Fig. 5 is consistent with the presence of a protoheme-containing, cytochrome *o* oxidase (difference spectrum maxima and minima at 413 and 435 nm, respectively [26,28]).

The observations reported above that indicated the absence of the reaction-center-associated cytochrome *c*-552/*c*-558 peptide in chemotrophically grown *Rps. viridis* prompted us to investigate whether any reaction center components are present in the membranes isolated from such cells. Fig. 6A shows a densitometer scan of membranes from phototrophically grown *Rps. viridis* after gel electrophoresis in the presence of SDS and subsequent staining for protein with Coomassie brilliant blue. Bands are observed at 38, 35, 28 and 24 kDa, the positions expected for the cytochrome *c*-552/*c*-558 peptide and the so-called H, M and L subunits of the reaction center [27]. A densitometer scan of a Coomassie-stained gel, run under identical conditions, of membranes isolated from chemotrophically grown *Rps. viridis* (Fig. 6B) showed no detectable 38 kDa band, confirming the absence of the cytochrome *c*-552/*c*-558 peptide, diminished amounts of the 35 and 28 kDa peptides and little detectable 24 kDa peptide. These results suggest the possibility that the entire reaction center is not synthesized in chemotrophically grown *Rps. viridis*. Further evidence to support this conclusion came from the absence of any observable EPR signal at $g = 1.82$ (characteristic of the reduced reaction center primary quinone-Fe^{2+} complex [29]) when dithionite-reduced membranes isolated from chemotrophically grown cells were examined at 8 K (data not shown).

It was mentioned above that membrane pre-

parations from chemotrophically grown *Rps. viridis* cells showed no detectable 14 kDa, heme-*c*-containing peptide, while membrane-containing fractions from phototrophically grown cells showed considerable amounts of this component (Fig. 3), likely to be cytochrome c_2. This surprising observation caused us to examine the soluble fractions of both chemotrophically and phototrophically grown cells for cytochrome c_2. Soluble extracts of phototrophically grown cells contained cytochrome c_2, with reduced and oxidized spectra and chromatographic behavior identical to those reported in the literature [30]. However, no cytochrome c_2 could be detected in the soluble extract from chemotrophically grown cells. Not only was no cytochrome c_2 detected in the crude soluble extract of chemotrophically grown cells, but no heme-containing components could be detected, even after concentration of the crude extract on a CM-Sephadex column (Fig. 7). In contrast, all of

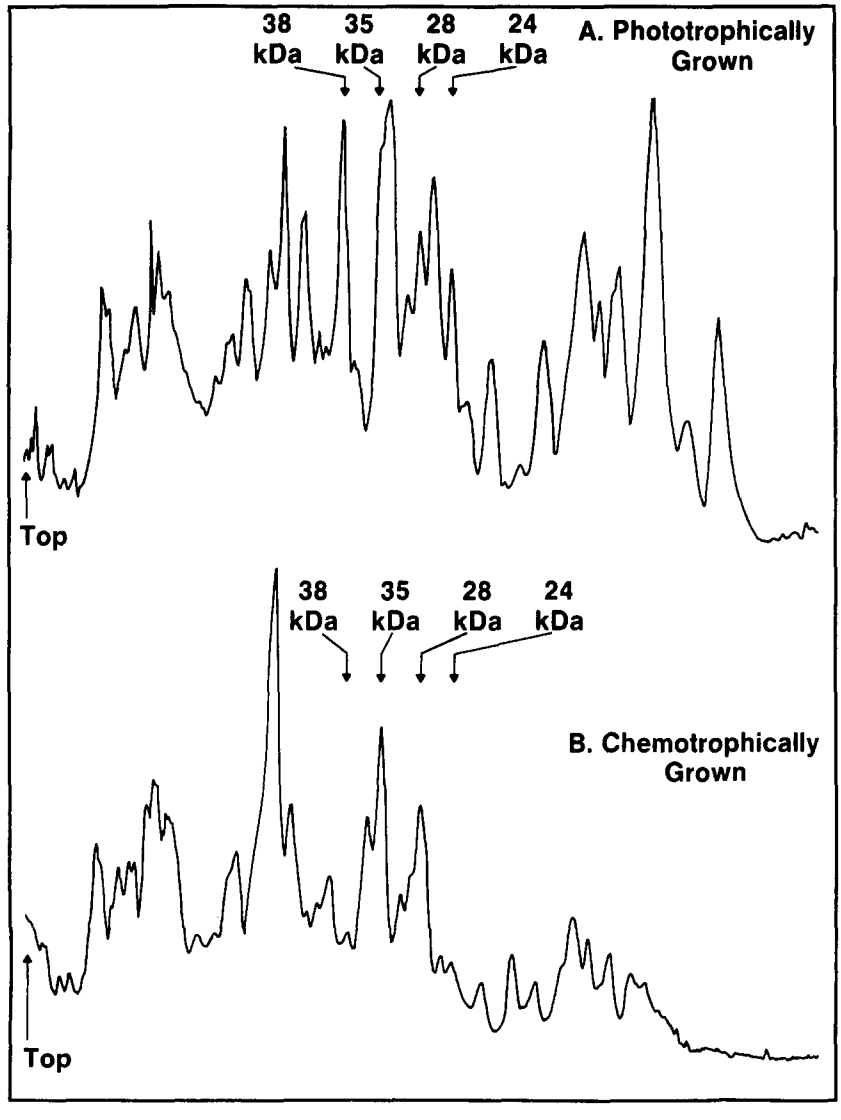

Fig. 6. Membrane-bound peptides of *Rps. viridis*. Membranes isolated from either phototrophically (A, 0.038 mg protein) or chemotrophically (B, 0.03 mg protein) *Rps. viridis* were subjected to polyacrylamide gel electrophoresis in the presence of SDS. After staining for protein, the gels were scanned with a densitometer at 580 nm.

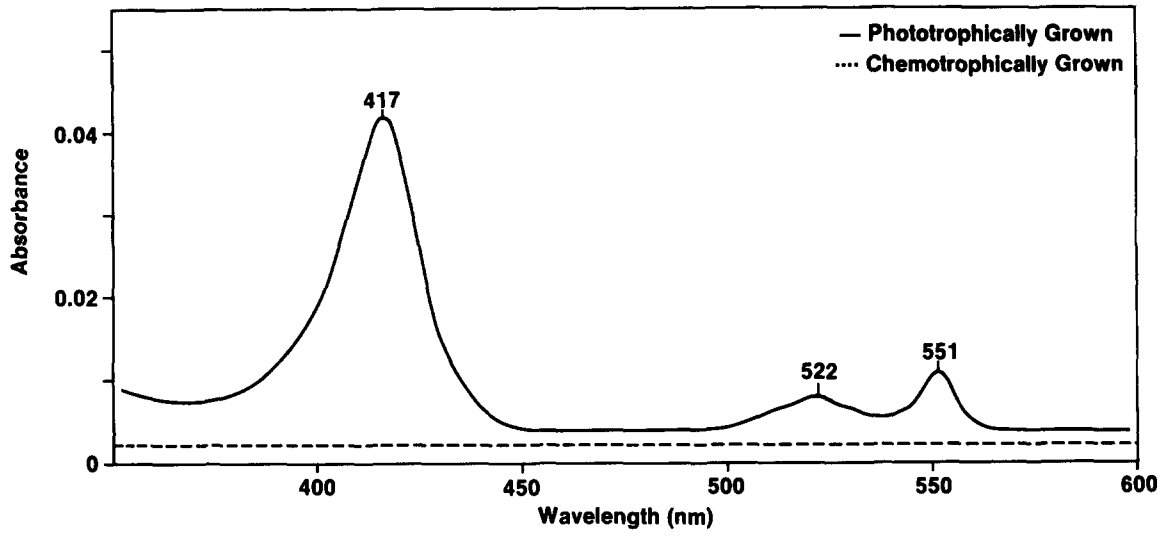

Fig. 7. The absence of cytochrome c_2 in chemotrophically grown *Rps. viridis*. The supernatants arising from sonication of equal wet weight samples of either chemotrophically or phototrophically grown cells were passed over a CM-Sephadex column and eluted with 10 mM Mes buffer (pH 7.0)/100 mM NaCl. Cytochrome-containing fractions from phototrophically grown cells were pooled and the spectrum taken after reduction with ascorbate plus PMS (———). A spectrum of the same fractions from chemotrophically grown cells (— — —) revealed no detectable cytochrome.

the cytochrome c_2 in the soluble extract of photosynthetically grown *Rps. viridis* bound to a CM-Sephadex column.

Although no cytochrome c_2 could be detected in chemotrophically grown *Rps. viridis*, a soluble *b*-type cytochrome not previously observed in phototrophically grown cells was found. Fig. 8 shows the spectra of both the oxidized and reduced forms of a partially purified preparation of this cytochrome. Heme analysis indicated the

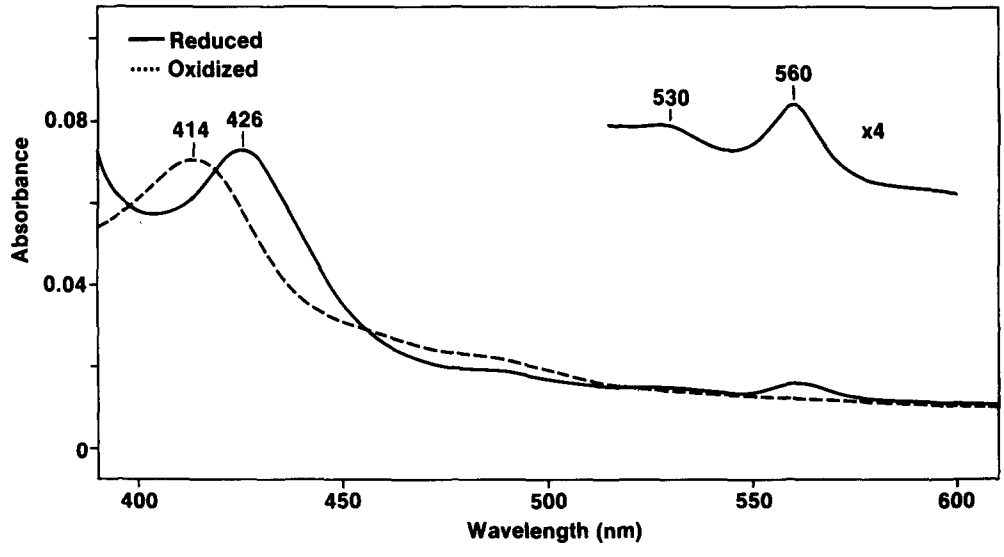

Fig. 8. Absorbance spectrum of *Rps. viridis* cytochrome *b*-560. The spectrum of the protein in 10 mM Mes buffer (pH 6.5) was recorded in the oxidized form (— — —) and after reduction with sodium dithionite (———). Spectral resolution, 1 nm.

presence of protoheme and the absence of heme *c* in the newly discovered cytochrome. The heme analysis results and the spectral characteristics of the reduced cytochrome (Soret band maximum at 426 nm and α-band maximum at 560 nm) are both consistent with the designation of the protein as a *b*-type cytochrome. We have not yet purified the protein to homogenity, but we have reached the stage where only a single heme-containing component is detected during gel filtration chromatography on Sephadex G-75 or during ion-exchange chromatography on DEAE-cellulose. Preliminary molecular weight estimation by gel-filtration chromatography under non-denaturing conditions indicated $M_r = 40\,000 \pm 2000$ for cytochrome *b*-560. The most prominent Coomassie blue-staining band detected after gel electrophoresis of the cytochrome in the presence of SDS had $M_r = 21\,000 \pm 2000$, suggesting the possibility that cytochrome *b*-560 may be dimeric. An oxidation-reduction titration of partially purified cytochrome *b*-560 yielded a E_m value of -20 ± 10 mV at pH 6.0.

Discussion

In *Rps. viridis*, as in other purple phototrophic bacteria [31], it appears that light and/or O_2 regulate the synthesis of components of the bacterium's light-harvesting antenna complexes and of its photosynthetic electron transport chain. Membranes isolated from chemotrophically grown *Rps. viridis* contain only minimal amounts of BChl *b* and no detectable reaction-center components. Consistent with the cells' use of O_2 as a terminal electron acceptor, membranes isolated from chemotrophically grown cells have at least two putative terminal oxidases. Spectra data suggest the presence of a cytochrome *d* oxidase and CO-difference spectra indicate the likely presence of a protoheme-containing, cytochrome *o* oxidase. Chemotrophically grown cells also contain a soluble *b*-type cytochrome (α-band maximum = 560 nm; M_r = 41 000; $E_m = -20$ mV), of unknown function, that was not detected in phototrophically grown cells.

Immunoblotting data have established the presence, in both chemotrophically and phototrophically grown *Rps. viridis*, of one component of the cytochrome bc_1 complex, cytochrome c_1. The absence of an α-band at 552–553 nm, expected for cytochrome c_1 [10,11,32], in the hydroquinone-reduced minus no addition difference spectrum of membranes isolated from chemotrophically grown cells (Fig. 2) probably results from the low concentration of the cytochrome (Table I) and the possible presence some residual ferricyanide in the membrane fraction (see Materials and Methods). The latter would prevent hydroquinone addition from lowering E_h sufficiently to reduce the cytochrome fully. Difference spectra indicate the likely presence of several *b*-type cytochromes in chemotrophically grown *Rps. viridis*. Although one of these may be a cytochrome *o* oxidase, the difference spectra are consistent with the two *b* cytochromes characteristic of cytochrome bc_1 complexes [32] also being present in these membranes. It thus appears likely that chemotrophically grown, like phototrophically grown [10,11], *Rps. viridis*, contains a membrane-bound cytochrome bc_1 complex. We have not yet been able to detect unambiguously an EPR signal characteristic of the third component of cytochrome bc_1 complexes – the Rieske iron-sulfur protein [32] – in membranes isolated from chemotrophically grown cells because of overlapping EPR signals from several iron-sulfur clusters present in these membranes.

The most surprising result of these investigations was the apparent absence of cytochrome c_2 in chemotrophically grown *Rps. viridis*. If the role of cytochrome c_2 were confined to serving as the electron donor to the reaction-center-associated cytochrome *c*-558 [9], its absence in chemotrophically grown cells lacking this reaction-center-associated cytochrome could be readily understood. However, in all photosynthetic purple non-sulfur bacteria studied to date, cytochrome c_2 is thought to serve as the electron acceptor for the cytochrome bc_1 complex [32]. As the cytochrome bc_1 complex appears to be present in both phototrophically and chemotrophically grown *Rps. viridis*, it might have been expected that cytochrome c_2 would be present under both conditions of growth. It should be pointed out that cytochrome c_2 was, until recently, considered essential for the growth of *Rhodobacter capsulatus* (formerly *Rhodopseudonomas capsulata*) under both phototrophic and chemotrophic growth con-

ditions [33]. In this BChl-*a*-containing, photosynthetic purple non-sulfur bacterium, cytochrome c_2 was thought to transfer electrons from the cytochrome bc_1 complex to either the reaction center (during photosynthesis) or to the terminal oxidase (during respiration). Recently, however, mutants of *Rb. capsulatus* lacking cytochrome c_2 were constructed and shown to be capable of both phototrophic and chemotrophic growth [33]. It was suggested that alternative electron transfer pathways, not involving cytochrome c_2, were present in *Rb. capsulatus*, perhaps involving a direct interaction between the cytochrome bc_1 complex and other membrane-bound electron transfer complexes [34]. A similar situation may occur in chemotrophically grown *Rps. viridis* or, alternately, a soluble electron carrier that functionally replaces cytochrome c_2 may be synthesized under aerobic conditions. It is highly unlikely that this role is filled by cytochrome *b*-560, since the E_m value of this soluble protein (-20 mV at pH 6.0) is so different from that of cytochrome c_2 ($E'_m = +295$, Ref. 30). Experiments to identify the electron acceptor for the cytochrome bc_1 complex in chemotrophically grown *Rps. viridis* are continuing in our laboratory.

Acknowledgements

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