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EPR characterization of the cytochrome b-c_1 complex from *Rhodobacter sphaeroides*

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EPR characteristics of cytochrome c_1, cytochromes b-565 and b-562, the iron-sulfur cluster, and an antimycin-sensitive ubisemiquinone radical of purified cytochrome b-c_1 complex of *Rhodobacter sphaeroides* have been studied. The EPR specra of cytochrome c_1 shows a signal at $g = 3.36$ flanked with shoulders. The oxidized form of cytochrome b-562 shows a broad EPR signal at $g = 3.49$, while oxidized cytochrome b-565 shows a signal at $g = 3.76$, similar to those of two b cytochromes in the mitochondrial complex. The distribution of cytochromes b-565 and b-562 in the isolated complex is 44 and 56%, respectively. Antimycin and 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) have little effect on the $g = 3.76$ signal, but they cause a slight downfield and upfield shifts of the $g = 3.49$ signal, respectively. 5-Undecyl-6-hydroxyl-4,7-dioxobenzothiazole (UHDBT) shifts the $g = 3.49$ signal downfield to $g = 3.56$ and sharpens the $g = 3.76$ signal slightly. Myxothiazol causes an upfield shift of both $g = 3.49$ and $g = 3.76$ signals. EPR characteristics of the reduced iron-sulfur cluster in bacterial cytochrome b-c_1 complex are: $g_x = 1.8$ with a small shoulder at $g = 1.76$, $g_y = 1.89$ and $g_z = 2.02$, similar to those observed with the mitochondrial enzyme. The $g_x = 1.8$ signal decreased and the shoulder increased concurrently as the redox potential decreased, indicating that the environment of the iron-sulfur cluster is sensitive to the redox state of the complex. UHDBT sharpens the g_z and and shifts it downfield from $g = 2.02$ to 2.03, and shifts g_x upfield from $g = 1.80$ to 1.78. UHDBT also causes an upfield shift of g_y but to a much lesser extent compared to the other two signals. Addition of DBMIB causes a downfield shift of the g_y from 1.89 to 1.94 and broadens the g_x signal with an upfield to $g =$ 1.75. Myxothiazol and antimycin show little effect on the g_y and g_z signals, but they broaden and shift the g_x signal upfield to $g = 1.74$. However, the myxothiazol effect is partially reversed by UHDBT. An antimycin-sensitive ubisemiquinone radical was detected in the cytochrome b-c_1 complex. At pH 8.4, the antimycin-sensitive ubisemiquinone radical has a maximal concentration of 0.66 mol per mol complex at 100 mV. The concentration of antimycin sensitive Q-radical correlates well with the enzymatic activity of the complex. The antimycin-sensitive ubisemiquinone radical exhibits a pH dependency of approx. 60 mV/pH unit, between pH 8.0 and 9.0, suggesting the possible involvement of a dissociable group, with a pK_a between 8.0 and 9.0, in Q-binding.

Introduction

The cytochrome b-c_1 complex (formerly known as cytochrome b-c_2 complex) [1,2] which catalyzes electron transfer from ubiquinol to cytochrome c_2 in the cyclic electron transfer system of the photosynthetic bacterium *Rhodobacter sphaeroides*, has been isolated and characterized [3–7]. The complex is functionally analogous to mitochondrial ubiquinol-cytochrome c reductase [8,9]. The isolated cytochrome b-c_1 complex contains four polypeptides with molecular masses of 43, 30, 24 and 15 kDa [3,5]. The first three subunits are cytochromes b [10,11] and c_1 [12], and the iron-sulfur protein. The smallest molecular weight subunit and cytochrome b were identified as the Q-binding proteins in this complex by photoaffinity labeling using an azido-Q derivative, 3-azido-2-methyl-5-methoxy-6[3,7-dimethyl [^{3}H]octyl]-1,4-benzoquinone [13]. Cytochromes b and c_1 from this complex have been isolated, purified to homo-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; Q_2H_2, 2,3-dimethoxyl-5-methyl-6-gerany,6-benzoquinol; Q, ubiquinone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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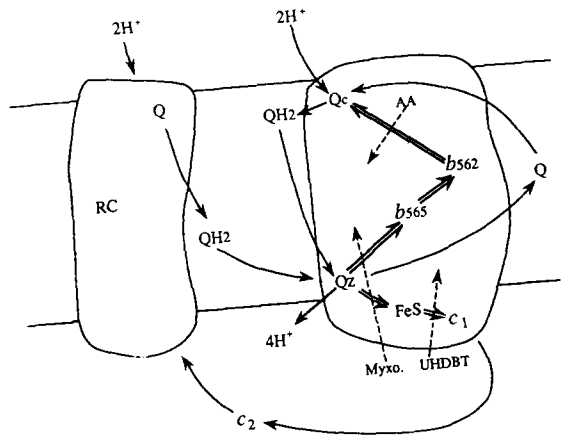

Scheme. I. Current view of electron transfer pathway and the action site of inhibitors in the cytochrome b-c_1 complex.

geneity, and characterized [10–12]. Because it has a simpler subunit structure but has properties similar to those of the mitochondrial enzyme, this bacterial cytochrome b-c_1 complex is an ideal model for structure function studies of this particular electron transfer region. Scheme I, adopted from Crofts et al. [14], summarizes the current view of the electron transfer pathway and the action site of inhibitors [15,16] in the cytochrome b-c_1 complex.

Although the EPR characteristics of cytochromes b and c_1 and the iron-sulfur protein in the isolated *R. sphaeroides* cytochrome b-c_1 complex have been reported [17] to be similar to those of their mitochondrial counterparts, the effect of specific inhibitors on the EPR characteristics of these redox components has not been examined. Two ubisemiquinone radicals, one which is sensitive to antimycin and another which is not, were detected in chromatophores [18] of this photosynthetic bacterium. Similar studies on the thermodynamic properties of these two radicals in the purified cytochrome b-c_1 complex [19,20] have not been made. Herein we report a method for large-scale preparation of the cytochrome b-c_1 complex from *R. sphaeroides* wild-type, and the EPR characterization of cytochromes b and c_1, the iron-sulfur protein, and the antimycin-sensitive ubisemiquinone radical in the cytochrome b-c_1 complex under various conditions.

Materials and Methods

Materials

Horse cytochrome c, type III, antimycin, and *n*-dodecyl β-D-maltoside were from Sigma; 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) from Aldrich; and myxothiazol from Boehringer-Mannheim, F.R.G. 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) [21], 2,3-dimethoxyl-5-methyl-6-gerany,6-benzoquinol (Q_2H_2) [22], and dodecyl maltoside [23] were synthesized according to previously reported procedures.

The cell culture of *Rhodobacter sphaeroides* wild-type strain NCIB8253 was obtained from Dr. Robert Niderman, Department of Biochemistry, Rutgers University. The growth conditions were exactly as reported [24] for *R. sphaeroides* R-26.

Isolation of cytochrome b-c_1 complex from Rhodobacter sphaeroides wild type

144 g of frozen cell paste was thawed and mixed with 432 ml of 20 mM Tris-succinate (pH 8.0) containing 1 mM EDTA. Cells were broken with a French pressure cell press, at a pressure of 1000 psi and flow rate of 20 ml/min. DNase (2.88 mg) and phenylmethylsulfonyl fluoride (PMSF) (360 mg in 14 ml ethanol) were added to the broken cell suspension before it was centrifuged at $17000 \times g$ for 15 min. The precipitates were discarded and the supernatant was centrifuged at $200000 \times g$ for 90 min. The precipitates thus obtained were suspended with 50 mM Tris-HCl (pH 8.0) containing 1 mM $MgSO_4$ and 1 mM PMSF (TMP buffer) to the final volume of 400 ml, and centrifuged at $200000 \times g$ for 90 min. The precipitate (chromatophore) obtained was homogenized with buffer containing 10% glycerol and adjusted to a protein concentration of 10 mg per ml with the same buffer. The chromatophores thus obtained can be frozen at −80°C for about 1 month.

The cytochrome b-c_1 complex was solubilized from chromatophore by Triton X-100 and purified with DEAE-Bio-Gel A and DEAE-Sepharose 6 B column chromatography in the presence of 0.01% dodecyl maltoside. To the chromatophore suspension was added Triton X-100 (70 μl of 15% Triton X-100 per ml suspension) and stirred for 30 min at 0°C before the mixture was centrifuged at $27000 \times g$ for 30 min. The hard precipitates at the bottom of the centrifuge tubes were discarded and the loose pellets and supernatant were collected and NaCl was added (25 μl of 4 M NaCl per ml solution). After stirring for 1 h at 0°C, the mixture was centrifuged at $200000 \times g$ for 90 min. The supernatant obtained was applied to a DEAE-Bio-Gel A column equilibrated with, in sequence, 100 ml 4 M NaCl, 100 ml 1 M Tris-HCl (pH 8.0) and 700 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM $MgSO_4$, 0.01% dodecyl maltoside (TMD buffer), and 100 mM NaCl. The column was washed with, in sequence, 1000 ml of TMD buffer containing 100 mM NaCl, 200 ml of TMD buffer containing 150 mM NaCl, and 500 ml of TMD buffer containing 200 mM NaCl. The crude cytochrome b-c_1 complex was eluted from the column with TMD buffer containing 300 mM NaCl. The collected b-c_1 complex was diluted with equal volume of TMD buffer containing 100 mM NaCl and applied to a DEAE-Sepharose 6 B column equilibrated with the same buffer. The column was washed with 100 ml each of TMD

TABLE I

Summary of purification data

Treatments	Protein mg	*b* nmol	c_1 nmol	Activity at 23°C	
				units [a]	yield
Chromatophores					
+ Triton X-100	4800	4080	-	6965	100
Triton X-100 extract	1059	2162	-	3800	55
DEAE-Bio-Gel A					
300 mM NaCl eluate	85	648	360	1825	26
DEAE-Sepharose 6B					
400 mM NaCl eluate	27	415	230	734	11

[a] μmol cytochrome *c* reduced per min per mg protein.

buffer containing 200 mM NaCl and 300 mM NaCl. The pure cytochrome b-c_1 complex was eluted from the column with TMD buffer containing 400 mM NaCl and concentrated by Amicon membrane concentrator to about 10 mg/ml. The concentrated solution was mixed with glycerol to a final concentration of 10%, and stored at −80°C until use. The purification data are summarized in Table I.

EPR spectra were obtained with a Bruker ER 200D spectrometer equipped with an Oxford Continuous Flow Cryostat. The details of instrument settings are given in the legends of figures.

Potentiometric titration was carried out anaerobically in the presence of 20 μM redox-mediating dyes as described by Dutton [25]. The redox mediators used were: 1,4-benzoquinone, 1,4-naphthoquinone, duroquinone, indigocarmine, indigo tetrasulfonate, phenosafranin and safranin O. For quantitative estimation of the ubisemiquinone radical and iron-sulfur cluster of the Rieske protein, 3-maleimidiomethyl-1-proxyl and ferredoxin were used as the spin quantization standard.

Protein [26], cytochromes *b* [27] and c_1 [28], phospholipids [29] and ubiquinone [30] were determined according to the reported methods. Electrophoresis was carried out according to Laemmli [31], except 2 M urea was included in the separating gel.

Assay conditions. The purified cytochrome b-c_1 complex was diluted in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 0.1% dodecyl maltoside to a cytochrome *b* concentration of 2 μM. An appropriate amount of enzyme solution was added to a assay mixture containing 1.0 ml of 50 mM sodium/potassium phosphate buffer (pH 7.0) 1 mM EDTA, 50 μM cytochrome *c* and 25 μM Q_2H_2. Ubiquinol-cytochrome *c* reductase activity was measured by the reduction of cytochrome *c* (the increase of the absorbance at 550 nm) at 23°C in a Cary spectrophotometer, model 219. A millimolar extinction coefficient of 18.5 was used in calculation. The nonenzymatic oxidation of Q_2H_2 was determined under the same conditions in the absence of enzyme.

Results and Discussion

Properties of the cytochrome b-c_1 complex

The cytochrome b-c_1 complex of wild-type *R. sphaeroides* was prepared by a method involving Triton X-100 solubilization followed by DEAE-Bio-Gel A and DEAE-Sepharose 6B column chromatography in the presence of 0.01% dodecyl maltoside. This method is developed by the adoption of methods of Yu et al. [5] and Ljungdahl et al. [6]. The isolated cytochrome b-c_1 complex contains four subunits with apparent molecular masses of 43000, 30000, 21000, and 15000 Da analyzed by SDS-PAGE (Fig. 1). The purity and subunit structure are comparable to those of most purified preparations [6,7] of the cytochrome b-c_1 complex from *R. sphaeroides*.

The major advantage of the present method over the method of Ljungdahl et al. [6] is on its low cost and suitability for large-scale operation. As indicated in Table I, about 11% of the cytochrome b-c_1 complex present in chromatophores was recovered in the final step which is about the same as that reported for the method of Ljungdahl et al. [6]. The use of Triton X-100 instead of dodecyl maltoside (0.66 mg/mg protein) to solubilize the cytochrome b-c_1 complex from chromatophores greatly reduces the cost, especially when a large quantity of the cytochrome b-c_1 complex is needed. The cost for the present method is only a quarter of that of the Ljungdahl et al. [6], or Andrews et al. [7]. Replacing the sucrose gradient centrifugation step used in Ljungdahl et al. [6] for the preparation of chromatophores

Fig. 1. SDS-PAGE of purified cytochrome b-c_1 complex. Lane A, 10 μl of cytochrome b-c_1 complex (5 mg/ml) in 50 mM Tris-HCl buffer (pH 8.0) containing 0.01% of dodecyl maltoside and 10% glycerol was treated with 40 μl of 4% SDS and 5% 2-ME for 2 h at 37°C before being subjected to electrophoresis. Lane B, the molecular weight reference proteins used were phosphorylase *b* (97000) bovine serum albumin (66200) ovalbumin (21000) and lysozyme (14400).

TABLE II

Chemical composition of the cytochrome b-c_1 complexes of R. sphaeroides wild-type

Component	Concentrations (nmol/mg)	
Preparation:	present method	Ljungdahl et al.
Cytochrome *b*	15.3	13.5
Cytochrome c_1	8.5	8.5
Iron-sulfur cluster	7.2	7.1
Phospholipid (phosphorus)	82.9	149.7
Ubiquinone-10	11.2	10.8
Enzymatic activity		
Spec. act. (μmol *c* reduced /min per mg) at 23°C	27.2	27.8
TN (s^{-1})	50.4	52.2 (128) [a]

[a] Reported by Ljungdahl et al. [6].

with differential centrifugations made the large-scale preparation possible.

Assayed under our described conditions the isolated complex catalyzes electron transfer from ubiquinol to cytochrome *c* with specific activity of 27.2 μmol cytochrome *c* reduced per min per mg protein at 23°C. This is lower than the activity reported for the cytochrome *b*-c_1 complex by Ljungdahl et al. [6]. This discrepancy is probably due to the conditions employed in the activity assay, because the enzymatic activities for cytochrome *b*-c_1 complexes prepared, side by side, from the same batch of cell using the present method and that of Ljungdahl et al. [6] are the same when assayed under our described conditions. Addition of asolectin or phospholipid prepared from chromatophores to the isolated complex, prepared either by the present procedure or according to Ljungdahl et al. [6], does not increase the activity, suggesting that the amount of phospholipid present in the isolated complex is sufficient for maximal activity. A preparation with a much lower phospholipid content has also been shown to be active [7].

Table II compares the chemical composition of cytochrome *b*-c_1 complexes prepared with the present method and with the method of Ljungdahl et al. Both preparations contain about the same amounts of cytochromes *b* and c_1, nonheme iron-sulfur protein, and ubiquinone-10. However, the phospholipid content in the cytochrome *b*-c_1 complex obtained with the present method is only about half of that prepared with the method of Ljungdahl et al. [6]. The Q_{10} content in the isolated cytochrome *b*-c_1 complexes obtained by these two methods is higher than that of the mitochondrial enzyme, in which a unit stoichiometry between Q and c_1 is obtained for the most active preparation. It is not known whether this more than stoichiometric amount of Q present in the bacterial enzyme results from the presence of dodecyl maltoside or from the intrinsic property of the preparation. Since dodecyl maltoside behaves like phospholipid in maintaining the enzymatic activity, it is likely that part of the Q_{10} detected in the isolated complex is dissolved in detergent and not really bound to protein. Further investigation is needed before the two populations of Q, one protein bound and the other detergent associated, can be established. The concentration of iron-sulfur cluster, estimated chemically or by spin quantification, is lower than other redox components. This lower-than-stoichiometric amount of iron-sulfur cluster might partially contribute to the lower activity of the system comparing to mitochondrial enzyme.

EPR characteristics of cytochromes b and c_1 in the cytochrome b-c_1 complex

Like the mitochondrial cytochrome *b*-c_1 complex, the bacterial complex contains two spectral distinguishable *b* cytochromes: *b*-565 and *b*-562 [5,8], and a *c*-type cytochrome, cytochrome c_1. The EPR spectra of cytochrome c_1, obtained by measuring the difference spectra between the fully oxidized and ascorbate-reduced samples, shows a signal at $g = 3.36$ (Fig. 2), which are similar to those observed in the R-26 preparation [17], except that the wild-type enzyme has a slight lower *g* value with less apparent shoulders on the both sides. The characteristics of EPR spectra of Rs. cytochrome c_1 are very similar to those observed in cytochrome c_1 of beef heart cytochome *b*-c_1 complex.

To eliminate complexity caused by the overlapping of the cytochrome c_1 signal with that of cytochrome *b*,

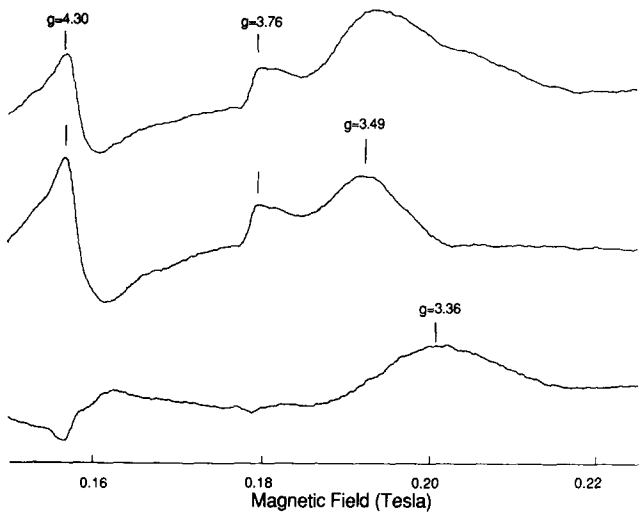

Fig. 2. The EPR spectra of cytochromes *b* and c_1 in the cytochrome *b*-c_1 complex. Isolated cytochrome *b*-c_1 complex, 8 mg/ml, in 50 mM Tris-acetate buffer (pH 8.0) was used. Spectrum A is the oxidized cytochrome *b*-c_1 complex which shows cytochromes *b*-562, *b*-565 and c_1. Spectrum B is the ascorbate reduced complex which represents cytochromes *b*-562 and *b*-565. Spectrum C represents the difference spectrum between A and B, which is the EPR signal of cytochrome c_1. The instrument settings were as follows: microwave frequency, 9.45 GHz; microwave power, 20 mW; modulation amplitude, 20 G; time constant, 0.5 s; scan rate, 5 G/s; temperature 14 K.

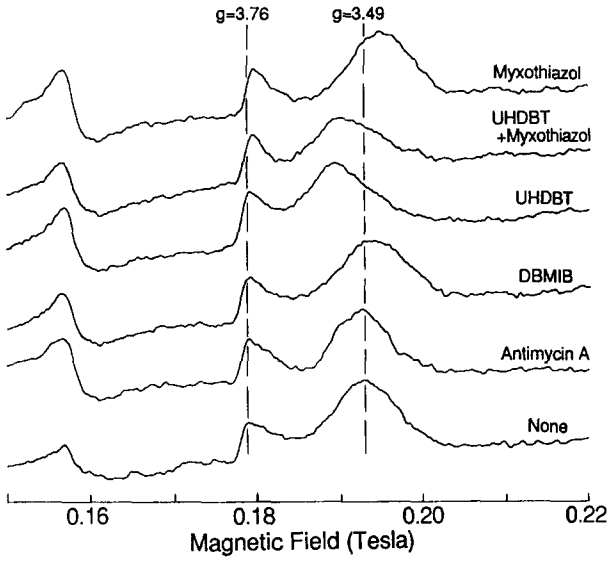

Fig. 3. The EPR spectra of *b* cytochromes in the cytochrome *b*-c_1 complex in the presence of various inhibitors. Isolated cytochrome *b*-c_1 complex, 8 mg/ml, in 50 mM Tris-acetate buffer (pH 8.0) which has been reduced with 1 mM sodium ascorbate, was treated with or without a 2 molar excess of inhibitors, individually or in combination, as indicated. The samples were incubated at 0°C for 10 min before being placed in EPR tubes and frozen in liquid nitrogen. The g_z region of cytochromes *b* were examined at 10 K. The instrument settings were the same as those in Fig. 2.

the EPR spectra of *b* cytochromes were taken after the cytochrome *b*-c_1 complex is reduced with sodium ascorbate. Three signals: a broad one at $g = 3.49$, a sharper peak at $g = 3.76$, and rather smaller one at $g = 4.3$ are observed in the ascorbate-reduced cytochrome *b*-c_1 complex (last spectrum of Fig. 3). When the complex is partially reduced with dithionite until all the *b*-562 is reduced (-50 mV), and subjected to EPR analysis, no $g = 3.49$ signal is observed, indicating that this signal belongs to cytochrome *b*-562 and the signal at $g = 3.76$ belongs to cytochrome *b*-565. The $g = 3.76$ signal disappears when all the cytochrome *b* in the preparation is reduced by dithionite. Similar EPR spectra were reported for cytochromes *b* in mitochondrial cytochrome *b*-c_1 complex [32–34]. The $g = 4.3$ signal is possibly due to nonspecifically bound iron(III) present in the preparation because the peak persists even alter all the cytochrome *b* in the complex is fully reduced by dithionite. Also, the $g = 4.3$ signal is much smaller if the spectra are corrected for the buffer solution.

According to Salerno [34], low-spin *b*-type cytochromes should have three EPR signals, one at $g = 3$ to 4 and one at g value around 1.6 to 1.9, and another having the *g* value near or below 1.0. However, the last two signals are too broad and too weak to be detected under the conditions used.

In contrast to mitochondrial cytochrome *b*-c_1 complex, which shows no high-spin heme EPR signal ($g = 6$), a significant amount of high-spin heme signal is observed in bacterial cytochrome *b*-c_1 complex (data not shown). The magnitude of the $g = 6$ signal is generally higher in cytochrome *b*-c_1 complex preparations with lower enzymatic activities. When the cytochrome *b*-c_1 complex was kept at room temperature, the enzymatic activity decreased as the incubation time increased, and the size of the $g = 6$ signal increased. The increase in the high-spin heme signal is at the expense of both low spin heme signals of cytochromes *b*-562 and *b*-565. That the conversion of low-spin cytochrome b_6 to high-spin cytochrome b_6 results from the loss of its sixth ligand has been suggested [35]. Whether or not the conversion of low-spin cytochrome *b* to high-spin cytochrome *b* in the bacterial cytochrome *b*-c_1 complex follows the same mechanism as that suggested for cytochrome b_6 in the b_6-*f* complex remains to be investigated.

The overlapping in absorption spectra between and the lack of true extinction coefficients for cytochromes *b*-562 and *b*-565 make the precise quantization of these cytochromes in the isolated complex difficult [36]. EPR spectra analysis has been successfully used to measure cytochromes *b*-565 and *b*-562 in the mitochondrial cytochrome *b*-c_1 complex [37]. We have adapted their method to estimate the amount of these two *b* cytochromes in the purified bacterial complex. The relative distribution is 56 and 44%, respectively, for *b*-562 and *b*-565. This distribution agrees with the average ratio of cytochrome *b* to cytochrome c_1 of 1.8 in isolated complexes, assuming one mol cytochrome *b*-562 and 0.8 mol cytochrome *b*-565 per mol of cytochrome c_1. A similar ratio of 1.10 : 0.86 : 1.00 for cytochromes *b*-562, *b*-565 and c_1 is estimated from the EPR spectra of these cytochromes. The EPR spectra of cytochrome c_1 is obtained by subtracting the EPR spectra of the ascorbate-reduced cytochrome *b*-c_1 complex from the EPR spectra of the oxidized complex. If we further assume that both cytochrome *b*-565 and *b*-562 are present in an equal molar ratio in intact membrane, as in the mitochondrial system, then the unequal distribution of these two *b* cytochromes in the isolated *b*-c_1 complex would suggest that part of cytochrome *b*-565 was lost or converted into the high-spin form during the isolation procedure. The ratio of cytochromes *b* to c_1 in isolated cytochrome *b*-c_1 complex of *R. sphaeroides* R-26 (prepared by the Triton-calcium phosphate method) is slightly lower (1.5) and the distribution between cytochrome *b*-562 and *b*-565 was estimated to be 66 and 34%. Apparently, more loss in cytochrome *b*-565 occurs in the R-26 preparation. This may be partially responsible for the lower activity of cytochrome *b*-c_1 complex prepared from the R-26. Although a ratio of 2.0 for cytochrome *b* to c_1 in isolated bacterial complex from wild-type has been reported [3], a ratio of 1.7 to 1.8 is commonly obtained. Part of this discrepancy can be attributed to the use of different absorption coefficients

in the calculation of the concentrations of these cytochromes. Although the b/c_1 ratio of the cytochrome b-c_1 complex of wild-type *R. sphaeroides* is similar to that obtained for the beef enzyme, the activity is still much lower. The reason for this is not clear. Perhaps some essential component(s) besides cytochrome b has become limiting in the isolated complex. The iron-sulfur cluster, but not its apo-protein, and the specific bound phospholipid may be the components lost or denatured during preparation. The loss or denaturation of these essential components may lead to the easier conversion of low-spin cytochrome b to high-spin cytochrome b and may be causing the decrease in activity.

Effect of electron transfer inhibitors on EPR characteristics of cytochromes b

Fig. 3 shows the effect of various inhibitors [15,16], such as antimycin [38], DBMIB [39], myxothiazole [40], and UHDBT [41], on EPR characteristics of cytochromes b-562 and b-565 in cytochrome b-c_1 complex. Antimycin and DBMIB have little effect on the $g = 3.76$ signal (cytochrome b-565), but the former causes a slight downfield shift and the latter causes a slight upfield shift of the $g = 3.49$ signal (cytochrome b-562), suggesting that the binding site of these two inhibitors is closer to cytochrome b-562 [38,42] than to cytochrome b-565. The binding site of antimycin in the cytochrome b-c_1 complex has not yet been established. Photoaffinity labeling studies [43] have indicated that antimycin is bound primarily to a small molecular weight protein subunit in the mitochondrial cytochrome b-c_1 complex, but such a subunit is absent from the *R. sphaeroides* b-c_1 complex. More recently, inhibitor resistant mutant studies [44] have found that a single glycine mutation in the cytochrome b protein has rendered yeast enzyme antimycin-resistant, suggesting an involvement of cytochrome b in antimycin binding. The observation of an antimycin effect on EPR characteristics of cytochrome b-562 confirms the involvement of cytochrome b protein in the antimycin binding.

Myxothiazol a Q_0 site inhibitor, which has been shown to cause a spectral red shift of cytochrome b-565 [45] causes upfield shifts of both $g = 3.49$ and $g = 3.76$ signals to $g = 3.354$ and $g = 3.74$, respectively. It also sharpens the $g = 3.74$ signal slightly. These results indicate that the myxothiazol binding site is close to both b cytochromes. These results are also consistent with the evidence obtained from the mutation study in yeast in which mutations that confer myxothiazol resistance have been located to mitochondrial cytochrome b gene [46].

UHDBT which shows little effect on the EPR characteristics of mitochondrial cytochromes b causes a significant downfield shift of the $g = 3.49$ signal to $g = 3.56$ and sharpens slightly the $g = 3.76$ signal, indicating that the microenvironment of b cytochromes in the bacterial b-c_1 complex may be slightly different from that in the mitochondrial system. Since UHDBT is known to affect ubiquinol oxidation [41,47] and cytochrome b-565 is the immediate electron acceptor for ubiquinol [48] whose first electron has been delivered to the iron-sulfur protein, it is not surprising to see the effect of UHDBT on cytochrome b-565. The significant effect of UHDBT on cytochrome b-562 is rather unexpected. One possibility is that the distance between cytochromes b-565 and b-562 is rather small, and the binding of inhibitor is strong enough to cause a conformational change to modify both heme environments. It was reported [49] that UHDBT inhibition results from the perturbation of a specific cardiolipin molecule which is tightly associated with a phospholipid-dependent protein(s) presumably cytochrome b protein in ubiquinol-cytochrome c reductase. The effect of UHDBT on both cytochrome b signals further suggests that this specific cardiolipin molecule is associated with cytochrome b protein at a site near to both heme groups. The perturbation of phospholipid environments upon the binding of UHDBT to specific cardiolipin molecules will thus alter or modify the heme environments of both b cytochromes.

Addition of myxothiazol to a UHDBT-treated cytochrome b-c_1 complex did not revert the EPR characteristics to those of a sample treated only with myxothiazol, suggesting that either the effect of myxothiazol on b cytochromes is weaker than that of UHDBT [45,50], or the binding site of these two inhibitors is not significantly overlapped.

EPR characteristics of the iron-sulfur cluster in the cytochrome b-c_1 complex

Fig. 4 shows the EPR spectra of the iron-sulfur cluster in ascorbate-reduced cytochrome b-c_1 complex in the presence and absence of various inhibitors. The iron-sulfur protein, generally referred as Rieske's iron-sulfur protein [51], in isolated *R. sphaeroides* cytochrome b-c_1 complex gives EPR signals at $g = 2.02$, 1.89, and 1.80. These signals are similar to those observed in other cytochrome b-c_1 complexes.

Addition of UHDBT to ascorbate-reduced cytochrome b-c_1 complex sharpens and shifts g_z to 2.03 and shifts g_y to 1.87. The effect of inhibitors on the g_x of iron-sulfur cluster will be discussed in the next section. Myxothiazol and antimycin have little effect on g_z and g_y regions. The effect of UHDBT on g_z and g_y region was reversed by addition of myxothiazol. This is different from the effect of UHDBT on cytochrome b-562, which was not reversed by the addition of myxothiazol. These results suggest again that these two inhibitors do not bind to the same site. Addition of DBMIB causes a upfield shift of g_z and downfield shift of the g_y from 1.89 to 1.94 similar to the effect on the mitochondrial enzyme.

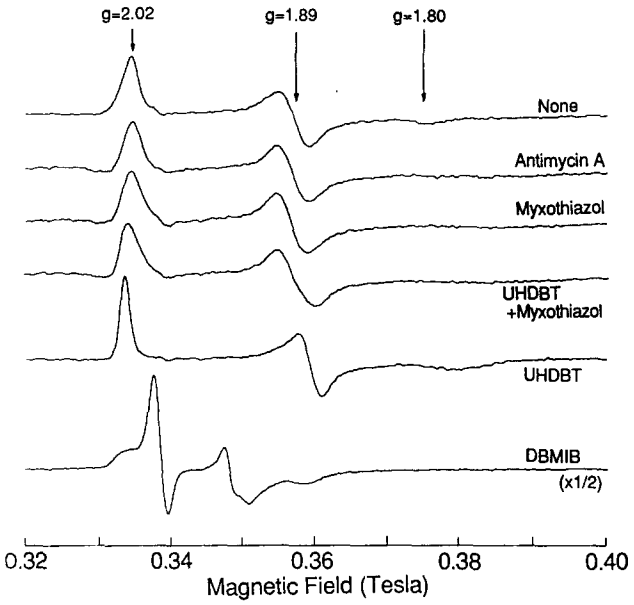

Fig. 4. The EPR spectra of the iron-sulfur cluster in the cytochrome b-c_1 complex in the presence and absence of inhibitors. The ascorbate reduced cytochrome b-c_1 complex was prepared as described in Fig. 3. The EPR spectra were taken at 15 K, with the following instrument settings: microwave frequency, 9.45 GHz; microwave power, 6.3 mW; modulation amplitude, 6.3 G; time constant, 0.5 s; scan rate 4 G/s.

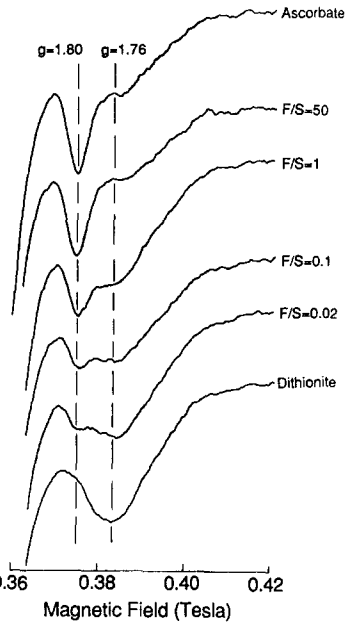

Fig. 5. Effect of redox potential on the g_x of the iron-sulfur cluster in the cytochrome b-c_1 complex. Fully oxidized cytochrome b-c_1 complex, 8 mg/ml, in 50 mM sodium/potassium phosphate (pH 7.0) was mixed with a catalytic amount of succinate-cytochrome c reductase. The sample was divided into 0.3 ml portions and 20 μl of 1 M ascorbate, 0.5 M dithionite or 0.5 M of the fumarate/succinate (F/S = 0.1 to 100) mixture was added. The EPR spectra were taken at conditions identical to those described for Fig. 4.

Since it has been reported that in cytochrome b-c_1 complexes of beef heart [52,53], yeast mitochondria [54], photosynthetic bacteria [55] and *P. denitrificans* [56] the line shape of the g_x of the iron-sulfur cluster is dependent on the redox state of the complex, it is important to see whether or not the g_x of the iron-sulfur protein of this purified bacterial complex is also affected by a change of redox potential. This is shown in Fig. 5. When the complex is reduced with a limiting amount of ascorbate, the g_x is found at $g = 1.80$ with a small shoulder at $g = 1.76$. When the redox potential of the complex decreases, the shoulder at $g = 1.76$ increases at the expense of signal at $g = 1.80$. Different redox potentials were obtained by adding mixtures with various fumarate/succinate ratios in the presence of catalytic amounts of mitochondrial succinate-cytochrome c reductase. When the complex is fully reduced by dithionite, the g_x is a broad line at 1.76. It was reported [53] that removal of Q from beef heart mitochondria broadened the g_x signal; whether or not the bacterial cytochrome b-c_1 shows similar behavior cannot be established because the procedure for reversible removal of Q from the complex has not yet been developed.

Fig. 6 summarizes the effect of various inhibitors on the g_x region of the EPR spectra of the iron-sulfur cluster in the cytochrome b-c_1 complex. Similar to beef heart [52] and yeast [57] enzymes, UHDBT shifts g_x from 1.80 to 1.76. Addition of myxothiazol broadens the line shape of the g_x and causes an upfield shift to $g_x = 1$ 76. Addition of myxothiazole to UHDBT-treated sample partially restores the g_x line shape to that of a sample treated only with myxothiazol ($g_x = 1.76$), suggesting that the effect of UHDBT is not as strong as

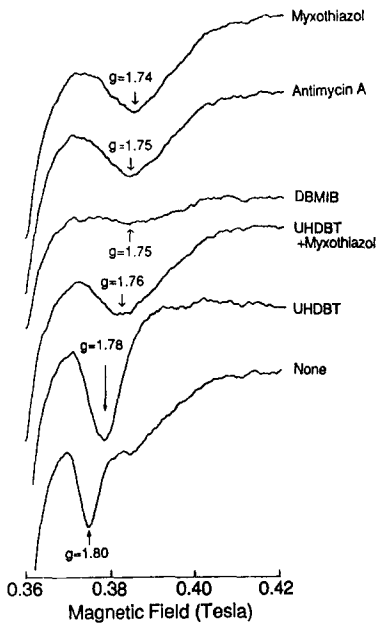

Fig. 6. The EPR characteristics of the g_x of the iron-sulfur protein in cytochrome b-c_1 complexes treated with various inhibitors. Sample treatments were the same as those shown in Fig. 3. The conditions for the EPR measurement were the same as described in Fig. 4.

that of myxothiazol in respect to the effect on the iron-sulfur center. One plausible explanation is that the binding of UHDBT to specific cardiolipin molecule causes conformational change of cytochrome *b*, which in turn induces the change in the iron-sulfur protein. From the prediction of the Q-cycle [58] and the recent identification of cytochrome *b* as one of the Q-binding sites [13], one can visualize that either on cytochrome *b*-565 or in its proximity a quinol oxidation site must be formed. In fact, one can even speculate that it is a quinol-cytochrome *b*-565 complex rather than quinol alone that serves as substrate for the iron-sulfur cluster. This way once the first electron is released to the iron-sulfur protein, the second electron is immediately transferred to cytochrome *b*-565. If this deduction is correct, then cytochrome *b*-565 and the iron-sulfur cluster must be rather close. The idea that cytochrome *b*-565 and the iron-sulfur cluster forms a quinol oxidation site has been speculated [35,45].

UHDBT also has a stronger effect on the iron-sulfur protein than DBMIB does. Upon addition of UHDBT to the DBMIB-treated sample, the DBMIB-altered EPR signal at 1.94 is returned to 1.89 (data not shown). A similar phenomenon has been reported in the cytochrome b_6-*f*-complex [59]. As indicated in Fig. 6, antimycin shows an effect similar to that of myxothiazol on the g_x of the iron-sulfur cluster. This is different from the reported observation in beef heart mitochondria [53] in which antimycin showed little effect on the g_x of the iron-sulfur cluster. One plausible explanation is the difference in phospholipid environments. According to Tsai and Palmer [60], the binding of antimycin to yeast cytochrome *b*-c_1 complex is phospholipid-dependent whereas binding of antimycin to beef heart cytochrome *b*-c_1 complex is not. Since there is no evidence that indicates a direct interaction between antimycin and the iron-sulfur protein, the effect is difficult to explain. However, since a single mutation in the cytochrome *b* protein can change the yeast mitochondrial *b*-c_1 complex to become insensitive to antimycin, it is possible that the binding of antimycin to cytochrome *b* may indirectly affect the iron-sulfur cluster. Since the signal line shape of the g_x of the iron-sulfur cluster is redox-potential-dependent [41,53], it is possible that the change of protein conformation with redox state of a close, interacting protein (cytochrome *b*) of the iron-sulfur cluster indirectly induces the change of the g_x of the iron-sulfur protein.

EPR characteristics of the antimycin-sensitive ubisemiquinone radical in the cytochrome b-c_1 complex

Fig. 7 shows the detection of ubisemiquinone radical in the cytochrome *b*-c_1 complex in the presence and absence of antimycin at 12 and 630 μW. When the complex is reduced with a fumarate/succinate mixture (10 : 1) in the presence of a catalytic amount of succinate-cytochrome *c* reductase, an EPR signal at $g = 2.005$, with a line width of 7.5 G, is observed. Since the line shape was identical when the spectra were recorded over a wide range of microwave power, it is safe to assume that only one radical is detected. When a 2-fold molar excess of antimycin is added to the sample, the signal completely disappears, indicating that ubisemiquinone radical detected in isolated cytochrome *b*-c_1 complex is antimycin-sensitive. Under the experimental conditions the antimycin-insensitive radical is not observed [18,61]. No effort in this work was devoted to the antimycin-insensitive radical which was reported to be detected under specific conditions in chromatophores [18]. The antimycin-sensitive ubisemiquinone radical probably corresponds to Q_c [19,20] in the bacterial cytochrome *b*-c_1 complex or Q_i (or QPc or Qc) in the mitochondrial *b*-c_1 complex. The antimycin-sensitive ubisemiquinone radical was first described as QPc [19] in the isolated cytochrome *b*-c_1 complex and as Qc [20] in succinate-cytochrome *c* reductase.

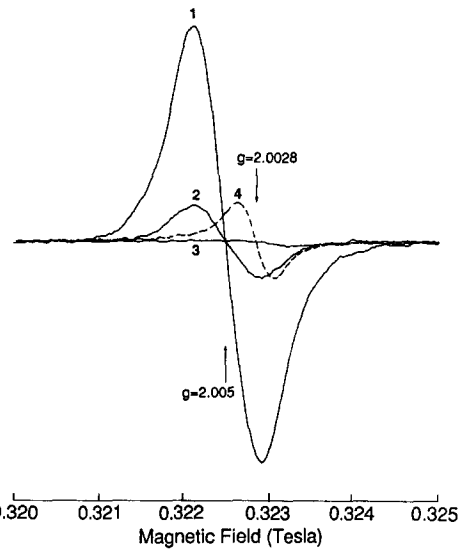

Fig. 7. EPR spectra of the antimycin-sensitive radical in the cytochrome *b*-c_1 complex. Oxidized cytochrome *b*-c_1 complex, 10 mg/ml, in 50 mM Tris-acetate buffer (pH 8.0) was mixed with one-tenth volume of the fumarate/succinate mixture (10 : 1). The final concentration of succinate was 5 mM. The sample was divided into two equal portions: one was treated with a 2-molar excess of antimycin (curve 3) and one was not (curves 1 and 2). Curve 1 was measured at 630 μW and curve 2 at 12 μW. The dashed line (curve 4) is the weak pitch signal, $g = 2.0028$, which was included for comparison. EPR instrument settings were as follows: microwave frequency, 9.32 GHz; modulation amplitude, 5 G; microwave power, 630 μW or 12 μW; time constant, 0.5 s; scan rate, 0.4 G/s; temperature, 105 K.

Redox behavior of the antimycin-sensitive ubisemiquinone radical

Fig. 8 shows the redox titration of the antimycin-sensitive ubisemiquinone radical at pH 8.4. When the antimycin-sensitive ubisemiquinone radical is measured at different redox potentials, a maximum signal height

($g = 2.005$) is obtained at 100 mV. Using the reported formulation [18,61], a half-height width of 118 mV in the redox titration curve (see Fig. 8) indicates 0.63 mol ubisemiquinone per mol of Q. Thus the maximal ubisemiquinone concentration would be 0.82 mol per mol complex (based on cytochrome c_1), if all of the 1.3 mol Q per mol complex present the cytochrome b-c_1 complex is in the protein bound form. When the concentration of the antimycin-sensitive ubisemiquinone radical in isolated cytochrome b-c_1 complex is determined by double integration of the signal using 3-(maleimidomethyl)proxyl as the spin reference, 0.66 mol ubisemiquinone per mol cytochrome b-c_1 complex is obtained. This is close to the value of 0.82 mol ubisemiquinone per mol Q estimated from the width of the half-height of the redox potential titration, although a part of this deviation may have been caused by the presence of heterogeneous Q in the cytochrome b-c_1 complex. Some of Q may not be bound to protein tightly, as described in the previous section that some Q may be merely dissolved in the bound detergent.

Fig. 9 shows the effect of pH on the EPR signal of the antimycin-sensitive ubisemiquinone radical. Below pH 7.0 very little Q-radical signal is detected. The amplitude of the signal is relatively constant from pH 7.4 to pH 8.0; beyond that, the signal decreases as the pH increases. From Fig. 9 one can calculate the pH dependency of E_m of Q radical. The E_m decreases by approx. 60 mV per unit pH increase. A similar result was also obtained when redox titrations were carried out at different pH levels (data not shown). Although the midpoint potential and the EPR characteristics of antimycin sensitive ubisemiquinone radical of R. sphaeroides cytochrome b-c_1 complex at temperature

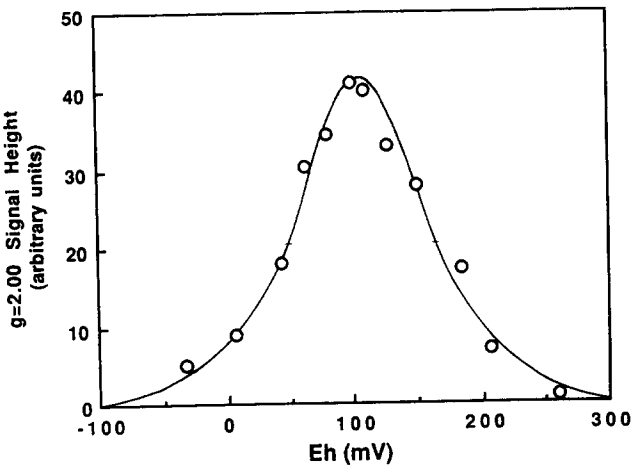

Fig. 8. Redox titration of the antimycin-sensitive ubisemiquinone radical in the cytochrome b-c_1 complex at pH 8.4. The purified cytochrome b-c_1 complex, 5 mg/ml, in 100 mM Tricine-NaOH (pH 8.4) was subjected to anaerobic redox titration in the presence of 20KM redox dyes as described in Materials and Methods. The conditions for EPR measurement were the same as those given in Fig. 7.

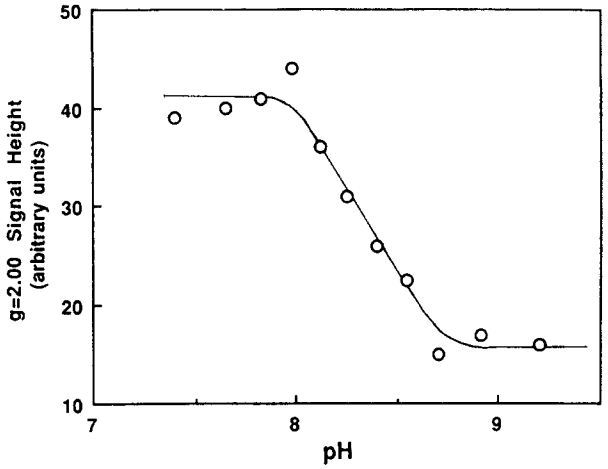

Fig. 9. pH-dependent EPR signal of ubisemiquinone in purified cytochrome b-c_1 complex. The complex was prepared in a high protein concentration and diluted with 100 mM of different buffer systems to a protein concentration of about 5 mg/ml. The buffer systems used to cover different pH ranges were: potassium phosphate (pH 7.1–8.9), Mops (pH 7.0–8.0), Tricine-NaOH (pH 7.6–8.4), glycylglycine (pH 8.5–9.0), glycine-NaOH (pH 9.0–9.5). The conditions for EPR measurement were the same as those given in Fig. 7, except that the redox potential of the system was kept at 100 mV.

below 175 K is similar to those of mitochondrial cytochrome b-c_1 complex, at temperature higher than 175 K the ubisemiquinone radical of *R. sphaeroides* cytochrome complex is fast relaxing. Unlike mitochondrial complex, very little ubisemiquinone radical is detected in the bacterial complex at room temperature. The absence of non-prothetic group bearing polypeptides in the bacterial complex may at least contribute part of these difference.

Correlation between the antimycin-sensitive ubisemiquinone radical concentration and enzymatic activity

Although a ubisemiquinone radical has been detected in cytochrome b-c_1 complexes from various sources, direct correlation of ubisemiquinone concentration with enzymatic activity has not been reported. Cytochrome b-c_1 complexes having different enzymatic activities were prepared by incubating the enzyme at 30 °C for different periods. As indicated in Fig. 10, the ubiquinol-cytochrome c reductase activity decrease correlated well with the amount of antimycin-sensitive radical decrease, indicating that the antimycin-sensitive ubisemiquinone radical (Q_c) is indeed involved in the catalytic activity of the complex. As described in the previous section, the partially inactivated cytochrome b-c_1 complex generally possesses a higher concentration of high-spin heme signal than the higher activity preparation. The correlation between the appearance of a high-spin heme signal and the activity loss is, however, not very apparent. No change in cytochrome c_1 and iron-sulfur cluster is observed when the 30 °C-treated complex is compared with the untreated sample, suggesting that these compo-

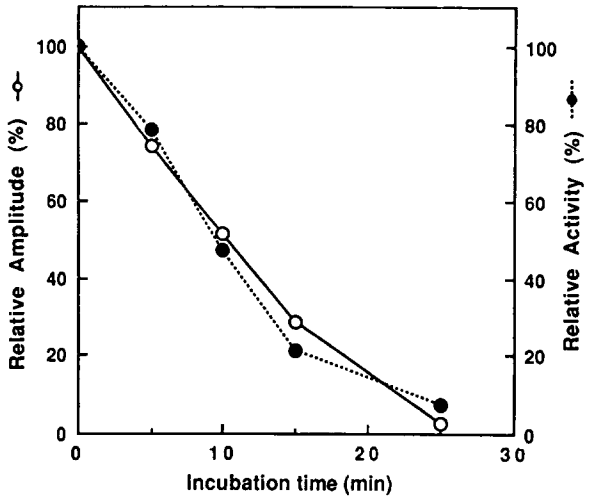

Fig. 10. Correlation between enzymatic activity and the EPR signal of the antimycin-sensitive ubisemiquinone radical. Purified cytochrome b-c_1 complex, 10 mg/ml, in 100 mM Mops (pH 7.8) was mixed with an equal volume of a mixture containing 300 mM fumarate and 4 mM succinate, and divided into 0.6 ml aliquots. All aliquots were incubated in a water bath at 30 °C. At given time intervals, the aliquots were removed from the water bath; 20 μl was used for activity measurement and the rest of the sample was mixed with 5 μl of succinate-cytochrome c reductase (1 mg/ml). After incubation at room temperature for 5 min, the samples were frozen in liquid nitrogen for EPR measurement. The conditions for EPR measurement were the same as those described for Fig. 4. The solid circles (●) represent activity and the open circles (○) represent the antimycin-sensitive radical intensity. The activity and the intensity of the ubisemiquinone radical signal of the sample before heat treatment were taken as 100%.

nents are not responsible for the activity loss during incubation at elevated temperatures.

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