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Functional Characterization of the Lesion in the Ubiquinol: Cytochrome *c* Oxidoreductase Complex Isolated from the Nonphotosynthetic Strain R126 of *Rhodobacter capsulatus*

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Abstract

The cytochrome bc_1 complexes from the nonphotosynthetic strain R126 of *Rhodobacter capsulatus* and from its revertant MR126 were purified. Between both preparations, no difference could be observed in the stoichiometries of the cytochromes, in their spectral properties, and in their midpoint redox potentials. Both also showed identical polypeptide patterns after electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate. The ubiquinol: cytochrome *c* oxidoreductase activity was strongly inhibited in the complex from the mutant compared to the one from the revertant. So was the oxidant-induced extra reduction of cytochrome *b*. Both preparations, however, showed an antimycin-induced red shift of cytochrome *b*, as well as antimycin-sensitive reduction of cytochrome *b* by ubiquinol. In accordance with a preceding study of chromatophores (Robertson *et al.* (1986). *J. Biol. Chem.* **261**, 584–591), it is concluded that the mutation affects specifically the ubiquinol oxidizing site, leaving the ubiquinol reducing site unchanged.

Key Words: Cytochrome bc_1 complex; ubiquinol oxidation; ubiquinone reduction; electron transport; *Rhodobacter capsulatus*; photosynthetic bacteria.

Introduction

In purple, nonsulfur, photosynthetic bacteria a key role in electron transfer is played by ubiquinol: cytochrome *c* oxidoreductase, a multienzyme complex.

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It accepts electrons from the ubiquinone pool reduced by the reaction center, and returns them via cyt c_2,[4] the electron donor to the reaction center. Its structural composition and catalytic behavior clearly indicate its analogy to other, similar complexes in the respiratory or photosynthetic membranes of mitochondria, chloroplasts, and other bacteria (for reviews, see Dutton and Prince, 1978; Hauska *et al.*, 1983; Crofts and Wraight, 1983; Melandri and Venturoli, 1984). This universality of bc_1 complexes has increased the interest in photosynthetic bacteria as a relatively easily handled system for kinetic and genetic studies.

The bc_1 complex has been isolated in active form from the purple, photosynthetic bacteria *Rhodobacter capsulatus* and *sphaeroides* (Gabellini *et al.*, 1982; Takamiya *et al.*, 1982; Yu *et al.*, 1984; see also Hauska, 1986, and Ljungdahl *et al.*, 1986). It is composed of a *b*-type cytochrome, containing two heme groups of different redox potentials (cyt *b*561 and *b*566), a *c*-type cytochrome (cyt c_1), and a high-potential FeS protein, called the Rieske FeS protein. No counterparts to the mitochondrial core proteins were found. The activities of the best isolates resembled the ones observed in membranes.

Detailed kinetic information on the interaction of the complex in membranes with the ubiquinone pool and cyt c_2, as well as on electron-transfer steps among the components of the complex, have been obtained using flash photometry under controlled redox conditions (Crofts *et al.*, 1983; Robertson and Dutton, 1988; Venturoli *et al.*, 1988). Ubiquinol is thought to be oxidized in a concerted two-electron reaction on an oxidizing site of the complex (Q_0, also called Q_z), releasing one electron to a high-potential chain (Rieske FeS center, cyt c_1, and cyt c_2), and one electron to a low-potential chain (cyt *b*566 and cyt *b*561). Following two turnovers of the Q_0 site (or one turnover in a dimeric bc_1), two electrons are returned to the ubiquinone pool via the cyt *b* chain at a ubiquinol reducing site (Q_r, also called Q_i or Q_c; Glaser *et al.*, 1984; Robertson *et al.*, 1984; Venturoli *et al.*, 1988). Specific inhibitors have been identified for these two sites: myxothiazol, 5-(*N*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), and 2-(*N*-undecyl)-3-hydroxy-1,4-naphthoquinone (UHNQ) interfere with the quinol oxidation site Q_0 (Meinhardt and Crofts, 1982), and antimycin A inhibits the oxidation of cyt *b*561 at the quinone reduction site Q_r (Crofts and Wraight, 1983). This inhibitor specificity is identical to the mitochondrial bc_1 (see Link and von Jagow, 1986). In spite of detailed experimentation, it is still a matter of debate whether the two sites are physically distinct, as postulated in the so-called Q cycle (Mitchell, 1976), or reflect two interchangeable, functional

[4]Abbreviations: Bchl, bacteriochlorophyll; UQ_3H_2, ubiquinol-3; cyt, cytochrome; bc_1, cytochrome bc_1 complex.

states of the same catalytic center, as formulated in the *b* cycle or semiquinone cycle (Wikström and Krab, 1986).

Many mutants of *Rb. capsulatus* impaired in photosynthetic electron transfer have been isolated and characterized (Marrs, 1978; Zannoni and Marrs, 1981). Among them strain R126 deserves special attention since the lesion affects the bc_1, all the other structural and functional properties of the photosynthetic membranes remaining unaffected (Zannoni and Marrs, 1981). Chromatophores from R126 are totally blocked in flash-induced reduction of cyt *b* in the presence of antimycin A, and rapid dark rereduction of cyt c_1 and c_2 (Robertson *et al.*, 1986). Cyt *b*561 reduction could, however, be demonstrated in R126 after ubiquinone extraction or at alkaline pH. Both conditions favor the reduction of cyt *b*561 through an antimycin A-sensitive pathway, which is interpreted as the reversal of the reaction at the Q_r site (Glaser *et al.*, 1984; Robertson *et al.*, 1984). On the other hand, the effect of ubiquinol on the EPR spectrum of the Rieske FeS center is lost in R126. These results indicate that the genetic lesion affects the Q_0 site, and that the two sites are independent from each other (Robertson *et al.*, 1986). We have adopted the method of Gabellini *et al.*, 1982 (see also Hauska, 1986) to purify the complex from the mutant R126 and from the revertant MR126.

In this paper we compare the subunit composition of these two preparations, the thermodynamic and spectral properties of their cytochromes, as well as their catalytic properties. The results demonstrate that the mutant complex is highly inhibited in ubiquinol : cyt *c* oxidoreductase activity, while the activity of the revertant is normal, although both preparations give identical subunit patterns on SDS-PAGE. Moreover, it is shown that the functional block involves the Q_0 site of the complex only. A part of these results has been presented elsewhere (Fernández-Velasco *et al.*, 1986).

Materials and Methods

Culture of Bacteria and Preparation of Chromatophores

Mutant R126 is a nonphotosynthetic strain of *Rb. capsulatus*. It is a green derivative of strain Y11 (Zannoni and Marrs, 1981), a nonphotosynthetic mutant obtained by nitrosoguanidine mutagenesis from strain Z1 (Marrs *et al.*, 1972). MR126 is a photosynthetically competent strain constructed from R126 using a gene transfer agent. Both strains, obtained from the collection of B. Marrs, were a generous gift of D. Zannoni. They were repurified from single colonies and selected for obligatory aerobic, or facultative phototrophic growth, respectively. A single stock culture of each, maintained in 66% (v/v) glycerol at −80°C, was used throughout

this work. For control experiments the parent strain Z1, the wild-type strain 37b4 (through the generosity of G. Drews, Freiburg, Germany), and the green strain of Gabellini (1988) were also used.

All strains were grown in the dark, in the malate medium of Ormerod *et al.*, (1961), in 1.2-litre Fernbach bottles under low oxygen tension, i.e., without aeration at low shaker agitation (50 rpm). Mutant and revertant grew with the same duplication time of 4.5 h and were harvested when the OD of the culture approximately reached 1.5 at 660 nm and 2.3 at 855 nm. Under these conditions the photosynthetic apparatus was normally synthesized in both strains. Occasionally the revertant was grown photoheterotrophically to the same density in the same medium, in closed Roux bottles, with a light intensity of 40 W m^{-2}. These different conditions did not affect the properties of the purified bc_1. Cells harvested by centrifugation were washed in 50 mM glycylglycine, pH 7.4, and were stored at $-80°C$.

Chromatophores were prepared by rupture of thawed cells, suspended in glycylglycine buffer to an OD of 80 at 660 nm, with a French press operating at 1000 kg cm^{-2}. Cell debris was separated by centrifugation at 15,000 rpm in an SS-34 Sorvall rotor for 20 min (repeated twice), and the chromatophores were sedimented by ultracentrifugation at 250,000 $\times$ g for 90 min. The supernatant was carefully discarded together with the upper, light layer of the sediment. To remove extrinsic proteins, the remaining membrane fraction was washed twice with 2 M NaBr, 0.2 M sucrose, and 50 mM glycylglycine, pH 7.4, as described by Gabellini *et al.* (1982). Independently of the strain and growing conditions, chromatophores were obtained with a protein/Bchl ratio of 11–12 (mg/mg).

Purification of the bc_1 Complex

The method of Gabellini *et al.* (1982) was modified as follows: (1) Triton X-100 was omitted from all steps; see Gabellini and Hauska (1983). (2) Any delays between purification steps involving detergents were avoided. (3) The chromatophore concentration during solubilization was equivalent to 0.4 mg Bchl/ml (identical results, however, were obtained with 0.2 or 0.8 mg/ml). (4) The chromatophore suspension was directly poured into the performed detergent mixture. (5) After incubation and ultracentrifugation the supernatant was kept, carefully discarding the pellet including the loosely packed layer; the octylglucoside concentration was then raised to 60 mM, and the preparation was incubated for a further 60 min (N. Gabellini, personal communication). Subsequently, the complex was precipitated with ammonium sulfate between 45 and 60% saturation, by dropwise addition of saturated solution at 4°C. After stirring for 30 min, the precipitate was collected by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor for 10 min, and

immediately resuspended in 0.5–2.5 ml of a buffer containing 80 mM octylglucoside, 0.25% (w/v) cholate, 30 mg/ml soybean lecithin, and 50 mM glycylglycine, pH 7.4 (previously clarified by 10 min sonication under nitrogen on ice); final purification was obtained by centrifugation on a discontinuous sucrose gradient (20 h at 40,000 rpm in a Beckman SW 41 Ti rotor at 4°C).

The overall yield of the purified bc_1 was about 2 nmol cyt c_1 per liter of cell culture. The enzymatic activities of this preparation, stored at −80°C were stable for months.

SDS Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed after Laemmli (1970). The gels were stained for protein with Coomassie blue, and for heme after Thomas *et al.* (1976). Immunoblotting was carried out as described in the kit of Biorad (USA), using the protein A-peroxidase conjugate. Monospecific antibodies against the purified subunits of bc_1 were employed (Gabellini *et al.*, 1985).

Differential Spectroscopy and Redox Titrations

Absorption spectra were recorded using a Jasco Uvidec 610 spectrophotometer (3 nm bandwidth). Cytochrome contents were routinely estimated by the redox difference of the α-peaks (Gabellini and Hauska, 1983). Differential spectra were obtained by computer subtraction of absolute spectra. For redox titrations the anaerobic cuvette previously described for kinetic studies (Venturoli *et al.*, 1986) was utilized. The extents of reduced cytochromes were estimated from differential spectra during the course of titration, using as reference a linear baseline drawn below the α-peaks, i.e., between 543 and 558 for cyt c_1, and between 547 and 570 for cyt *b* as follows: cyt c_1 in both chromatophores and purified complex was measured at 552 nm, cytochromes of *b* type in the purified complex at 560 nm, whereas in chromatophores the high-potential heme was measured at 560 nm and the low-potential heme at 559 or, with the same results for midpoint redox potential, at 565 nm; cf. García *et al.* (1987). The buffer was 50 mM glycylglycine, pH 7.0, containing 10 mM KCl and the following redox mediators: 1,2-naphthoquinone, 1,4-naphthoquinone, *p*-benzoquinone, phenazine methosulfate, and phenazine ethosulfate, 20 μM each; 40 μM duroquinone; 100 μM diaminodurene; 5 μM pyocyanine; and 10 μM 5-hydroxy-1,4-naphthoquinone. For titrations of the isolated bc_1 the buffer additionally contained detergents as in the sucrose density gradient for purification. For titration of cytochromes in the chromatophores, valinomycin and nigericin, 10 μM each, were present.

Catalitic Activities

The ubiquinol:cyt *c* oxidoreductase activity was measured at 25°C utilizing a Sigma-ZW II dual-wavelength spectrophotometer, provided with magnetic stirring. The assay mixture contained 50 mM glycylglycine, pH 7.4, 25 μM horse heart cyt *c*, 40 μM UQ_3H_2, and bc_1 equivalent to 5–10 nM cyt c_1. The enzymatic reaction was started by adding bc_1. Nonenzymatic activity was subtracted. In the case of the revertant it was about 5% of the total activity. Initial rates were taken, the activity slowing off during prolonged measurement. This is partially due to an inactivation of the complex, i.e., a preincubation of bc_1 in the absence of UQ_3H_2 for 5 min at 25°C produced 50% inhibition. Addition of inhibitors occurred during the first 20 sec where linearity is maintained.

Partial redox reactions of the bc_1 were measured with the same setup, except that the bc_1 concentration was equivalent to about 1 μM cyt c_1, and that the mixture contained the same concentration of detergents as the sucrose gradient during purification.

Bchl was measured according to Clayton (1963), and protein according to Lowry *et al.* (1951). For the purified bc_1, the protein standard (bovine serum albumin) contained the same amount of detergents as the bc_1 samples. UQ_3 was a generous gift of G. Lenaz. Chemical reduction to UQ_3H_2 was performed after Rieske (1967), and was measured by the UV spectrum (Crane and Barr, 1971).

Results

Purification of the Complex

The bc_1 on the discontinuous sucrose gradient was contained in a sharp band at about 40% (w/v) sucrose, well separated from other pigmented bands. After 20-h centrifugation the position of this band did not depend on the strain, on the amount of protein loaded (varied by a factor of 8), on the condition of growth (semiaerobic or photosynthetic for strain MR126), or on the chromatophore concentration during solubilization (varied by a factor of 4).

The purified bc_1 preparations were contaminated with antenna complexes belonging to both LH I and LH II (absorption peaks of Bchl at 588, 802, and 856, with a soulder at 875 nm, and of carotenoids at 375, 412, 454, and 496 nm). Typically the preparations contained about 5 Bchl per cyt c_1, corresponding to a 50-fold enrichment with respect to chromatophores. The yield was about 20% for both strains, based on cyt c_1.

Spectral and Thermodynamic Characterization

The redox difference spectra in the α-band region of the cytochromes in the purified complex were identical in R126 and MR126, whether the latter was grown semiaerobically or photosynthetically. They resembled those of the active complex from a green strain of *Rb. capsulatus* previously characterized (Gabellini *et al.*, 1982; Gabellini and Hauska, 1983; Gabellini, 1988). Cyt c_1 had the α-peak at 552, and total cyt *b* at 560 nm. In the chromatophores of both strains the high-potential cyt *b* α-peak was found at 560 nm and the low-potential cyt *b* showed a double peak at 559 and 565 nm. In the purified complex of both strains the ratio of cyt *b*/cyt c_1 was 1.4, and the cyt c_1/protein ratio was 6.5 nmol/mg.

Redox titration at pH 7.0 was reversible in all cases and gave midpoint potentials of +320 mV for cyt c_1 (not shown), and of about +75 and −100 mV for high- and low-potential cyt *b*, respectively, with both bc_1 preparations (Fig. 1). The forms of cyt *b* titrated with $n = 1$ and gave the same contributions to the overall change in both strains. The reversion of the functional lesion in R126, therefore, is not accompanied by any change in the relative amounts, the spectral properties, or the redox potentials of the cytochromes in isolated bc_1. This conclusion was confirmed by redox titrations of chromatophores, which again gave identical results for mutant and revertant. For both, the midpoint potentials of cyt *b* were +55 and −140 mV, and the ones for cyt c_1 and cyt c_2 were +300 and +340 mV, respectively (after deconvolution into two Nernst components with $n = 1$, not shown). It is noteworthy that the midpoint potential of cyt c_1 for the

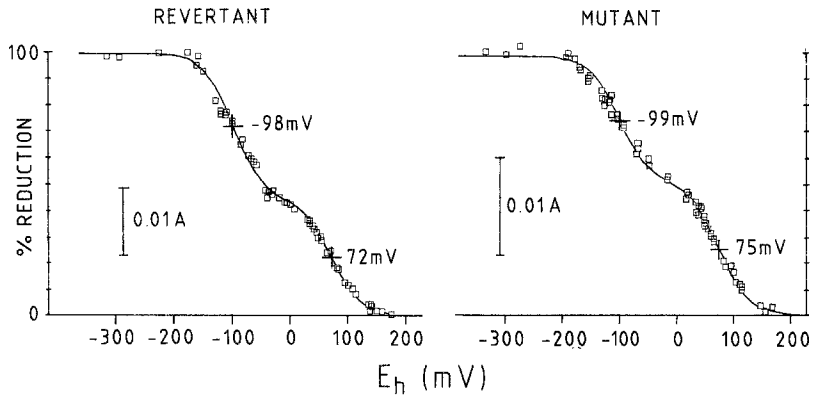

Fig. 1. Redox titrations at pH 7.0 of cytochromes *b* in the purified bc_1 complex of mutant and revertant. In both cases the cells were grown semiaerobically. Titrations were performed as described in Materials and Methods. The bc_1 concentration was equivalent to 1.7 and 1.2 μM cyt c_1 for the revertant and mutant, respectively. The pH was controlled also at the end of the titration. The continuous line is computer fitted for two components with $n = 1$.

mutant and the revertant was found to be somewhat more positive than previously reported for isolated bc_1 (Gabellini *et al.*, 1982), or for chromatophores (Wood, 1980; García *et al.*, 1987).

Subunit Composition

The bc_1 preparations were analyzed on SDS-PAGE. Coomassie blue-stained protein bands are seen in Fig. 2. Cyt c_1, cyt b, and the Rieske FeS protein were identified by staining for heme, and by immunoblotting with specific antibodies (not shown; see Gabellini *et al.*, 1985). The mobilities of these three main proteins were affected by the temperature during denaturation, the temperature during electrophoresis, and the presence of mercaptoethanol in the sample buffer. Routinely the denaturation step was performed

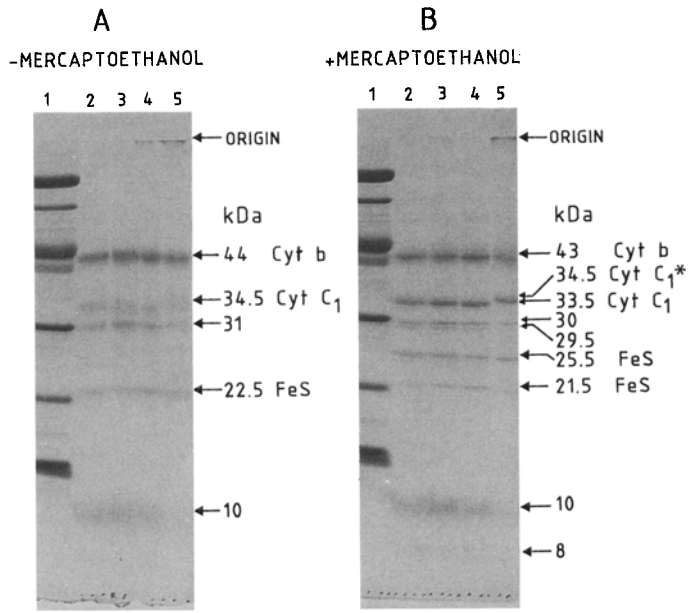

Fig. 2. SDS-PAGE of the bc_1 complex from mutant and revertant, and from the green strain described by Gabellini (1988). The lanes are: (1) MW standards: 92, 66, 45, 31, 21, and 14.5 kDa; (2) mutant R126 grown aerobically; (3) revertant MR126 grown aerobically; (4) revertant MR126 grown photosynthetically; (5) green strain grown photosynthetically (courtesy N. Gabellini). In all cases the amount of bc_1 complex was equivalent to 100 pmol cyt c_1. The samples were denatured at room temperature for 3 h with 6 μg SDS/μg protein. For lower SDS/protein ratios, slightly different mobilities of the protein bands were observed. Running gel was 14% and stacking gel was 3.8% (w/v) acrylamide. Bis-acrylamide was 2.6% of total acrylamide. The run was performed in a cold chamber at 4°C for 3.5 h with a current of 40 mA for a 100 × 100 × 1 mm slab. Gel A: denaturation buffer without HSEtOH. Gel B: with 5% (w/v) HSEtOH. *, green strain.

at room temperature for 3 h, since boiling caused aggregation of cyt *b*, which then did not enter the running gel. However, for identification of the Rieske FeS protein the samples were boiled for 10 min, since only then a single band at 25 kDa was observed (not shown). For samples denatured at room temperature, this protein was always present in two forms, at 25.5 and 21.5 kDa (Fig. 2B), both reacting with the specific antibody. In samples denatured at room temperature in the absence of mercaptoethanol (Fig. 2A), cyt *b* exhibited two bands, a major one at 44 and a minor one at 40 kDa, not easily seen with Coomassie blue. Both of them reacted with the specific antibody. These species were converted to a single band by mercaptoethanol corresponding to 40 or 43 kDa, when the run was performed at room temperature (not shown) or in the cold room (Fig. 2B), respectively. Under these conditions the band could no longer be stained for heme.

Cyt c_1 is found for both the mutant and the revertant as a diffuse band centered at 34.5 kDa in the absence of mercaptoethanol (Fig. 2A), and as a sharp band at 33.5 kDa in its presence (Fig. 2B). Interestingly, cyt c_1 in the bc_1 from a green strain of *Rb. capsulatus* (Gabellini *et al.*, 1982; Gabellini, 1988) migrated somewhat more slowly. Its position in the presence of mercaptoethanol corresponded to 34.5 kDa (Fig. 2B, lane 5).

To investigate whether this difference in mobility has any relevance to the R126 mutation, the mobility of cyt c_1 of R126, and of MR126, was compared to the one of the parent strain Z1 (Marrs *et al.*, 1972). As shown in Fig. 3 by immunoblotting with an antiserum specific for cyt c_1, the mobility of this protein is greater in R126, MR126, and Z1 (lanes 1, 2, and 4), while in the green strain of Gabellini (lanes 3 and 6) and in the wild-type strain 37b4 (lane 5) it is less. The same was observed by heme staining of SDS-PAGE (not shown). These observations exclude any connection of the observed difference with the lesion in the R126 mutant, as is already indicated by the unchanged cyt c_1 mobility in the revertant MR126.

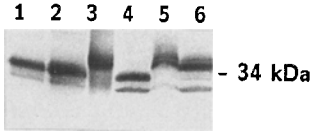

Fig. 3. Immunoblot of cyt c_1 in different strains of *Rb. capsulatus*. SDS-PAGE was as for Fig. 2. After electroblotting, the nitrocellulose filter was incubated with 100 μl of an antiserum specific for cyt c_1 in 30 ml saline solution and was decorated with protein A-peroxidase conjugate as described in the kit of Bio-Rad (USA). The lanes are: (1) bc_1 complex from the mutant R126; (2) bc_1 complex from the revertant MR126; (3) bc_1 complex from the green strain (courtesy N. Gabellini); (4) chromatophores of the parent strain Z1; (5) chromatophores of the wt strain 37b4 (courtesy G. Drews); (6) chromatophores of the green strain. The complexes were equivalent to 100 pmol of cyt c_1, and the chromatophores to 20 μg Bchl each.

The identical polypeptide pattern in complexes from the mutant and the revertant strain demonstrates that the mutation does not involve major deletions in the structure of the proteins of the bc_1 complex, nor alterations in their biosynthetic processes.

Minor bands with M_r = 30, 29, 10, and 8 kDa (the last 2 are diffuse) were also present on the gels (cf. Gabellini *et al.* 1982; Takamiya *et al.*, 1982; Ljungdahl *et al.*, 1986). The position of the smaller polypeptides is dependent on the conditions of electrophoresis, especially the temperature. The presence of antenna pigment in the preparation suggests that they may represent corresponding polypeptides (Drews, 1985, but see Yu and Yu, 1987). The larger two might reflect reaction-center proteins (Gabellini *et al.*, 1982).

Catalytic Activity

The overall oxidoreductase activities of the two complexes are compared in Table I. The ubiquinol : cyt *c* oxidoreductase activity was severely inhibited in the purified complex from the mutant strain. The bc_1 complex from the revertant strain had a turnover number higher than that previously reported for a green strain of *R. capsulatus* (Gabellini *et al.*, 1982). The activity of the revertant was highly sensitive to antimycin A (10 μM) and myxothiazol (10 μM). The small, residual activity of the mutant was also partially inhibited by antimycin A, but was insensitive to myxothiazol.

Spectral Effects of Antimycin and Myxothiazol

Myxothiazol and antimycin have been shown to bind to the isolated bc_1 complexes (von Jagow and Engel, 1981, Gabellini and Hauska, 1983). Both inhibitors cause a spectral red shift of the α-band of reduced cyt *b*. Spectral effects by both inhibitors very similar to the ones reported by Gabellini and Hauska (1983) for a green strain were obtained for both the mutant and the revertant (not shown). The red shift caused by antimycin was identical in mutant and revertant (peak and trough in the differential spectrum were

Table I. Activity of Ubiquinol : Cyt *c* Oxidoreductase of the bc_1 Complexes Purified from Mutant R126 and Revertant MR126 Strains[a]

	Revertant MR126	Mutant R126
Activity (μmol cyt *c* red. per nmol cyt c_1 and *h*)	155	8
Corresponding turnover number (sec^{-1})	43	2
Inhibition by antimycin A (10 μM)	90–95%	40%
Inhibition by myxothiazol (10 μM)	90–95%	< 10%

[a]For reaction conditions, see Materials and Methods. The detergents contained in the enzyme addition (1–5 μl/ml reaction mixture) slightly enhance the nonenzymatic reaction, as does myxothiazol in EtOH. The values presented are accordingly corrected.

at 563 and 556 nm, respectively, measured at −320 mV and pH 7.0). The effect of myxothiazol was smaller in the mutant and slightly different, which is being further investigated.

Partial Reactions

The reducibility of the cyt *b* components of the complexes by exogenous ubiquinol is demonstrated in Figs. 4 and 5. In the presence of 1.5 mM ascorbate, which keeps cyt c_1 and the Rieske FeS center reduced, UQ_3H_2 (50 μM) caused a partial reduction of cyt *b* (about 23% of total change observed as dithionite minus ascorbate). No further reduction was observed by a second addition of UQ_3H_2. This reduction pathway was present in both the revertant and the mutant strain (Fig. 4a and e), and was strongly inhibited by antimycin A (Fig. 4b and f), but was insensitive to myxothiazol (Fig. 4c and g). It occurs via the Q_r site of the complex (Gabellini *et al.*, 1982; Gabellini and Hauska, 1983; Glaser *et al.*, 1984; Link and von Jagow, 1986; Robertson and Dutton, 1988; Venturoli *et al.*, 1988) and remains intact in the mutant strain R126.

A pulse of ferricyanide (100 μM final concentration) added to the revertant complex after addition of antimycin A caused a further reduction of cyt *b* (about 35–40% of the total absorbance change observed as dithionite

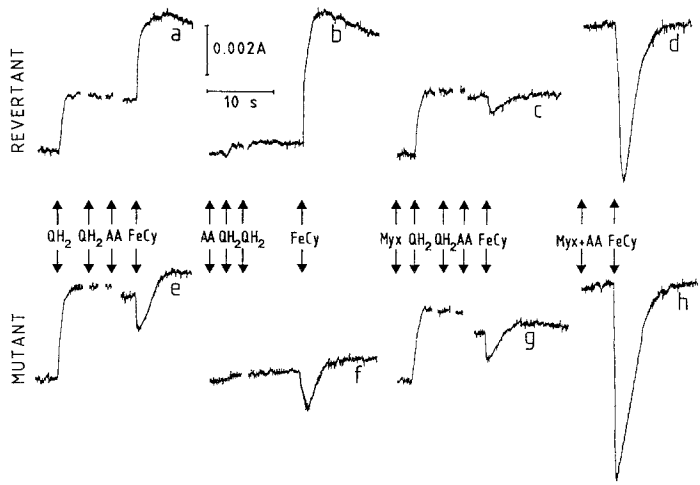

Fig. 4. Partial redox reactions of the revertant and mutant bc_1 complexes. Changes of cyt *b* (traces a, b, c, e, f, and g) and cyt *c*1 (traces d and h) were monitored at 560 minus 570 nm and 552 minus 570 nm, respectively. Traces a–d: revertant; traces e–h: mutant. The concentration of the complex was equivalent to 0.7 and 1.0 μM cyt *b* for the revertant and mutant, respectively. The additions during the experiment as indicated by arrows were: QH_2, 50 μM UQ_3H_2; FeCy, 100 μM ferricyanide; AA, 10 μM antimycin A; Myx, 10 μM myxothiazol. Ascorbate (1.5 mM) was present in all cases. Instrument time constant 50 ms, spectral band width 1.5 nm.

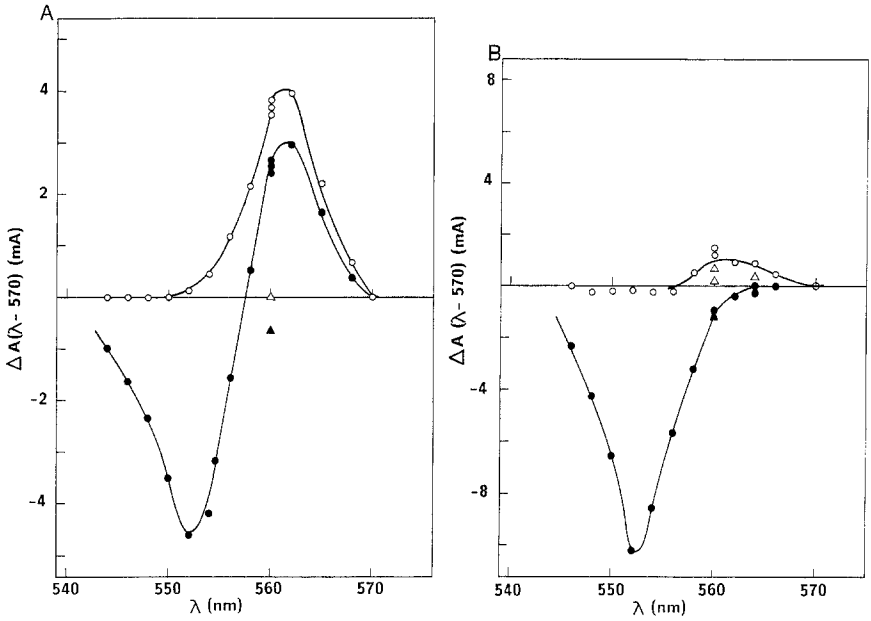

Fig. 5. Time-resolved spectra of the oxidant-induced redox reactions of the preparations purified from the revertant (A) and the mutant (B). The reaction is started by addition of ferricyanide (100 μM) in a reaction mixture containing 1.5 mM ascorbate, 100 μM UQ_3H_2, and 20 μM antimycin A. Other conditions were as for Fig. 4. The reference wavelength was 570 nm. The extents of the charge after 1 sec (●) and after 7 sec (○) are shown. Changes with 10 μM myxothiazol additionally present at 1 sec (▲) and 7 sec (△) are also indicated.

minus ascorbate). The total level of UQ_3H_2-dependent reduction reached the same final amplitude irrespective of whether antimycin A was added after or before the addition of ubiquinol (Fig. 4a and b). The oxidant-induced reduction of cyt *b* was totally inhibited by myxothiazol (the small absorbance transient observed in Fig. 4c reflects cyt c_1 redox reactions; see Fig. 5A). This inhibition was independent of the order of addition of antimycin A and myxothiazol (not shown). This reduction pathway occurs via the Q_0 site of the complex (Gabellini *et al.*, 1982; Gabellini and Hauska, 1983; Link and von Jagow, 1986; Robertson and Dutton, 1988; Venturoli *et al.*, 1986, 1988). The mutant complex behaved in a markedly different fashion. Addition of ferricyanide, after a fast, transient absorption decrease due to oxidation of cyt c_1, induced a small and slow increase in absorbance (Fig. 4e and f), which is partially inhibited by myxothiazol (Fig. 4g).

The transient oxidation of cyt c_1 by ferricyanide in the presence of excess ubiquinol appeared to be comparable in the two preparations (Fig. 4d and h), in the absence (not shown) and the presence of inhibitors. The rereduction of cyt c_1 was always much faster than the reoxidation of cyt *b*. This allowed

for a spectral resolution of the ferricyanide-induced absorbance changes. The results for the revertant and the mutant preparations are compared in Fig. 5. In the revertant the spectra recorded within 1 sec after the addition of ferricyanide indicated a fast oxidation of cyt c_1 and a fast reduction of cyt b, while spectra evaluated after about 7 sec showed a clear spectrum of cyt b only. On the contrary, in the preparation from the mutant strain the fast oxidation of cyt c_1 was not accompanied by any observable cyt b reduction. A small amount of reduced cyt b, much smaller than in the revertant, was observed only 7 sec after the ferricyanide pulse. This small, slow reduction probably resembles results of flash experiments obtained with whole chromatophores of R126, which demonstrate a slow reduction of cyt b only after repetitive flash activation (Robertson *et al.*, 1986).

Discussion

In this study we have systematically compared preparations of the bc_1 complex from the nonphotosynthetic mutant strain R126 of *Rb. capsulatus* with those from its photosynthetically competent revertant MR126. Spectral and thermodynamic characterizations do not indicate any significant alterations in amount and properties of the cytochrome components present. Both preparations contain three main protein subunits whose M_r are the same on SDS-PAGE. These polypeptides of 43, 33.5, and 25.5 kDa have been identified as the apoproteins of cyt b, cyt c_1, and the Rieske FeS protein, respectively (Gabellini *et al.*, 1982). The M_r values correspond to molecular weights computed from the nucleotide sequences of the structural genes (Gabellini and Sebald, 1986), which are 48,100; 30,100; and 21,000, respectively. The M_r has been determined for all strains tested, including the arsenate resistant Z1, the strain from which the nonphotosynthetic mutant R126 was derived (Marrs *et al.*, 1972). The slight difference in mobility of cyt c_1 to other strains of *Rb. capsulatus* is already found in the parent strain Z1 (Fig. 3), and therefore is not connected with the genetic lesion in the mutant R126. Polypeptides of smaller M_r, as found in other preparations of bc_1 complexes, are also present in both the mutant and revertant. Whether these belong to the complexes is uncertain, however. They may well be contaminations (Gabellini *et al.*, 1982; Gabellini, 1988). It is noteworthy, however, that a polypeptide of about 13 kDa seems to be an additional constituent of the bc_1 complex prepared from *Rb. sphaeroides* (Ljungdahl *et al.*, 1986; Yu and Yu, 1987; Purvis *et al.*, 1990; Andrews *et al.*, 1990).

In spite of this structural identity, the bc_1 complex from the mutant strain is largely impaired in catalytic activity. The small, residual ubiquinol : cyt c oxidoreductase activity in the mutant complex must be different from

the original pathway, since, although inhibited by antimycin, it is insensitive to myxothiazol. Partial reactions of the purified mutant complex with ubiquinol and ferricyanide indicate clearly that cyt *b* is still accessible to reduced ubiquinol at the antimycin-sensitive quinone reduction site (Q_r), while cyt *b* reduction via the myxothiazol-sensitive quinol oxidation site (Q_0) is largely blocked. This conclusion is in full agreement with the studies on intact chromatophores (Robertson *et al.*, 1986). The function of this site is restored in the revertant strain MR126, since light-induced electron flow in membranes of this strain is normal (Robertson *et al.*, 1986), and the isolated bc_1 complex behaves as described for wild-type preparations (Gabellini *et al.*, 1982).

The mutation in strain R126 has been located in the structural gene for cyt *b* by Daldal *et al.* (1989) recently. The change of a single base pair results in the replacement of glycine-158 by aspartate. Interestingly, this change falls into a stretch of less than 20 amino acids where most of the mutations to the Q_0 site inhibitor-resistant strains cluster. This stretch therefore very likely forms at least part of the quinol-binding pocket at the Q_0 site. As has been pointed out before (Robertson *et al.*, 1986), the fact that the Q_r site is left intact by the mutation close to the Q_0 site provides conclusive evidence for a complete functional independence of the Q_0 and the Q_r site and a strong argument in favor of the Q-cycle mechanism (Mitchell, 1976).

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