The branched respiratory chain of heterotrophically dark-grown *Chloroflexus aurantiacus*

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The respiratory electron-transport chain of heterotrophically dark-grown *Chloroflexus aurantiacus* has been investigated. Membranes isolated from these cells have been shown to contain at least three *c*-type cytochromes ($E_{m,7\,0}$ 255, 180, and 10 mV), three *b*-type cytochromes ($E_{m,7\,0}$ of 210, 60 and −65 mV) and two cytochromes of the *a* type with $E_{m,7\,0}$ of 330 and 190 mV. Spectroscopic evidence from CO-difference spectra, CN^--difference spectra and spectra at fixed oxidation-reduction potentials suggests that the two *a*-type components may be analogous to cytochromes *a* and a_3 of mitochondria. The analyses of the effects induced by CN^-, myxothiazol and antimycin A on both steady-state respiratory activities and semi-rapid oxidation-reduction kinetic patterns of *c*- and *a*-type cytochromes indicate the presence of a branched respiratory chain. Growth of *Chloroflexus* in medium lacking added copper diminished the concentration of the *a*-type cytochromes but not those of cytochromes of the *b* and *c* type.

Cytochrome a *Cytochrome* a_3 *Cytochrome* b *Cytochrome* c *Respiration* *Thermophilic bacterium* (Chloroflexus aurantiacus)

1. INTRODUCTION

The thermophilic green photosynthetic bacterium *Chloroflexus aurantiacus* is a facultative photoheterotroph. When grown anaerobically in the light, the cells contain bacteriochlorophyll (BChl) *a*, associated with the cytoplasmic membrane (CM), plus BChl *c* which is enclosed in oblong bodies (chlorosomes) attached to the inner surface of the CM [1]; under aerobic dark conditions the number of BChl *c*-containing vesicles declines and pigment synthesis is repressed [1]. Similarly to purple non-sulphur bacteria, the cytochrome composition of *Chloroflexus* varies with the conditions of growth [2]. Indeed, the haem *c*/protohaem ratio decreases up to several-fold from phototrophic to chemotrophic growth [3]. In addition, aeration leads to the development of what could possibly be a terminal membrane-bound oxidase with an *a*-type haem [3].

Recently, the plasma membrane-bound electron-transfer components in heterotrophically light-grown cells of *Chloroflexus* have been examined [4]. It was shown that this thermophile contains several cytochromes (cyt.) of the *b* and *c* type, along with multiple ferredoxin-like centres with resonances at $g = 1.93$, $g = 2.017$ and $g = 1.90$ (Rieske iron-sulphur centre) [4]. These observations led to the suggestion of the presence of the redox elements of a cyt. b/c_1 complex in this bacterium.

Here, the respiratory electron-transfer chain of heterotrophically dark-grown cells of *Chloroflexus* has been investigated. It is concluded that the *Chloroflexus* respiratory apparatus is formed by a branched chain leading to two oxidases, namely: (i) a cyt. *c* oxidase of the aa_3 type and (ii) an alternative oxidase, presumably of the *b* type.

2. MATERIALS AND METHODS

2.1. *Organism cultivation and membrane isolation*

The medium used for chemoheterotrophic growth of *C. aurantiacus* strain J-10-f1 was that

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described by Pierson and Castenholz [5] except for copper-limited cultures in which copper was omitted. Dark-grown cells were cultivated in a 14 l fermentor (Microferm; New Brunswick Scientific Co., NJ) at 55°C for 24 h with stirring at 200 rpm, and bubbled with humidified air at 6 l/min. Plasma membrane fragments were prepared as described in [4].

2.2. *Optical spectroscopy*

Cytochrome spectra were obtained with a computer-linked dual-beam spectrophotometer (Jasco Uvidec-610 KDB-101). Dark potentiometric titrations and absorption changes of the membrane-bound cytochromes were performed in a medium containing *N*-tris(hydroxymethyl)methylglycine (Tricine) buffer (50 mM, pH 7.0) plus 50 mM KCl using a dual-wavelength spectrophotometer (Sigma ZW-11) equipped with a rapid-mixing apparatus (mixing time approx. 0.05 s) as previously described [4]. E_0' values were assigned on the basis of a computer-assisted analysis as in [6]. Estimation of the amounts of cytochrome was made by using the following extinction coefficient and wavelength pairs: cyt. *c*, 552–540 nm, ϵ = 19 $mM^{-1} \cdot cm^{-1}$; cyt. *b*, 560–575 nm, ϵ = 22 $mM^{-1} \cdot cm^{-1}$; cyt. *a*, 602–630 nm, ϵ = 19 $mM^{-1} \cdot cm^{-1}$ [7].

2.3. *Steady-state resporatory activities*

Rates of oxygen consumption were measured polarographically at 35°C with a Clark-type oxygen electrode.

2.4. *Other assays*

Proteins were determined by using the method of Lowry et al. [8].

3. RESULTS AND DISCUSSION

3.1. *Cytochrome absorption spectra and dark-equilibrium potentiometry*

Fig.1. shows the cytochrome spectra of membrane fragments isolated from dark-grown cells of *Chloroflexus*. As previously shown in photosynthetically grown cells [4], absorption maxima indicative of *b*-type (530 and 560 nm) and *c*-type (554 and 520 nm) haems in both dithionite- and Na-ascorbate-reduced difference spectra are present. According to a previous report [3], a large absorption band due to a presumed *a*-type component is also evident at 602 nm. Fig.1 also indicates that addition of Na-ascorbate to membranes treated with 1 mM KCN causes the reduction of about 30% of the dithionite-reducible *b*-type complement along with the appearance of a new absorption band at 597 nm (spectrum c). Since the CO-difference spectrum of *Chloroflexus* membranes (dashed trace) suggests that a component forms a complex with CO, with absorption properties similar to those of mitochondrial cyt. *a* (reduced α_{max} at 605 nm; reduced + CO α_{max} at 592 nm) [7], it is reasonable to suggest that the new peak at 597 nm corresponds to a second *a*-type component contributing to the broad absorption

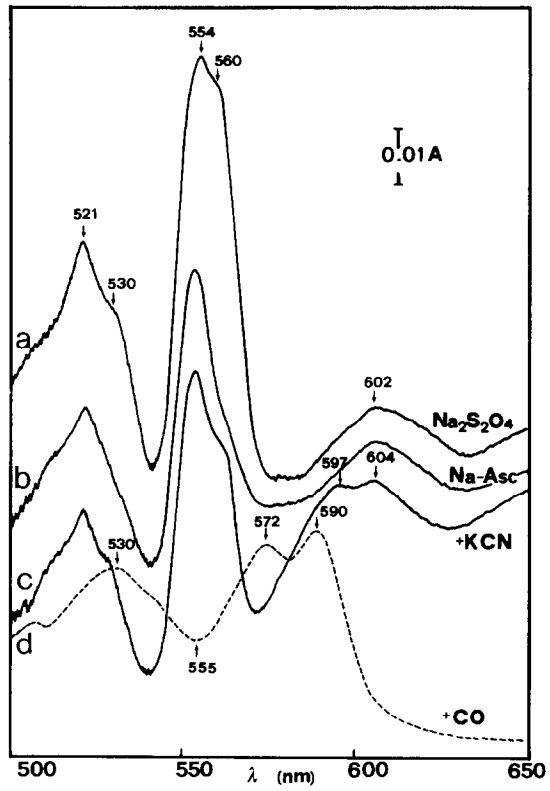

Fig.1. Cytochrome difference spectra of membranes from dark-grown cells of *C. aurantiacus*. (a,b) Dithionite-reduced and ascorbate-reduced minus ferricyanide-oxidized difference spectra, respectively; (c) as in (b) plus 1 mM cyanide; (d) dithionite-reduced plus CO minus dithionite-reduced difference spectrum. The protein concentrations were about 2 mg/ml. Spectrophotometer settings: *t*, 25°C; bandwidth, 1 nm; time constant, 1 s; scanning speed, 40 nm/min.

band at 602 nm. The additional CO-binding haem, with broad peaks at 530–535 and 570–575 nm and trough at 555 nm, is tentatively assigned to an *'o'*-type cytochrome.

The above interpretation of the CN^--difference spectra is essentially based on previous observations with purified mitochondrial cytochrome oxidase (cf. [7]) and may not be applicable to bacterial membrane preparations. A potentiometric titration was therefore carried out (at 602–630 nm) to provide additional evidence for the presence of two *a*-type haems. Such a titration has previously been shown to distinguish between cyt. *a* and a_3 in mitochondrial [9] and bacterial systems [10,11]. The results of such a titration (fig.2) show that there are indeed two components present with $n = 1$ and $E_{m,7.0}$ of 330 ± 10 and 190 ± 7 mV. Their relative contributions to the total absorbance signal at 602–630 nm were approx. 55 and 45%, respectively.

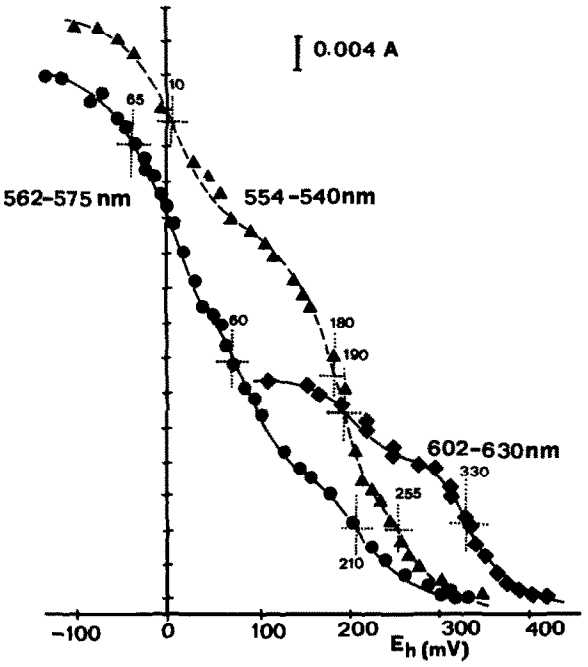

Fig.2. Dark-equilibrium potentiometric titrations of membranes from dark-grown cells of *C. aurantiacus*. Redox titrations at 554–540 nm (▲---▲), 562–575 nm (●——●) and 602–630 nm (◆——◆) are shown. Mid-point potentials at pH 7.0 from best-fit procedures are indicated. In (▲---▲,●——●) and (◆——◆), the protein concentrations were about 2.8 and 2.6 mg/ml, respectively.

The dark-potentiometric titrations reported in fig.2 show that also membranes from aerobically grown *Chloroflexus* contain several haems of the *b* and *c* type. Titrations at 562–575 nm could be resolved into 3 components, each component behaving as an $n = 1$ theoretical Nernst curve, with $E_{m,7.0}$ of 210 ± 5, 60 ± 10 and -65 ± 7 mV; their relative contribution to the total absorption change were approx. 28, 36 and 36% for cyt.(s) *b*210, *b*60 and $b-65$, respectively. The results of titrations at 554–540 nm could be fitted to a 3-component Nernst curve ($n = 1$), to which were assigned $E_{m,7.0}$ values of 255 ± 5, 180 ± 10 and 10 ± 15 mV. Their relative contributions to the total absorbance change were approx. 15, 40 and 45% for cyt. *c*255, *c*180 and *c*10, respectively. In this respect, the *c*-type complement of aerobic membranes is quite similar to that previously found in photosynthetically grown *Chloroflexus* [4]. However, in membranes from the latter type of cells, the *c*-type haems are present in a ratio close to unity, whereas the above-reported data with aerobic membranes suggest a ratio of 0.3 : 1 : 1, due to the low contribution of cyt. *c*255 to the total absorbance change at 554–540 nm. Conversely, apart from a generalized increase of the protohaem content (on a molar/protein basis), the relative ratio of the *b*-type haems in respiratory membranes is analogous to that previously found in photosynthetic cells. The detection by redox potentiometry of two cyts *a* was of special interest, because the aa_3 pair involves the presence of a mitochondrial type cytochrome oxidase which has not been described previously in membranes from *Chloroflexus* [3,4]. In fig.3, difference spectra at fixed oxidation-reduction potentials are shown, in order to characterize these components further. After subtraction of the spectrum recorded at 440 mV (sample 98% oxidized) from that obtained at 274 mV (cyt. *a*330 approx. 80% reduced), a significant band at 607 nm is evident. Other bands at 521 and 552 nm, characteristic of *c*-type haem are also present (fig.3, trace a). In fig.3, the difference between the spectra obtained at 57 and 440 mV (trace b, cyt. *a*330 + cyt. *a*190 reduced) and 57 and 274 mV (trace c, cyt. *a*190) is also shown. It is apparent that cyt. *a*190 + cyt. *a*330 show a large band at 602 nm clearly distinct from that of cyt. *a*190 (trace c) which is at 597 nm.

Mitochondrial cyt. aa_3 oxidase is a copper-

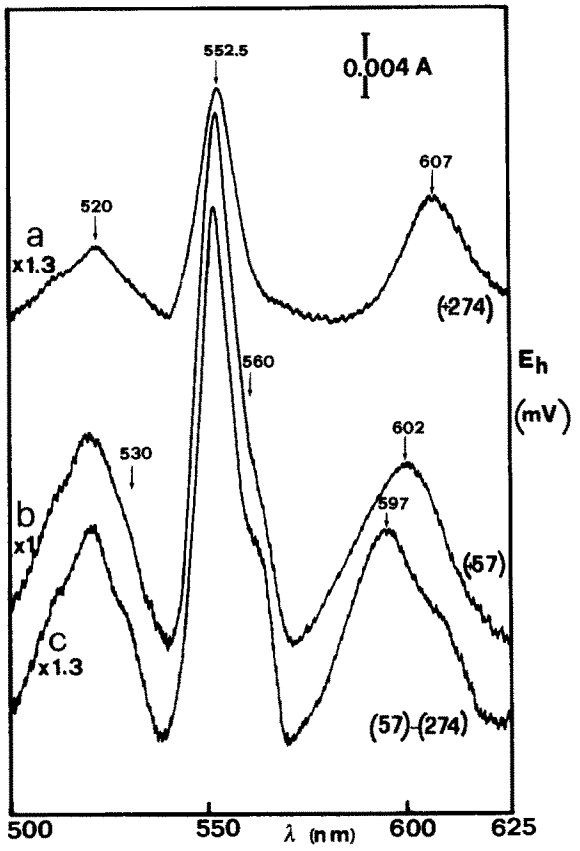

Fig.3. Cytochrome difference spectra at fixed oxidation-reduction potentials of membranes from aerobically grown *C. aurantiacus*. In (a), the difference spectrum obtained at 440 and 274 mV is shown. Traces (b) and (c) indicate the differences between spectra obtained at 57 and 440 mV, and 57 and 274 mV. See text for more details. Protein concentration was approx. 2.6 mg/ml.

containing complex and it has been shown that when yeast cells are grown under conditions of copper limitation the cytochrome oxidase level of the cells decreases [12,13]. Studies with dark-grown *Chloroflexus* show that omission of copper from the growth medium (see section 2) leads to a fall in the cytochrome aa_3 content of the cells of about 65% (on a molar/protein basis; not shown).

3.2. *Steady-state respiratory activities and semi-rapid oxidation-reduction kinetic analysis*

The results shown in fig.4 demonstrate that NADH-oxidase activity of *C. aurantiacus* membrane fragments gives a biphasic KCN-inhibition curve with a first K_i of 4 μM and a second K_i of

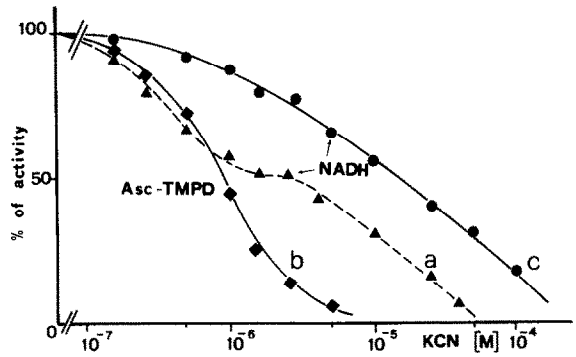

Fig.4. Cyanide inhibition of ascorbate-*N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD) and NADH-oxidation activities in membranes from either control or copper-limited cultures of aerobically grown *C. aurantiacus*. Control cultures (◆——◆, ▲---▲). Copper-limited culture (●——●). Activities: NADH oxidation, 6.5 and 20 μmol O_2 reduced · h^{-1} · mg protein^{-1} in (▲---▲) and (●——●), respectively; ascorbate-TMPD oxidation, 120 and 55 μmol O_2 reduced · h^{-1} · mg protein^{-1} in control and copper-limited cultures, respectively. Additions: NADH, 1 mM; ascorbate, 4 mM; TMPD, 250 μM.

0.12 mM (curve a). In contrast, the cyt. *c* oxidase activity, measured as ascorbate-*N,N,N'N'*-tetramethyl-*p*-phenylenediamine oxidation, shows a monophasic inhibition pattern (curve b) with a $K_i = 8$ μM suggesting that alternative terminal oxidases with different sensitivities to cyanide exist. This conclusion is consistent with the results in curve c (fig.4), obtained with membranes from *Chloroflexus* grown under conditions of copper limitation. Membranes from this type of cell show an NADH-oxidase sensitivity to KCN (K_i = 0.1 mM) similar to that of the less sensitive component of the biphasic NADH-oxidation curve in normal cells. Since the total respiratory activity is 3-times higher in membranes from copper-limited cultures than normal membranes, the conclusion is likely that only the cyt. *c* oxidase-dependent activity is affected by copper limitation. In fig.5 (A, traces a–c), the reduction kinetics of the *c*-type cytochromes (signal 554–540 nm) as a function of antimycin A and myxothiazol concentrations are shown. The traces clearly show that in membranes pretreated with 1 mM KCN, reduction of a large part (approx. 70%) of the dithionite-reducible *c*-type species takes place in less than 1 s. Traces b and c indicate that cyt. *c* reduction is affected by antimycin A and/or myxothiazol (20 μM each). A synergistic effect of these antibiotics is also evi-

dent. Part B of fig.5 shows that under steady-state NADH-dependent respiration, cyt.(s) *a* and cyt.(s) *c* are slightly reduced (14 and 22%, respectively). This suggests that an active electron-transport flow operates which maintains a major fraction of *a*- and *c*-type cytochromes in an oxidized state. Conversely, a strong reduction of cyt.(s) *c* and cyt.(s) *a* (70 and 90%, respectively), is observed after the onset of anaerobiosis. Part C of fig.5 shows that complete restoration of the steady-state respiratory reduction levels can be achieved in about 100 ms by an oxygen pulse (magnetic stirring of the cuvette; mixing time approx. 0.05 s). Acceleration of the reoxidation time is observed in the presence of antimycin A plus myxothiazol (20 μM each) (dashed traces).

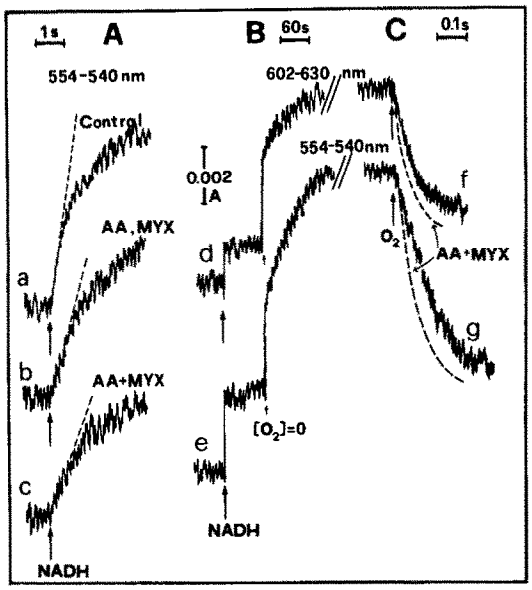

Fig.5. Effects of antimycin A and myxothiazol on the oxidation-reduction kinetics of cyt. *c* and cyt. *a*. Traces in (a-c) show reduction of cyt.(s) *c* (554–540 nm) by NADH in the presence of 1 mM KCN. Traces (d,e) show reduction of cyt.(s) *a* (602–630 nm) and cyt.(s) *c* (554–540 nm), respectively, by NADH under aerobic and/or anaerobic conditions. Traces (f,g) show reoxidation by oxygen of cyt.(s) *a* and cyt.(s) *c* in the presence (– – –) or absence (——) of antimycin A plus myxothiazol. AA, antimycin A; MYX, myxothiazol; $[O_2] = 0$, anaerobiosis. Additions: AA, 20 μM; MYX, 20 μM; NADH, 1 mM. Protein concentrations were approx. 0.35 and 1 mg/ml in (a-c,e,g) and (d,f), respectively. The total cytochrome content in the assay was estimated by addition of few crystals of sodium dithionite at the end of each experiment.

It is noteworthy that, in spite of the thermophilic nature of *Chloroflexus* (optimal growth temperature at 55°C), all measurements of respiratory electron transport reported in this study have been performed at 35°C. These experimental conditions have been chosen deliberately in order to avoid heat inactivation of the inhibitors. Additional experiments revealed that 3–4-times activation of the respiratory activities takes place raising the temperature from 35 to 55°C. The latter finding suggests that the electron-transport rates reported in figs 4 and 5 might be an underestimation of the actual in vivo situation.

4. CONCLUSION

This study indicates that in membrane fragments isolated from dark-grown cells of *C. aurantiacus* an *a*-type cytochrome oxidase develops which possesses some of the properties described for bacterial and mitochondrial oxidases of the aa_3 type [14]. It has two haem components that can be resolved by KCN-difference spectra, CO-difference spectra, and by potentiometric titrations. The two haems have mid-point potentials of 190 and 330 mV, and are present in a ratio close to unity. According to previous work [3,4], no cyt.(s) *c* corresponding to mitochondrial and bacterial soluble cyt. *c* (formerly cyt. c_2 in photosynthetic bacteria) was found in aerobic membranes of *Chloroflexus*, nor in the soluble fraction (not shown). A noteworthy observation is the detection of two *b*-type haems with $E_{m,7.0}$ of 60 and -65 mV plus a *c*-type haem with $E_{m,7.0}$ of 180 mV. These values may be compared with those of cyt. *b*562, cyt. *b*566 and cyt. c_1, respectively, the 3 haems associated with mitochondrial and bacterial b/c_1 complexes [15]. The presence of the haem-type elements of the b/c_1 complex, previously suggested in photoheterotrophic cells [4], is strongly supported by the observation (experiments of fig.5) that electron transfer in *Chloroflexus* is sensitive to antimycin A and myxothiazol, two specific inhibitors of electron flow catalyzed by the ubiquinol-cyt. *c* oxidoreductase [15].

At present, the arrangement of the electron-transfer components in aerobic membranes of *Chloroflexus* is only partially understood. The inhibitory data tend to indicate that the electron

transport of *Chloroflexus* is branched since two oxidase-containing pathways may be distinguished by KCN sensitivity (K_i values of 4 μM and 0.12 mM). Reduced TMPD is oxidized exclusively via the most KCN-sensitive pathway ($K_i = 4\ \mu M$) leading to an oxidase which is of the aa_3 type. This conclusion is supported by evidence that in membranes from copper-limited cultures, respiration is largely insensitive to low KCN concentrations (shown in fig.3).

The cyt. aa_3 oxidase of *Chloroflexus* is thought to be responsible for catalyzing oxidation of the major part of the membrane-bound cyts *c* although the role of the different *c*-type species in electron transfer remains unclear. In this respect, the findding that the amount of cyt. *c*255 is considerably repressed in aerobic membranes compared to photosynthetic membranes is interpreted here as an indication of its insignificant involvement in respiration. A previous report [16] indicated that the direct electron donor to the photosynthetic reaction centre is a cyt. *c* with the α-band at 554 nm. Cyt. *c*554 contains two haems with $E_{m,8.0}$ of 265 and 140 mV [17]. Therefore, the haem at 255 mV found in aerobic membranes is likely to be part of the cyt. *c*554 haem complement. The lack of evidence for the presence of a *c*-type haem at 140 mV in intact membranes from *Chloroflexus* might be due to overlapping signals.

The results obtained from the CO-difference spectra show the typical pattern of peaks and troughs for cyt. *b*-CO and cyt. *a*-CO complexes (fig.1). Cyt. *a*-CO complex might be due to a mitochondrial-like haem of the *a* type, whereas the cyt. *b*-CO complex is tentatively assigned to an *o*-type cytochrome [14]. This latter cytochrome was not observed in *Chloroflexus* grown at high light intensity, probably because of the strong cyt. *c*-CO binding activity present [3,4]. Since the cytochrome redox titrations indicate that a substantial part of the *b*-type signal titrates at a high mid-point potential, it will clearly be of interest to determine the role of such *b*-type cytochrome(s) as potential oxidase(s) and as potential CO-binding component(s).

In conclusion, the present and previous results indicate that, with respect to the chemical and structural composition of its antenna system [1], *Chloroflexus* is a classic example of a green bacterium, whereas in its primary photochemistry [18] and electron transport (this work) it displays many characteristics of the non-sulphur purple bacterium *Rhodobacter sphaeroides* (cf. [2]).

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