Characterization of cytochrome *b* in the isolated ubiquinol–cytochrome c_2 oxidoreductase from *Rhodopseudomonas sphaeroides* GA

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Extinction coefficients for cytochrome *b* and c_1 in the isolated cytochrome bc_1 complex from *Rhodopseudomonas sphaeroides* GA have been determined. They are 25 $mM^{-1}.cm^{-1}$ at 561 nm for cytochrome *b* and 17.4 $mM^{-1}.cm^{-1}$ at 553 nM for cytochrome c_1, for the difference between the reduced and the oxidized state. Cytochrome *b* is present in two forms in the complex. One form has an E_{m7} of 50 mV, an α-peak of 557 nm at liquid N_2 temperature and of 561 nm at RT, which is red-shifted by antimycin A. The other form has an E_{m7} of -90 mV, a double α-peak of 555 and 561 nm at liquid N_2 temperature corresponding to 559 and 566 nm at RT. The absorption at 566 nm is red-shifted by myxothiazol. The two shifts are independent of each other. Both midpoint potentials of cytochromes *b* are pH-dependent. The redox center compositions of the cytochrome bc_1 complexes from *Rhodopseudomonas sphaeroides* and from mitochondria are identical.

Bacterial bc_1 *complex* Rps. sphaeroides *Cytochrome* b *Myxothiazol* *Antimycin A* *Ubiquinol–cytochrome* c *oxidoreductase*

1. INTRODUCTION

A cytochrome bc_1 complex with ubiquinol–cytochrome c_2 oxidoreductase activity has been isolated recently from *Rhodopseudomonas sphaeroides* GA [1]. The preparation was sensitive to antimycin A and UHDBT. It contained two hemes *b* and substoichiometric amounts of the Rieske FeS center and of ubiquinone/cytochrome c_1. On SDS–PAGE 3 main polypeptides of M_r 40000, 34000 and 25000 were resolved. Two midpoint potentials of 50 and -60 mV at pH 7 could be ascribed to cytochrome *b* from redox titrations. These are similar to midpoint potentials of cytochromes *b* in the parent chromatophore membrane [2,3], the lower corresponding to a component with an α-peak at 566 nm plus a shoulder at 559 nm, the higher corresponding to a component with an α-peak at 561 nm. Such a spectral heterogeneity of cytochrome *b* has not been found at first in the isolated cytochrome bc_1 complex [1], the α-peak remaining at 561 nm throughout the redox titration.

Here, we provide unequivocal evidence for the two forms of cytochrome *b* in the isolated complex, which in addition to redox titration [1] is based on the independent spectral effects of antimycin A and myxothiazol, and on low temperature spectroscopy.

2. MATERIALS AND METHODS

The cytochrome bc_1 complex was isolated as described [1], except that Triton X-100 was avoided for resuspending the pellet after ammonium sulfate fractionation. Instead the octylglucoside concentration was raised to 120 mM in the suspending mixture. In this case the complex banded at 30% sucrose in the subsequent density gradient centrifugation [1]. Oxidoreductase activity and oxidant-induced reduction were measured as described [1], with the specifications given in the

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legends. With ubiquinol-2 a maximal turnover of 20 s^{-1} was found for cytochrome c_1 in the complex. Low temperature spectra and the spectral shifts caused by myxothiazol and antimycin A were measured in an Aminco DW2 UV/Vis spectrophotometer as described in the legends. Pyridine hemochrome was prepared and assayed as in [4]. Myxothiazol was a kind gift from Dr Thierbach (Braunschweig). Other sources for materials were listed in [1,5].

3. RESULTS AND DISCUSSION

From the redox difference spectra and the corresponding pyridine hemochrome spectra in fig.1 extinction coefficients of 17.5 $mM^{-1}.cm^{-1}$ at 553 nm for cytochrome c_1, and of 25 $mM^{-1}.cm^{-1}$ at 561 nm for cytochrome b in the isolated complex can be calculated, which are similar to the values known for cytochromes b and c_1 in the mitochondrial cytochrome bc_1 complex [4,6,7]. Two hemes b/heme c are present in the bacterial complex (0.6 and 0.3 μM in fig.1), confirming [1]. No reliable measurement is known for cytochrome b and c_1 in the chromatophore membrane [8].

In [1] redox heterogeneity of cytochrome b was found, which was not paralleled by spectral heterogeneity, however. The α-peak remained at

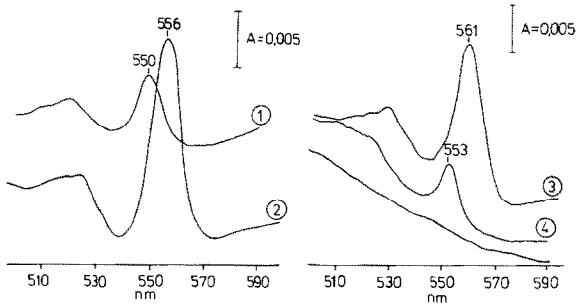

Fig.1. Pyridine hemochrome and cytochrome spectra. The measurements are described in section 2. Pyridine hemochromes were prepared as in [4]: (1) protein bound pyridine hemochrome, dithionite-reduced minus ferricyanide-oxidized; (2) acetone-soluble pyridine hemochrome, dithionite-reduced minus ferricyanide-oxidized; (3) cytochrome bc_1 complex, dithionite minus ascorbate; (4) cytochrome bc_1 complex, ascorbate minus ferricyanide. A baseline is included in the right part of the figure.

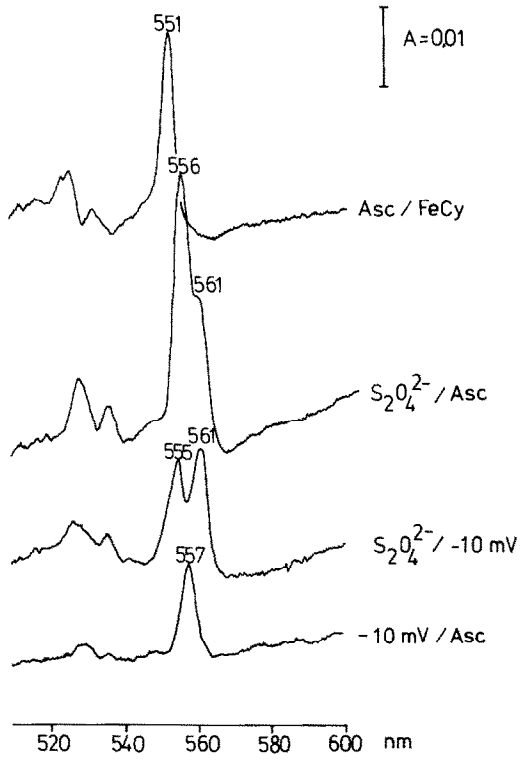

Fig.2. Low temperature redox difference spectra. The spectra were recorded with the low temperature equipment for the Aminco DW2 UV/Vis spectrophotometer over liquid nitrogen. The 2 mm-cuvettes were used. The cytochrome bc_1 complex was diluted to 0.4 μM cytochrome c_1 in 5 mM glycylglycine (pH 7.4), 20% (v/v) glycerol, 20 μM duroquinone. Before freezing the solution was either oxidized by ferricyanide (FeCy), or reduced by ascorbate (Asc) or dithionite, or was poised to an E_h of -10 mV. Difference spectra between sample and reference cuvettes were taken as indicated.

561 nm during redox titrations. When Triton X-100 is left out during the preparation of the complex a shoulder at 559 nm with the main peak at 566 nm was detectable at low potentials. A clear spectral resolution of the two forms of cytochrome b in the complex is possible at low temperature (fig.2). In liquid nitrogen the component with the higher midpoint potential shows an α-peak at 557 nm, the component with the lower midpoint shows two peaks at 555 and 561 nm, of almost equal height. The two components probably represent cytochrome b-561 and cytochrome b-566 (second peak at 559 nm at RT) in the chromatophore

membrane, recently resolved by redox difference, and flash spectroscopy [3,9]. Spectra of two forms of cytochrome *b* have been reported before for a less purified preparation of the cytochrome bc_1 complex [10], but they do not match our results.

Redox potential measurements [1] were repeated with our modified preparation, avoiding Triton X-100. The two E_{m7}-values match exactly the values reported for the cytochromes *b* in chromatophores [2,3], and both are pH-dependent (table 1). In chromatophores at least one cytochrome *b* has a pH-dependent midpoint potential [11].

The cytochromes *b*-561 and *b*-566 in the bacterial complex correspond to the cytochromes *b*-562 and *b*-565 in the mitochondrial complex, which have E_{m7}-values of 92 and −34 mV, respectively [7,12]. Also these midpoint potentials are pH-dependent [13].

In fig.3 spectral shifts of the α-peaks of cytochrome *b* in the isolated complex by antimycin A and myxothiazol are shown. Both cause a red shift, but the effects are observed in the absence as well as in the presence of the other antibiotic. Thus they seem to be independent of each other, antimycin affecting the component resembling *b*-561 and myxothiazol the component resembling *b*-566. These results are identical to a report for the isolated mitochondrial cytochrome bc_1 complex [14]. Independent spectral changes of cytochrome *b* by antimycin A and myxothiazol have also been reported for the chromatophore membrane [15] but a blue shift was observed with myxothiazol in contrast to the red shift with antimycin A, which has been found before [16]. Since, also in the mitochondrial membrane, the spectral change caused by myxothiazol [17] does not resemble the one found for the isolated bc_1 complex [14], we must conclude that cytochrome *b*-566 changes during isolation of the complexes from the membrane, even if Triton X-100 is avoided. The spectral shift by myxothiazol is best observable at slightly acidic pH (see [15]).

Table 1

pH-Dependence of the midpoint potentials of cytochrome *b*

	Midpoint potential (mV)	
pH	b_h	b_l
6.0	+100	− 47
7.0	+ 50	− 90
8.0	+ 35	−110

Redox titration of the isolated cytochrome bc_1 complex was carried out as in [1]. The E_m-values were obtained with the assumption of two $n = 1$ components present in equal amounts [1]. The pH was adjusted with glycylglycine, Hepes and MES at 50 mM total buffer concentration; b_h and b_l stand for high-potential and low potential cytochrome *b*, respectively

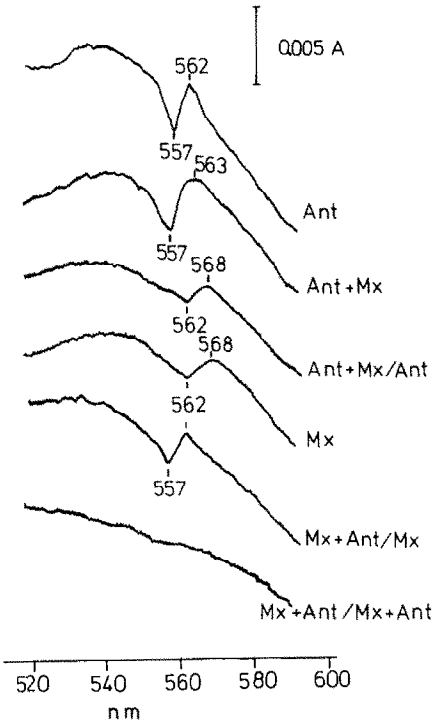

Fig.3. Shifts of the α-peaks of the cytochromes *b* by antimycin A and myxothiazol. The difference spectra were recorded at RT with the cytochrome bc_1 complex in 50 mM Na-acetate (pH 5.8), 0.9 μM in cytochrome c_1. The complex was reduced by dithionite, antimycin A was added to 10 μM to the sample cuvette and the difference spectrum to the reference cuvette without antimycin A was recorded (Ant). Then myxothiazol was added to 10 μM to the sample cuvette (Ant + Mx), followed by addition of antimycin to the reference cuvette (Ant + Mx/Ant). In a second set of recordings (lower 3 spectra) the sequence of additions was changed as indicated.

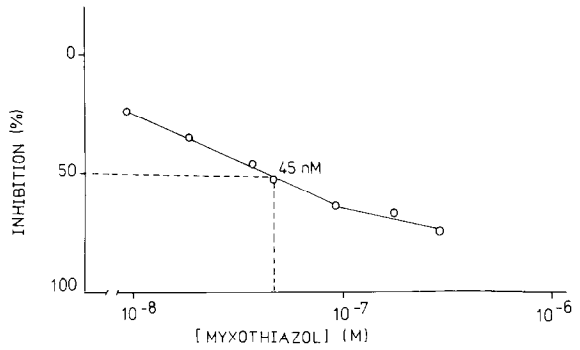

Fig.4. Inhibition of ubiquinol–cytochrome *c* oxidoreductase activity by myxothiazol. The reaction mixture contained the cytochrome bc_1 complex, 37 nM in cytochrome c_1, 50 mM MES–NaOH (pH 6.2), 20 μM cytochrome *c* from horse heart, and the indicated concentrations of myxothiazol. The reaction was started by the addition of ubiquinol-3 to 50 μM. The uninhibited rate was 15 μmol cytochrome *c* reduced . nmol cytochrome $c_1^{-1} . h^{-1}$.

Myxothiazol is an inhibitor of the ubiquinol–cytochrome *c* oxidoreductase activity of the isolated complex, almost as potent as antimycin A [1]; 50% inhibition is reached at a molar ratio of myxothiazol to cytochrome c_1 of 1.3 (fig.4). Fig.5 shows that myxothiazol inhibits ferricyanide-induced reduction of cytochrome *b*, but does not inhibit the antimycin A-sensitive, direct reduction of cytochrome *b* by ubiquinol. The results in fig.5 fit a Q-cycle [18] or a b-cycle [19] mechanism for the ubiquinol–cytochrome *c* oxidoreductase, where myxothiazol inhibits the reduction of low-potential cytochrome *b*-566 by ubisemiquinone, formed from ubiquinol by oxidation with the Rieske FeS center, and antimycin A inhibits the reversible reoxidation of the high-potential cytochrome *b*-561 by ubiquinone in a different site or state. Exactly the same conclusion as to the sites of inhibition of the two antibiotics has been reached before in [15] for the chromatophore membrane, and in [14] for the isolated, mitochondrial cytochrome bc_1 complex.

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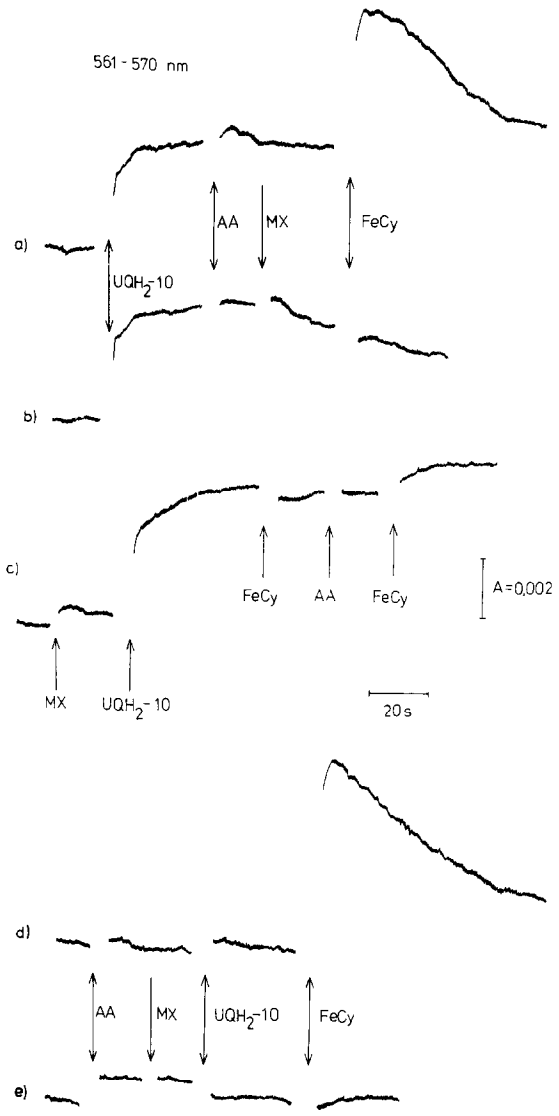

Fig.5. Inhibition of oxidant-induced reduction of cytochrome *b* by myxothiazol. The traces were recorded in the dual wavelength mode with 561 nm as the measuring and 570 nm as the reference wavelength. The cytochrome bc_1 complex was diluted to 0.54 μM in 50 mM glycylglycine (pH 7.4). A 1-ml cuvette was used without stirring. The additions during the experiments (a–e) were as indicated by arrows: Ubiquinol-10 (UQH_2-10), 50 mM in 10% Triton X-100 to 250 μM; antimycin A to 10 μM (AA); myxothiazol to 45 μM (MX); ferricyanide to 100 μM (FeCy). The spectral changes after addition of UQH_2-10 in traces a, b and c correspond to about 25%, the changes after addition of ferricyanide in traces a and d correspond to 30 and 40% of the dithionite-reducible cytochrome *b*.

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