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Membrane-Associated Cytochrome c_y of *Rhodobacter capsulatus* Is an Electron Carrier from the Cytochrome bc_1 Complex to the Cytochrome *c* Oxidase during Respiration

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We have recently established that the facultative phototrophic bacterium *Rhodobacter capsulatus* has two different pathways for reduction of the photooxidized reaction center during photosynthesis (F. E. Jenney and F. Daldal, EMBO J. 12:1283–1292, 1993; F. E. Jenney, R. C. Prince, and F. Daldal, Biochemistry 33:2496–2502, 1994). One pathway is via the well-characterized, water-soluble cytochrome c_2 (cyt c_2), and the other is via a novel membrane-associated *c*-type cytochrome named cyt c_y. In this work, we probed the role of cyt c_y in respiratory electron transport by isolating a set of *R. capsulatus* mutants lacking either cyt c_2 or cyt c_y, in the presence or in the absence of a functional quinol oxidase-dependent alternate respiratory pathway. The growth and inhibitor sensitivity patterns of these mutants, their respiratory rates in the presence of specific inhibitors, and the oxidation-reduction kinetics of *c*-type cytochromes monitored under appropriate conditions demonstrated that cyt c_y, like cyt c_2, connects the bc_1 complex and the cyt *c* oxidase during respiratory electron transport. Whether cyt c_2 and cyt c_y are the only electron carriers between these two energy-transducing membrane complexes of *R. capsulatus* is unknown.

Rhodobacter capsulatus is a facultative phototroph that can grow by anoxygenic photosynthesis, aerobic dark respiration, or anaerobic dark growth in the presence of dimethyl sulfoxide (DMSO) (4, 9, 32). In this soil bacterium, photosynthetic electron transport involves a cyclic pathway between the photochemical reaction center (RC) and the ubihydroquinone:cytochrome *c* (cyt *c*) oxidoreductase (bc_1 complex) (20, 23). These membrane-bound energy-transducing components are connected to each other via a ubiquinone-ubihydroquinone pool and several *c*-type cytochromes (cyt c_2 and cyt c_y) which act as electron carriers (Fig. 1) (12, 13). On the other hand, respiratory electron transport pathways are branched at the quinone pool (19, 30). One branch is similar to that found in mitochondria and involves the bc_1 complex and a cyt *c* oxidase (C_{ox}, also called cytochrome oxidase b_{410} [31] or cyt *cb* oxidase [11]). The other branch is independent of the bc_1 complex and depends on a quinol oxidase (Q_{ox}, also called alternative oxidase b_{260}) (Fig. 1) (16, 34). Previous biochemical and genetic studies have demonstrated that in *R. capsulatus*, photoheterotrophic (photosynthetic) growth (Ps^+ phenotype) is abolished by the absence of a functional bc_1 complex, while chemoheterotrophic (respiratory) growth (Res^+ phenotype) still continues via Q_{ox} (7, 28). Moreover, two independent mutations, one blocking C_{ox} and the other blocking Q_{ox}, are required to arrest the respiratory growth of this bacterium (19, 31), in agreement with the pathways shown in Fig. 1.

Earlier, electron transfer from the bc_1 complex to the RC during photosynthetic growth (22) and to the C_{ox} during respiratory growth (36) was thought to be mediated solely by the soluble electron carrier cyt c_2, encoded by *cycA* (26). However, the ability of a cyt c_2^- mutant of *R. capsulatus* to grow phototrophically (6, 21) and of a cyt c_2^- Q_{ox}^- double mutant to grow chemoheterotrophically (5) indicated the presence of additional electron carriers capable of replacing functionally cyt c_2 both in photosynthesis and in respiration (see Fig. 1 in reference 5). The molecular nature of one such carrier was first suggested by the works of Jones et al. (14) and Zannoni et al. (37), which described the photooxidation of a *c*-type heme in a mutant lacking both cyt c_1 and cyt c_2 (23). More recent genetic (12) and biochemical (13) studies identified this component as a novel membrane-associated *c*-type cytochrome named cyt c_y, encoded by *cycY* (Fig. 1). Deletion of *cycY*, like that of *cycA*, had no major effect on the photosynthetic growth ability of *R. capsulatus*, but inactivation of both cyt c_2 and cyt c_y yielded a Ps^- mutant which could be complemented to Ps^+ growth by either *cycA* or *cycY* (12). This finding demonstrated the existence of two independent electron transfer pathways, between the bc_1 complex and the RC, which function simultaneously in a wild-type *R. capsulatus* strain such as MT1131 (Fig. 1) (13). These findings established that cyt c_y is also an electron carrier between the bc_1 complex and RC during photosynthesis and led us to examine whether it plays any role in electron transfer from the bc_1 complex to C_{ox} during respiration.

In this present work, we first constructed a series of mutants lacking either cyt c_2 or cyt c_y in the presence or in the absence of a Q_{ox}-dependent alternative respiratory pathway and examined their ability to grow by respiration via the bc_1 complex-dependent branch. We then measured different respiratory rates and oxidoreduction kinetics of *c*-type cytochromes in appropriate mutants in order to probe the role of cyt c_y as an electron carrier in respiration. The overall data indicate that cyt c_y can mediate electron transfer between the bc_1 complex and the C_{ox} at a rate sufficient to support respiratory growth of *R. capsulatus* in the absence of cyt c_2. Whether cyt c_2 and cyt c_y are the only electron carriers operating between these two energy-transducing complexes is unknown.

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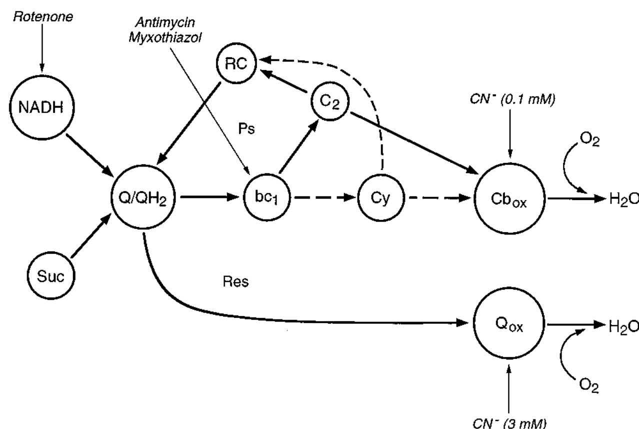

FIG. 1. Electron transport pathways between various energy transduction components involved in photosynthetic and respiratory growth of *R. capsulatus*. Ps, photoheterotrophic growth; Res, chemoheterotrophic (semiaerobic) growth; NADH, NADH dehydrogenase; Suc, succinate dehydrogenase; Q/QH$_2$, ubiquinone/ubihydroquinone pool; Rc, photochemical reaction center; bc$_1$, bc_1 complex; C$_2$, cyt c_2; C$_y$, cyt c_y; Cb$_{ox}$, cyt *cb* oxidase; Q$_{ox}$, quinoloxidase. Dashed arrows indicate cyt c_y-dependent pathways. Rotenone, antimycin, myxothiazol, and cyanide (CN$^-$; 0.1 and 3 mM) are inhibitors of NADH dehydrogenase, bc_1 complex, cyt *cb* oxidase, and Q$_{ox}$, respectively.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic techniques. The *R. capsulatus* mutants used are listed in Table 1, and their properties relevant to this work are indicated. Strains MT1131 (wild type [wt] with respect to its cytochrome content) (18), MT-G4/S4 (c_2^-) (6), FJ1 (c_y^-), and FJ2 (c_2^- c_y^-) (12) have already been described. The double mutants SL1 (c_y^- Q_{ox}^-) and SL2 (c_y^- C_{ox}^-) and the triple mutant SL4 (c_2^- c_y^- C_{ox}^-) were isolated by using the gene transfer agent (27) and introducing the *cycY*::*spe* allele of cyt c_y from pFJ66 (12) into the previously described mutants M6G (Q_{ox}^-), M7G (C_{ox}^-), and M7G-G4/S4 (c_2^- C_{ox}^-) (5). All strains were grown chemoheterotrophically in MPYE rich medium as described in Zannoni et al. (37), and all genetic techniques were as described by Jenney and Daldal (12). Resistance to the bc_1 complex inhibitor myxothiazol (5×10^{-6} M) was tested as previously described (8), and the Nadi phenotype (ability of the C_{ox} to catalyze the reaction α-naphthol + dimethyl-*p*-phenylenediamine + $O_2 \rightarrow$ indophenol blue + H_2O) was determined as described by Marrs and Gest (19).

Biochemical and spectroscopic techniques. Membrane fragments were prepared either in 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.0) containing 100 mM KCl for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (13) or in 50 mM MOPS buffer (pH 7.2) containing 5 mM $MgCl_2$ for spectroscopic measurements, using a French pressure cell as reported by Zannoni et al. (37). Protein content of the samples was determined by the method of Bradford (2) or that of Lowry et al. (17), and bacteriochlorophyll concentration was measured by the optical density of acetone-methanol (7:2) extracts, using an extinction coefficient ε_{775} of 75 mM^{-1} cm^{-1} (3). SDS-PAGE was performed by the method of Schaegger and von Jagow (25) as described by Jenney et al. (13). Respiratory rates were measured by polarography at 28°C with a Clark-type oxygen electrode, using membrane fragments resuspended to ca. 0.2 mg/ml in 50 mM MOPS buffer (pH 7.2)–5 mM $MgCl_2$. Optical difference spectra of the reduced-minus-oxidized samples were obtained at room temperature, using either a Jasco 7800 or a Hitachi U3210 spectrophotometer. Kinetic changes in the absorption of *c*-type cytochromes were monitored at 551 minus 540 nm, using a Sigma ZW-11 dual-wavelength spectrophotometer equipped with a rapid mixing apparatus (mixing $t_{1/2}$ of ca. 100 ms) as described previously (29). The amounts of the *b*- and *c*-type cytochromes were estimated by using the extinction coefficients $\varepsilon_{561-575}$ of 22 mM^{-1} cm^{-1} and $\varepsilon_{551-540}$ of 19 mM^{-1} cm^{-1}, respectively.

RESULTS

Isolation of *R. capsulatus* cyt c_y^- mutants in various terminal oxidase-deficient backgrounds. Mutants lacking the newly discovered cyt c_y were isolated to assess its respiratory role in different terminal oxidase-deficient backgrounds, using strains M6G (Q_{ox}^-) and M7G (C_{ox}^-) (5, 19) (Fig. 1), as described in Materials and Methods. The cyt *c* content of the double mutants SL1 (Q_{ox}^- c_y^-) and SL2 (C_{ox}^- c_y^-) was examined by using Schaegger-type SDS-polyacrylamide gel stained with 3,3′,5,5′-tetramethylbenzidine (Fig. 2), and it was found that cyt c_y was absent in both SL1 (compare lanes 6 and 10) and SL2 (compare lanes 7 and 11). In addition, a 32-kDa *c*-type cytochrome (named cyt c_p) associated with the C_{ox} (11) was also absent in SL2. Both mutants grew by respiration and responded appropriately to the bc_1 complex inhibitor myxothiazol (Table 1), indicating that cyt c_y is not essential for Res$^+$ growth in the presence of cyt c_2 in both Q_{ox}^- and C_{ox}^- backgrounds (Fig. 1).

Next, the role of cyt c_y was probed in the absence of cyt c_2 by isolating cyt c_2^- and cyt c_y^- derivatives of SL1 and SL2 or M6G-G4/S4 (Q_{ox}^- c_2^-) and M7G-G4/S4 (C_{ox}^- c_2^-), respectively, using MPYE as a permissive medium under Res$^+$ growth conditions. The triple mutant SL4 (C_{ox}^- c_2^- c_y^-) (Fig. 2, lane 12) was readily obtained with either SL2 (lane 11) or M7G-G4/S4 (lane 9) as a recipient. It was Res$^+$ and myxothiozol resistant (Table 1), confirming that cyt c_2 and cyt c_y are not involved in the Q_{ox}-dependent respiratory pathway of *R. capsulatus* (Fig. 1). On the other hand, using neither SL1 nor M6G-G4/S4 were we able to obtain a triple mutant lacking Q_{ox} and both of cyt c_2 and c_y, on either MPYE rich or medium A minimal medium (with malate or succinate as a carbon

TABLE 1. *R. capsulatus* strains used

Strain	Genotype[a]	Relevant phenotype[b]	Origin
MT1131	*crtD121*	wt	18
Y262		Gene transfer agent overproducer	27
MT-G4/S4	Δ(*cycA*::*kan*)	cyt c_2^- Ps$^+$ Res$^+$ Myx^r Nadi$^+$ DMSO$^{+/-}$	6
FJ1	Δ(*cycY*::*spe*)	cyt c^{y-} Ps$^+$ Res$^+$ Myx^r Nadi$^+$ DMSO$^+$	12
FJ2	Δ(*cycA*::*kan*) Δ(*cycY*::*spe*)	cyt c_2^- cyt c_y^- Ps$^-$ Res$^+$ Myx^r Nadi$^+$ DMSO$^{+/-}$	13
M6G	*qox*	Q^{ox-} Ps$^+$ Res$^+$ Myx^s Nadi$^+$ DMSO$^{+/-}$	19
M7G	*cox*	C_{ox}^- Ps$^+$ Res$^+$ Myx^r Nadi$^-$ DMSO$^{+/-}$	19
M6G-G4/S4	Δ(*cycA*::*kan*) *qox*	cyt c^{2-} Q_{ox}^- Ps$^+$ Res$^+$ Myx^s Nadi$^+$ DMSO$^+$	5
M7G-G4/S4	Δ(*cycA*::*kan*) *cox*	cyt c_2^- C_{ox}^- Ps$^+$ Res$^+$ Myx^r Nadi$^-$ DMSO$^{+/-}$	5
SL1	Δ(*cycY*::*spe*) *qox*	cyt c^{y-} Q_{ox}^- Ps$^+$ Res$^+$ Myx^s Nadi$^+$ DMSO$^+$	This work
SL2	Δ(*cycY*::*spe*) *cox*	cyt c_y^- C_{ox}^- Ps$^+$ Res$^+$ Myx^r Nadi$^-$ DMSO$^+$	This work
SL4	Δ(*cycA*::*kan*) *cox* Δ(*cycY*::*spe*)	cyt c_2^- cyt c_y^- C_{ox}^- Ps$^-$ Res$^+$ Myx^r Nadi$^-$ DMSO$^+$	This work

[a] All strains except Y262 carry the *crtD121* mutation and therefore are "green." All gene designations are as described previously (5). MT1131 is a green derivative of SB1003 constructed by using gene transfer agent (36) and is wt with respect to its cytochrome contents.

[b] Myx^r and Myx^s refer to resistance and sensitivity of respiratory (chemoheterotrophic) growth on MPYE rich medium to myxothiazol (5×10^{-6} M). All Ps$^+$ strains are also Myx^s under photoheterotrophic growth conditions. DMSO refer to anaerobic dark growth on MPYE plates supplemented with 80 mM DMSO; + and +/− indicate vigorous and poor growth, respectively, on these plates observed after 4 days of anaerobic dark incubation at 35°C.

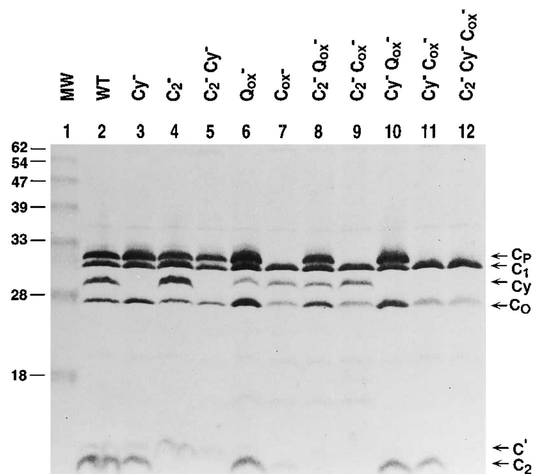

FIG. 2. SDS-PAGE (25) of chromatophore membranes of various *R. capsulatus* cyt c_2^- and cyt c_y^- mutants grown in MPYE by respiration. Approximately 200 μg of total protein was used in each case; after electrophoresis, the gel was stained with 3,3′,5,5′-tetramethylbenzidine-H_2O_2 to visualize the *c*-type cytochromes present in different mutants. Lane 1 contains a prestained protein ladder (molecular weight [mw] standards) from BRL/Gibco (Gaithersburg, Md.); sizes are indicated in kilodaltons at the left. Lanes 2 to 12 correspond to MT1131 (wt), FJ1 (c_y^-), MT-G4/S4 (c_2^-), FJ2 (c_2^- c_y^-), M6G (Q_{ox}^-), M7G (C_{ox}^-), M6G-G4/S4 (Q_{ox}^- c_2^-), M7G-G4/S4 (C_{ox}^- c_2^-), SL1 (Q_{ox}^- c_y^-), SL2 (C_{ox}^- c_y^-), and SL4 (C_{ox}^- c_2^- c_y^-), respectively.

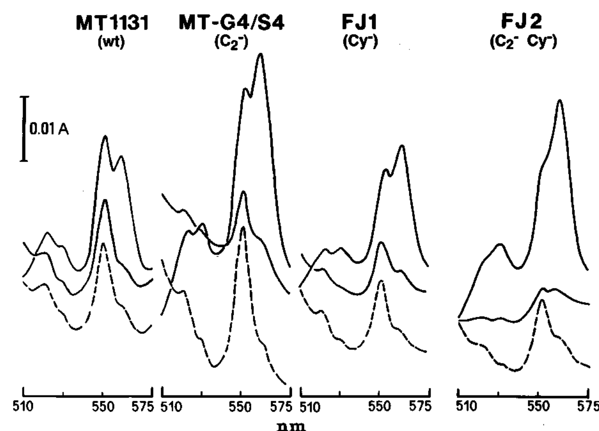

FIG. 3. Reduced-minus-oxidized absorption difference spectra recorded at 25°C of chromatophore membranes from *R. capsulatus* MT1131, MT-G4/S4, FJ1, and FJ2. Membrane fragments were resuspended at 0.4 mg of protein per ml in 50 mM MOPS (pH 7.2), oxidized with the addition of a few crystals of potassium ferricyanide, and reduced similarly with a few crystals of asc-DAD (10 μM) (dashed traces), NADH (middle traces), and dithionite (upper traces), successively.

source), under Res^+ or Ps^+ growth conditions (Table 1). Moreover, we also failed to isolate such a mutant by using DMSO-dependent anaerobic dark growth (33) but noticed that various mutants lacking different cyt *c*s are affected differently in their growth via this pathway (Table 1). Currently, the electron transfer components of *R. capsulatus* involved in DMSO-dependent anaerobic dark growth are not well known (9, 24). Thus, to what extent this pathway is a permissive medium for a triple mutant, lacking both cyt c_2 and cyt c_y and the Q_{ox} terminal oxidase, remains unclear. In any event, our inability to obtain such a triple mutant, in addition to the myxothiazol insensitivity of the respiratory growth of FJ2 (c_2^- c_y^-) (Table 1), strongly suggests, but do not prove, that cyt c_2 and cyt c_y may be the only electron carriers from the bc_1 complex to C_{ox}, like the photocyclic electron pathways of *R. capsulatus* (Fig. 1) (12, 13).

Cytochrome content of membranes from various *R. capsulatus* mutants. The total *b*- and *c*-type cytochrome content of membranes isolated from *R. capsulatus* MT1131 (wt), MT-G4/S4 (c_2^-), FJ1 (c_y^-), and FJ2 (c_2^- c_y^-) grown by respiration in MPYE medium was estimated from reduced-minus-oxidized absorption difference spectra (Fig. 3). The cyt *b*/*c* ratio (on a molar/protein basis) increases from 0.77 ± 0.05 in wild-type membranes (MT1131) to 1.45 ± 0.19 in FJ2 lacking both cyt c_2 and cyt c_y. This result indicates that while the *b*-type heme content remains approximately the same (2.55 ± 0.35 nmol/mg of protein), the amount of *c*-type cytochromes drops from a maximum of 3.5 ± 0.2 nmol/mg of protein in MT1131 (wt) to a minimum of 1.87 ± 0.1 nmol/mg of protein in FJ1 (c_y^-) and FJ2 (c_2^- c_y^-), as a result of the genetic elimination of various cyt *c*s. Comparable data were also obtained with SL1 (c_y^- Q_{ox}^-), SL2 (c_y^- C_{ox}^-), and SL4 (c_2^- c_y^- C_{ox}^-) lacking either C_{ox} or Q_{ox} (Table 1, Fig. 2, and data not shown). On the other hand, the amounts of *c*-type hemes reduced by NADH versus ascorbate–2,3,5,6-tetramethyl 1,4-phenylenediamine (asc-DAD) (expressed as NADH/asc-DAD ratio), under steady-state respiratory conditions, were similar for MT1131 and FJ1 (0.73 ± 0.03) and different from that for MT-G4/S4 (0.4 ± 0.02) and for FJ2. The fact that in MT-G4/S4 the amount of *c*-type hemes reduced by asc-DAD was higher than that reduced by NADH suggests that elimination of cyt c_2, rather than that of c_y, has a stronger effect on NADH-induced reduction of *c*-type hemes under the conditions used here (Fig. 3).

Respiratory electron flow via cyt c_2 versus cyt c_y. Electron flow through the respiratory chain can be initiated or blocked by using specific substrates or inhibitors (Fig. 1). For example, while addition of NADH or succinate initiates electron transport at the level of the respiratory dehydrogenases, inhibitors like antimycin A or myxothiazol block it at the bc_1 complex (4). In Table 2, the rates of oxygen uptake depending on NADH, succinate, and C_{ox} in membranes from various Q_{ox}^+ (MT1131, MT-G4/S4, FJ1, and FJ2) and Q_{ox}^- (M6G, M6G-G4/S4, and SL1) strains are presented. In all Q_{ox}^+ mutants, the substrates tested were rapidly oxidized, and the oxygen uptake activities measured were sensitive to antimycin A or to myxothiazol (5 μM each). However, in FJ2 these activities were less sensitive to the bc_1 complex inhibitors than in MT-G4/S4 and FJ1, especially when measured by using succinate, indicating that bc_1-dependent electron transfer still continues in vitro in the absence of cyt c_2 or cyt c_y, but appears to be more restricted when both cytochromes are missing.

Next, considering that the electron transport abilities of the bc_1 complex-dependent and the Q_{ox}-dependent respiratory branches partially overlap under steady-state conditions, the rates of O_2 uptake depending on NADH, succinate, and C_{ox} were also determined by using membranes from various Q_{ox}^- mutants (Table 2). The data clearly indicated that all respiratory activities monitored were strongly inhibited by 5 μM myxothiazol or 0.1 mM cyanide. On the other hand, in the absence of the cyt c_2 (M6G-G4/S4) or cyt c_y (SL1), electron transfer from the bc_1 complex to C_{ox} still continued, although an approximately 30 to 40% decrease in the steady-state respiratory activities, measured by using succinate or NADH as the substrate, was observed. Thus, both cyt c_2 and cyt c_y are major electron carriers between the bc_1 complex and C_{ox} in *R. capsulatus*. Furthermore, the total C_{ox} activity measured in the presence of reduced DAD (a membrane-permeable electron

TABLE 2. NADH, succinate, and C_{ox} activities in membrane fragments from various *R. capsulatus* mutants grown chemoheterotrophically in MPYE medium[a]

Electron donor	Inhibitor	Activity (μmol of O_2/h/mg of protein)[b]						
		MT1131 (wt)	MT-G4/S4 (c_2^-)	FJ1 (c_y^-)	FJ2 (c_2^- c_y^-)	M6G (Q_{ox}^-)	M6G-G4/S4 (Q_{ox}^- c_2^-)	SL1 (Q_{ox}^- c_y^-)
NADH		4.2	7.9	10.4	13.3	6.5	3.8	3.1
	Rot	0.3	0.4	0.5	0.4	0.9	0.5	0.6
	Myx	3.4	6.5	9.2	11.0	0.2	0.3	0.1
	AA	3.4	6.3	8.0	11.0	0.6	0.8	0.8
	KCN	3.2	6.3	8.6	10.8	0.9	0.5	0.3
Succinate		4.7	3.5	2.8	3.5	5.0	3.4	4.0
Succinate-cyt *c*		6.0	6.3	5.0	6.5	5.0	5.5	5.1
Succinate	Myx	3.8	2.8	2.0	3.5	0.1	0.1	0.1
	AA	1.4	1.4	0.9	2.8	1.2	0.7	0.9
	KCN	3.1	2.6	1.7	3.0	0.2	0.2	0.2
Ascorbate-cyt *c*		7.8	6.8	9.7	7.7	28.0	10.2	13.5
	KCN	2.0	1.1	1.4	1.9	2.9	0.9	1.5
Asc-DAD		3.2	2.2	2.2	1.1	34.7	17.5	27.0
Asc-DAD	KCN	0.9	0.5	0.6	0.2	1.3	0.9	1.6

[a] Various inhibitors and substrates were added at the following concentrations: rotenone (Rot), 5 μM; myxothiazol (Myx), 5 μM; antimycin A (AA), 5 μM; potassium cyanide (KCN), 100 μM; horse heart cyt *c*, 50 μM; and DAD, 20 μM.

[b] Mean of two membrane determinations.

carrier) was 22 and 50% less in SL1 and M6G-G4/S4, respectively, than in M6G, suggesting that the electron flow to C_{ox} via cyt c_2 is higher than that via cyt c_y.

Finally, the C_{ox} activity present in membranes from the Q_{ox}^- mutants (M6G, SL1, and M6G-G4/S4) was approximately 8 to 12 times higher than that in Q_{ox}^+ strains (MT1131, FJ1, and MT-G4/S4) (Table 2), possibly to counterbalance the lack of the Q_{ox} activity which catalyzes, under the in vitro conditions used here, most (80 to 85%) of the electron transport activity. Similarly, NADH respiration resistant to cyanide (0.1 mM) and antimycin A or myxothiazol (5 μM each) in MT-G4/S4, FJ1, and FJ2 was two- to threefold higher than that in the wt strain MT1131, indicating that electron flow via the Q_{ox}-dependent branch was enhanced by the absence of either cyt c_2 or cyt c_y or both. A similar increase of the bc_1-independent respiratory flow has also been observed (35) in *R. capsulatus* mutant MT-GS18 lacking both the cyt c_2 and the bc_1 complex (23).

Kinetics of cyt *c* oxidoreduction in membranes from various *R. capsulatus* strains lacking the cyt c_2 or cyt c_y. The kinetics of NADH-induced cyt *c* reduction in membranes from various *R. capsulatus* mutants were monitored in the presence of oxygen to assess directly the involvement of cyt c_y in electron transfer from the bc_1 complex to C_{ox} (Fig. 4) in the presence (first four traces) or in the absence (last three traces) of Q_{ox}. Upon addition of NADH, approximately 50% of the total reducible *c*-type hemes in membranes were rapidly reduced in MT1131 (Fig. 4A). This respiration-dependent steady-state reduction of cyt *c* was further increased to 70 and 80% by addition of 0.1 and 3 mM cyanide, blocking the C_{ox} and Q_{ox} activities, respectively. On the other hand, cyt *c* reduction was decreased to approximately 40% by addition of antimycin A plus myxothiazol (5 μM each), leading to inhibition of the bc_1 complex (Fig. 4A, dashed traces). Thus, a large fraction of the *c*-type heme complement of a wt strain is in rapid equilibrium with the respiratory electron transport system. Similar oxidoreduction kinetics were observed with FJ1 (c_y^-) ($t_{1/2}$ of 200 ms), whereas a slightly slower rate was seen with MT-G4/S4 (c_2^-) ($t_{1/2}$ of 400 ms). In contrast, membranes from FJ2 (c_2^- c_y^-) catalyzed a markedly slow cyt *c* reduction ($t_{1/2}$ of >1 min) which was largely insensitive to cyanide (Fig. 4A). Whether cyt c_y is an electron carrier between the bc_1 complex and C_{ox} was further probed by using Q_{ox}^- mutants (M6G, SL1, and M6G-G4/S4) (Fig. 4A, last three traces). As before, addition of antimycin A plus myxothiazol under steady-state respiration led to an almost complete reoxidation of cyt *c* in these strains, while addition of cyanide approximately doubled the percent reduction of cyt *c* in M6G-G4/S4 and SL1, indicating that cyt c_y and cyt c_2 are in equilibrium with C_{ox} (Fig. 4A). Moreover, as in MT-G4/S4, the rate of cyt *c* reduction in membranes from M6G-G4/S4 was slower ($t_{1/2}$ of <400 ms) than that from SL1 and M6G ($t_{1/2}$ of <200 ms, close to that of the mixing apparatus), again suggesting that electron transfer to C_{ox} via the soluble cyt c_2 is a better route than that via the membrane-associated cyt c_y.

It should be noted that traces very similar to those observed for FJ2 without any inhibitor (Fig. 4A) were also seen with antimycin A- and myxothiazol-treated membranes in all strains tested here (Fig. 4B). Thus, on a larger time scale, some cyt *c* reduction can be observed in membrane fragments in all cases, including FJ2 (c_2^- c_y^-). However, this activity detected in vitro has no physiological significance since it is similar to that observed in the presence of antimycin and myxothiazol, which are known to abolish bc_1 complex-dependent respiratory growth of *R. capsulatus* Q_{ox}^- mutants (Table 1) (5).

DISCUSSION

In this work, a biochemical genetic approach was used to analyze the role of cyt c_y in respiratory electron transport of *R. capsulatus* (12, 32). We demonstrated that in the absence of the Q_{ox}-dependent alternate respiratory pathway, *R. capsulatus* mutants lacking either cyt c_2 or cyt c_y (M6G-G4/S4 or SL1) are still able to grow by respiration in a manner absolutely dependent on the bc_1 complex, as indicated by their sensitivity to myxothiazol (Table 1). This finding demonstrates that either one of these two cytochromes is sufficient to support the bc_1-dependent respiratory growth, even though the NADH-dependent and myxothiazol-sensitive electron flow becomes rate limiting, as measured in vitro (Fig. 4A). Thus, as in the case of photosynthesis (12), cyt c_2 and cyt c_y are alternate electron carriers in respiration, and only the inactivation of both of

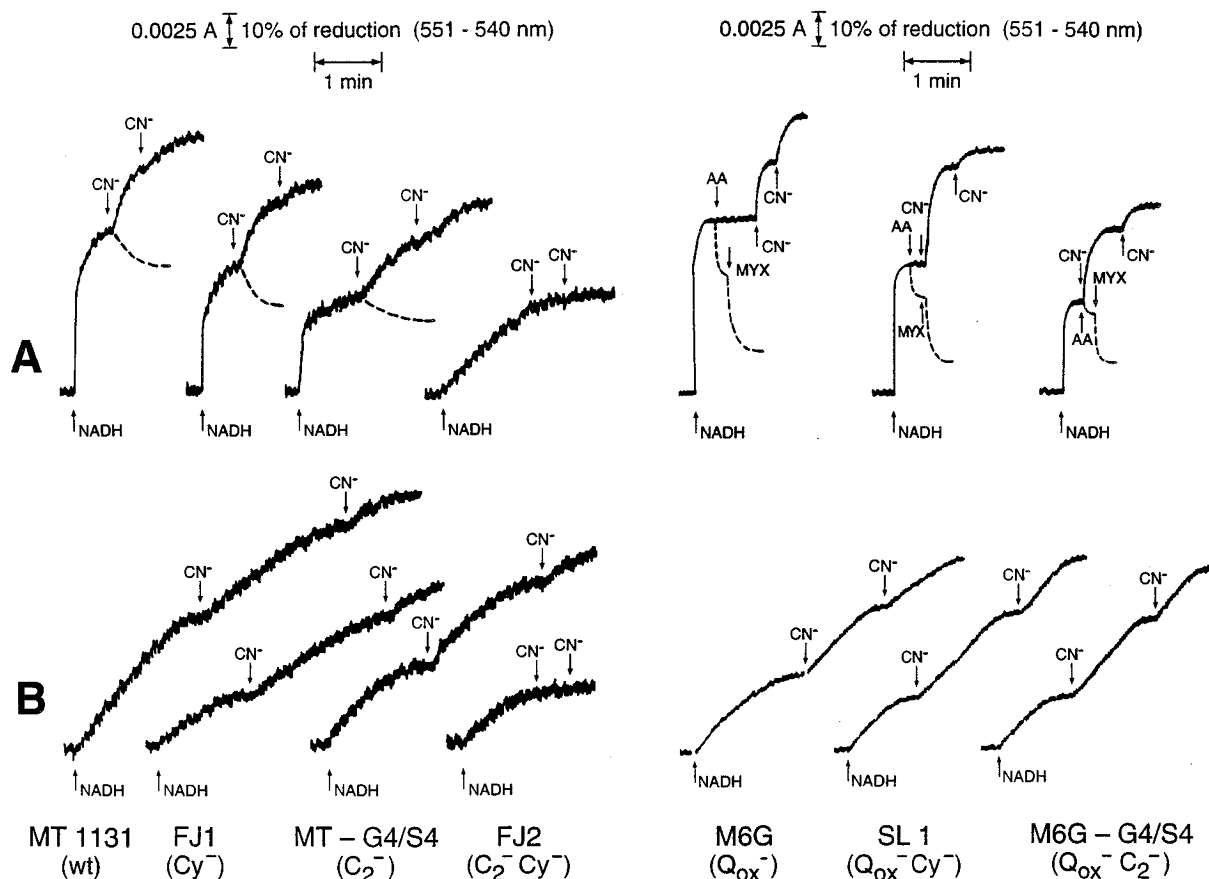

FIG. 4. Effects of cyanide (CN^-), antimycin A (AA), and myxothiazol (MYX) on the NADH-dependent reduction of *c*-type hemes monitored at 551 minus 540 nm in chromatophore membranes from *R. capsulatus* Q_{ox}^+ strains MT1131, FJ1, MT-G4/S4, and FJ2 (A, first four traces) and Q_{ox}^- strains M6G SL1, and M6G-G4/S4 (last three traces). Dashed traces represent the patterns of cyt *c* oxidoreduction observed upon addition of antimycin A plus myxothiazol as a control for the contribution of the bc_1 complex-dependent electron transfer (note that no response was observed with FJ2). In all experiments, the cyanide additions were as follows: 0.1 mM, first addition; 3 mM, second addition. The traces in panel B were obtained by adding antimycin A plus myxothiazol (5 μM each) before NADH and illustrate the nonphysiological, bc_1 complex-independent slow electron transport to *c*-type hemes.

them, as in FJ2, can render the respiratory electron flow insensitive to inhibitors of the bc_1 complex in a Q_{ox}^+ background (Table 1). The absence of physiologically relevant, cyanide- and myxothiazol-sensitive, and rapid cyt *c* reduction in FJ2 (Fig. 4), taken together with our inability to obtain a triple mutant lacking both the cyt c_2 and cyt c_y and Q_{ox} (Table 1), strongly suggests, but does not prove, that cyt c_2 and cyt c_y are the only carriers available in vivo between the bc_1 complex and C_{ox}. Also, we failed to obtain a bc_1^- Q_{ox}^- double mutant during an earlier work (5), again suggesting that in the absence of the Q_{ox}-dependent alternate pathway, the bc_1 complex and cyt c_2 or cyt c_y become essential for respiratory growth. A more definitive conclusion about the number of the electron carriers should await the isolation of an appropriate mutant in which some of these components are conditionally inactivated.

Interestingly, restriction of the bc_1 complex-dependent pathway by deletion of cyt c_2 or cyt c_y only moderately increases the electron flow via the Q_{ox}-dependent branch (2- to 3-fold), but elimination of Q_{ox} greatly increases the C_{ox} activity present in membranes of appropriate mutants (8- to 12-fold) (Fig. 1, lanes 6, 8, and 10; Table 2). While the molecular events governing these changes are not yet known, they nonetheless indicate that in *R. capsulatus*, electron flow via the two branches of respiration is regulated. Finally, it has recently been shown that *R. capsulatus* (11) and *R. sphaeroides* (10) contain a novel form of C_{ox} (called cyt *cb* oxidase) which has, in addition to its low- and high-spin cyt *b*s two c-type cytochromes (cyt c_p and cyt c_o) with an apparent redox midpoint potential at pH 7 of +265 and 320 mV (11). Although the purified enzyme can oxidize reduced *R. capsulatus* cyt c_2, neither the nature of its preferred electron donor(s) in vivo nor the pathway of electron transfer through it is yet known (11).

The discovery of two structurally distinct electron carriers operating between the RC, bc_1 complex, and C_{ox} raises the intriguing question of how the soluble cyt c_2 and the membrane-associated cyt c_y interact with their physiological partners. One possibility is that these cytochromes operate in parallel, binding interchangeably to the bc_1 complex and to C_{ox} either at the same or a different site. The other possibility is that they interact with different subpopulations of the bc_1 complex and C_{ox}, one dedicated primarily to cyt c_2 and the other dedicated primarily to cyt c_y. If so, then these subpopulations must coexist in wt membranes and be able to support the bc_1-dependent respiratory growth of *R. capsulatus*. However, whether they are confined to different topological regions of the cytoplasmic membrane or whether they form structural supercomplexes, as purified earlier from *Paracoccus denitrificans* (1) and thermophilic bacterium PS3 (15), needs to be explored. While the experiments reported here cannot discriminate between these two possibilities, the different cyt *c* reduc-

tion kinetics observed with cyt c_2 and cyt c_y indicate that these electron carriers interact with their donors and acceptors in different ways. For example, cyt c_2 is more rapidly reduced and oxidized than cyt c_y, which is less accessible to the respiratory substrates (Fig. 4A; compare FJ1 and SL1 with MT-G4/S4 and M6G-G4/S4).

In summary, this work clearly indicates that both cyt c_2 and cyt c_y are electron carriers between the bc_1 complex and C_{ox} during the respiratory growth of *R. capsulatus*. Further work is necessary to probe for the presence of additional carriers and to characterize more completely the respiratory electron transport pathways of facultative phototrophs such as *Rhodobacter* spp. as a model organism for studying cellular energy transduction.

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