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Electron flow and heme–heme interaction between cytochromes *b*-558, *b*-595 and *d* in a terminal oxidase of *Escherichia coli*

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The ESR signals of the cytochromes in the *Escherichia coli* terminal oxidase cytochrome *d* complex were studied at cryogenic temperature. The intensities and *g* values of the rhombic high-spin signals changed when the electronic state of cytochrome *d* was changed from the oxidized state to the reduced or oxygen-binding or CO-binding state. These rhombic signals were therefore assigned to cytochrome *b*-595, which is located near cytochrome *d* in the oxidase complex. This assignment was supported by the finding that the E_m value of the rhombic signals differed from that of cytochrome *d* (Hata, A. et al. (1985) Biochim. Biophys. Acta 810, 62–72). Photolysis and ligand-exchange experiments with the reduced CO complex of the oxidase were performed in the presence of oxygen at −140 °C. The ESR spectra of three intermediate forms trapped by controlled low temperatures were detected. These forms were designated as the oxygen-binding intermediate I (ESR-silent), oxygen-binding intermediate II (giving ESR signals at $g = 6.3$, 5.5 and 2.15), and oxygen-binding intermediate III (giving signals at $g = 6.3$, 5.5 and 6.0). From these results, electron flow in the cytochrome *d* complex is proposed to proceed in the order, cytochrome *b*-558 → cytochrome *b*-595 → cytochrome *d* → O_2. A model of the mechanism of four-electron chemistry for oxidation of ubiquinol-8 and formation of H_2O by the cytochrome *d* complex is presented.

Introduction

The cytochrome *d* complex is one of the two terminal oxidases of the aerobic respiratory chain in *Escherichia coli* [1] and oxidizes ubiquinol-8, which mediates transfer of reducing equivalents from dehydrogenases [2]. The complex is unique among terminal oxidases reported in various organisms with respect to its high affinity for oxygen [1] and the high stability of the oxygen-binding form of cytochrome *d* [3,4]. The purified complex [1,5] consists of two polypeptide subunits; the 57 kDa subunit contains a *b*-type heme (cytochrome *b*-558), and the 43 kDa one contains a *d*-type heme (cytochrome *d*) [6]. Recently, another *b*-type heme (cytochrome *b*-595) has been shown to be associated with the 43 kDa subunit in comparable amount to cytochrome *b*-558 [7]. However, the function of cytochrome *b*-595 is not yet understood.

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Abbreviations: E_h, ambient oxidation-reduction potential of the system; E_m, oxidation-reduction midpoint potential; Mops, 4-morpholinepropanesulfonic acid.

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We analyzed the ESR spectra of the purified cytochrome *d* complex at liquid helium temperature [4]. Potentiometric titration showed that the oxidized cytochrome *b*-558 gives an axial high-spin ESR signal at $g = 6.0$ and that the oxidized cytochrome *d* gives low-spin signals at $g = 2.5$ and 2.3. Results also showed that the rhombic high-spin signals reported in the oxidase complex do not arise from the cytochrome *d*. Furthermore, we concluded that the cytochrome *d* is present in a diamagnetic oxygen-binding form under air-oxidized conditions, since the cytochrome *d* is ESR-silent.

Poole et al. studied the low-temperature absorption spectra after photolysis [8] of the reduced CO complex in the presence of oxygen. They showed that the CO ligand of the cytochrome *d* was specifically exchanged with oxygen by a laser line of 632.8 nm at $-131°$C [9]. By ESR study, they also found that the laser photolysis and ligand exchange of the CO-ligated cytochrome *d* complex preceded the oxidation of at least two kinetically separable high-spin cytochromes [10]. They assigned the rhombic high-spin signals to cytochrome *d* and the axial signal near $g = 6.0$ to *b*-type cytochrome(s). However, this assignment did not agree with our potentiometric study of the rhombic high-spin and the low-spin signals [4]. In this study, we further analyzed the rhombic high-spin ESR signals by the method of ligand binding and laser photolysis of the CO complex in the presence of oxygen. We found changes of ESR signals in both the high-spin (*b*-type cytochromes) and low-spin (cytochrome *d*) regions after photolysis. We concluded that the electron transfer pathway for reduction of dioxygen involves at least three heme species in the oxidase complex and that the rhombic high-spin signals arise from the middle component, cytochrome *b*-595.

Materials and Methods

Preparation of membrane vesicles and purified cytochrome d complex

To obtain cytochrome *d*-enriched cells, *E. coli* K12 strain MR43L (F^-, *gal, recA, thi, lac*) was grown in 0.5% DL-lactate medium with 0.5% polypeptone under oxygen-limited conditions [1]. Cytoplasmic membrane vesicles and purified cytochrome *d* complex were prepared as described previously [4].

Photolysis of the CO-ligated oxidase and ESR measurements

Samples of CO-ligated cytochrome *d* complex in the presence of oxygen were prepared by the method of Chance [8]. *E. coli* cytoplasmic membrane vesicles prepared from cytochrome *d* complex-enriched cells were suspended at a final concentration of 5.6 mg protein/ml in a solution of 30% ethylene glycol in 20 mM Mops/KOH (pH 7.4). The mixture was transferred to an ESR tube with an air-tight rubber cap, and the tube was degassed and filled with argon gas. After repeated gas phase exchange, the tube was degassed and filled with CO gas. Then 1 M potassium succinate was added to give a final concentration of 10 mM. The CO gas phase was again changed, and the tube was stood for 15 min at room temperature to allow formation of the reduced CO complex of the oxidase. The tube was then cooled to $-23°$C in a temperature-controlled ethanol bath, the rubber cap was opened to supply atmospheric oxygen by stirring with 30 strokes of a coiled wire in dim light. Then the tube was rapidly frozen to $-78°$C in solid CO_2/ethanol solution and stored in liquid nitrogen until use.

ESR measurements and temperature control of the ESR cavity were performed with an X-band Bruker ER 200D spectrometer (Bruker, F.R.G.) equipped with a liquid-helium cryostat (Model ESR-900, Oxford Instruments, U.K.). After spectral measurements of the CO-ligated form, samples were warmed to $-140°$C in the ESR cavity, and irradiated for 5 min with a beam from a 0.95 mW He-Ne laser (wavelength 632.8 nm) to photolyze the cytochrome *d*-CO complex and form the oxygen-binding form of cytochrome *d* [11]. After further incubation at $-140°$C for 10 min, the sample was cooled to 10 K and its ESR spectra were obtained at a microwave power of 2 mW. The same sample was incubated at the desired temperature for 10 min before each further ESR measurement.

Other methods

Absorption spectra were measured in an air-tight cuvette in a Shimazu 2000D spectrophotometer.

The contents of cytochromes were determined from the oxidation-reduction difference spectra at 20 °C in a Hitachi 557 dual-wavelength spectrophotometer. The molar absorption coefficient of the cytochrome *d* used was 18.5 (629–648 nm) $cm^{-1} \cdot mM^{-1}$ [1]. Protein was determined by the method of Lowry et al. [12] with bovine serum albumin as a standard.

Results

Rhombic high-spin signals interact with cytochrome d

For reconsideration of the assignment of the rhombic high-spin signals, which were observed previously in the E_h range between 40 and 200 mV under anaerobic conditions (Ref. 4, Fig. 9), the effects of various ligands on the ESR spectrum of the cytochrome *d* complex were studied. Fig. 1a shows a spectrum with rhombic high-spin signals (indicated by arrows) at 120 mV. At this oxidation-reduction potential, only about 1% of the cytochrome *d* was calculated to be in the oxidized form. Fig. 1b is a spectrum of the oxidized cytochrome *d* complex at 310 mV, showing weak signals of rhombic high-spin with different *g* values. From comparison of Figs. 1a and 1b, it seems impossible to assign the rhombic signals to oxidized cytochrome *d*. We have already reported large low-spin signals of oxidized cytochrome *d* under the same conditions as for Fig. 1b [4]. Fig. 1c is a spectrum measured under air-oxidized conditions, in which no distinguishable rhombic high-spin signal is observed. Under the conditions for Fig. 1c, almost all the cytochromes *b*-558 and *b*-595 were in the oxidized forms and cytochrome *d* was in the oxygen-binding form, as observed in the optical spectrum (data not shown).

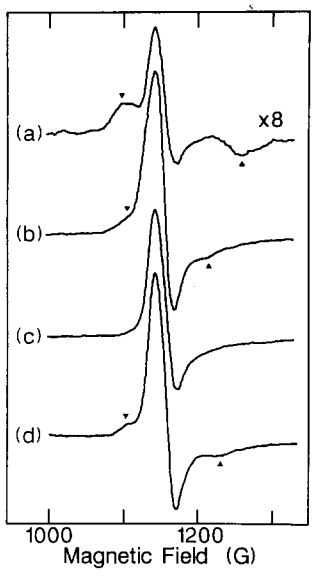

Fig. 1. ESR signals of the cytochrome *d* complex in *E. coli* membrane vesicles. (a) under anaerobic conditions at 120 mV; (b) under anaerobic conditions at 310 mV; (c) under air-oxidized conditions; (d) CO complex made from an air-oxidized sample. The cytochrome *d* complex was suspended in ethylene glycol solution. The electron mediators used were as described previously [4]. Samples contained 22.1 μM cytochrome *d* complex.

To confirm the effect of ligands to cytochrome *d* on the rhombic signals, we prepared the CO complex of this oxidase having oxidized cytochromes *b*-558 and *b*-595. The cytochrome *d* of an oxygen-binding form has a reducing equivalent to bind dioxygen [4], and so the bound oxygen can be replaced by carbon monoxide [5]. After the purified cytochrome *d* complex was blown with CO gas for 10 min with stirring, the absorption spectrum changed to that of the CO complex (data not shown). The cytochrome *d* in this CO complex was easily changed to the oxygen-binding form by addition of atmospheric oxygen (not shown), and its spectrum differed from that of the cytochrome *d* in dithionite reduced CO-complex as shown in Figs. 5 and 6 of Ref. 5. The cytochrome *d* in the CO complex made from the air-oxidized cytochrome *d* complex showed a broader absorption peak with a shoulder at 680 nm. These differences may be caused by interaction with the cytochrome *d* and the *b*-type cytochrome(s). Fig. 1d is an ESR spectrum of the CO complex made from the air-oxidized cytochrome *d* complex (Fig. 1c), showing distinct rhombic signals with *g* values slightly different from those in Fig. 1a (reduced cytochrome *d*) or Fig. 1b (oxidized cytochrome *d*). Figs. 1c and d indicate that changes in the electronic density of cytochrome *d* affect the electronic state of the rhombic high-spin heme, which suggests that the two hemes are located fairly close to each other. As discussed later, we assigned the rhombic high-spin signals to the cytochrome *b*-595, which is located in the 43 kDa subunit with the cytochrome *d*.

Photolysis of the CO complex in the presence of oxygen

To study the roles of the three heme components (axial and rhombic high-spin hemes and a low-spin heme) in the reduction of ligated oxygen, intermediates were trapped under the conditions of controlled low temperature following photolysis and ligand exchange of the reduced CO complex in the presence of oxygen. Immediately after laser photolysis at $-140\,^{\circ}C$, no specific change of the ESR signal was observed (Fig. 2b) from that of its reduced CO complex (Fig. 2a) as reported by Kumar et al. [10], except for slight development of rhombic high-spin signals. This ESR-silent form of the oxidase was previously examined by Poole et al. [9] and suggested to be ligated by oxygen, since it showed a characteristic absorption peak near 650 nm. Full development of the heme oxidations required elevation of the temperature to $-120\,^{\circ}C$. This form gave rhombic high-spin peaks at $g = 6.3$ and 5.5, and a broad low-spin signal at $g = 2.15$ (Fig. 2c). The broad low-spin signal was different from that of cytochrome *d* ($g = 2.5$ and 2.3) observed in potentiometric analysis under anaerobic conditions [4] and seemed to be due to an intermediate form of the cytochrome *d*-O_2 complex which had accepted one electron from the rhombic high-spin heme. After incubation at $-40\,^{\circ}C$, the sample showed rhombic high-spin signals and a higher axial signal at $g = 6.0$ (Fig. 2d). The low-spin signal of the one-electron-reduced intermediate cytochrome *d*-O_2 complex had disappeared, showing that further reduction of the intermediate form of the cytochrome *d*-O_2 complex had occurred. These results indicate that the electron flow from the rhombic high-spin heme to the cytochrome *d*-O_2 complex can occur at $-120\,^{\circ}C$ and that that from the axial high-spin heme to the cytochrome *d*-O_2 complex can occur at $-40\,^{\circ}C$.

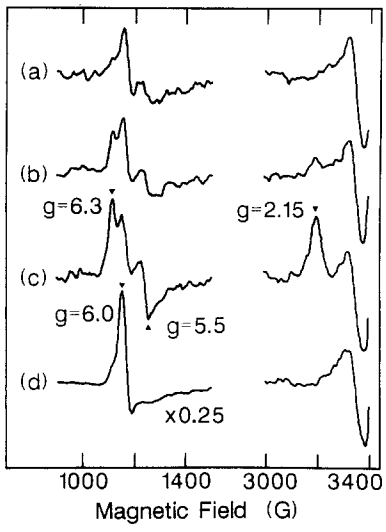

Fig. 2. ESR signals after CO photolysis of the cytochrome *d* complex. (a) reduced CO complex; (b) after photolysis at $-140\,^{\circ}C$; (c) after incubation at $-120\,^{\circ}C$; (d) after further incubation at $-40\,^{\circ}C$. Other conditions were as described for Fig. 1.

Discussion

Kumar et al. [10] proposed that rhombic high-spin ESR signals are due to cytochrome *d* in the oxidase complex. However, we have shown that the cytochrome *d* gives low-spin signals and that the cytochrome *b*-558 gives an axial high-spin signal [4]. From the present study we conclude that the rhombic high-spin signals are attributable to cytochrome *b*-595, for the following two reasons. The first reason is based on their dependence on the oxidation-reduction potential under anaerobic condition. As we reported previously [4], the rhombic high-spin signals were observed in the E_h range between 40 and 200 mV in the membrane preparation. Further rise in E_h resulted in decrease of their intensities, making estimation of their E_m values difficult. The heights of the rhombic signals were more than 50% of that of the axial signal at lower E_h (less than 50 mV), and less than 10% of that of the latter at higher E_h (greater than 150 mV). This suggests that the E_m value of the rhombic high-spin heme should be a little lower than that of the axial high-spin heme. According to Lorence et al. [13], the E_m value of cytochrome *b*-595 is about 30 mV lower than that of cytochrome *b*-558. Thus, we consider that the rhombic high-spin signals originate from the oxidized cytochrome *b*-595 and the axial high-spin signal from the oxidized cytochrome *b*-558. The second reason is evidence for a heme–heme interaction between the rhombic high-spin heme and the cytochrome *d* (see Fig. 1). Green et al. proved genetically [6] that cytochrome *b*-558 is located in the larger subunit, while cytochromes *b*-595 and *d*

are both located in the smaller subunit. It seems reasonable to assume that cytochrome *b*-595 interacts with cytochrome *d* located close to it.

Our assignment of the rhombic high-spin heme as cytochrome *b*-595 is further supported by its role in electron transfer (Fig. 2). The CO-photolysis experiment showed that the rhombic high-spin heme transfers electrons from the axial high-spin heme to the cytochrome *d*. Kranz and Gennis have suggested that the cytochrome *b*-558 accepts electrons from ubiquinol-1 [14]. These results indicate that the axial high-spin heme is due to the cytochrome *b*-558 and the rhombic heme is the cytochrome *b*-595. Furthermore, in the study by low-temperature spectrophotometry, Williams and Poole have shown that the cytochrome a_1 of *Acetobacter pasteurianus* (identical to cytochrome *b*-595) transfers electrons from the *b*-type cytochrome to the cytochrome *d* [15].

The results of laser photolysis (Fig. 2) can be summarized as follows. Laser photolysis of the reduced CO-ligated cytochrome *d* complex in the presence of oxygen causes a ligand exchange reaction at $-140\,^{\circ}C$ as shown below:

$$(b\text{-}558)^{2+}\cdot(b\text{-}595)^{2+}\cdot(d^{2+}\cdot CO)$$

$$\text{CO} \swarrow \quad \searrow O_2$$

$$(b\text{-}558)^{2+}\cdot(b\text{-}595)^{2+}\cdot(d^{2+}\cdot O_2) \qquad (1)$$

The cytochrome *d* becomes a diamagnetic oxygen-binding form (see Refs. 4, 9 and Fig. 2b). These results indicate that the oxygen-binding form of cytochrome *d* is a starting cytochrome *d*-O_2 complex for the reduction of the ligated dioxygen. On incubation at $-120\,^{\circ}C$, electron transport from the cytochrome *b*-595 (the rhombic high-spin heme) occurs, and the cytochrome *b*-595 becomes spin-active (Fig. 2c). At the same time, a new low-spin signal at $g = 2.15$ appears. It seems reasonable to assume that the new species becomes spin-active by accepting one electron from the cytochrome *b*-595, and is a one-electron-reduced cytochrome *d*-O_2 complex. These reactions are summarized as follows:

$$(b\text{-}558)^{2+}\cdot(b\text{-}595)^{2+}\cdot(d^{2+}\cdot O_2)$$

$$\downarrow \qquad (2)$$

$$(b\text{-}558)^{2+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)^{-}$$

This reduced intermediate form of cytochrome *d*-O_2 complex may correspond to the intermediate form of mitochondrial cytochrome *c* oxidase, giving an ESR signal at $g = 5.0$ [16]. The next step of electron transport occurs at about $-40\,^{\circ}C$ from cytochrome *b*-558 via cytochrome *b*-595 to the reduced intermediate form of the cytochrome *d*-O_2 complex. The cytochrome *b*-558 is oxidized and becomes spin-active, and the intermediate form of cytochrome *d*-O_2 complex is further reduced and becomes ESR-silent (Fig. 2d). Namely,

$$(b\text{-}558)^{2+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)^{-}$$

$$\downarrow \qquad (3)$$

$$(b\text{-}558)^{3+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)^{2-}$$

Our ESR study demonstrated the occurrence of the three redox intermediates of the cytochrome *d* complex shown in Eqns. 1–3. Here we designate the oxygen-binding intermediates I, II, and III as $(b\text{-}558)^{2+}\cdot(b\text{-}595)^{2+}\cdot(d^{2+}\cdot O_2)$, $(b\text{-}558)^{2+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)^{-}$, and $(b\text{-}558)^{3+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)^{2-}$, respectively.

Under physiological conditions of limited oxygen tension, most of the cytochrome *d* complex is thought to be the oxygen-binding intermediate I. Electron transport from cytochrome *b*-595 to (cytochrome $d^{2+}\cdot O_2$) results in the formation of the oxygen-binding intermediate II as shown in Fig. 3, reaction A. This intermediate is subjected to consecutive electron transfer from cytochrome *b*-558 via cytochrome *b*-595 to (cytochrome $d^{2+}\cdot O_2)^{-}$ to form the oxygen-binding intermediate III (Fig. 3, reaction B). Then we assume that the oxygen-binding intermediate III can react with ubiquinol-8 (UQH_2) to form a putative intermediate of highly reduced state (Fig. 3, reaction C). This reaction takes place because cytochrome *b*-558 has an E_m value similar to that of cytochrome *b*-595 and so rapid electron transfer from cytochrome *b*-558 to cytochrome *b*-595 occurs. This putative intermediate accepts four protons to generate two molecules of water followed by binding of dioxygen, leading to the formation of $(b\text{-}558)^{3+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)$ (Fig. 3, reactions D and E). The product, $(b\text{-}558)^{3+}\cdot(b\text{-}595)^{3+}\cdot(d^{2+}\cdot O_2)$, is known to be the air-oxidized cytochrome *d* complex [4], or can be designated as

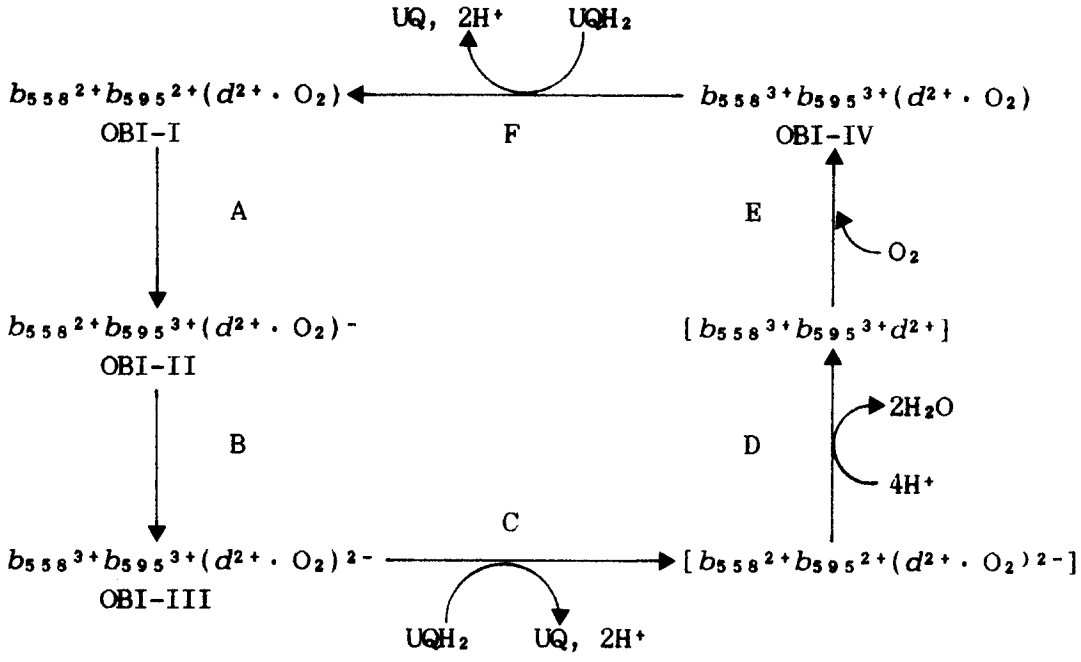

Fig. 3. A model for oxidation-reduction reaction cycle in the cytochrome *d* complex. Abbreviations used: OB-I, OBI-II, OBI-III and OBI-IV represent oxygen-binding intermediates I, II, III and IV, respectively; UQH_2 and UQ represent ubiquinol-8 and ubiquinone-8, respectively.

the oxygen-binding intermediate IV. The oxygen-binding intermediate IV reacts with ubiquinol-8 to give the oxygen-binding intermediate I for initiation of the next redox cycle (Fig. 3, reaction F).

This model is plausible so far as the results of the present ESR study are concerned, but is not unique in explaining the data. It must be verified by protein chemical studies and rotation-vibration spectroscopy.

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References

1 Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3375–3381
2 Anraku, Y., and Gennis, R.B. (1987) Trends Biochem. Sci. 12, 262–266.
3 Poole, R.K., Kumar, C., Salmon, I. and Chance, B. (1983) J. Gen. Microbiol. 129, 1335–1344
4 Hata, A., Kirino, Y., Matsuura, K., Itoh, S., Hiyama, T., Konishi, K., Kita, K. and Anraku, Y. (1985) Biochim. Biophys. Acta 810, 62–72
5 Miller, M.J. and Gennis, R.B. (1983) J. Biol. Chem. 258, 9159–9165
6 Green, G.N., Kranz, R.G., Lorence, R.M. and Gennis, R.B. (1984) J. Biol. Chem. 259, 7994–7997
7 Lorence, R.M., Koland, J.G. and Gennis, R.B. (1986) Biochemistry 25, 2314–2321
8 Chance, B. (1978) Methods Enzymol. 54, 102–111
9 Poole, R.K., Salmon, I. and Chance, B. (1983) J. Gen. Microbiol. 129, 1345–1355
10 Kumar, C., Poole, R.K., Salmon, I. and Chance, B. (1985) FEBS Lett. 190, 227–231

11 Poole, R.K., Sivaram, A., Salmon, I. and Chance, B. (1982) FEBS Lett. 141, 237–241
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Lorence, R.M., Green, G.N. and Gennis, R.B. (1984) J. Bacteriol. 157, 115–121
14 Kranz, R.G., and Gennis, R.B. (1984) J. Biol. Chem. 259, 7998–8003
15 Williams, H.D., and Poole, R.K. (1986) Biochem. Soc. Trans. 14, 1215–1216
16 Shaw, R.W., Hansen, R.E. and Beinert, H. (1979) Biochim. Biophys. Acta 548, 386–396