Roles of the Soluble Cytochrome c_2 and Membrane-Associated Cytochrome c_y of *Rhodobacter capsulatus* in Photosynthetic Electron Transfer†

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ABSTRACT: Genetic evidence indicates that *Rhodobacter capsulatus* has two different pathways for reduction of the photooxidized reaction center (RC) [Jenney, F. E., & Daldal, F. (1993) *EMBO J. 12*, 1283–1292]. One pathway is via the water soluble cytochrome (cyt) c_2, and the other is via a novel, membrane-associated *c*-type cytochrome, cyt c_y, now believed to be identical to the cyt c_x of Jones et al. [Jones, M. R., et al. (1990) *Biochim. Biophys. Acta 975*, 59–66] and c_{354} of Zannoni et al. [Zannoni, D., et al. (1992) *Arch. Microbiol. 157*, 367–374]. Mutants lacking either cyt c_2, cyt c_y, or the bc_1 complex, as well as various combinations, were utilized to probe the functional role of these cytochromes in electron transfer. Data obtained by monitoring flash induced electron transfer kinetics in the RC, cyt *c* pool, cyt *b*, and the carotenoid band shift indicate that there are two pathways for electron transfer from the bc_1 complex to the RC in *R. capsulatus*, one via cyt c_2 and the other through cyt c_y. The two pathways show strikingly different kinetics for RC reduction and cyt *c* oxidation, and both are present in the wild-type strain MT-1131. After genetic inactivation of both cyt c_2 and cyt c_y there remains no flash oxidizible *c*-type cytochrome, and inactivation of cyt c_y rather than cyt c_2 has a more pronounced effect on the extent of the light-induced membrane potential under the conditions tested. Finally, heme-stained SDS–PAGE and flash spectroscopy experiments indicate that cyt c_y is detectable in strains lacking the bc_1 complex when grown on minimal growth medium but not on rich medium. These findings complement the earlier genetic data and further establish that cyt c_y is the electron carrier permitting soluble cyt c_2-independent photosynthetic growth in *R. capsulatus*.

The purple, nonsulfur Gram-negative facultative photosynthetic bacteria, in particular *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, are exceptional model systems for elucidating the identities and functions of proteins involved in electron transport (Clayton & Sistrom, 1978; Prince, 1990; Deisenhofer & Michel, 1991; Gennis et al., 1993; Daldal & Zannoni, 1993) using both biochemical and genetic tools (Daldal, 1990; Scolnik & Marrs, 1987; Donohue & Kaplan, 1991). Specific inactivation of genes encoding electron transfer proteins and examination of the resulting effects on either the respiratory or photosynthetic electron transport chain in either whole cells or inside-out membrane vesicles (chromatophores) is readily feasible in this system (Daldal, 1990; Scolnik & Marrs, 1987; Donohue & Kaplan, 1991). Previous studies have clearly demonstrated that the genetic elimination of the quinol:cyt c_2 oxidoreductase (bc_1 complex)[1] leads to the arrest of photosynthesis (Ps^-) without affecting respiration (Daldal, 1987; Marrs & Gest, 1973; Baccarini-Melandri & Zannoni, 1978). However, in the absence of cyt c_2, *R. capsulatus* still grows photosynthetically via an alternate electron carrier functioning between the bc_1 complex and the reaction center to complete cyclic electron flow (Daldal et al., 1986; Figure 1).

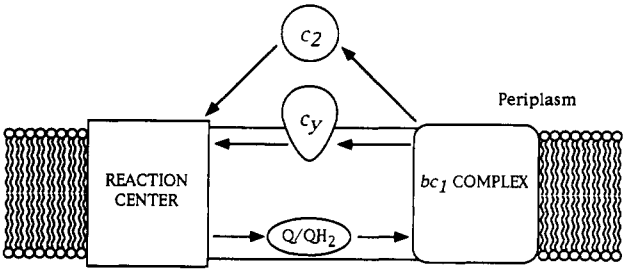

FIGURE 1: Model of the proposed branched photosynthetic electron transport chain of *R. capsulatus*. Reaction center, photochemical reaction center; Q/QH_2, quinone/quinol pool; bc_1 complex, quinol:cyt c_2 oxidoreductase; c_y, membrane-associated cytochrome c_y; c_2, soluble cytochrome c_2. Arrows indicate direction of electron flow. The figure as drawn indicates the branched electron transfer chain in wild-type *R. capsulatus* (MT-1131), where both cyt c_2 and cyt c_y connect the bc_1 complex to the reaction center (see text). The electron transfer chain in *R. capsulatus* strain FJ1 (cyt c_y^-), as well as that in wild-type *R. sphaeroides* [where, barring a mutation which overexpresses the soluble isozyme of cyt c_2, there appears to be no membrane-associated homologue of cyt c_y (Donohue et al., 1988; Jenney & Daldal, 1993)] utilize only the soluble cyt c_2. The electron transfer chain in *R. capsulatus* MT-G4/S4 (cyt c_2^-) utilizes only the membrane-bound carrier cytochrome c_y. This state can also be constructed in a cyt c_2^- derivative of *R. sphaeroides* by providing the *R. capsulatus cycY* gene in *trans* (Jenney & Daldal, 1993). It is possible that in wild-type *R. capsulatus* there are two separate pools of bc_1 complexes and reaction centers, one connected by only cyt c_2 and the other by only cyt c_y, separated either physically or functionally. The nature of the interactions between cyt c_y and both the bc_1 complex and the reaction center is presently unknown.

Various lines of evidence implicated a membrane-bound carrier in electron transfer between the bc_1 complex and the reaction center (Prince et al., 1986; Prince & Daldal, 1987; Jones et al., 1990) rather than another water soluble,

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[1] Abbreviations: cyt, cytochrome; bc_1 complex, quinol:cytochrome c_2 oxidoreductase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ps, photosynthetic; Q/QH_2, quinone/quinol pool; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethylbenzidine; UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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Table 1: *Rhodobacter* Strains Used

strain	genotype	phenotype	source
MT-1131	*crt*D121, Rif^R	wild type[a]	Davidson et al. (1987)
MT-G4/S4	*crt*D121, Rif^R, Δ(*cycA*::*kan*)	cyt c_2^-, Ps$^+$	Daldal et al. (1986)
MT-CC1	*crt*D121, Rif^R, Δ(*fbcC*::*kan*)	cyt c_1^-, Ps$^-$	Tokito and Daldal (1992)
MT-GS18	*crt*D121, Rif^R, Δ(*cycA*::*kan*), Δ(*fbcBC*::*spec*)	cyt c_2^-, cyt bc_1^-, Ps$^-$	Prince and Daldal (1987)
FJ1	*crt*D121, Rif^R, Δ(*cycY*::*spe*)	cyt c_y^-, Ps$^+$	Jenney and Daldal (1993)
FJ2	*crt*D121, Rif^R, Δ(*cycA*::*kan*), Δ(*cycY*::*spe*)	cyt c_2^-, cyt c_y^-, Ps$^-$	Jenney and Daldal (1993)
FJ3	*crt*D121, Rif^R, Δ(*fbcC*::*kan*), Δ(*cycY*::*spe*)	cyt c_1^-, cyt c_y^-, Ps$^-$	this work

[a] MT-1131 is referred to as "wild type" throughout this work as it is wild type in terms of the relevant growth phenotypes and cytochrome complement.

periplasmic protein such as the isocytochrome c_2 described in the closely related *R. sphaeroides* (Donohue et al., 1988). Recent genetic work (Jenney & Daldal, 1993) identified this component as a novel membrane-associated *c*-type cytochrome cyt c_y. Inactivation of either *cycA* (encoding cyt c_2) or *cycY* (encoding cyt c_y) alone has no major effect on Ps$^+$ growth (Daldal et al., 1986; Jenney & Daldal, 1993), although inactivation of both results in a Ps$^-$ phenotype, which is complemented to Ps$^+$ growth by either *cycA* or *cycY* provided in *trans* (Jenney & Daldal, 1993). The predicted amino acid sequence, reduced minus oxidized absorption difference spectra, and heme-stained SDS–PAGE analysis suggest that cyt c_y is a novel membrane-associated *c*-type cytochrome (Jenney & Daldal, 1993). These genetic and biochemical data strongly support the hypothesis that cyt c_y functions in the photosynthetic electron transport chain of *R. capsulatus*.

In the present work we utilize a number of deletion strains, which lack alone or in some combination the bc_1 complex, cyt c_2, or cyt c_y, and kinetic (flash) absorption spectroscopy to measure the responses of various components of the electron transport chain to a train of actinic flashes. We find that cyt c_y can act as a donor to the photooxidized reaction center in both the presence and the absence of cyt c_2 and that it is probably identical to the cyt c_x described by (Jones et al. (1990) and the c_{354} by Zannoni et al. (1992). We now resolve for the first time the kinetics of cyt c_2 versus cyt c_y oxidation and reduction in both the presence and the absence of the bc_1 complex. We show that these kinetics are significantly different and that under the experimental conditions used here cyt c_2 and cyt c_y are the only reductants of the reaction center in *R. capsulatus*. We also demonstrate that in the absence of the bc_1 complex the presence of cyt c_y in chromatophore membranes is conditionally dependent on the nature or composition of the growth medium used. Although the molecular basis of this observation is unknown, it reconciles the earlier conflicting spectroscopic experiments using the same strains (Prince & Daldal, 1987; versus Jones et al., 1990; Zannoni et al., 1992).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Molecular Genetic Techniques. The various *R. capsulatus* strains used in this work are described in Table 1. All strains were grown aerobically in either Sistrom's minimal medium A or in enriched MPYE medium as described (Jenney & Daldal, 1993). All molecular genetic techniques have also been previously described (Daldal et al., 1986; Jenney & Daldal, 1993).

Biochemical and Spectroscopic Techniques. Chromatophores (Clayton & Sistrom 1978) were prepared in 20 mM MOPS buffer, pH 7.0/100 mM KCl using a French pressure cell as described (Jenney & Daldal, 1993). Protein concentrations were measured by the method of Lowry (Lowry et al., 1951), and 16.5% SDS–polyacrylamide gels were run according to Schägger & von Jagow (1987) 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. (1976). For flash-activated kinetic spectroscopy the chromatophore bacteriochlorophyll concentrations were determined using a molar extinction coefficient of $\epsilon_{mM855} = 100\ mM^{-1}\ cm^{-1}$, and chromatophores were diluted to approximately 20 μM bacteriochlorophyll. Samples were subjected to a train of eight actinic flashes (greater than 90% saturating) and 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 $E_h \approx +150$ mV. Spectroscopic measurements were performed as previously described on a double-beam spectrophotometer (Prince et al., 1986) using the wavelength pairs of 605–540 nm for monitoring changes in the reaction center, 550–540 nm for cytochromes *c*, 560–572 nm for cytochrome *b*, and 490–475 nm for the carotenoid band shift. The redox titration of flash-activated cytochrome *c* oxidation was performed as previously described (Prince et al., 1986) in 20 mM MOPS, pH 7.0/100 mM KCl. The membrane potential uncoupler valinomycin was used at 4 μM, and several inhibitors of bc_1 complex turnover such as antimycin (1 μM), myxothiazol (1 μM), and UHDBT (≈1 μM) were used where indicated to block electron transport in order to reveal the full extent of oxidation of the various components.

RESULTS

Photosynthetic Electron Transport via Cyt c_2 and Cyt c_y. The goal of this study was to examine the roles of cyt c_2 and cyt c_y in photosynthetic electron transfer. Using genetic techniques (Daldal et al., 1986; Jenney & Daldal, 1993), we previously documented the existence of two distinct electron carriers, cyt c_2 and cyt c_y, between the bc_1 complex and the reaction center (Figure 1). In this work several appropriate strains (MT-1131 wild type; FJ1 cyt c_y^-; MT-G4/S4 cyt c_2^-; FJ2 cyt c_y^- cyt c_2^-; FJ3 cyt c_y^- cyt c_1^-; and MT-GS18 cyt c_1^- cyt c_2^-) were utilized to dissect these dual pathways. The genotypes and photosynthetic growth phenotypes of these strains are summarized in Table 1. The strains FJ2 and MT-GS18 demonstrate that at least one of the two genes, *cycA* (cyt c_2) or *cycY* (cyt c_y), as well as the bc_1 complex are required for photosynthetic growth of *R. capsulatus* (Jenney & Daldal, 1993; Daldal et al., 1987).

The kinetics of electron transfer between the various components involved (Figure 1), and the electrogenic events accompanying these reactions, were monitored in chromatophores derived from the different strains in the presence of antimycin and valinomycin (except for the carotenoid band shift measurements where no inhibitors were used). Figure 2 shows the absorbance changes of the photochemical reaction

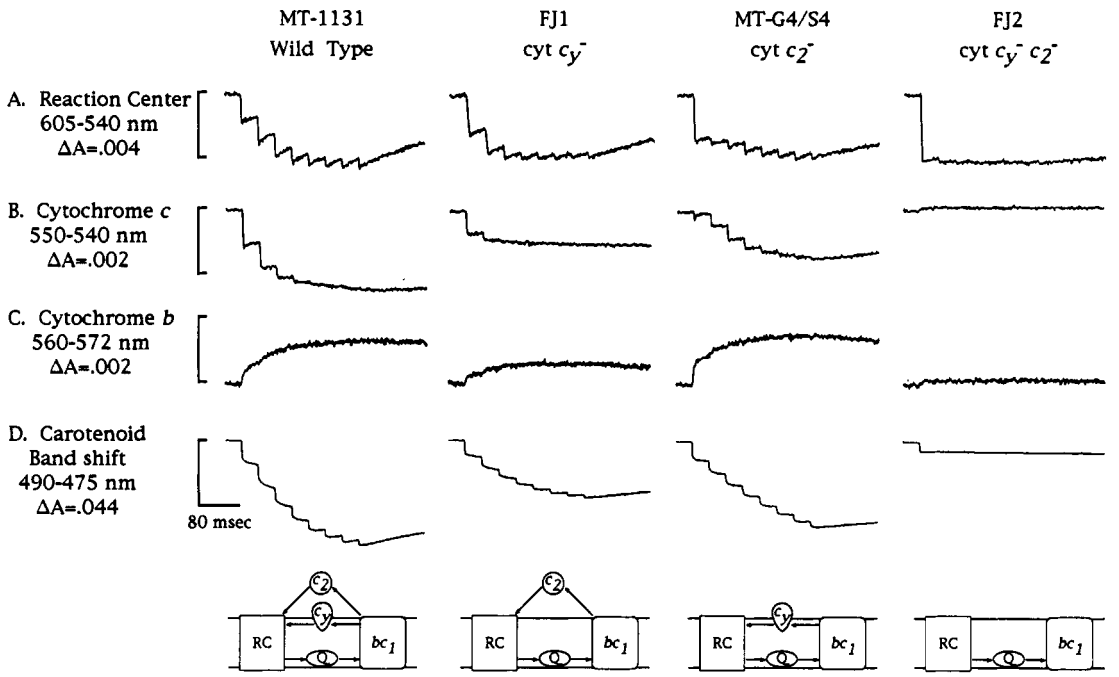

FIGURE 2: Flash-induced electron transfer kinetics of the various components present in chromatophore membranes in 20 mM MOPS, pH 7.0/100 mM KCl. Traces are absorbance changes in response to a train of eight saturating actinic flashes, conditions as described under Experimental Procedures. Chromatophores were prepared from cells grown semi-aerobically in Sistrom's minimal medium A. Responses are from (Row A) reaction center (605–540 nm); (Row B) cytochrome *c* (550–540 nm); (Row C) cytochrome *b* (560–572 nm); and (Row D) carotenoid bandshift (490–475 nm). MT-1131, wild type; FJ1, cyt c_y^-; MT-G4/S4, cyt c_2^-; FJ2, 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. At the bottom are figures adapted from Figure 1 depicting a model of the electron transport chain in each strain. RC, photochemical reaction center; Q, quinone/quinol pool; bc_1, quinol:cyt c_2 oxidoreductase; c_y, membrane-associated cytochrome c_y; c_2, soluble cytochrome c_2. Arrows indicate direction of electron flow.

center, the *c*-type cytochromes, and the *b*-type cytochromes in response to a train of eight saturating (12 μs at half-width) actinic flashes spaced 32 ms apart, with oxidation showing as a decrease in absorbance at the appropriate wavelength, reduction as an increase. Row A shows the responses of the reaction center primary donor (bacteriochlorophyll special pair, $P \rightarrow P^+$). The traces represent, for each flash, a rapid oxidation (bleaching of 605 nm) followed by a rapid re-reduction (by a *c*-type cytochrome, cyt c_2 or cyt c_y, see below) faster than can be measured with this device; thus it is impossible to see the complete extent of reaction center oxidation on any flash. This is followed by a slower reduction of some of the remaining oxidized reaction centers. The data for the wild-type strain MT-1131 and the cyt c_2^- strain MT-G4/S4 are essentially identical to those reported previously (Prince & Daldal, 1987) and are only included here for comparison.

The traces for strain FJ1 (cyt c_y^-) indicate that the kinetics of reaction center re-reduction differ from wild type and MT-G4/S4 (cyt c_2^-) (Figure 2, row A). As previously observed in both chromatophores and whole cells (Prince et al., 1986), the extent of reaction center re-reduction after the first flash is less in MT-G4/S4 (here 33% of total reaction center oxidation) than in wild type (62%). However, on the second and third flashes an almost equal amount of reaction center re-reduction was observed with MT-G4/S4 whereas the amount of reaction center re-reduction in wild type is decreased by about 50% with each consecutive flash. FJ1 behaves qualitatively more like wild type than MT-G4/S4 in that there is more reaction center re-reduction after the first flash in FJ1, and this amount decreases substantially with each consecutive flash. However, in FJ1 there is significantly less re-reduction of reaction center on the first flash than in wild type. Consistent with its Ps^- phenotype, the trace for FJ2 (cyt c_2^- c_y^-) shows no reaction center re-reduction after any flash, indicating no reductant is present.

The kinetics of cyt *c* oxidation in FJ1 (cyt c_y^-) are also more similar to wild type than to MT-G4/S4 (Figure 2, row B). As in wild type the oxidized cyt c_2 is not completely re-reduced after the first flash (as expected in the presence of antimycin). However, similar to MT-G4/S4 under these conditions, there is significantly less oxidizable cytochrome *c* in FJ1 than in MT-1131, approximately 50% based on the total amount of *c*-type cytochrome oxidation. As observed previously (Prince et al., 1986), after the first flash in MT-G4/S4 virtually all of the photooxidized *c*-type cytochromes are re-reduced in the presence of antimycin, and only on subsequent flashes are the *c*-type cytochromes re-reduced to a lesser extent.

In FJ2 (cyt c_2^- c_y^-), consistent with the lack of reaction center re-reduction, no significant cyt *c* oxidation is observed (Figure 2, row B). FJ2 does, however, contain an active bc_1 complex based on a functional assay using an artificial substrate as in Atta-Asafo-Adjei and Daldal, (1991) and on Western blots using monoclonal antibodies against cyt c_1 (data not shown). These flash data indicate that there are no carriers other than cyt c_2 and cyt c_y connecting the bc_1 complex and the reaction center under these conditions. This is further demonstrated by rows C and D of Figure 2 which show that there is also no cyt *b* reduction and that only phase I of the carotenoid band shift is present. Phase I represents charge separation due to the oxidation of the reaction center bacteriochlorophyll special pair by light. Therefore, in contrast to what we have suggested previously and in agreement with Jones et al. (1990) and Zannoni et al. (1992), the bc_1 complex cannot directly donate electrons to the reaction center in these in situ conditions.

Flash data obtained using chromatophores of the four strains depicted in Figure 2 are essentially the same whether the cells

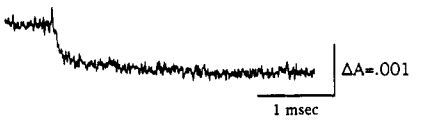

FIGURE 3: Flash-induced oxidation kinetics of cytochrome c_2 (550–540 nm) in chromatophores from enriched MPYE grown FJ3 cells (cyt c_1^- cyt c_y^-) in the presence of 4 μM valinomycin and $\approx$1 μM UHDBT. The trace represents an averaging of 100 single flashes at a time constant of 3 μs. The estimated half-time of oxidation of cyt c_2 (the sole remaining flash-oxidizible c-type cytochrome) is $t_{1/2} \approx$ 250 μs.

are grown semiaerobically or photosynthetically (except the Ps$^-$ FJ2) in MPYE broth (data not shown). The only major difference observed was that the overall extent of cyt b reduction is lower in chromatophores derived from MPYE semiaerobically grown cells relative to the same strains grown semiaerobically in minimal medium A (data not shown). Figure 2 rows C and D demonstrate the responses of cyt b of the bc_1 complex and the carotenoid band shift respectively in these four strains. FJ1 (cyt c_y^-) shows an apparent decrease of $\approx$40% in both traces relative to wild type and MT-G4/S4. This difference is reproducible from one batch of strains to another, although less extensive in chromatophores derived from cells grown in enriched MPYE medium (data not shown). Whether this effect is due to an artifact of chromatophore preparation, a change in the amount of functioning bc_1 complex, or less connection between the bc_1 complex and cyt c_2 is not yet known. As it has been shown that ionic strength can affect the interaction between the soluble cyt c_2 and both the photosynthetic reaction center and the bc_1 complex (Overfield & Wraight, 1980; Knaff et al., 1990; Güner et al., 1993), chromatophores from both wild-type MT-1131 and FJ1 (cyt c_y^-) were prepared in 2 mM MOPS, pH 7.0/10 mM KCl. The carotenoid bandshifts in these two strains are essentially the same in both the lower and higher ionic strength buffers, although a little more cyt b reduction is observed in chromatophores isolated in the lower ionic strength buffer (data not shown), indicating that this observation is probably not due to ionic strength effects.

Figure 3 demonstrates for the first time the rate of oxidation of cyt c_2 alone in *R. capsulatus* with an average of 100 single flashes measured at 550–540 nm in the strain FJ3, which lacks both cyt c_1 and cyt c_y. At this resolution the trace indicates the half-time of cyt c_2 oxidation to be approximately $\leq$250 μs, which is slower than the $t_{1/2} \leq 100$ μs observed earlier with MT-G4/S4 (Prince et al., 1986), originally attributed to cyt c_1 oxidation but now known to be a combination of cyt c_1 and cyt c_y oxidation.

Effect of Inhibitors of the bc_1 Complex on Cyt c_y Oxidation. The bc_1 complex is essential for photosynthetic growth (Daldal et al., 1987), and a number of inhibitors active at different sites of this enzyme are known. Antimycin binds at the Q_i (or quinone reduction) site and inhibits the oxidation of cyt b, myxothiazol binds and inhibits at the Q_o (or quinol oxidation) site, and UHDBT inhibits electron transfer from the Rieske iron–sulfur protein to cyt c_1 of the complex (Crofts, 1985). Thus using these inhibitors, one can control the number of electrons flowing through the bc_1 complex. For example, antimycin allows only a single turnover at the Q_o site as the Q cycle requires a counterpart reduction of a quinone at the Q_i site (Robertson & Dutton, 1988; Trumpower, 1990; Mitchell, 1975; Prince, 1990). Using myxothiazol provides one less electron as the Q cycle is now completely blocked, and UHDBT leaves only one electron from cyt c_1 available for reducing the carriers cyt c_2 or cyt c_y.

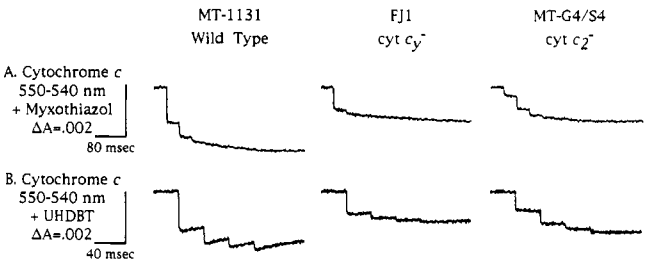

FIGURE 4: Traces are absorbance changes in response to a train of eight saturating actinic flashes (row A) or 4 flashes (row B), conditions as described under Experimental Procedures. Chromatophores were prepared from cells grown semiaerobically in Sistrom's minimal medium A. Responses are from cytochrome c (550–540 nm) in the presence of (Row A) 1 μM myxothiazol; (Row B) $\approx$1 μM UHDBT. MT-1131, wild type; FJ1, cyt c_y^-; MT-G4/S4, cyt c_2^-.

Figure 4 demonstrates the effects of these different inhibitors on c-type cytochrome oxidation in different strains. In the presence of myxothiazol or UHDBT the pool of c-type cytochromes is reduced to a lesser extent than in the presence of antimycin (compare with Figure 2, row B) as expected from the known activity of these inhibitors. As described earlier, this effect is particularly visible in MT-G4/S4 (Prince et al., 1986) and indicates that cyt c_y reduction occurs via the bc_1 complex. The difference in the oxidation/reduction kinetics of cyt c_2 (in FJ1) and cyt c_y (in MT-G4/S4) is also seen in the presence of the inhibitor UHDBT, which, completely inhibiting the bc_1 complex, allows better observation of total c-type cytochrome oxidation. Even in the presence of the inhibitor UHDBT the amount of c-type cytochrome remaining oxidized after the first flash is significantly less in MT-G4/S4 than in FJ1 (i.e., cyt c_y versus cyt c_2), indicating that under our conditions the total amount of electron transfer from the bc_1 complex to cyt c_y is more efficient than electron transfer to cyt c_2.

Effects of Growth Medium and the bc_1 Complex on Properties of cyt c_y. In order to better characterize the roles of cyt c_2 and cyt c_y and the relationship of cyt c_y to the previously described cyt c_x (Jones et al., 1990) and cyt c_{354} (Zannoni et al., 1992) in *R. capsulatus*, a redox titration of flash-oxidizable cyt c_y was performed. Figure 5 shows the dependence of the oxidation of c-type cytochromes (measured at 550–540 nm) on ambient potential in chromatophores from minimal medium A grown MT-GS18 which lack cyt c_1 and c_2 but still possess cyt c_y under these growth conditions. These data follow Nernstian behavior with a midpoint of +349 mV at pH 7.0, consistent with earlier studies (Jones et al., 1990; Zannoni et al., 1992). Since this component is eliminated in FJ2 (cyt c_2^- cyt c_y^-), we conclude that cyt c_x (Jones et al., 1990) and cyt c_{354} (Zannoni et al., 1992) detected spectroscopically and cyt c_y detected genetically all represent the same component.

A growth medium effect on the presence of cyt c_y in the membranes has been proposed as a possible explanation for the earlier conflicting results related to the presence or absence of a c-type cytochrome able to donate electrons to the reaction center in MT-GS18 (Zannoni et al., 1992). A more complete analysis using SDS–PAGE gels stained specifically for heme-catalyzed peroxidase activity using TMBZ and H_2O_2 (Thomas et al., 1976) of the growth medium dependence for the presence of cyt c_y in membranes indicates that this dependence is confined to strains lacking the bc_1 complex. Figure 6 demonstrates that in MT-G4/S4 the $\approx$30-kDa heme-staining band which correlates with the presence of the *cycY* gene (Jenney & Daldal, 1993) is present regardless of the growth medium used. However, this is not the case in membranes from cells lacking the bc_1 complex. Chromatophores of MT-

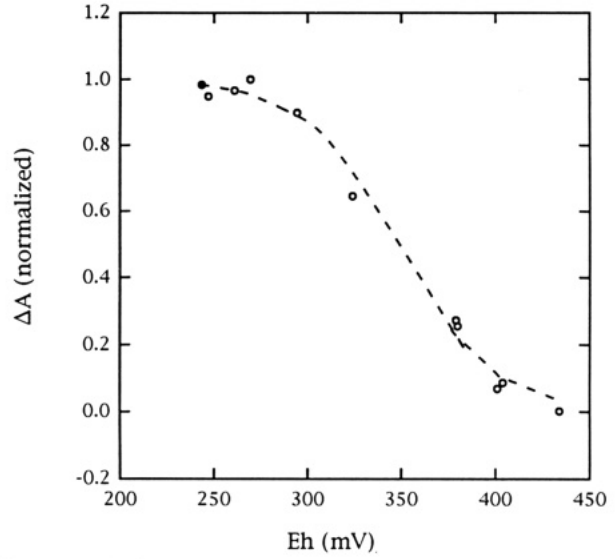

FIGURE 5: Redox titration of *c*-type cytochrome photooxidation in chromatophores from minimal medium A grown MT-GS18 (cyt c_1^- cyt c_2^-). The extent of oxidation of cyt c_y (the sole remaining flash oxidizable cytochrome *c*) was measured at 550–540 nm at various ambient potentials. The data were fit to a single $n = 1$ Nernst curve (dotted curve) with an E_m of +349 mV using the program Sigmaplot (Jandel Scientific).

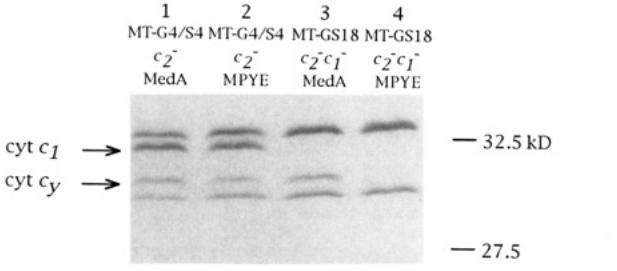

FIGURE 6: SDS–PAGE gel [16.5%, according to Schägger and von Jagow (1987)] stained for *c*-type cytochromes by the method of Thomas et al. (1976). Molecular mass markers are in kilodaltons. The ≈30-kDa bands associated with cyt c_1 and cyt c_y (Jenney & Daldal, 1993) are indicated by arrows. (Lanes 1 and 2) MT-G4/S4 (cyt c_2^-) chromatophores derived from minimal medium A and enriched MPYE medium grown cells respectively; (lanes 3 and 4) MT-GS18 (cyt c_1^- cyt c_2^-) chromatophores derived from minimal medium A and enriched MPYE medium grown cells, respectively. Note that the heme-staining band which correlates with the presence of the *cycY* gene is missing in MT-GS18 only when cells are grown on enriched medium.

GS18 (cyt c_2^- bc_1^-) derived from minimal medium A grown cells possess the band associated with cyt c_y (Figure 6, lane 3) whereas the same strain grown on enriched MPYE medium lacks this heme-stainable band (lane 4). The other heme-staining bands of ≈25 and 18 kDa which were reported to correlate with the *cycY* gene in Jenney and Daldal (1993) appear to be artifacts of the SDS–PAGE system used in that work (data not shown).

The cyt c_y that is present in membranes derived from minimal medium grown MT-GS18 cells (cyt bc_1^- cyt c_2^-) is photooxidizible (Figure 7). The traces for MT-G4/S4 (cyt c_2^-) indicate that there is no difference in *c*-type cytochrome oxidation in membranes derived from cells grown in either minimal growth medium (Figure 2, row B) or in rich MPYE medium (Figure 7). However, in chromatophores derived from MT-GS18 cells grown in minimal medium there is clearly oxidation of a *c*-type cytochrome by the flash-oxidized reaction center which is not detectable in the same strain grown in enriched MPYE medium (Figure 7). Further, note that the kinetics of this oxidation are very different from those seen with MT-G4/S4. While the absence of the bc_1 complex has no apparent effect (as fast as we can measure) on the rate of cyt c_2 oxidation on the first flash [compare FJ1 (cyt c_y^-) in Figure 4 with FJ3 (cyt c_1^- c_y^-) of Figure 7], the lack of a bc_1 complex considerably slows cyt c_y oxidation [compare MT-G4/S4 (cyt c_2^-) in Figure 4 to MT-GS18 (cyt bc_1^- c_2^-) in Figure 7]. The molecular basis for the absence of cyt c_y in rich medium grown membranes lacking the bc_1 complex and for the very different oxidation kinetics of cyt c_y in the absence of the bc_1 complex is presently unknown.

DISCUSSION

Previous genetic and biochemical studies indicated that another membrane-associated electron carrier, cyt c_y, exists in *R. capsulatus* in addition to the periplasmic, water soluble cyt c_2 (Daldal et al., 1986; Prince et al., 1986; Jenney & Daldal, 1993). The mutant FJ2 which lacks both of these cytochromes is Ps$^-$, but it is complemented to Ps$^+$ growth by either the *cycA* gene (encoding cyt c_2) or the *cycY* gene (encoding cyt c_y) provided in *trans* (Jenney & Daldal, 1993). The spectroscopic data obtained here using various strains lacking bc_1 complex, cyt c_2, and/or cyt c_y support the genetic findings and indicate that cyt c_y is the component which completes cyclic electron flow in the absence of cyt c_2 in *R. capsulatus* (Figure 1). Further, inactivation of either the *cycA* gene or *cycY* gene alone causes a significant change in both the extent and kinetics of the flash-oxidizible cytochrome *c* pool indicating that both pathways, one via a soluble carrier and one via a membrane-bound carrier, are operational in the wild type. Only these two carriers connect the bc_1 complex to the reaction center in *R. capsulatus* as demonstrated by FJ2 which lacks both of these carriers. We have been unable to isolate spontaneous revertants to Ps$^+$ growth from the Ps$^-$ FJ2 (cyt c_2^- cyt c_y^-) even after treatment with the mutagens ethyl methane sulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (data not shown; and Jenney & Daldal, 1993). Thus no component like the iso-cyt c_2 described in *R. sphaeroides* (Donohue et al., 1988) has yet been detected in *R. capsulatus*. Genetic data also support this idea; 100% of the antibiotic resistant transductants produced by inactivating either *cycA* or *cycY* are Ps$^+$, indicating that no mutation is required for expression of the nondeleted gene (Jenney & Daldal, 1993).

These results indicate that the photosynthetic electron transport chain in wild-type *R. capsulatus* is branched and thus quite different from earlier models and from that in *R. sphaeroides* (Figure 1), raising the question of the structure of the branched photosynthetic electron transport chain. One possibility is that cyt c_y and cyt c_2 could operate in parallel, interchangeably reducing the reaction center at the same (or a different) site and also possibly exchanging electrons with each other. Another possibility is that there are two different pools of bc_1 complexes and reaction centers, one of which interacts primarily with cyt c_y and one with cyt c_2, and that both pools are present in the wild type. Such pools, if they exist, need not necessarily be spatially separated but simply connected by the different carriers (Figure 1). Both of these models require that either carrier alone can complete the cycle in the absence of the other, consistent with the photosynthesis proficient phenotypes of the cyt c_2^- and cyt c_y^- single mutants. While our data cannot discriminate between these possibilities, the fact that mutant FJ2 (cyt c_2^- cyt c_y^-) contains no flash-oxidizible *c*-type cytochrome indicates that our earlier hypothesis that the bc_1 complex could function as a direct donor to the reaction center (Prince et al., 1986) is invalid in situ,

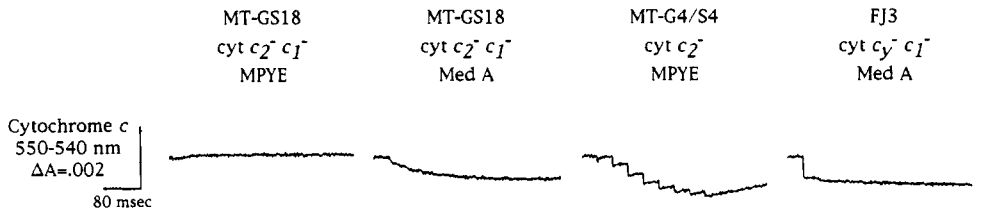

FIGURE 7: Traces are absorbance changes in response to a train of eight saturating actinic flashes, conditions as described under Experimental Procedures. Chromatophores were prepared from cells grown semiaerobically in Sistrom's minimal medium A or enriched MPYE medium as indicated. Responses are from cytochrome c (550–540 nm). MT-GS18, cyt c_1^- cyt c_2^-; MT-G4/S4, cyt c_2^-; FJ3, cyt c_1^- cyt c_y^-.

despite the evidence of Venturoli et al. (1990) documenting in vitro electron transfer between the bc_1 complex and the reaction center.

Earlier observations indicated that photooxidation of c-type cytochromes in wild-type MT-1131 had biphasic kinetics, exhibiting a fast phase attributed to oxidation of cyt c_2 by the oxidized reaction center and a slower phase attributed to oxidation of cyt c_1 by the oxidized cyt c_2 (Crofts, 1985; Prince et al., 1982; Meinhardt & Crofts, 1982). Experiments by Prince et al. (1986) with the cyt c_2^- mutant MT-G4/S4 showed that this strain surprisingly exhibited only the fast phase of c oxidation in the absence of cyt c_2, although the extent is smaller than in wild type, which was interpreted as meaning that the fast and slow phases actually represented oxidation of cyt c_1 and cyt c_2, respectively. However, the data from Prince et al. (1986) combined with the data in this work are consistent with the idea that the oxidation of cyt c_y by the photooxidized reaction center is actually part of the fast phase of the biphasic kinetic. The half-time of cyt c_2 oxidation in the cyt c_1^- cyt c_y^- strain FJ3 is $t_{1/2} < 250$ μs (Figure 3) implying that in the wild type the slow phase corresponds to cyt c_2 oxidation. Thus it would seem that although the amount of cyt c_y that is interacting with the reaction center may be small, it is an efficient donor. The kinetic data presented here demonstrate that these two carriers, the membrane-associated cyt c_y and the soluble cyt c_2, interact with their substrates in a fundamentally different way. The connection between the bc_1 complex and the reaction center via cyt c_y can perhaps be described as "tighter" than the connection via cyt c_2, based on the lower amount of cyt c re-reduction and cyt b reduction in FJ1 (cyt c_y^-) compared to MT-G4/S4 (cyt c_2^-) (Figure 2). Measurement of the rate of electron transfer at a much faster time scale is necessary to further characterize these reactions.

One of the puzzles associated with the analysis of the cyt c_2-independent electron transfer pathway has been why cyt c_y was not detected in the cyt c_2^- bc_1^- strain MT-GS18 in the initial analysis (Prince & Daldal, 1987), while flash oxidation of a c-type cytochrome (cyt c_x $E_{m7} \approx +360$ mV) was later observed in the same strain by Jones et al. (1990). Zannoni et al. (1992) showed that a membrane-bound c-type cytochrome (cyt c_{354} $E_{m7} \approx +354$ mV) could be flash-oxidized in membranes of MT-GS18 only when grown on minimal RCV medium and not in rich medium. Further they still detected a cytochrome with $E_{m7} \approx +354$ mV in dark equilibrium redox titrations of membranes from both minimal and rich medium grown cells of MT-GS18 (Zannoni et al., 1992). They proposed that differences in culture conditions, specifically the nature of the growth medium (minimal versus rich), gave rise to this difference in the c-type cytochrome complement (Zannoni et al., 1992). We show here, in agreement with these earlier findings, that cyt c_y could not be detected by SDS–PAGE in chromatophores from MT-GS18 cells grown on enriched MPYE medium, though it is detectable in chromatophores from cells grown in minimal medium A as well as in cells containing bc_1 complex (MT-G4/S4) grown in either growth medium (Figure 6). SDS–PAGE analysis (Figure 6) as well as redox titrations further indicated that there are actually two components with midpoint potentials around +350 mV, one of which is cyt c_y and the other one is a c-type cytochrome component of the cytochrome oxidase 410 in *R. capsulatus* (Zannoni et al., 1992; Gray et al., 1994). Why cyt c_y is lacking in cyt bc_1^- enriched MPYE medium grown cells, and at what level this control is effected, is unknown.

The presence of the bc_1 complex has a second effect on cyt c_y as demonstrated by Figure 7. The rate of oxidation of cyt c_y is much faster in the presence of the bc_1 complex (MT-G4/S4) than in the absence (MT-GS18). For example, it was reported by Jones et al. (1990) that the $t_{1/2}$ for c-type cytochrome oxidation in MT-GS18 is <400 μs compared to $t_{1/2} < 100$ μs for cyt c_y measured in MT-G4/S4 (Prince et al., 1986). This indicates that cyt c_y alone is not interacting with the reaction center in the same manner as when bc_1 is present, raising the possibility that the bc_1 complex and cyt c_y may be closely associated in some manner in the membrane, such that a subset of these membrane proteins may form a bc_1/cyt c_y/reaction center supercomplex (although not necessarily limited to these three components). Such a close association could also explain the rapid and complete re-reduction of cyt c_y in MT-G4/S4 as opposed to cyt c_2 in FJ1, as well as the lack of cyt c_y in cyt bc_1^- cells under certain growth conditions. The existence of cyt c_2 attached to the reaction center as a single mobile unit (Prince et al., 1978) or in the form of a "kinetic supercomplex" with the bc_1 complex has already been proposed for cyt c_2 in *R. sphaeroides* (Joliot et al., 1989).

An important question raised by this work is why there are two electron carriers, one soluble and one membrane-associated acting between the bc_1 complex and the reaction center in *R. capsulatus*. One possibility is that they may have separate functions; for example, in different growth conditions they could be differentially expressed or utilized. Another possibility is that cyt c_y may play a role in some other metabolic pathway(s) and may only incidentally function in the photosynthetic electron transport chain. Whether cyt c_y has a role in electron transfer pathways other than photosynthesis remains to be tested, and of particular interest is the possible role of cyt c_y in respiration. Daldal (1988), using mutants in the terminal quinol oxidase (strain M6G) in combination with deletion of the *cycA* gene encoding cyt c_2, showed that this strain was still able to grow via aerobic respiration in the absence of cyt c_2 in a cyt bc_1 dependent manner. It is plausible that cyt c_y is also connecting the bc_1 complex to the cytochrome oxidase complex in the same way it connects the bc_1 complex to the reaction center, and experiments are in progress to address this question.

More work is necessary to better understand the role of this branched electron transport chain and whether similar branches exist in other organisms. Similar membrane-associated c-type cytochromes have recently been described in some bacteria such as *CycM* in *Bradyrhizobium japonicum*

(Bott et al., 1991), the c_{551} in *Chlorobium vibrioforme* (Okkels et al., 1992), and the c_{550} of *Bacillus subtilis* (von Wachenfeldt & Hederstedt, 1993). A newly reported membrane-associated *c*-type cytochrome in *Nitrobacter winogradskyi* may also be similar to cyt c_y based on its proposed function and membrane location (Nomoto et al., 1993). Thus, membrane-associated electron carriers such as cyt c_y may be more widely spread and may play a larger role in electron transport chains than has previously been suspected.

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