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The membrane-bound cytochrome c_y of *Rhodobacter capsulatus* can serve as an electron donor to the photosynthetic reaction center of *Rhodobacter sphaeroides*

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Abstract

Rhodobacter capsulatus has two different pathways for reduction of the photo-oxidized reaction center, one using water-soluble cytochrome c_2, the other via membrane-associated cytochrome c_y. *Rhodobacter sphaeroides* differs in that it lacks a cytochrome c_y homologue capable of functioning in photosynthetic electron transfer; cytochrome c_2 is thus the sole electron carrier, and is required for photosynthetic (Ps^+) growth. Genetic evidence indicates that cytochrome c_y of *R. capsulatus* can complement a Ps^- cytochrome-c_2-deficient mutant of *R. sphaeroides* (Jenney, F.E. and Daldal, F. (1993) EMBO J. 12, 1283–1292). Here, we show that it transfers electrons from the cytochrome bc_1 complex to the reaction center in *R. sphaeroides*, albeit at a lower rate than that catalyzed by the endogenous cytochrome c_2. When cytochrome c_y is expressed in *R. sphaeroides* in the presence of cytochrome c_2, there is an increase in the amount of photo-oxidizible *c*-type cytochrome. In the absence of cytochrome c_2, electron transfer via cytochrome c_y shows significantly different kinetics for reaction center reduction and cytochrome *c* oxidation. These findings further establish that cytochrome c_y, the electron carrier permitting soluble cytochrome c_2-independent photosynthetic growth in *R. capsulatus*, can function in a similar capacity in *R. sphaeroides*.

Keywords: Membrane-associated *c*-type cytochrome; Photosynthetic bacterium; Trans expression; Electron transfer; Cytochrome *c*

1. Introduction

In addition to the well-known soluble electron carrier cytochrome c_2, the presence of an alternative, membrane-bound electron carrier connecting the cytochrome bc_1 complex to the photochemical reaction center has been demonstrated in *Rhodobacter capsulatus* using both genetic and biochemical means [1–5]. Jones et al. were first to indicate that a *c*-type cytochrome, called c_x was involved in this pathway [6] and that there was no direct electron transfer from the bc_1 complex to the reaction center as proposed initially [7]. The cytochrome c_x proposed by Jones et al. [6] was recently shown to be identical to the novel membrane-bound cytochrome c_y [3,4]. Cytochrome c_y functions in photosynthetic electron transfer in *R. capsulatus* both in the presence and absence of soluble cytochrome c_2 (Fig. 1A). Recent data indicate that cytochrome c_y also functions in a similar manner in respiratory electron transfer, connecting the cytochrome bc_1 complex to the terminal cytochrome oxidase [8]. On the other hand, the closely related *R. sphaeroides* differs in that photosynthetic electron transfer is mediated solely by cytochrome c_2 [9] (Fig. 1B). Thus, while the *R. capsulatus* cytochrome c_2 deletion mutant MT-G4/S4 is proficient for photosynthetic growth (i.e., Ps^+) [1], the equivalent cytochrome c_2^- *R. sphaeroides* mutant $Gadc_2$ is not (i.e., Ps^-) (see for example [9,10]). However, spontaneous

Abbreviations: cyt, cytochrome; bc_1 complex, ubihydroquinone:cytochrome c_2 oxidoreductase; kDa, kilodalton; Mops, 3-[*N*-morpholino]propanesulfonic acid; Ps, photosynthetic; Q/QH_2, quinone/quinol pool; Rc c_y, *Rhodobacter capsulatus* membrane-bound cytochrome c_y; RC, photochemical reaction center; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Spe^R, spectinomycin resistance; Tet^R, tetracycline resistance; TMBZ, 3,3′,5,5′-tetramethylbenzidine; UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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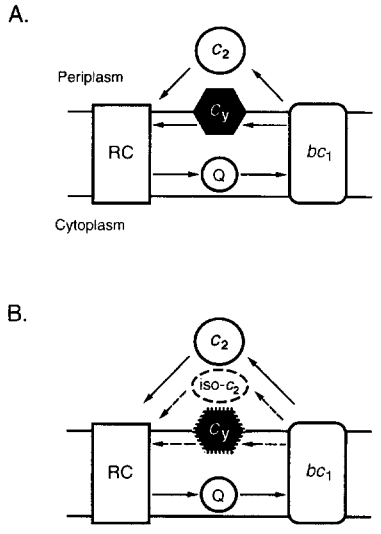

Fig. 1. Models for photosynthetic electron transport in the purple non-sulfur bacteria *R. capsulatus* (A) and *R. sphaeroides* (B). RC, bc_1, c_2, c_y, iso-c_2 and Q correspond to the photochemical reaction center, the bc_1 complex, the cytochromes c_2, c_y, and iso-c_2 and the quinone pool, respectively. Solid lines represent components and pathways present in wild-type cells, and dotted lines are those that require either a mutation (iso-c_2) or transcomplementation (c_y) for function.

Ps^+ pseudorevertants of *R. sphaeroides* cytochrome c_2^- mutants can be readily obtained, and have been shown to overproduce a low abundance soluble *c*-type cytochrome called isocytochrome c_2 [11,12] (Fig. 1B). The *R. sphaeroides* cytochrome c_2^- mutant $Gadc_2$, was utilized to clone the *R. capsulatus cycY* gene (encoding cytochrome c_y) by complementation to Ps^+ growth with an *R. capsulatus* chromosomal library [3]. The present work describes the electron transfer kinetics of *R. capsulatus* cytochrome c_y in *R. sphaeroides*, and further elucidates its role as an electron carrier between the *R. sphaeroides* reaction center and the cytochrome bc_1 complex, both in the presence, and absence of the endogenous cytochrome c_2.

2. Materials and methods

2.1. Bacterial strains and molecular genetic techniques

The various *R. sphaeroides* strains and plasmids used in this work are described in Table 1. All strains were grown semi-aerobically in Sistrom's minimal medium A as described [3]. All molecular genetic techniques have also been described previously [1,3].

2.2. Biochemical and spectroscopic techniques

R. sphaeroides cultures (1.5 liters) were grown semi-aerobically in 2-liter flasks, shaken at 140 rpm for 24 h at 35°C in the dark, followed by 6 h growth without shaking. Chromatophores [13] were prepared in 100 mM KCl, 20 mM Mops buffer (pH 7.0) using a French pressure cell as described [4]. Protein concentrations were measured by the method of Lowry et al. [14]. The 16.5% SDS-polyacrylamide gels were run according to Schägger and Von Jagow [15], and specifically stained for *c*-type cytochromes via their endogenous peroxidase activity using tetramethylbenzidine (TMBZ) and H_2O_2 as described by Thomas et al. [16]. For flash-activated kinetic spectroscopy the chromatophore concentrations were normalized to contain equivalent amounts of photobleachable photochemical reaction centers, as monitored by the wavelength pair 605–540 nm. Samples were reduced with a slight excess of sodium ascorbate and allowed to equilibrate in the dark for at least 30 min before use, poising the ambient redox potential around E_h +150 mV. Spectroscopic measurements were performed as previously described on a double-beam spectrophotometer [2,4] using the wavelength pairs of 605–540 nm for monitoring absorbance changes for the reaction center, 550–540 nm for cytochromes *c*, and 490–475 nm for the carotenoid bandshift. The membrane potential uncoupler valinomycin was used at 4 μM,

Table 1
Rhodobacter strains and plasmids used

Strain	Genotype	Phenotype	Reference
R. capsulatus			
MT-1131 [a]	*crt*D121, Rif^R	wild type	[27]
FJ1	*crt*D121, Rif^R, Δ(*cycY*::*spe*)	cyt c_y^-, Spe^R, Ps^+	[3]
R. sphaeroides			
Ga	*crt*	wild type	[23]
$Gadc_2$	*crt*, Δ(*cycA*::*spe*)	cyt c_2^-, Spe^R, Ps^-	[10]
Ga/pFJ63	*crt*, Rc *cycY*	wild type, Rc cyt c_y^+	this work
$Gadc_2$/pFJ63	*crt*, Δ(*cycA*::*spe*), Rc *cycY*	cyt c_2^-, Spe^R, Rc cyt c_y^+ Ps^+	this work
Plasmid			
pFJ63	*cycY* on 1.2 kb *Mlu* I-*Hin*dIII insert	Tet^R	[3]

[a] The *R. capsulatus* strain MT-1131 (Rif^R *crtD*) and the *R. sphaeroides* strain Ga (*crt*) are referred to as 'wild type' throughout this work as they are wild-type in terms of the relevant growth phenotypes and cytochrome complement. These strains are green derivatives (*crtD121* derivatives of *R. capsulatus* SB1003 [28,29] and *crt* for *R. sphaeroides* [28,29]) lacking the carotenoid pigments which absorb light in the same range as the *c*-type cytochromes (α-max ≈ 550 nm), making spectroscopic analysis of cytochromes simpler.

and the cytochrome bc_1 complex inhibitor antimycin (1 μM) was used, where indicated, to block electron transport in order to reveal the full extent of oxidation of the various components.

3. Results

The SDS-PAGE gel in Fig. 2 compares the *c*-type cytochrome complement of *R. capsulatus* and *R. sphaeroides* chromatophore membranes. As indicated (Lane 1), two of the four major *R. capsulatus* bands, cytochromes c_p and c_o, are subunits of the terminal *cb*-type cytochrome oxidase [17,8]. The other two are cytochrome c_1 of the bc_1 complex, and cytochrome c_y, which is readily identified by comparing the wild-type *R. capsulatus* strain (MT-1131, lane 1) and its cytochrome c_y^- derivative (FJ1, lane 2) [4]. The soluble cytochromes c_2 and c', which are trapped in chromatophore vesicles, are also present in these membrane preparations, but the region of the gel where they are located is not shown in this figure. Other than cytochrome c_2, the identities of the major *R. sphaeroides* heme-stainable bands (Ga, Lane 3) are not known, but they probably represent cytochrome c_1 [18] and the two cytochromes of the *R. sphaeroides cb*-type cytochrome *c* oxidase [19]. Introduction of the *R. capsulatus cycY* gene encoding cytochrome c_y into *R. sphaeroides* (Ga/pFJ63 and $Gadc_2$/pFJ63, lane 4 and 5, respectively) results in the appearance of a new, approximately 30 kDa band in heme-stained SDS-PAGE gels (see also [3]). As reported earlier, expression of cytochrome c_y causes an increase in the total amount of *c*-type cytochrome, based on reduced-minus-oxidized absorption difference spectra, only in the membrane fraction and not in the soluble fraction in both species [3]. It was further shown that cytochrome c_y is present at higher levels in *R. sphaeroides* strains carrying plasmid pFJ63 than in the equivalent *R. capsulatus* strains, based on these spectra [3]. The heme stain being not precisely quantifiable, this increased level is not immediately obvious in SDS-PAGE gels stained to saturation with TMBZ for heme detection (Fig. 2, compare lanes 1 and 4). Finally, this figure demonstrates that cytochrome c_y is produced in *R. sphaeroides* to similar amounts in the presence or absence of the endogenous cytochrome c_2 (compare lanes 4 and 5).

In order to assess the role of *R. capsulatus* cytochrome c_y as an electron carrier in *R. sphaeroides*, the kinetics of electron transfer reactions between the reaction center and the *c*-type cytochromes, and the electrogenic events accompanying these reactions were monitored in chromatophores prepared from the appropriate strains (Fig. 3). Antimycin and valinomycin were present in those experiments that monitored the redox events associated with the reaction center and *c*-type cytochromes. Oxidation is seen as a decrease in absorbance at the appropriate wavelength and reduction as an increase. Fig. 3 rows A and B show

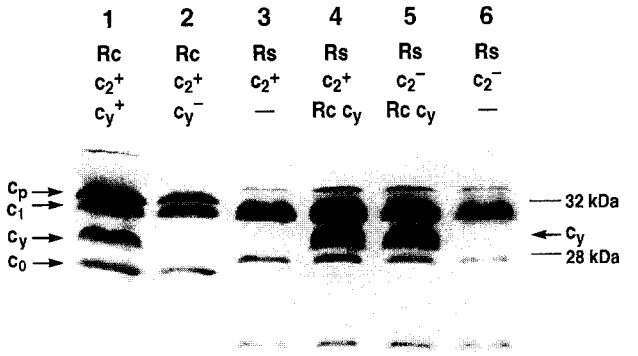

Fig. 2. SDS-PAGE gel (16.5%, according to Schägger and Von Jagow [15]) stained for *c*-type cytochromes by the method of Thomas et al. [16]. Approximate molecular mass markers are in kDa, and each lane contains 250 μg total protein. The approx. 30 kDa bands associated with cytochrome c_1 and cytochrome c_y [3], and the two *c*-type cytochrome components of the terminal cytochrome *c* oxidase of *cb*-type, c_p and c_o [17] are indicated by arrows. All chromatophores are derived from minimal Medium A-grown cells. **Lane 1**: wild-type *R. capsulatus* MT-1131 (cyt c_2^+, cyt c_y^+); **Lane 2**: *R. capsulatus* FJ1 (cyt c_2^+, cyt c_y^-); **Lane 3**: wild-type *R. sphaeroides* strain Ga (cyt c_2^+); **Lane 4**: *R. sphaeroides* Ga/pFJ63 (cyt c_2^+, Rc cyt c_y^+); **Lane 5**: *R. sphaeroides* $Gadc_2$/pFJ63 (cyt c_2^-, Rc cyt c_y^+); **Lane 6**: *R. sphaeroides* $Gadc_2$ (cyt c_2^-).

the absorbance changes associated with the reaction center and the *c*-type cytochromes, respectively.

Row A shows the responses of the reaction center primary donor (bacteriochlorophyll special pair, $P \rightarrow P^+$). In the wild-type strain (Ga), the first flash in the trace induces a rapid photo-oxidation of the reaction center special pair which is followed by a rapid re-reduction by a *c*-type cytochrome, faster than can be measured in this device [20]. In the absence of inhibitors, the oxidized cytochromes would be re-reduced by the cytochrome bc_1 complex. However, under these conditions where antimycin (which inhibits electron transfer within the cytochrome bc_1 complex) is present, reduction is restricted to electron donation from the Rieske iron-sulfur center only. Re-reduction of photo-oxidized cytochrome *c* thus ceases after one or two turnovers, and its full oxidation can be seen. Once the cytochromes are fully oxidized there is no further rapid electron donation to the photo-oxidized reaction centers, and the full extent of reaction center oxidation is then revealed after the fourth flash. As expected, essentially no reaction center re-reduction is detectable in the Ps^- mutant $Gadc_2$, while an appreciable amount of reaction center re-reduction is present when the same mutant harbors *R. capsulatus cycY* (strain $Gadc_2$/pFJ63) which is consistent with its Ps^+ growth phenotype (Table 1). Note that the amount of reaction center re-reduction in the latter *R. sphaeroides* strain is less than that in the strains containing cytochrome c_2, similar to the data seen with the cytochrome c_2^- cytochrome c_y^+ Ps^+ *R. capsulatus* strain MT-G4/S4 [4]. Expression of cytochrome c_y in the presence of cytochrome c_2 (Ga/pFJ63) results in an approx.

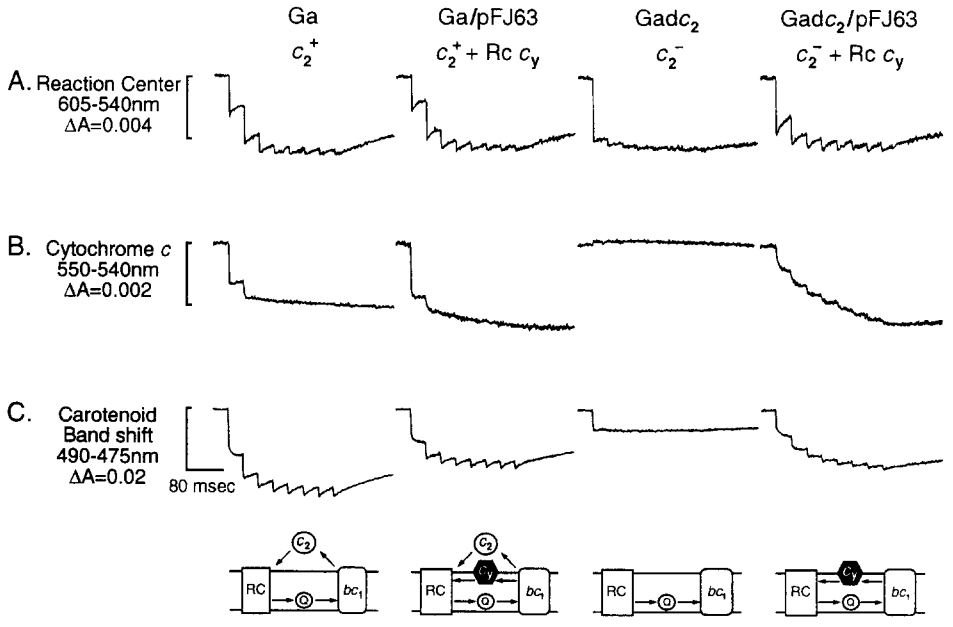

Fig. 3. Flash-induced electron transfer kinetics of the various components present in chromatophore membranes, prepared from cells grown semi-aerobically in Sistrom's minimal Medium A, resuspended in 100 mM KCl, 20 mM Mops (pH 7.0) and reduced by addition of sodium ascorbate. Traces are absorbance changes in response to a train of eight saturating actinic flashes, experimental conditions are as described in Materials and Methods. Responses are: from row A, reaction center (605–540 nm); row B, cytochrome *c* (550–540 nm); and **Row C**, carotenoid bandshift (490–475nm). Ga: wild-type; Ga/pFJ63 (Rc cyt c_y^+); Gadc$_2$ (cyt c_2^-); Gadc$_2$/pFJ63 (Rc cyt c_y^+ cyt c_2^-). Measurements of the carotenoid bandshift were made in the absence of inhibitors and uncouplers; 1 μM antimycin and 4 μM valinomycin were present during the other measurements.

20% increase in the extent of reaction center re-reduction after the initial flash.

The cytochrome traces in row B of Fig. 3 demonstrate that in the absence of cytochrome c_2 (Gadc$_2$), there is no photo-oxidizible *c*-type cytochrome detectable under the growth conditions used here, consistent with previous reports [9]. In the wild-type strain (Ga), cytochrome c_2 is oxidized by the reaction center, but in the presence of antimycin it is only reduced by the cytochrome bc_1 complex after the first flash, and to a very small extent after the second flash. Expression of *R. capsulatus* cytochrome c_y in a wild-type *R. sphaeroides* background (Ga/pFJ63, i.e., in the presence of cytochrome c_2) results in an approximately 38% increase in the total amount of flash-oxidizible *c*-type cytochromes. Interestingly, re-reduction of the *c*-type cytochrome pool still occurs mainly after the first flash in this strain, similar to the wild type, but different from the situation in wild-type *R. capsulatus* [4]. Cytochrome oxidation in the wild-type *R. capsulatus* strain MT-1131 resembles a mixture of the traces observed with a strain containing only cytochrome c_2 (such as FJ1) and the cytochrome c_y only strain MT-G4/S4 [4]. On the other hand, in the absence of cytochrome c_2, cytochrome c_y alone behaves much differently in *R. sphaeroides*. In Gadc$_2$/pFJ63 the cytochrome c_y pool requires all eight flashes to be completely photo-oxidized, and the oxidation is much slower (Fig. 3 row B).

Fig. 3 row C exhibits the carotenoid band shift (monitored at 490–475 nm) in the absence of inhibitors and ionophores. The carotenoid bandshift is a response to the transmembrane potential generated by electron transfer [20]. As expected, only Phase I of the bandshift is visible in Gadc$_2$, concomitant with the oxidation of the reaction center after the first flash. In the wild-type Ga, Phase I is followed by Phase II, which represents cytochrome oxidation, and then the rather slower Phase III, due to electronic events in the cytochrome bc_1 complex and approximately equal in extent to the sum of Phases I + II. The addition of cytochrome c_y in Ga/pFJ63 has little effect on the ratios of the bandshift phases, but it is noteworthy that the presence of cytochrome c_y in the cytochrome c_2 deletion strain Gadc$_2$/pFJ63 allows a substantial Phase III, indicating that cytochrome c_y is catalyzing electron flow from the cytochrome bc_1 complex to the reaction center (Fig. 4). As expected, addition of the cytochrome bc_1 complex inhibitor UHDBT eliminates the slow Phase III of the bandshift in this strain. Note that the data related to the

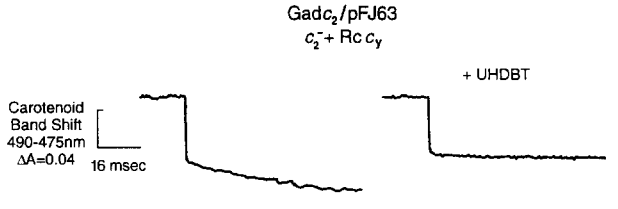

Fig. 4. Single flash-induced carotenoid bandshift in membranes of *R. sphaeroides* Gadc$_2$/pFJ63 (Rc cyt c_y^+ cyt c_2^-). The first trace represents the average of four single flashes with a time constant of 1 ms in the absence of inhibitors, the second trace is in the presence of approx. 1 μM UHDBT.

carotenoid bandshift are shown only to illustrate the relative contributions of the three phases in the various mutants. The absolute size of the bandshift varies with the amount of the light-harvesting II complex, which is dependent on subtle differences in oxygenation during growth.

4. Discussion

Although the heme-stained SDS-PAGE patterns of the chromatophore membranes in Fig. 2 are different, the *c*-type cytochrome complement of *R. capsulatus* and *R. sphaeroides* are similar in several ways. In the 32 to 28 kDa range the former species has at least four, and the latter species at least three major *c*-type cytochrome bands. Of these bands, one corresponds to the cytochrome c_1 subunit of the cytochrome bc_1 complex in both cases [21,18]. Both species contain a terminal cytochrome *c* oxidase of *cb*-type which has two *c*-type cytochrome subunits [17,19], though *R. sphaeroides* also possesses a terminal cytochrome aa_3 oxidase which has not been found in *R. capsulatus* [22]. As the chromatophores used in this work were derived from semi-aerobically grown cells, the *cb*-type cytochrome *c* oxidase is likely to be present [19]. Thus, two of these heme-stainable bands would represent the two *c*-type cytochrome subunits of this complex. On the other hand, a striking difference between these two closely related species is the absence in *R. sphaeroides* of the approx. 30 kDa band corresponding to the cytochrome c_y of *R. capsulatus* (Fig. 2). The identity of the remaining minor bands, and in particular that of the approx. 20 kDa band seen only in *R. sphaeroides* (Fig. 2, lanes 3 to 6), is unknown. Whether this latter is a degradation product, or it corresponds to an uncharacterized *c*-type cytochrome remains to be seen.

The data presented in Fig. 3 demonstrate that *R. capsulatus* cytochrome c_y can transfer electrons to the flash-oxidized *R. sphaeroides* reaction center in the cytochrome c_2^-, cytochrome c_y^+ strain $Gadc_2$/pFJ63 (row B). These data are consistent with the Ps^+ phenotype of this strain. As expected, the cytochrome c_2^- Ps^- strain $Gadc_2$ shows no photo-oxidation of *c*-type cytochrome. On the other hand, similar to the situation in the cytochrome c_2^- cytochrome c_y^+ *R. capsulatus* strain MT-G4/S4 [2,4], cytochrome c_y reduces the reaction center to a lesser extent than cytochrome c_2 after each flash in $Gadc_2$/pFJ63, and these reaction centers continue to function until the cytochrome c_y pool is depleted of electrons (Fig. 3 row A). Note that the kinetics of cytochrome c_y oxidation are quite different from those of cytochrome c_2 (Fig. 3). Furthermore, the increase in cytochrome *c* oxidation is approximately twice that of the increase in the amount of reaction center reduction upon addition of cytochrome c_y in Ga/pFJ63, possibly due to other reductants present in the chromatophore preparations. Since the cytochrome bc_1 complex inhibitor antimycin was present during these experiments, it is unlikely that cytochrome c_y is being re-reduced by the cytochrome bc_1 complex after each of the eight flashes in $Gadc_2$/pFJ63. Thus, the slow kinetics of oxidation indicate that cytochrome c_y is a poor substrate for the *R. sphaeroides* cytochrome bc_1 complex under these conditions, as it is not re-reduced even after the first two flashes. In contrast, almost 100% of cytochrome c_y is re-reduced after the first flash in the similar *R. capsulatus* strain MT-G4/S4 [2,4]. The observation that cytochrome c_y appears to be a "poorer substrate" for the *R. sphaeroides* cytochrome bc_1 complex than for that of *R. capsulatus* is reminiscent of the inability of an *R. capsulatus* double mutant lacking both the cytochrome bc_1 complex and cytochrome c_2, and complemented with *R. sphaeroides* cytochrome bc_1 complex (MT-GS18/pGAB294) to grow photosynthetically on minimal media, as observed previously [23].

Although expression of cytochrome c_y in *R. sphaeroides* Ga/pFJ63 causes an increase in the photo-oxidizible cytochrome *c* pool, cytochrome c_2 appears to be the preferred donor to the reaction center when both cytochromes are present (Ga/pFJ63) based on the similarity of the trace to that of wild type (Ga). Nonetheless, cytochrome c_y is present to the same extent both in the presence and absence of cytochrome c_2, based on the heme-stained SDS-PAGE analysis (Fig. 2). This is in contrast to the data for wild-type *R. capsulatus* MT-1131, where cytochrome c_y and cytochrome c_2 each represent approximately 50% of the photo-oxidizible *c*-type cytochrome pool [4]. Thus, when both carriers are artificially present in *R. sphaeroides*, the photosynthetic electron transfer chain is not branched to as great an extent as that in *R. capsulatus* (Fig. 1).

No evidence yet exists for a cytochrome c_y homologue in *R. sphaeroides* that can function in either photosynthesis or respiration. Cytochrome c_y homologues of the non-photosynthetic bacteria such as cytochrome *c*-552 in *Paracoccus denitrificans* [24] and CycM in *Bradyrhizobium japonicum* [25] are of approximately 20 kDa molecular mass, and are believed to connect the cytochrome bc_1 complex to a terminal cytochrome *c* oxidase [26,25]. Cytochrome c_y has also a role in respiratory electron transfer in *R. capsulatus* [8], but during aerobic growth *R. sphaeroides* uses a cytochrome aa_3-type terminal oxidase [22,19], unlike *R. capsulatus* whose growth depends on a *cb*-type cytochrome *c* oxidase [17]. *R. sphaeroides* also contain a similar *cb*-type cytochrome *c* oxidase when grown under microaerophilic conditions [19] and may also possess a cytochrome c_y homologue that functions as an electron donor to cytochrome oxidase. However, whether the induction of this oxidase is accompanied by that of a cytochrome c_y homologue, and whether this corresponds to the *c*-type cytochrome of approx. 20 kDa seen in Fig. 2 is not yet clear.

Finally, it should be stressed that how membrane-attached electron carriers like cytochrome c_y interact with

their membrane-attached physiological partners in *Rhodobacter* species, and how these bacteria regulate electron transfer chain components in response to environmental changes are completely unknown, and need to be studied to obtain a more complete picture of electron transfer pathways.

5. Note added in proof

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Very recently, a gene highly homologous to *R. capsulatus cycY* has been found in *R. sphaeroides* and has kindly been made available to us by Dr. S. Kaplan.

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