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Assignment of ESR signals of *Escherichia coli* terminal oxidase complexes

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The ESR signals of all the major components of the aerobic respiratory chain of *Escherichia coli* were measured and assigned at liquid helium temperature. Cytochrome *b*-556 gives a weak high-spin signal at $g = 6.0$. The terminal oxidase cytochrome *b*-562 · *o* complex gives signals at $g = 6.0$, 3.0 and 2.26, and the terminal oxidase cytochrome *b*-558 · *d* complex gives signals at $g = 6.0$, 2.5 and 2.3. A signal derived from cupric ions in the purified cytochrome *b*-562 · *o* complex was observed near $g = 2.0$. It was shown by the effects of KCN or NaN_3 on cytochromes under the air-oxidized conditions that cytochrome *o* has a high-spin heme and cytochrome *d* has a low-spin heme. The E'_m values for cytochromes *b*-558 and *d*, respectively, determined by potentiometric titration of the ESR signals were 140 and 240 mV in the membrane preparation, and 30 and 240 mV in the purified preparation. The oxidized cytochrome *d* gave intense low-spin signals at $g = 2.5$ and 2.3, while cytochrome *d* under the air-oxidized conditions gave corresponding signals of only very low intensity. These results suggested that most of the cytochrome *d* under the air-oxidized conditions contains a diamagnetic iron atom with a bound dioxygen.

Introduction

The respiratory chain of aerobically grown *E. coli* contains one cytochrome *d* and four types of cytochrome *b* [1]. Cytochrome *b*-556 catalyzes electron transport from dehydrogenase(s) to ubiquinone-8 [2]. The cytochrome *b*-562 · *o* complex is the sole terminal oxidase of cells in the early exponential phase of aerobic growth. Under culture conditions with a limited oxygen supply, the cytochrome *b*-558 · *d* complex is synthesized adaptively as the major terminal oxidase and these oxidase activities branch at the site of ubiquinone-8. Both oxidase complexes have ubiquinol oxidase activity [1,3], and generate a membrane potential of −145 mV when reconstituted into liposomes [1,4].

We established procedures for purification of cytochrome *b*-556 [5], the cytochrome *b*-562 · *o* complex [3] and the cytochrome *b*-558 · *d* complex [1] to near homogeneity. The cytochrome *b*-558 · *d* complex has two hemes, while the cytochrome *b*-562 · *o* complex has two copper atoms in addition to two hemes [1,3]. Moreover, the former

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Abbreviations: E_h, ambient oxidation-reduction potential of the system; E'_m, standard oxidation-reduction potential at neutral pH; EDTA, ethylenediaminetetraacetate; Mops, 4-morpholinepropanesulphonic acid.

complex has a 10-times higher affinity for oxygen and is less sensitive to KCN or NaN_3 than the latter complex.

Pudek and Bragg [6] found that the cytochrome *d* of *E. coli* membrane vesicles had three forms which were spectrophotometrically distinguishable by potentiometric titration under anaerobic conditions. Koland et al. [7] examined purified cytochrome *b*-558 · *d* complex under anaerobic conditions, and confirmed that the reduced cytochrome *d* had an absorption band near 630 nm, while oxidized cytochrome *d* had no remarkable absorption band in the 600–700 nm range, but that cytochrome *d* under the air-oxidized conditions * had an absorption peak at 645 nm. Recently, Poole et al. [8] proposed a hypothesis that the cytochrome *d*, which has an absorption peak near 650 nm, is an intermediate in the reaction of reduced cytochrome *d* with oxygen. But the electronic state of cytochrome *d* under the air-oxidized conditions has not been characterized at a molecular level.

In this paper, we report the ESR spectra of purified cytochromes of the aerobic respiratory chain of *E. coli* and the assignment of the ESR signals from cytoplasmic membranes to each cytochrome. We found for the first time that the spin-active form of terminal oxidase, cytochrome *d*, is in the low-spin state in cytoplasmic membrane preparations and purified preparations. We also describe the ESR signals of cytochrome *b*-558 · *d* complex in different anaerobic oxidation-reduction states either in a purified or a membrane preparation, and determine the E'_m values of cytochromes *b*-558 and *d*. The comparison of ESR signals of cytochrome *d* under the anaerobically oxidized conditions and the air-oxidized conditions showed that the majority of cytochrome *d* under the air-oxidized conditions is cytochrome *d* in its oxygen-binding form.

* A preparation of cytochrome *b*-558 · *d* complex or cytochrome *d* "under the air-oxidized conditions" denotes a preparation that was obtained reproducibly under given conditions in the atmosphere. In this report, we show that the cytochrome *d* under the air-oxidized conditions consists of the oxygen-binding form of cytochrome *d*, which is the principal constituent, and the oxidized form of cytochrome *d*.

Materials and Methods

Chemicals. Ubiquinone-1 was a generous gift from Dr. T. Murata, Takeda Chemicals Co., Osaka. Pyocyanin was prepared from phenazine methosulfate by the method of McIlwan [9]. Other chemicals used were described previously [1] and were of analytical grade.

Strains and growth of cells. E. coli K12 strains MR43L (F^-, *gal, recA, thi, lac*) [1] and KL251/ORF4 [3] were used. Strain SASX41B (*hemA41, metB1, relA1, spoT1*) [10] was a generous gift from Dr. B. Bachmann.

Cells were grown aerobically at 37°C in a Magnaferm Fermentor (New Brunswick Scientific Co.) in 0.5% DL-lactate medium [3] with 1.0% Casamino Acids (Difco) or 0.5% polypeptone. For oxygen-limited growth, the rate of aeration was controlled as described previously [1]. For culture of SASX41B, 0.5% glucose was used as a carbon source in place of lactate.

The low-spin signals at $g = 3.0$ and 2.26 from membranes of MR43L harvested in the early exponential phase of aerobic growth were too weak to examine. Therefore, KL251/ORF4, which is known to contain about twice as much cytochrome *b*-562 · *o* complex as MR43L, due to the gene dosage effect of the episome [3], was used for measuring the low spin signals from membrane vesicles.

Preparation of membranes. Cytoplasmic membrane vesicles were prepared from freshly grown cells by the method of Yamato et al. [11] with a slight modification [5].

The membrane preparations used for Figs. 8–10 contained 3.0 nmol heme *b* and 2.6 nmol heme *d* per mg of protein. The purified cytochrome *b*-558 · *d* complex [1] contained equimolar amounts of cytochrome *b*-558 and cytochrome *d*. Accordingly, more than 85% of the total *b*-type hemes in these membrane preparations were regarded as cytochrome *b*-558. Because of its high concentration of cytochrome *b*-558, the membrane preparation that contained three high spin hemes, cytochromes *b*-558, *b*-556 and *o*, could be used for quantitative potentiometric measurement of the E'_m value of cytochrome *b*-558.

Preparations of purified cytochromes. Cytochrome *b*-556 [5], the cytochrome *b*-562 · *o* com-

plex [3] and the cytochrome *b*-558 · *d* complex [1] were purified as reported previously.

The specific contents of hemes used were 28.0 nmol heme per mg of protein (cytochrome *b*-556), 11.8 nmol *b*-type heme per mg of protein (cytochrome *b*-562 · *o* complex) and 6.1–9.5 nmol *b*-type heme per mg of protein (cytochrome *b*-558 · *d* complex).

Electron spin resonance (ESR) measurements. ESR spectra were recorded with an X-band JES-FE3XG spectrometer (JEOL, Tokyo), or with an X-band Bruker ER 200D spectrometer (Bruker, F.R.G.). With the JES-FE3XG apparatus, measurements were made at a microwave power of 10 μW, modulation amplitude of 20 G at 100 kHz and at liquid helium temperature (4.2 K), unless otherwise mentioned. With this microwave power, the signals reported were almost free from microwave saturation, but microwave preamplification with a solid-state amplifier (Narda, U.S.A.) was required before signal detection. The Bruker ER 200D apparatus was equipped with a liquid-helium cryostat (Model ESR-900, Oxford Instruments, U.K.). A minicomputer system was used for subtraction of background signals from the spectra obtained. Measurement conditions were mentioned in each figure legend. The receiver gain used is shown in each figure. An NMR gaussmeter was used to measure the magnetic field and then to determine *g* values. All the *g* values except for $g = 5.6$ were determined at the peak of each signal. The *g* value of 5.6 was determined at the trough of its signal.

Potentiometric titration. Oxidation and reduction potentials were measured in a cell similar to that described by Dutton [12] under anaerobic conditions. The medium used was the buffer (100 mM Mops/KOH buffer (pH 7.4) for purified preparations, and that with 3 mM potassium EDTA for membrane preparations) with the following mediators at 40 μM: 2,3,5,6-tetramethylphenylenediamine ($E'_m = 260$ mV), 2,5-dimethyl-1,4-benzoquinone ($E'_m = 180$ mV), phenazine methosulfate ($E'_m = 70$ mV), pyocyanin ($E'_m = -30$ mV), 2-hydroxy-1,4-naphthoquinone ($E'_m = -130$ mV). The cell containing the cytochrome *b*-558-*d* complex and the medium was firstly deoxygenated by passage of argon gas over the solution with stirring, and exhaustively deoxygenated by poising at about −100 mV under continuous passage of argon gas, before the titration began. Ambient redox potentials (E_h) were monitored with a Pt-Ag/AgCl electrode (Type PS-165F, Toa Denpa, Tokyo). Desired potentials were attained by adding a small volume of saturated sodium dithionite or 100 mM potassium ferricyanide solution using a microsyringe. When the potential became stabilized, 0.2 ml of the sample solution was transferred anaerobically to a sample tube previously flushed with argon. The tube was immediately frozen in liquid nitrogen and transferred to a cryostat. The sample tubes used were quartz tubes of 4 ± 0.05 mm outer diameter.

Determination of cytochrome contents. The contents of cytochromes were determined from the absorption spectra at 20°C (Hitachi 557 dual wavelength spectrophotometer). For calculation of the concentrations of total *b*-type cytochromes, cytochrome *b*-558 and cytochrome *d*, molar extinction coefficients of 17.5 (560–580 nm) [13], 14.8 (560–580 nm) [1] and 18.8 (629–648 nm) [1] $cm^{-1} \cdot mM^{-1}$, respectively, for the difference spectra of reduced minus air-oxidized samples were used. The content of cytochrome *o* was calculated using a value for the molar extinction coefficient of 145 $cm^{-1} \cdot mM^{-1}$ from the CO-difference spectra at the wavelength pair, 416–430 nm [3].

Other method. Protein was determined by the method of Lowry et al. [14] with bovine serum albumin as a standard.

Results

ESR signals of cytochrome b-556 and cytochrome b-562 · o complex under the air-oxidized conditions

Fig. 1a shows the ESR signals from the cytoplasmic membranes of strain KL251/ORF4, which was harvested in the early exponential phase of aerobic growth with high-oxygen partial pressure. This membrane preparation contained 14.9 μM cytochrome *b*-556 and 11.0 μM cytochrome *b*-562 · *o* complex. Signals with *g* values of 6.0, 4.3, 3.0 and 2.26 were observed. All the signals except for $g = 4.3$ were not detected from the cytoplasmic membranes of heme-deficient strain SASX41B (Fig. 1c), indicating that they are due to cytochromes in the aerobic respiratory chain. The signal observed at $g = 4.3$ is most likely due to free ferric

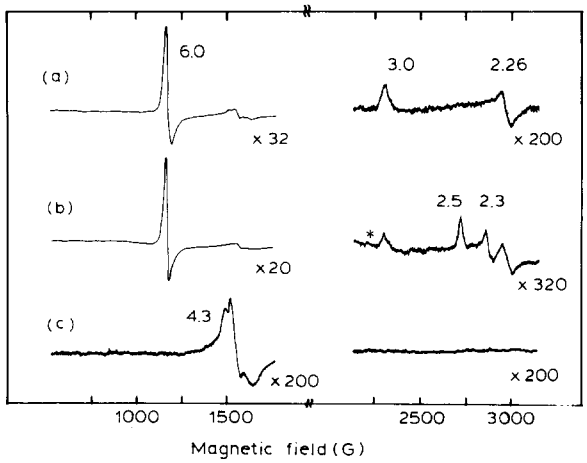

Fig. 1. ESR signals of cytochromes in cytoplasmic membrane vesicles under the air-oxidized conditions. The conditions for ESR measurements were as follows: microwave power, 10 μW; modulation amplitude, 20 G at 100 kHz; temperature, 4.2 K; magnetic field, $\pm$1000 G, the spectrum indicated by an asterisk was measured with a microwave power of 40 μW. (a) Membrane vesicles containing the cytochrome *b*-556 and the cytochrome *b*-562 · *o* complex, from KL251/ORF4 harvested in the early exponential phase of aerobic growth under the condition of high oxygen partial pressure (26.1 mg protein/ml); (b) the cytochrome *b*-558 · *d* complex enriched membrane vesicles, from MR43L harvested in the early stationary phase of aerobic growth under the condition of low-oxygen partial pressure (26.8 mg protein/ml); (c) membrane vesicles from heme-deficient strain, SASX41B (22.6 mg protein/ml). Cytochrome content of each membrane vesicle was mentioned in the text.

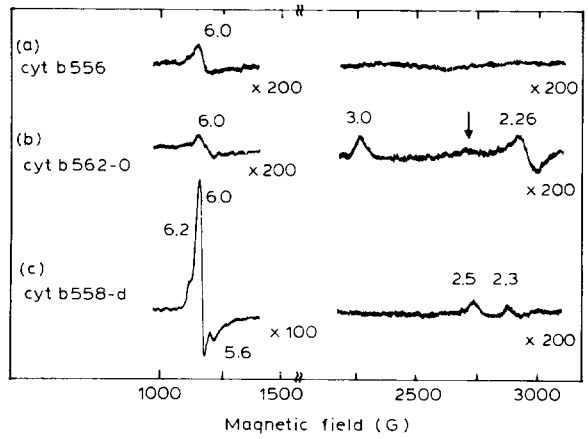

Fig. 2. ESR signals of purified cytochromes under the air-oxidized conditions. Conditions for measurements were as described for Fig. 1. Purified cytochromes were dialysed against 100 mM potassium phosphate buffer (pH 6.8) for more than 3 h at 4°C before use. The cytochrome contents of purified cytochromes used were as follows: (a) cytochrome *b*-556, 14 μM; (b) cytochrome *b*-562 · *o* complex, 2.3 μM; (c) cytochrome *b*-558 · *d* complex, 3.2 μM.

ions, as reported before [15].

For further assignment, ESR signals from purified cytochromes were examined (Fig. 2). Purified cytochrome *b*-556 gives a weak high spin signal with a g value of $g = 6.0$ (Fig. 2a) and the purified cytochrome *b*-562 · *o* complex gives high ($g = 6.0$) and low ($g = 3.0$, 2.26) spin signals (Fig. 2b). In some cases, a weak signal at $g = 2.5$ (Fig. 2b, arrow) was observed.

The purified cytochrome *b*-562 · *o* complex contains two copper atoms per complex [3]. With a purified preparation, we observed a broad ESR signal near $g = 2.0$ due to cupric ions (Fig. 3), which resembles the signal detected from mitochondrial cytochrome *c* oxidase [16]. This signal of cupric ions had a lower g value than that reported by Ingledew et al. [17] from a membrane preparation of *E. coli* at 30 K. No further quantitative characterization of this signal was carried out because a signal from radicals at $g = 2.0$ is superimposed on the signal from cupric ions, and both signals are susceptible to microwave saturation (Fig. 3).

ESR signals of cytochrome b-558 · d complex under the air-oxidized conditions

Fig. 1b is the spectrum from cytoplasmic membranes of strain MR43L, which was grown under the condition of limited oxygen supply and harvested in the early stationary phase. This membrane preparation contained 20.1 μM cytochrome *b*-558 · *d* complex in addition to 5.90 μM cytochrome *b*-556 and 2.49 μM cytochrome *b*-562 · *o* complex. A strong high-spin signal at $g = 6.0$ and low-spin signals at $g = 3.0$, 2.5, 2.3 and 2.26 were detected.

The purified cytochrome *b*-558 · *d* complex gives the high-spin signal at $g = 6.0$ with small rhombic signals at $g = 6.2$ and 5.6, and low-spin signals at $g = 2.5$ and 2.3 (Fig. 2c). These rhombic high-spin signals, which were observed reproducibly after solubilization, may be due to an environmental change caused by solubilization.

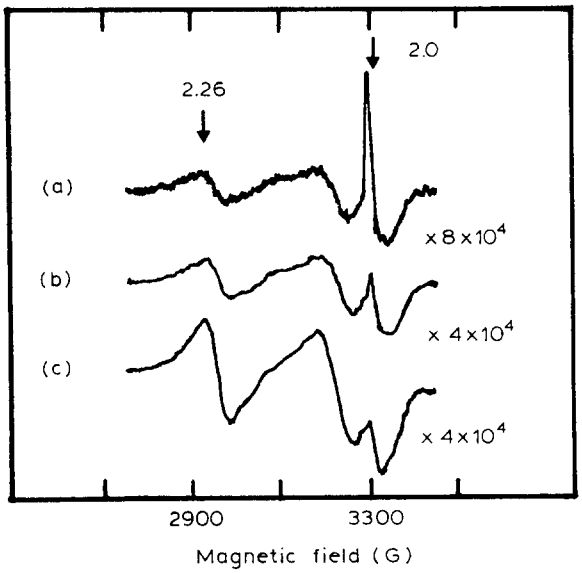

Fig. 3. ESR signals from purified cytochrome *b*-562·*o* complex near $g = 2.0$. Conditions for measurements were: modulation amplitude, 40 G at 100 kHz; magnetic field, ±2000 G; temperature, 11 K; microwave power, (a) 10 μW; (b) 100 μW; and (c) 1 mW. The sample was dialysed as in Fig. 2. The cytochrome content of the sample used was 21.1 μM.

Effects of KCN and NaN_3

Since respiratory inhibitors such as potassium cyanide and sodium azide are known to react with cytochrome *o* and cytochrome *d* in *Escherichia coli* [1,2,18], we examined their effects on the ESR spectra. The spectra from membranes containing the cytochrome *b*-562·*o* complex as the only terminal oxidase complex with or without inhibitor are shown in Figs. 4A and 5A. Upon addition of 10 mM KCN, the signal intensity from the high

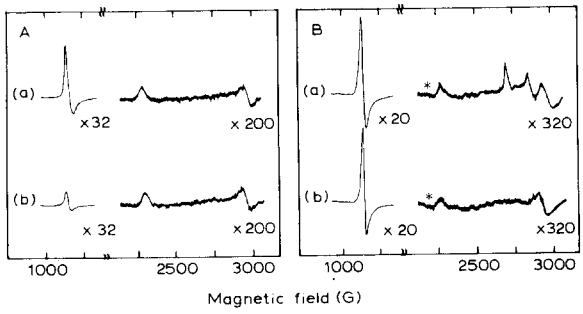

Fig. 4. Effects of the addition of 10 mM KCN on ESR signals. KCN was added to each membrane preparation and the mixtures were incubated for 5 min at 20°C. Conditions for ESR measurements and the cytochrome content of each sample were as in Fig. 1. (A) Spectra from membrane vesicles containing the cytochrome *b*-562·*o* complex as the sole terminal oxidase complex (26.1 mg protein per ml). (B) Spectra from membrane vesicles containing the cytochrome *b*-558·*d* complex as the major terminal oxidase complex (26.8 mg protein per ml). (a) Control; (b) with 10 mM KCN.

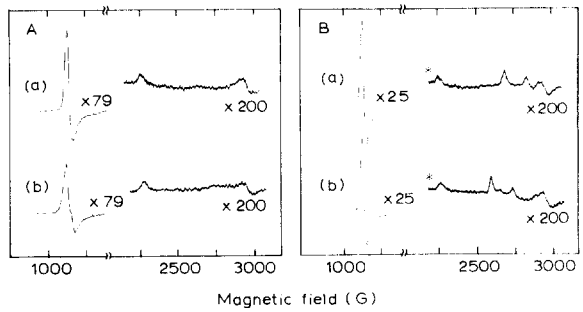

Fig. 5. Effects of the addition of 50 mM NaN_3 on ESR signals. NaN_3 was added to each membrane preparation and the mixtures were incubated for 5 min at 20°C. Conditions for ESR measurements and the cytochrome content of each sample were as in Fig. 1. (A) Spectra from membrane vesicles containing cytochrome *b*-562·*o* complex as the sole terminal oxidase complex (17.4 mg protein per ml). (B) Spectra from membrane vesicles containing cytochrome *b*-558·*d* complex as the major terminal oxidase complex (20.1 mg protein per ml). (a) Control; (b) with 50 mM NaN_3.

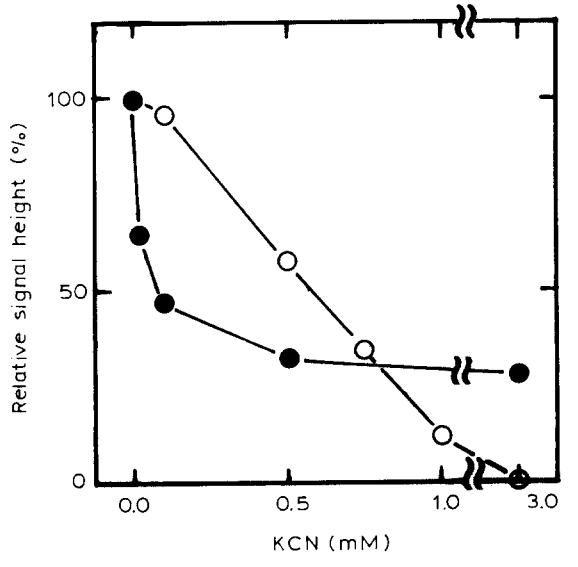

Fig. 6. Effect of KCN on ESR signals of cytochromes *o* and *d*. Experimental conditions were as in Fig. 4. Relative signal heights of the $g = 6.0$ signal for cytochrome *o* (●) and $g = 2.5$ signal for cytochrome *d* (○) were examined and plotted.

spin heme ($g = 6.0$) decreased, and the rhombicity of the g values of the two low-spin signals ($g = 3.0$, 2.26) decreased about 20 G (Fig. 4A-b). Addition of 50 mM NaN_3 broadened the high-spin signal (Fig. 5A-b). When 10 mM KCN was added to membranes containing the cytochrome *b*-558 · *d* complex as the major terminal oxidase complex, the intensities of the two low-spin signals ($g = 2.5$ and 2.3) from the cytochrome *b*-558 · *d* complex decreased (Fig. 4B-b). Upon addition of 50 mM azide, the signals from a low-spin heme shifted to lower magnetic field (Fig. 5B-b). These changes caused by addition of KCN depended on the concentrations of the inhibitory ligands (Fig. 6). These distinctive effects of KCN were also observed with the purified cytochrome oxidase complexes (Fig. 7). The high-spin ESR signal of the purified cytochrome *b*-562 · *o* complex (Fig. 7A-b) and the low-spin signals of the purified cytochrome *b*-558 · *d* complex (Fig. 7B-b) were decreased. The weakening of high-spin signals of the purified cytochrome *b*-558 · *d* complex was observed (Fig. 7B-b), suggesting that its high spin heme was more susceptible to 10 mM KCN after purification.

From these observations we propose that both terminal oxidase complexes of *E. coli* contain two hemes, one of which is sensitive and one of which is insensitive to inhibitory ligands, like cytochrome *c* oxidase in mitochondria [19]. Judging from the reactivities of the ligands with the terminal oxidases [1,2], the high-spin species of cytochrome *b*-562 · *o* complex, which is sensitive to the ligands, is attributable to cytochrome *o*, while the low-spin species of the cytochrome *b*-558 · *d* complex, which is sensitive to ligands, is attributable to cytochrome *d*.

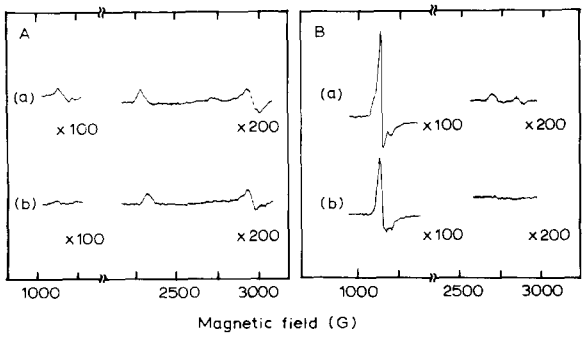

Fig. 7. Effect of 10 mM KCN on ESR signals of purified cytochrome oxidase complexes. Experimental conditions were as in Fig. 4. Purified cytochromes were dialysed against 100 mM potassium phosphate buffer (pH 6.8) for more than 3 h at 4°C before use. (A) Spectra from the cytochrome *b*-562 · *o* complex (3.6 μM). (B) Spectra from the cytochrome *b*-558 · *d* complex (1.9 μM). (a) Control; (b) with 10 mM KCN.

E'_m values of cytochromes b-558 and d determined from ESR signals

Fig. 8A shows the results of potentiometric analyses of ESR signals of the cytochrome *b*-558 · *d* complex in purified preparations. The signal heights of $g = 6.0$ and 2.5 were taken to measure the contents of oxidized cytochromes *b*-558 and *d*, respectively, as described previously. The height of each signal was determined and normalized taking the signal height of the oxidized form as 1.0. From the results in Fig. 8A the E'_m values of purified cytochromes *b*-558 and *d* were determined to be 30 and 240 mV, respectively. These values are in agreement with those reported by the potentiometric titration of the optical absorption spectra [1,7], as summarized in Table I.

Next, we examined the E'_m values of the cytochrome *b*-558 · *d* complex in membrane preparations (Fig. 8B). The E'_m value of cytochrome *b*-558 in membrane preparations was determined by measuring the signal intensity at $g = 6.0$: the membrane preparations used in this experiment contained other high-spin cytochromes, cytochro-

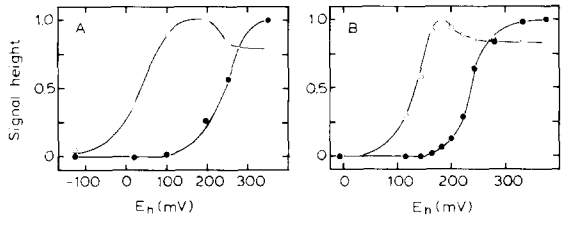

Fig. 8. Potentiometric analysis of ESR signals of cytochrome *b*-558 · *d* complex. ESR measurements were made at a microwave power of 1 mW, temperature at 8 K, and a modulation amplitude of 4 G at 100 kHz for high spin signals and 40 G at 100 kHz for low spin signals. The relative heights of the $g = 6.0$ signal (○) of oxidized cytochrome *b*-558 and the $g = 2.5$ signal (●) of oxidized cytochrome *d* are shown. (A) Purified preparation (3.5 μM) dialysed against 100 mM Mops/KOH buffer (pH 7.4) for 4 h at 4°C before use. (B) Membrane preparation, which contains much cytochrome *b*-558 · *d* complex (4.6 mg of protein/ml), dialysed against 100 mM Mops/KOH buffer (pH 7.4) with 3 mM potassium EDTA for 4 h at 4°C before use.

TABLE I

COMPARISON OF E'_m VALUES OF CYTOCHROMES *b*-558 AND *d*

Investigator	E'_m (mV)			
	in membrane preparation		in purified preparation	
	b-558	*d*	*b*-558	*d*
Present work	140	240	30	240
Kita et al. [1]			10	240
Koland et al. [7]			61	232
Lorence et al. [20]	180	260		
Pudek et al. [6]	165	260		
Reid et al. [21]		280		

mes *b*-556 and *o*, but the amounts of these were less than 15% of that of total *b*-type heme and could be neglected. The E'_m values of cytochromes *b*-558 and *d* in membrane preparations were found to be 140 and 240 mV, respectively (Fig. 8B), which were in agreement with the values reported before by the optical method (Table I).

No other spin-active forms of the cytochrome *b*-558 · *d* complex were observed in either preparation under any redox potential (for details, see Discussion).

Oxidation-reduction potential-dependent changes of ESR signals of cytochrome b-558

As shown in Fig. 8, we found that the signal heights of axial high spin hemes decreased to some extent when the E_h was raised to 200 mV or higher under standard conditions. They also decreased to the same extent when they were measured as in Fig. 8 but in the presence of 4 μM instead of 40 μM mediators (data not shown). Therefore, these decreases in the potentiometric titration curves did not seem to be due to an artifact caused by the reaction of added mediators with cytochrome *b*-558. One possible explanation for these observations is that the anisotropy of ESR signals of cytochrome *b*-558 was changed when the E_h was raised above 200 mV.

The weak rhombic signals at $g = 6.3$ and 5.7 reported by Poole et al. [15] were clearly observed in membrane preparations in the E_h range between 40 and 200 mV (Fig. 9a and b), but further

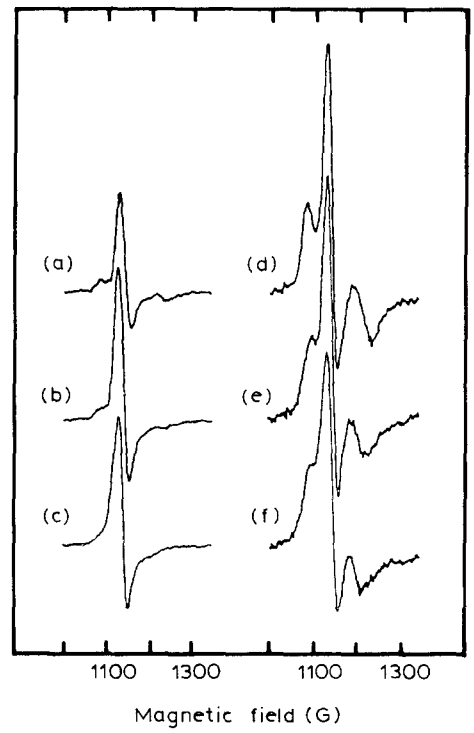

Fig. 9. Effect of the ambient redox potential on the rhombic high-spin signals of the cytochrome *b*-558 · *d* complex. Preparations used: (a)–(c), a membrane preparation which contains much cytochrome *b*-558 · *d* complex; (d)–(f), purified cytochrome *b*-558 · *d* complex. Experimental conditions were as in Fig. 8. Receiver gains for ESR measurements: (a)–(c), $1 \cdot 10^5$; (d)–(f), $5 \cdot 10^6$. Redox potentials (E_h) of samples: (a) 143 mV, (b) 163 mV, (c) 375 mV, (d) 100 mV, (e) 198 mV, (f) 352 mV.

rise of E_h resulted in decrease in their intensities (Fig. 9c). These rhombic signals at $g = 6.3$ and 5.7 were also observed from the purified preparations at low E_h values (Fig. 9d), whereas the ESR spectra from purified preparations observed at higher E_h values (Fig. 9f), lost these rhombic signals and showed different set of signals at $g =$ 6.2 and 5.6 * as reported in Fig. 2. We attributed the former set of weak rhombic high-spin signals at $g = 6.3$ and 5.7 to oxidized cytochrome *b*-558, based on the range of E_h values in which these

* The *g* value of 5.6 was determined at the trough of its signal (see Fig. 2), but the *g* value of 5.7 was determined at the middle of its signal [15].

ESR signals were observed and the E'_m values of cytochromes *b*-558 and *d* in the membrane preparation described before. We also supposed that this decrease in the rhombicity, depending on the redox potential, might be due to conformational change of cytochrome *b*-558 accompanying with change in the redox potential.

ESR signals of cytochrome d under the air-oxidized conditions

The low-spin signals assigned to cytochrome *d* were 10-times stronger when the cytochrome *b*-558 · *d* complex was oxidized under anaerobic conditions as mentioned in Materials and Methods (Fig. 10b and c) than when it was under the air-oxidized condition (Fig. 10a), although the high-spin signals that were assigned to cytochrome *b*-558 were not significantly different. The low-spin signals from the preparations under the air-oxidized conditions and the anaerobically oxidized conditions were probably due to the same chemical species, judging from the similarity in their ESR parameters and of their reactivities with KCN. The signal intensities of $g = 2.5$ and 2.3 of cytochrome *d* under the anaerobically oxidized conditions were decreased by KCN, like those under the air-oxidized conditions; the ESR signals of purified cytochrome *d* under the anaerobically oxidized conditions completely disappeared on addition of 0.5 mM KCN.

The weakened low-spin signals of cytochrome *d* under the air-oxidized conditions were not the result of an oxygen-broadening effect [22], since the intensities of these low spin signals did not decrease when the anaerobically oxidized preparations were exposed to atmospheric oxygen. Therefore, most of the cytochrome *d* under the air-oxidized conditions was spin inactive, and seemed to have ferrous heme *d*. However, the ferrous iron in most of the cytochrome *d* under the air-oxidized conditions was not chemically oxidizable. No increase in the intensity of low spin ESR signals was observed when 100 μM potassium ferricyanide, which increased the ambient E_h sufficiently, was added to the preparation. These findings suggest that most of the iron in cytochrome *d* under the air-oxidized conditions was present not as free ferrous iron, but bound in a diamagnetic compound, as observed in case of the iron in oxyhemoglobin [23]. Therefore, we considered that most of the cytochrome *d* under the air-oxidized conditions was present in the oxygen-binding form; that is, the diamagnetic heme *d* having a bound dioxygen.

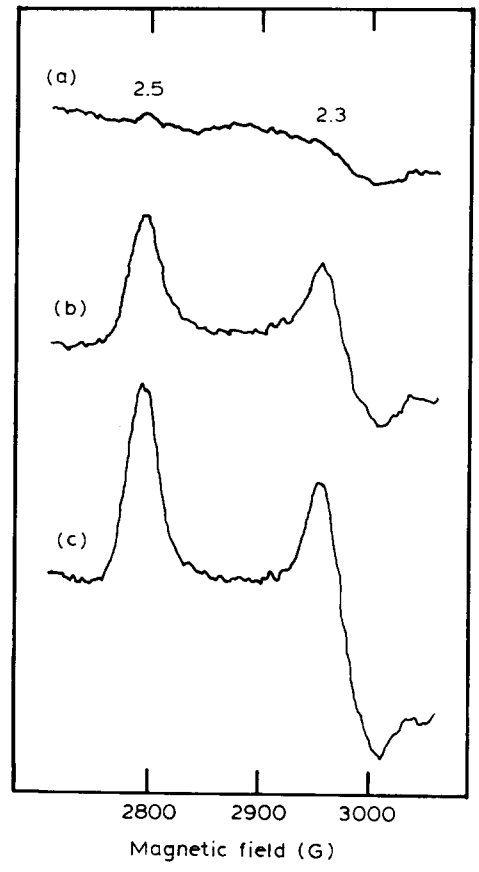

Fig. 10. ESR signals of cytochrome *d* in membrane preparations under the air-oxidized conditions and anaerobically oxidized conditions. Experimental conditions were as in Fig. 8. The receiver gain for ESR measurement was $1 \cdot 10^5$. Conditions for samples were as follows: (a) preparation under the air-oxidized conditions; (b) preparation under the anaerobically oxidized conditions, $E_h = 242$ mV; (c) preparation under the anaerobically oxidized conditions, $E_h = 333$ mV.

Discussion

This is the first report of the assignment of ESR signals of all the major cytochromes in the aerobic respiratory chain of *E. coli*. The *g* values of signals from purified cytochromes under the air-oxidized conditions corresponded well with those

of signals from membrane vesicles under the air-oxidized conditions. The cytochromes *b*-556, *b*-558 and *o* give a high spin signal at $g = 6.0$. The cytochrome *b*-562 gives low spin signals at $g = 3.0$ and 2.26, whereas the cytochrome *d* gives low spin signals at $g = 2.5$ and 2.3. It is noticeable that cytochrome *d*, terminal oxidase, has a low spin heme. The signal intensities at $g = 6.0$ of purified cytochromes *b*-556 and *o* per mole of heme (Figs. 2a and 2b) were about 5-times weaker than those from membrane preparation (Fig. 1a). This may be due to broadening of the signals by solubilization.

Here we propose that the cytochrome *o* under the air-oxidized conditions has a high-spin heme, judging from the effect of inhibitor ligands (Figs. 4, 5 and 7), as reported for a partially purified cytochrome *o* from *Pseudomonas aeruginosa* [24].

On the contrary, Poole [25] described that cytochrome *o* in *E. coli* has a low spin heme, based on the following observations: (1) the heme plane of cytochrome *o* in *E. coli* was perpendicular to the membrane [26]; (2) only the heme plane of a low spin species was perpendicular to the membrane [15]. However, the oriented multilayers of membrane preparations used by Poole et al. [15] most likely contained not only cytochrome *b*-556 and the cytochrome *b*-562 · *o* complex, but also the cytochrome *b*-558 · *d* complex, since they recorded weak low-spin signals at $g = 2.5$ and 2.3 (cf. Fig. 3 in Ref. 15). Therefore, the weak dependency of intensities of the signal at $g = 6.0$ from the oriented multilayers on the magnetic field angle, with which Poole et al. [15] determined that the heme plane of high spin species was inclined at 45° to the membrane plane, could be caused by different orientations of the three high spin heme components that were present in their preparations.

Fig. 6 shows that the ESR signal from cytochrome *o* under the air-oxidized conditions is more sensitive to KCN than that from cytochrome *d* under the air-oxidized conditions. Since all the reported spin-active species of hemoproteins are in the oxidized form, Fig. 6 indicates that the oxidized cytochrome *o* is more sensitive to KCN than the oxidized cytochrome *d*. We suppose that it is one of the reasons for the corresponding difference in sensitivities to KCN of the terminal oxidase activities [1].

It is rather curious that upon addition of KCN the ESR signals from terminal oxidase complexes of *E. coli* were appreciably reduced (Figs. 4 and 7), since CN-ligated forms of high-spin terminal oxidases are known to be in low spin states in mitochondria [19] and *Azotobacter vinelandii* [27]. To clarify these phenomena, we are presently examining the redox states of the *E. coli* terminal oxidase complexes in CN-ligated forms by resonance Raman spectroscopy.

In this work, the E'_m values of cytochrome *b*-558 and cytochrome *d* determined by potentiometric titration of ESR signals of their oxidized species agreed well with the values determined from the optical absorption spectra of the reduced species of these cytochromes (Table I). The present results confirmed that the reduced species, which were characterized by absorption peaks at 560 nm and 629 nm, were converted to oxidized species containing cytochrome *b*-558 in the high-spin state and cytochrome *d* in the low-spin state. Thus, we assigned this oxidized cytochrome *d* in the low spin state to the oxidized cytochrome *d* with no remarkable optical absorption band that was reported previously [6,7]. These results, obtained from both the optical absorption spectrum and the ESR spectrum, showed that under anaerobic conditions cytochrome *d* has only two electronic states (a reduced form and oxidized form) at all redox potentials.

Potentiometric analyses in Fig. 8 confirmed our assignment that the ligand-sensitive low spin signals at $g = 2.5$ and 2.3 of the cytochrome *b*-558 · *d* complex are due to the terminal oxidase, cytochrome *d*. This conclusion differs from previous reports [8,27], in which high-spin signal(s) was attributed to the cytochrome *d*. These low-spin signals have not been reported by others [15,28], possibly for two reasons. First, these signals are so weak that either membrane preparation from cells grown under strictly controlled low oxygen pressure, or concentrated purified preparation is needed for their clear detection. Second, cytochrome *d* in the membrane vesicles examined by Poole et al. [15] might be reduced by contaminating respiratory substrates, as rhombic high-spin signals at $g = 6.3$ and 5.7 were recorded (Fig. 7 in Ref. 15).

We found that about 90% of the cytochrome *d*

under the air-oxidized conditions is diamagnetic (Fig. 10). This chemical species was not observed under the condition of anaerobic titration. The purified cytochrome *b*-558 · *d* complex used in this work had an apparent molecular weight of 100000 (Konishi, K. and Anraku, Y., unpublished results), suggesting that it consisted of a monomer of the constituent polypeptides with one heme *b* and one heme *d*. Thus, at present, we consider that the diamagnetic chemical species is a cytochrome *d* with a bound dioxygen, and so we named it the oxygen-binding form. This is the first evidence showing that the majority of cytochrome *d* under the air-oxidized conditions is in the oxygen-binding form. We also consider that this oxygen-binding form is the chemical species of cytochrome *d* that has an absorption peak at 645 nm, which is proposed to be an intermediate in the reaction of reduced cytochrome *d* with oxygen by Poole et al. [8].

The remaining 10% of the cytochrome *d* under the air-oxidized conditions is in the oxidized form, since weak low-spin ESR signals of cytochrome *d* at $g = 2.5$ and 2.3 were detected from the preparations under the air-oxidized conditions (Fig. 10a). This view was supported by the fact that these low-spin signals detected from the preparation under the air-oxidized conditions are KCN-sensitive, like those from an anaerobically oxidized preparation.

Cytochrome *b*-558 was apparently in the oxidized form under conditions where cytochrome *d* in the cytochrome *b*-558 · *d* complex was in the oxygen-binding form, since we observed no difference between the intensities of the ESR signals of cytochrome *b*-558 from the preparations under the air-oxidized conditions and the anaerobically oxidized conditions. Therefore, the unique stability of the oxygen-binding form of cytochrome *d* under the air-oxidized conditions is supposed to result from the absence of further donation of reducing equivalents from the cytochrome *b*-558. The physiological meaning of the oxygen-binding form as an oxygen intermediate in the redox reaction of dioxygen requires further study. Since this oxygen-binding form is very stable at room temperature, it should be useful in physicochemical studies on dioxygen, which binds to cytochrome oxidase.

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