Biochimica et Biophysica Acta 891 (1987) 216–226
Elsevier

BBA 42520

Electron-transport chains of phototrophically and chemotrophically grown *Chloroflexus aurantiacus*

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(Received 2 October 1986)
(Revised manuscript received 23 December 1986)

Key words: Electron-transport chain; Cytochrome *c*; Cytochrome *b*; CO binding; Chemotrophic bacterium; (*C. aurantiacus*)

The membrane-bound electron-transfer chain components of both phototrophically and chemotrophically grown *Chloroflexus aurantiacus* have been characterized. Membranes isolated from chemotrophically grown *Chloroflexus* have been shown to contain at least three *c*-type cytochromes and at least three *b*-type cytochromes. In addition, these cells appear to lack a photochemical reaction center and the high potential (E_m = +260 mV) cytochrome *c*-554 that serves as the immediate donor to the reaction center in phototrophically grown *Chloroflexus*. Phototrophically grown cells contain a CO-binding *c*-type cytochrome, apparently absent in the chemotrophically grown cells. However, a different CO-binding component, which may function as the terminal oxidase, is present in chemotrophically grown cells.

Introduction

Chloroflexus aurantiacus, a thermophilic, photosynthetic gliding green bacterium, is capable of both phototrophic and chemotrophic growth [1]. Cells grown under phototrophic conditions contain bacteriochlorophyll *a* (BChl *a*), which is associated with the cytoplasmic membrane, plus BChl *c*, which is located in chlorosomes attached to the cytoplasmic side of the cell membrane [2,3]. It is now accepted that chlorosomes are antenna structures, serving to absorb and transfer light energy to the reaction center located in the cytoplasmic membrane [3]. During chemotrophic growth the number of chlorosomes declines and bacteriochlorophyll synthesis is repressed [1]. The induction and assembly of the pigment-protein polypeptides in phototrophically grown cells has recently been investigated and shown to be controlled by the oxygen tension [4]. The cytochrome composition of *Chloroflexus* has also been shown to vary with growth conditions [4–8], as had previously been demonstrated for purple non-sulfur bacteria [9]. The most dramatic change in heme content was a striking increase in the protoheme:

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Abbreviations: BChl, bacteriochlorophyll; Mops, 4-morpholinepropanesulphonic acid; PMS, phenazine methosulfate.

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heme *c* ratio observed after a transition of *Chloroflexus* from phototrophic to chemotrophic growth [7,8]. Increased oxygen concentrations have been reported to induce also the synthesis of a membrane-bound heme *a*-containing terminal oxidase [6,7]. As the electron-transfer chain composition of *Chloroflexus* appears to depend critically on the O_2-concentration during growth [4], we have extended the previous studies and utilized a variety of techniques to compare the cytochrome and iron-sulfur protein contents of phototrophically grown cells to those of essentially bacteriochlorophyll-free cells grown in the dark under O_2-saturated conditions.

Materials and Methods

Chloroflexus J-10-fl was grown anaerobically in the light as described previously [1]. Fully dark-adapted cells were grown chemotrophically as described in Ref. 4. Dissolved oxygen concentrations under aerobic growth conditions in the dark were monitored with a Clark-type electrode. BChl *a* and *c* were determined spectrophotometrically, after extraction with 7 : 2 (v/v) acetone/methanol, at 772 nm and 666 nm, respectively [10]. Phototrophically and chemotrophically grown *Chloroflexus* were disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0), containing 200 mM NaCl [11]. After centrifugation at 250000 × *g* for 2 h, the sedimented membrane fragments were resuspended in 20 mM Tris-HCl buffer.

Protein concentration was determined according to the method of Bradford [12] using bovine serum albumin as a standard. Heme *c* and protoheme were determined after extraction of pigments and lipids, followed by extraction of the protoheme with acetone/HCl [13]. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate was performed on 12% slab gels (1.5 mm thickness) prepared according to Laemmli [14]. Gels were stained for protein with Coomassie Brilliant Blue and for heme with 3,3′,5,5′-tetramethylbenzidine plus hydrogen peroxide [15]. Protein molecular weight standards were obtained from Bio-Rad.

Chloroflexus cytochrome *c*-554 was solubilized and purified as previously described [16]. Cytochrome *c*-554 was coupled to a Bio-Rad Affigel 10 affinity matrix using 4.65 mg of the cytochrome and 1 ml of Affigel 10, following the procedure outlined by the manufacturer [17]. Antiserum to cytochrome *c*-554 was prepared by injecting rabbits with 100 μg of cytochrome *c*-554 that had been purified by gel electrophoresis. The serum was chromatographed on CM Affi-Gel Blue (Bio-Rad) and subsequently chromatographed on the cytochrome *c*-554 affinity column before being utilized to probe Western blots. Antibody against cytochrome c_1 from *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas sphaeroides*) was a gift of Professor Chang-An Yu.

Absorbance spectra at ambient temperature were obtained using Aminco DW-2a or Perkin-Elmer Lambda 5 spectrophotometers. (Reduced + CO) minus (reduced) difference spectra of phototrophically grown cells were obtained using an Aminco DW-2a spectrophotometer, while those of chemotrophically grown cells were recorded on a Cary 2300 spectrophotometer controlled by an Apple IIe computer. The latter spectra represent the computer-subtracted difference between the +CO and −CO spectra of the same reduced sample. Oxidation-reduction titrations of membrane-bound components were performed electrochemically [18] in a thin layer cuvette, as described previously [19]. All E_m and n values represent the average from three titrations and were determined using a nonlinear least squares program run on an Apple II + computer. At each ambient potential optical spectra were measured from 500 to 650 nm.

Absorbance spectra at liquid nitrogen temperature were obtained using a Varian/Cary Model 219 spectrophotometer and a home-built low-temperature accessory. Membranes were suspended in 25 mM Mops buffer (pH 7.0) containing 25 mM KCl and 50% glycerol under an Ar atmosphere and were adjusted to desired E_h values by additions of buffered solutions of sodium dithionite or potassium ferricyanide. The redox potential was measured using Radiometer P101 Pt and K401 saturated calomel electrodes along with a Corning Model 701 digital pH meter. Phenazine methosulfate, phenazine ethosulfate, *o*-naphthoquinone sulfonate and 2-hydroxynaphthoquinone, each at 10 μM, were present as redox mediators. When the desired E_h was obtained, an aliquot was re-

moved with a gas-tight syringe, injected into a 3 mm pathlength cuvette continuously flushed with Ar and immediately frozen in liquid nitrogen. The spectrum prior to illumination was recorded and stored on an Apple II computer and the sample was illuminated for 1 min with white light from a 100 W tungsten halogen lamp focused on the cuvette. The spectrum of the illuminated sample was recorded and stored. Light-induced difference spectra were then obtained by computer subtraction. As a control, photooxidation of cytochrome *c*-552 was monitored in membranes isolated from *Rhodopseudomonas viridis* (poised at E_h values between +25 and −30 mV) at 77 K [20].

Electron paramagnetic resonance (EPR) spectra were obtained using a Varian E-109 spectrometer equipped with an Air Products Helitrans flexible helium transfer line and a Varian/Hewlett-Packard E-935 data acquisition system [21]. Membranes were washed once in buffer containing 1 mM EDTA to eliminate signals from Mn-containing species prior to EPR analysis.

Results

The optical absorbance spectrum of membrane preparations from chemotrophically grown *Chloroflexus* exhibits a marked decrease in the contributions from BChl *a* and *c* when compared to that of phototrophically grown cells [4,11,22]. Spectra of the membranes revealed no features attributable to either residual BChl *a* (bands at 800, 595 and 373 nm) or BChl *c* (bands at 740 and 470 nm). However, small amounts of BChl *a* (0.003 nmol per mg of membrane protein) could be detected in acetone/methanol extracts of the membranes. No BChl *c* was detected in the acetone/methanol extract. In phototrophically grown cells the BChl *a* content ranged from 14 to 16 mmol/mg cell protein [10].

As a first approach to characterizing the cytochrome composition of these membranes, a series of increasingly stronger chemical reductants were added and spectra were recorded in the wavelength region characteristic of α and β bands for reduced cytochromes. The hydroquinone minus ferricyanide difference spectrum, shown in Fig. 1, indicates the presence of a hydroquinone-reducible cytochrome with α and β band maxima at 551

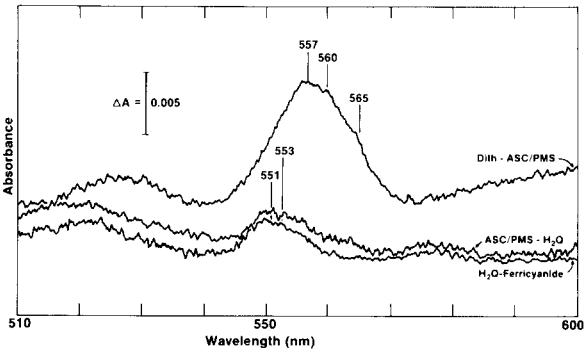

Fig. 1. Difference spectra of membrane-bound cytochromes from chemotrophically grown *Chloroflexus*. Membranes (50 μg protein/ml) were suspended in 20 mM Tris buffer (pH 7.0). Spectral resolution, 1 nm; optical pathlength, 1 cm, Hydroquinone (H_2Q), potassium ferricyanide, sodium ascorbate and sodium dithionite (Dith) were added as small solid aliquots until no further absorbance changes were detected. PMS was present at a final concentration of 5 μM.

nm and 523 nm, respectively. This hydroquinone minus ferricyanide difference spectrum differs significantly from that observed with membranes derived from phototrophically grown cells (data not shown), which shows a prominent alpha band maximum at 554–555 nm due to the presence of cytochrome *c*-554 ($E_m = +260$ mV; Ref. 8). The sodium ascorbate/PMS minus hydroquinone difference spectrum, which resembles the hydroquinone minus ferricyanide difference spectrum, is smaller in amplitude (Fig. 1). In membranes isolated from phototrophically grown cells, cytochrome *c*-554, which is only partially reduced by hydroquinone, becomes fully reduced upon addition of ascorbate/PMS (data not shown). Fig. 1 also shows the sodium dithionite minus ascorbate/PMS difference spectrum of the membranes. This difference spectrum, which is similar to that reported by Pierson [7], shows a broad absorbance band (centered between 557 and 561 nm), probably arising from the presence of multiple components. The presence of a maximum near 557 nm plus the asymmetry of the band at shorter wavelengths indicates the presence of *b*- and possibly *c*-type cytochrome components that are dithionite-reducible but not reduced by ascorbate/PMS. The dithionite minus ascorbate/PMS difference spectrum also appears to be somewhat asymmetric at wavelengths longer than the 557 nm

maxima, with a possible shoulder present at 565–566 nm. This spectrum differs considerably from that obtained with membranes isolated from phototrophically grown cells [7,8], which indicated no evidence for the presence of *b*-type cytochromes (however, see Ref. 5). No evidence for cytochromes *a* or *d* was detected in any of the difference spectra.

The results of a series of analyses of *Chloroflexus* membranes for protoheme, heme *a* and heme *c* indicate average heme *c* contents of 1.2 and 8.9 μmol per mg of membrane protein for chemotrophically and phototrophically grown cells, respectively (Table I). Analysis for protoheme showed an average protoheme content of 0.44 μmol per mg of membrane protein for chemotrophically grown cells, as compared to 0.31 μmol per mg of membrane protein for phototrophically grown cells. No heme *a* (less than 0.02 μmol/mg protein) was found in the phototrophically grown cells. Heme *a* (0.08 μmol/mg protein) was found in only one out of three samples of chemotrophically grown cells assayed, while the other two samples contained no detectable heme *a* (less than 0.02 μmol/mg protein) No heme *d* was detected in extracts from either phototrophically or chemotrophically grown cells.

The results of a typical electrochemical oxidation-reduction titration (carried out at pH 7.0) of membranes isolated from chemotrophically grown cells of *Chloroflexus* are shown in Fig. 2. These measurements were carried out at two wavelength pairs, 552–540 nm and 562–574 nm, in an attempt to maximize the contribution from either *c*- or

TABLE I

HEME CONTENT IN *CHLOROFLEXUS AURANTIACUS*

	(μmol heme/mg protein)	
	Phototrophically grown cells	Chemotrophically grown cells
Heme *c*	8.9	1.2
Protoheme	0.31	0.44
Heme *a*	< 0.02	⩽ 0.08 [a]
Heme *c*/protoheme	28	2.8

[a] All values are the average of three determinations, except that for heme *a* in chemotrophically grown cells. In this case, one sample contained 0.08 μg/mg protein, while the other two contained no detectable heme *a*.

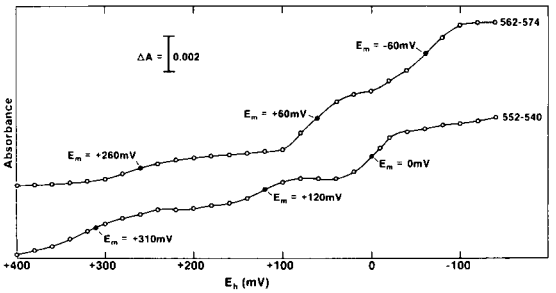

Fig. 2. Oxidation-reduction titrations of membranes from chemotrophically grown *Chloroflexus*. The titrations were conducted as described in Materials and Methods using a reaction mixture containing membranes (5 mg protein/ml) suspended in 20 mM Tris buffer (pH 7.0). The following oxidation-reduction mediators were also present: 20 μM ferricyanide; 50 μM hydroquinone; 50 μM diaminodurene; 50 μM 1,2-naphthoquinone; 100 μM duroquinone; 25 μM PMS; 25 μM phenazine ethosulfate and 25 μM anthroquinone disulfonate. The titration was conducted at 20 °C. Spectral resolution, 1 nm; optical pathlength, 0.3 mm.

b-type cytochromes, respectively. Three distinct features, corresponding to one-electron transfer components with E_m values of +310 mV (n = 0.85), +120 mV (n = 0.92) and 0 V (n = 0.93), were detected in redox titrations monitored at 552–540 nm (Fig. 2). No further absorbance changes at this wavelength pair were detected if the E_h value were increased from +400 to +450 mV or lowered from −150 to −200 mV (data not shown). Complete optical spectra, in the 500–600 nm region, of each of these components showed absorbance maxima at 551–552 nm for each component (data not shown), similar to features observed in the chemical difference spectra of Fig. 1. As the absorbance maxima of *c*-type cytochromes are in this wavelength region, these features are likely to indicate the presence of at least three membrane-bound *c*-type cytochromes. Based on the magnitudes of the absorbance increases associated with the reduction of the +310 and 0 V components, it would appear that these two components are in (2–3)-fold excess compared to the +120 mV component, assuming similar molar extinction coefficients for each component. In addition to these *c*-type cytochromes, Fig. 2 also demonstrates the presence of three components with E_m = +260 mV (n = 0.92), E_m = +60 mV

($n = 0.89$) and $E_m = -60$ mV ($n = 0.91$), respectively, that contribute to the absorbance at 562–574 nm, but not at 552–540 nm. No additional $\Delta A_{562-574}$ was observed at E_h values between +400 and +450 mV or between −150 and −250 mV. All three components exhibited absorbance maxima close to 560 nm. These results are consistent with the presence of at least three membrane-bound, *b*-type cytochromes. All titrations gave E_m values that were reproducible to within ±5 mV, n values that were reproducible to within ± 10% and were fully reversible. The E_m and n values obtained for all six components were independent of mediator concentrations over a 10-fold range (10–100 μM).

Apparent molecular weights for the membrane-bound, *c*-type cytochromes of *Chloroflexus* were determined by SDS-polyacrylamide gel electrophoresis followed by staining the gels for heme [15]. Two prominent heme-containing peptides were detected in the membranes of chemotrophically grown cells and four different heme-containing peptides were found in the membranes of phototrophically grown cells (Fig. 3). The apparent molecular weights are 36 and 24 kDa in the case of chemotrophically grown cells, while those from phototrophically grown cells are 43–45, 36, 24 and 19 kDa. The 43–45 kDa heme-containing peptide, present in phototrophically grown *Chloroflexus* cell membranes, corresponds to cytochrome *c*-554 [16]. The highest molecular-weight band is a dimer of cytochrome *c*-554 [4]. In agreement with earlier findings [4], no 43–45 kDa heme *c*-containing component was detectable in membranes isolated from chemotrophically grown cells, even in experiments utilizing larger amounts of membrane protein. These data, as well as the difference spectrum of Fig. 1, indicate that the cytochrome *c*-554 is not present in chemotrophically grown *Chloroflexus*. Further evidence for this conclusion comes from the immunoblotting experiments also shown in Fig. 3. While an affinity-purified antibody against cytochrome *c*-554 reacts specifically with cytochrome *c*-554 present in membranes isolated from phototrophically grown cells, membranes isolated from chemotrophically grown cells showed no immunochemical reactivity with anti-cytochrome *c*-554. These results strongly suggest that the cytochrome *c*-554 peptide is absent in membranes isolated from chemotrophically grown *Chloroflexus* [4].

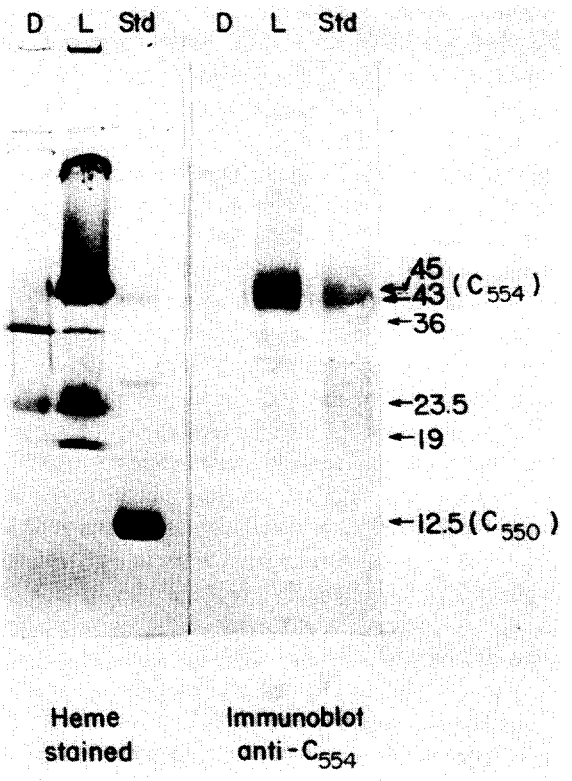

Fig. 3. Left-hand panel: the heme *c*-containing, membrane-bound polypeptides of chemotrophically and phototrophically grown *Chloroflexus*. Membranes equivalent to 100 μg protein in 30 μl volume were applied to the gels and stained for heme as described in Methods. Horse heart cytochrome *c* (*c*-550, $M_r = 12500$) was used as a standard (Std). D and L represent membranes isolated from dark- and light-grown cells, respectively. Right-hand panel: the immunological identification of cytochrome *c*-554 in whole cell homogenates of dark- (D) and light- (L) grown cells. Purified cytochrome *c*-554 was present in the standard lane. Membranes equivalent to 100 μg protein in 30 μg volume were applied to the gel, which was subsequently electroblotted to nitrocellulose paper and probed with the affinity-purified antibody against cytochrome *c*-554. Antisera reactions were visualized with ^{125}I-protein A and autoradiography.

The (reduced plus CO) minus reduced difference spectrum of membranes isolated from chemotrophically grown cells, shown in Fig. 4, shows that CO-binding to a heme component (difference maxima at 427, 556 and 587 nm) occurs in membranes reduced with ascorbate plus

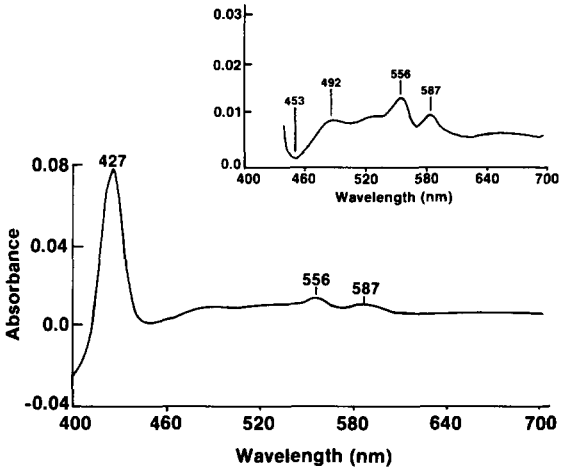

Fig. 4. CO difference spectrum of membranes from chemotrophically grown *Chloroflexus* cells. Membranes (20 μg protein/ml) were suspended in 20 mM Tris buffer (pH 7.0). The difference spectrum was recorded following exposure to CO for 10 min. Spectral resolution, 1 nm; pathlength, 1 cm. Ascorbate was added as small solid aliquots. PMS was present at a final concentration of 10 μM.

PMS. Similar results were obtained using hydroquinone or dithionite, instead of ascorbate plus PMS, as the reductant. The CO difference spectrum obtained with membranes isolated from phototrophically grown cells and reduced with dithionite (Fig. 5) is quite different. No absorbance changes were observed when CO was equilibrated with membranes from phototrophically grown cells that had been reduced with either ascorbate plus PMS or hydroquinone instead of dithionite. The CO-difference spectrum of membranes isolated from phototrophically grown cells is characteristic of Co-binding by a *c*-type cytochrome [23], as has been previously reported [5,7], while the CO difference spectrum of membranes isolated from chemotrophically grown cells resembles that reported for the so-called cytochrome a_1 [23].

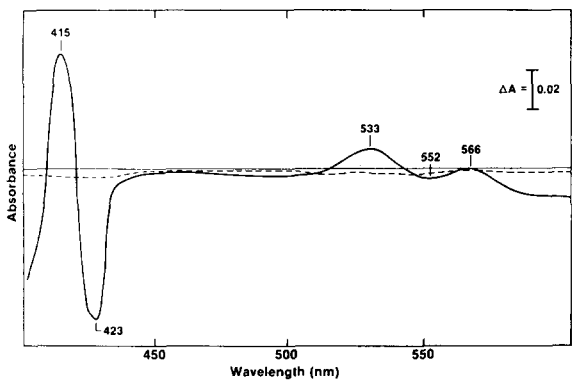

Fig. 5. CO difference spectrum of membranes from phototrophically grown *Chloroflexus*. Membranes (25 μg protein/ml) were suspended in 20 mM Tris buffer (pH 7.0). The difference spectrum was recorded following exposure to CO for 10 min. Spectral resolution, 1 nm; pathlength, 1 cm. Ascorbate or dithionite were added as small solid aliquots. PMS was present at a final concentration of 10 μM. The solid line represents the dithionite-reduced sample and the dashed line the ascorbate/PMS-reduced sample.

As low potential, CO-binding cytochromes *c* in the membranes of several other phototrophic bacteria are capable of undergoing photooxidation at cryogenic temperatures [20,24], an attempt was made to demonstrate cytochrome *c* photooxidation at 77 K in membranes isolated from phototrophically grown *Chloroflexus*. No photooxidation was observed in samples frozen at E_h values between +25 and −45 mV (data not shown). In control experiments using *Rps. viridis* membranes poised between +25 and −30 mV, photooxidation of cytochrome *c*-552 [20] at 77 K could readily be observed.

The EPR spectrum of membranes isolated from phototrophically grown *Chloroflexus* and frozen without addition of reductant is shown in Fig. 6. This spectrum contains a broad feature at $g = 1.89$, a value suggesting the presence of a reduced Rieske

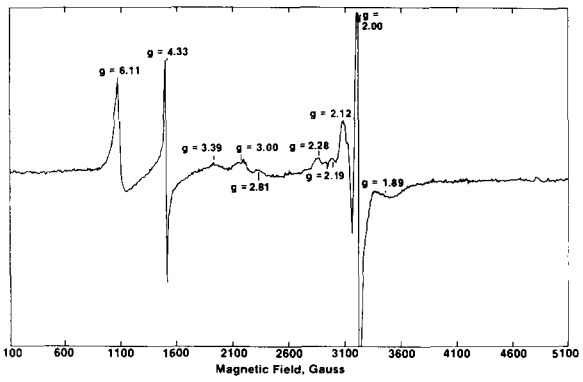

Fig. 6. EPR spectrum of membranes from phototrophically grown *Chloroflexus*. Membranes (10 mg protein/ml) were suspended in 10 mM Tris buffer (pH 7.0). Conditions of EPR spectroscopy: microwave frequency, 9.150 GHz; microwave power, 3 mW; modulation amplitude, 8 G; modulation frequency, 100 kHz and temperature, 15 K.

iron-sulfur protein [25–27] The relatively great width of this signal is likely to be due to overlapping signals from other membrane-bound species. No increase in the amplitude of the $g = 1.89$ feature was observed when dithionite was added, suggesting that the Rieske iron-sulfur protein was largely reduced in the isolated membranes. In addition to the EPR signal arising from the Rieske protein, a signal at $g = 2.0$, presumably arising from one or more free radicals, is present. Membranes isolated from phototrophically grown cells also exhibited unassigned EPR signals with g-values of 3.39, 3.00 and 2.81 probably due to oxidized low spin cytochromes, a $g = 4.33$ signal, presumably arising from non-specifically bound iron, and a feature at $g = 6.11$, presumably arising from an oxidized high-spin heme. A similar signal at $g = 6.11$ was also seen in membranes isolated from chemotrophically grown cells.

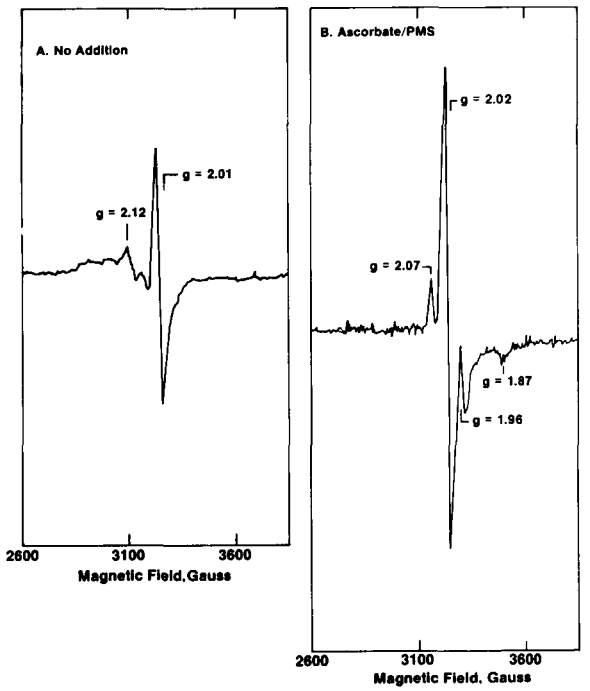

Fig. 7. EPR spectra of oxidized (A) and ascorbate/PMS-reduced (B) membranes from chemotrophically grown *Chloroflexus*. Membranes (equivalent to 5 mg protein/ml) were suspended in 20 mM Tris buffer (pH 7.0). Ascorbate was added as a solid. PMS was present at a concentration of 5 μM. Conditions of EPR spectroscopy were as in Fig. 6, except that the temperature was 10 K.

Fig. 7 shows EPR spectra of membranes isolated from chemotrophically grown *Chloroflexus* Fig. 7A shows the spectrum obtained prior to any addition and has prominent features at $g = 2.12$ and 2.01. Fig. 7B was obtained by the addition of ascorbate and PMS to the sample of Fig. 7A, and displays resonance at $g = 2.07$, 2.02, 1.96 and 1.87. An essentially identical set of resonances was observed if succinate replaced ascorbate/PMS as the added reductant (data not shown). Similar EPR signals have also been observed in phototrophically grown *Chloroflexus* [5]. EPR signals with g-values of 2.05 and 1.94 were observed in NADH-reduced membranes from chemotrophically grown cells (data not shown). No EPR signals characteristic of a reduced Rieske iron-sulfur protein could be detected in membranes isolated from chemotrophically grown cells. At this time it is not possible to make definite assignments of the other resonances of Figs. 6 and 7. Certainly these resonances, by virtue of their g-values and their appearance upon addition of different reductants, must arise from a variety of iron-sulfur proteins. Assignments of these resonances to individual components of electron-transfer complexes such as succinate dehydrogenase or NADH dehydrogenase [29,30] will require further experimentation which is beyond the scope of the current investigation.

Discussion

Oxidation-reduction titrations, shown above, suggest that membranes of chemotrophically grown *Chloroflexus* contain at least three *c*-type cytochromes with α band maxima at 551–552 nm. No component with an absorbance maximum at 554–555 nm was detected. The absence, in chemotrophically grown cells, of any heme-containing peptide with $M_r = 43\,000$ and of any cross reaction with antibody directed against cytochrome *c*-554 (Fig. 3) indicate that cytochrome *c*-554, present in phototrophically grown *Chloroflexus*, is not present in membranes of chemotrophically grown cells. In phototrophically grown cells of *Chloroflexus*, cytochrome *c*-554 functions in cyclic electron flow as the immediate donor to P-865 [16,31]. Since cyclic electron flow cannot function in membranes isolated from chemotrophically

grown cells due to the absence of the reaction center [11], the lack of cytochrome *c*-554 is not surprising. The high potential *c*-type cytochrome cytochrome ($E_m = +310$ mV; α band maximum at 551–552 nm), detected by oxidation-reduction titrations of membranes from chemotrophically grown cells (Fig. 2), apparently represents a *c*-type cytochrome, different from cytochrome *c*-554, which has not been observed previously in chemotrophically or phototrophically grown *Chloroflexus*. The function of this cytochrome is not known at this time. It is interesting to note that a similar situation occurs in the photosynthetic purple sulfur bacterium *Chromatium vinosum*, where the high potential ($E_m = +340$ mV) cytochrome *c*-555 that serves as an immediate donor to the reaction center in phototrophically grown cells is absent in chemotrophically grown cells, while a different high potential *c*-type cytochrome ($E_m = +330$ mV) is present in chemotrophically grown cells [21].

The *c*-type cytochrome with $E_m = +120$ mV may be identical to that with $E_m = +180$ mV observed in chemotrophically grown cells by Zannoni [6] and may possibly be related to the cytochrome c_1. Recent studies of phototrophically and chemotrophically grown *Chloroflexus* [5,6] suggest the presence of a cytochrome bc_1 complex in cells under both conditions of growth. One would thus expect to detect a cytochrome with spectral properties, molecular weight and midpoint potential similar to cytochrome c_1. If this $E_m = +120$ mV cytochrome were in fact cytochrome c_1, it would have an atypically low E_m value, as most cytochromes c_1 have E_m values near +270 mV [27]. However, cytochromes c_1 are usually approximately isopotential with the Rieske proteins in a given organism [27] and phototrophically grown *Chloroflexus* has been shown [5] to contain an unusually low potential Rieske iron-sulfur protein ($E_m = +100$ mV). Thus, a E_m value of +120 mV may not be inappropriate for cytochrome c_1 in this bacterium. Cytochromes c_1 generally have molecular weights of approx. 30 kDa [27]. Thus one would have expected that a peptide at $M_r = 30000$ to be detected on gels stained for heme. No heme-staining band near this molecular weight was detected in SDS-polyacrylamide gel electrophoresis gels of membranes isolated from either phototrophically or chemotrophically grown cells (Fig. 3). The heme-staining bands closest to $M_r = 30000$ are the 24 and the 36 kDa bands. These heme-proteins appear to be constitutive components of *Chloroflexus*, since they are present in membranes from both phototrophically and chemotrophically grown cells. It is also possible that some of the heme-containing peptides detected by SDS-polyacrylamide gel electrophoresis represent proteolytic degradation products, since no protease inhibitors were added in these experiments. No peptides were found in either membranes isolated from chemotrophically or phototrophically grown *Chloroflexus* that reacted with antibodies raised against cytochrome c_1 from *Rb. sphaeroides*. Thus, definitive proof for the presence of cytochrome c_1 in *Chloroflexus* remains to be obtained.

The low potential *c*-type cytochrome observed in chemotrophically grown cells in this work has a midpoint potential ($E_m = 0$ V) similar to those previously determined for a spectrally similar cytochrome in chemotrophically ($E_m = +10$ mV, Ref. 6) and phototrophically grown ($E_m = +20$ mV, Ref. 5) *Chloroflexus*. A low-potential, *c*-type cytochrome is almost certainly the component responsible for CO-binding in phototrophically grown cells (Fig. 5), as the difference spectrum resembles that expected for a *c*-type cytochrome [5,7,23] and CO-binding occurs in dithionite-reduced samples but not in hydroquinone or ascorbate/PMS-reduced samples. In the presence of ascorbate/PMS, the ambient potential is approximately +100 mV, a value low enough to completely reduce the $E_m = +310$ mV component and partially reduce the +120 mV component but not the $E_m = 0$ V component. However, dithionite is a sufficiently strong reductant to also reduce the $E_m = 0$ V component. No spectral changes attributable to CO-binding by a *c*-type cytochrome could be observed in membranes isolated from chemotrophically grown cells (also see Ref. 6), raising the possibility that this cytochrome is absent in chemotrophically grown cells and that different low potential ($0 \text{ mV} \leqslant E_m \leqslant -20$ mV) cytochromes are present under the two conditions of growth. Our results with phototrophically grown cells are consistent with earlier reports [5,7] of a CO-binding cytochrome *c* and extend the earlier

work by providing evidence that the lowest potential cytochrome *c* present in phototrophically grown cells is the only one that binds CO. Furthermore, we have demonstrated that this cytochrome *c* differs from low potential *c*-type cytochromes in bacteria such as *Rps. viridis* and *C. vinosum* in being unable to undergo photooxidation at cryogenic temperatures.

Optical spectra (Fig. 1), protoheme analysis (Table I) and electrochemical titrations (Fig. 2) indicate the presence of *b*-type cytochromes in the membranes of chemotrophically grown *Chloroflexus*. This is in dramatic contrast to the very small amounts of *b*-type cytochrome(s) present in phototrophically grown *Chloroflexus* (Table I; also see Refs. 7 and 8; however, see Ref. 5). Definitive evidence for two *b*-type cytochromes came from the electrochemical titrations (Fig. 2) which showed two major components ($E_m = +60$ and -60 mV). The additional component ($E_m = +260$ mV), detected in titrations at 562–574 nm is present in considerably lower amounts than the $+60$ and -60 mV components and may represent an '*o*'-type cytochrome [6]. Similar *b*-cytochrome titrations have been obtained by Zannoni [6]. One puzzling observation is the relatively large size of the total $\Delta A_{562-574}$ compared to the $\Delta A_{552-540}$ (Fig. 2) in view of the heme *c*/protoheme ratio (Table I) of the chemotrophically grown cells. One possible explanation for this apparent discrepancy may be that one of the components seen at 562–574 is a *c*-type cytochrome with a α-band maximum at an unusually long wavelength. However, additional data will be required to settle this question.

The E_m values, $+60$ and -60 mV, of the major cytochrome *b* components are similar to those reported for cytochrome *b*-562 and *b*-566, respectively, of the cytochrome bc_1 complexes of mitochondria and photosynthetic purple nonsulfur bacteria [27,33]. The observations that electron flow in both phototrophically [5] and chemotrophically [6] grown *Chloroflexus* is sensitive to antimycin A and myxothiazol, two specific inhibitors of electron flow through cytochrome bc_1 complexes [32], suggest the presence of such a complex in this bacterium. This conclusion was supported by the observation by Zannoni and Ingledew [5], of an EPR signal attributable to a Rieske iron-sulfur protein with $E_m = +100$ mV in membranes isolated from phototrophically grown *Chloroflexus*. We have also observed a Rieske protein (see Fig. 6 above and Ref. 33) in membranes isolated from phototrophically-grown cells but have been unable to detect a $g = 1.90$ EPR signal characteristic of the reduced Rieske protein in membranes isolated from chemotrophically grown cells of *Chloroflexus*. This could, in principle, be due to the presence of considerably smaller amounts of this protein in the chemotrophically grown cells membranes or because the $g = 1.90$ feature was obscured by other signals. If the former explanation were correct, then one would expect that the amounts of the $E_m = +60$ and -60 mV *b*-cytochromes would also be considerably lower in chemotrophically grown cells than in phototrophically-grown cells, since the Rieske iron-sulfur protein and each of the two *b*-hemes of cytochrome bc_1 complexes are generally present in a 1 : 1 : 1 ratio [27]. However, the reverse is true. Additional investigations to resolve this problem are currently underway in our laboratories.

A question of considerable interest is the identity of the terminal oxidase(s) in chemotrophically grown *Chloroflexus*. As reduced oxidases generally bind CO [23,34], it seemed reasonable to use CO difference spectra in identifying the terminal oxidase of chemotrophic *Chloroflexus*. The ascorbate/PMS-reduced CO difference spectrum (Fig. 4) indicates the presence of a CO-binding, heme-containing component in membranes isolated from chemotrophically grown cells. This spectrum is similar to those reported for so-called cytochrome a_1 oxidases [23,34] in having well-defined maxima in the absorbance difference spectra at 587 nm and 556 nm. However, the Soret band differences maximum, 427 nm, is at a somewhat shorter wavelength than the 435–445 nm region usually observed for 'cytochrome a_1'. Nevertheless, the difference spectrum of Fig. 4 corresponds considerably better to that reported for 'cytochrome a_1' than to that reported for cytochrome *o* [23,34]. As this CO difference spectrum was also observed with membranes that had been reduced with the weak reductant hydroquinone, ($E'_m = +290$ mV), the CO-binding component responsible for the absorbance changes must be of relatively high potential ($E_m \geq +250$ mV). The absence of sig-

nificant amounts of heme *a* (Table I) does not rule out the presence of an a_1 oxidase, as recent studies with *Escherichia coli* have identified the component designated as cytochrome a_1 to be an unusual high-spin, protoheme-containing species free of heme *a* [35,36].

Considerable evidence has been obtained in other laboratories for the presence of a cytochrome aa_3 oxidase in chemotrophically grown *Chloroflexus* [6,7]. However, we detected no heme *a* and could find neither optical absorbance changes characteristic of cytochrome aa_3 nor a Cu^{2+} EPR signal of the type found in Cu^{2+}-containing cytochrome aa_3 oxidases [37,38] in the membranes used in this study. The differences between the results obtained in this study and those from other laboratories may arise from the different growth conditions used for chemotrophic growth in these studies. In particular, the strictly dark, aerobic conditions for cell growth [4] used in this work resulted in cells free of cytochrome *c*-554 and virually free of BChl *c* and *a*, while those used by Zannoni [6] contained residual BChl and cytochrome *c*-554 (Zannoni, D., personal communication). It is known, for example, that in *E. coli* [39] and *Rb. sphaeroides* [40] the nature of the terminal oxidase is controlled by the O_2 concentration during growth. A similar situation appears to be the case in *Chloroflexus*.

Acknowledgements

The authors are grateful to Professor Chang-An Yu for his generous gift of antibodies against *Rb. sphaeroides* cytochrome c_1, to Jeff Trost for affinity purification of the antibody against cytochrome *c*-554 and to Dr. Charlotte Kämpf for helpful discussions. The authors would especially like to thank Dr. Beverly Pierson and Dr. David Zannoni for access to manuscripts prior to publication and for extensive and extremely helpful discussions. This work was supported by grants to D.B.K. from the National Science Foundation (PCM-8408564), to R.E.B. from the U.S. Department of Agriculture (84-CRCR-1-1523), to R.C.F. from the National Science Foundation (DMB-05077) and to R.W.S. from the National Science Foundation (PCM-9119188) and the Robert A. Welch Foundation (D-909).

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