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EPR studies of the cytochrome-*d* complex of *Escherichia coli*

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We have examined the thermodynamic and EPR properties of one of the ubiquinol oxidase systems (the cytochrome *d* complex) of *Escherichia coli*, and have assigned the EPR-detectable signals to the optically identified cytochromes. The axial high spin $g = 6.0$ signal has been assigned to cytochrome *d* based on the physicochemical properties of this signal and those of the optically defined cytochrome *d*. A rhombic low spin species at $g_{x,y,z} = 1.85, 2.3, 2.5$ exhibited similar properties but was present at only one-fifth the concentration of the axial high spin species. Both species have an E_{m7} of 260 mV and follow a −60 mV/pH unit dependence from pH 6 to 10. The rhombic high spin signal with $g_{y,z} = 5.5$ and 6.3 has been assigned to cytochrome *b*-595. This component has an E_{m7} of 136 mV and follows a −30 mV/pH unit dependence from pH 6 to 10. Lastly, the low spin $g_z = 3.3$ signal which titrates with an E_{m7} of 195 mV and follows a −40 mV/pH unit dependence from pH 6 to 10 has been assigned to cytochrome *b*-558. Spin quantitation of the high-spin signals indicates that cytochrome *d* and *b*-595 are present in approximately equal amounts. These observations are discussed in terms of the stoichiometry of the prosthetic groups and its implications on the mechanism of electron transport.

Introduction

The electron-transport chain of *Escherichia coli* is composed of a series of substrate dehydrogenases that reduce ubiquinone and two ubiquinol oxidases, the cytochrome-*o* and cytochrome-*d* complexes. Of the cytochromes found in *E. coli* membrane particles, all of them, with the exception of the *b* cytochrome associated with the succinate dehydrogenase, can be assigned to one of these two complexes [1–6]. Both of the oxidases can support cell growth under a variety of cell-culture conditions [7,8] and appear to form a redundant system. The production of the two oxidases is controlled by many factors, the most prevalent being the oxygen tension of the growth medium (see Ref. 9 for a review). The cytochrome-*d* complex, which is responsible for cyanide-resistant oxygen consumption, has been isolated and consists of two polypeptides having molecular weights of 43 000 and 57 000. Within this complex there are three optically distinct redox species; cytochrome *d*, cytochrome *b*-595 (formerly designated cytochrome a_1), and cytochrome *b*-558 [10,11].

The cytochrome-*d* oxidase has been studied extensively by optical spectroscopy. Cytochrome *d*, which has a chlorin as its chromophore [12], exhibits an absorption maximum at 625 nm in the reduced state. The redox midpoint potential (E_m) of cytochrome *d* is 260 mV at pH 7.0 and follows a −60 mV/pH unit dependence [13]. Cytochrome *b*-595 is an unusual *b*-type cytochrome with absorption peaks at 560 and 595 nm and a trough near 650 nm. The spectrum of this cytochrome is very similar to that of the high-spin *b*-type cytochrome found in cytochrome *c* peroxidase [14]. Cytochrome *b*-595 has an $E_{m7.0}$ of approx. 150 mV, which is also pH dependent, −40 mV/pH unit [13]. The $E_{m7.0}$ value of cytochrome *b*-558 is 180 mV and follows the same pH dependence as that of cytochrome *b*-595. Studies using carbon monoxide binding and coulometry have indicated that within the complex there are two molecules of cytochrome *d*, and one each of cytochrome *b*-595 and cytochrome *b*-558 [14].

Abbreviations: Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulphonic acid; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; Bis-Tris propane, (1,3-bis[tris(hydroxymethyl)methylamino]propane; DAD, 2,3,5,6-tetramethylparaphylenediamine; TW80, Tween-80.

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Although this enzyme has been studied extensively by optical spectroscopy, there have been only a few EPR studies performed. These studies have resulted in conflicting assignments of the EPR signals present in the cytochrome *d* complex. Previously, DerVartanian et al. [15] and Kauffman et al. [16] have assigned a high-spin axial signal near $g = 6$ to cytochrome *d*. Hata et al. [17] have assigned a low-spin rhombic species $g_{y,z}$ * = 2.3, 2.5 to cytochrome *d*. A high-spin rhombic signal $g_{y,z} = 5.5$ and 6.3 has also been assigned to cytochrome *d* by Kumar et al. [18]. The high spin axial signal at $g = 6$ has been assigned to cytochrome *b*-558 by Hata et al. and by Kumar et al. On the other hand the rhombic high-spin signal, $g_{y,z} = 5.5$ and 6.3, has also been assigned to cytochrome *b*-558 by Finlayson and Ingledew [19]. Recently the $g = 5.5$, 6.3 signal has been assigned to cytochrome *b*-595 by two groups [20,21] based on low-temperature carbon-monoxide flash-photolysis experiments. Thus, the assignments of signals due to cytochrome *b*-558 and cytochrome *d* are controversial with only the assignment of cytochrome *b*-595 showing any consensus. In the present work we have attempted to clarify the situation by reexamining the assignment of the EPR signals to the various cytochromes present in the cytochrome-*d* complex. We have adopted the combined methods of redox potentiometry, protein isolation, and genetic manipulation.

Materials and Methods

E. coli strains RG127[pNG2] and GR84N[pNG10] were grown in large batches as described previously [10]. The cells were harvested and kept frozen at −80 °C until use. Membrane vesicles were prepared as described in Ref. 4 with the exception that a 100 mM Hepes, 10 mM EDTA (pH 8.0) buffer was used. The cytochrome *d* complex was isolated as described by Miller and Gennis [10]. Cytochrome *b*-558 was isolated as described by Green et al. [22].

When isolated, the cytochrome *d* complex is in an inactive state in the detergent cholate. It may be reactivated by exchanging the detergent cholate for another detergent such as Tween-80 (TW80). Cholate was exchanged for Tween-80 by a 10-fold dilution of the cholate solubilized complex, in a solution of 0.1% TW80, 100 mM Hepes, 10 mM EDTA (pH 8), and was stored overnight at 4 °C. The complex was then concentrated using the Amicon ultra-filtration system with PM 30 membranes. The sample was subsequently diluted a second time, 1 : 10, in the same buffer detergent solution (TW80, Hepes, EDTA, pH 8) and concentrated again.

EPR samples were redox poised, under anaerobic conditions, as described by Dutton [23] and were rapidly frozen by immersion of the sample tubes in a 1 : 5 mixture of methylcyclohexane : isopentane which was precooled to approx. 81 K by liquid nitrogen. For each redox titration 40 μM of each of the following mediators were added: 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-naphthoquinone-4-sulfonate, duroquinone, 1,4-benzoquinone, and 2,3,5,6-tetramethylparaphenylenediamine (DAD), and either 10 or 20 μM pyocyanine. The buffer solutions consisted of 20 mM EDTA and 100 mM of the appropriate buffer Mes (pH 6), Mops (pH 7), Hepes or Hepps (pH 8.0), Bistris propane (pH 9) and Caps (pH 10). In all titrations the samples were initially fully reduced by the addition of sodium dithionite. In some cases, after the initial reduction, the sample was oxidized by the addition of potassium ferricyanide to obtain the fully oxidized state and followed by a reductive titration. Otherwise an oxidative titration was performed. EPR spectra were recorded using a Varian E-109 X-band spectrometer with a variable temperature cryostat (Air Products and Chemicals, Inc., LTD-3-110). The EPR spectrometer is coupled to an IBM personal computer which is used for data acquisition and spectral manipulation.

Spectra used for simulations were obtained under non-saturating conditions. The simulation program assumes gaussian line shapes and no hyperfine interactions as described in Ref. 24. The magnetic field sweep was divided into 500 points and integration around the angles theta (θ) and phi (ϕ) were performed in 200 loops. Simulations were calculated on the University of Pennsylvania School of Medicine Vax-8600 computer (Digital Electronics Corp., Maynard, MA) and then transferred to the IBM PC for comparison to the original spectra and plotting.

The EPR signals were quantitated by the method of Aasa and Vänngård [25]. The spectra of the signal to be quantitated and of a reference, ferrimyoglobin or ferrimyoglobin-azide, were obtained under non-saturating conditions. Both the reference and sample spectra were simulated and then the simulation was normalized to fit the original spectrum. The value obtained from the double integration of the normalized simulation was then used in the concentration calculation. The use of the simulation for the quantitation of the signals eliminated errors due to baseline drift of the spectrometer. The zero-field splitting parameter, *D*, was measured for cytochromes *d*, *b*-595, and ferrimyoglobin from the temperature dependence of the signal amplitude at $g =$ 6,6.3 and 6, respectively. In our measurements the zero-field splitting for cytochromes *d* and *b*-595 were found to be within 1 cm^{-1} of the value obtained for ferrimyoglobin. Therefore no correction was made to the concentration measured for the high spin cytochromes. The concentration of the reference samples was mea-

* The EPR signals have been labeled with g_x, g_y, g_z representing the minimum, middle and maximum g values, respectively, in accordance with the assignments generally used for heme proteins.

sured optically using 503 nm ($\varepsilon = 9.23$ $mM^{-1} \cdot cm^{-1}$) for ferrimyoglobin [26]] and 540 nm ($\varepsilon = 8.7$ $mM^{-1} \cdot cm^{-1}$) for ferrimyoglobin azide [27].

Results

EPR signals associated with the cytochrome-d oxidase

Previous studies of the cytochrome-*d* complex have been performed with membrane particles from cells which were enriched in this complex but still retained the cytochrome-*o* complex, making it difficult to assign conclusively signals to the cytochrome-*d* complex. To avoid this difficulty we have studied membrane particles from the strain RG127[pNG2] which is cytochrome *o* deficient and overproduces the cytochrome-*d* complex by 10–20 times the normal concentration. In Fig. 1 EPR spectra obtained from membrane particles isolated from the *E. coli* mutant RG127[pNG2] (traces A and B) and of the isolated cytochrome *d*-complex (traces C and D) are shown. The membrane particles and the isolated complex exhibit essentially the same EPR spectra with the exception of two signals seen in the membrane particles, the $g = 2.03$ signal, which most likely arises from the iron-sulfur cluster S-3 present in the succinate dehydrogenase [28] and a shoulder present on the higher magnetic-field side of the $g = 3.3$ signal. Several different species give rise to the signals seen in Fig. 1. In the membrane particles (trace A) two high-spin species can be distinguished. The first is an axial species, centered near $g = 6.0$. The second is a rhombic high-spin species which appears on both sides of the central peak with g values near 6.3 and 5.5. In the isolated complex (trace C) the central peak at $g = 6.0$, representing the axial high-spin species, is still quite prevalent. The rhombic signal, although still present as indicated by the trough at $g = 5.5$, has a smaller intensity than that observed in the membrane. The signal at $g = 4.3$ has been associated with non-specifically bound iron centers and is usually seen in most membrane preparations. Traces B and D show spectra obtained by expanding the magnetic field sweep of the spectrometer in the high-field region. In membranes (trace B) signals are seen at $g = 2.61$, 2.48, 2.35, 1.85, and 1.81. As we will discuss below, all of these signals titrated with the same redox midpoint potential and follow the same pH dependence as that of cytochrome *d*. Therefore we have

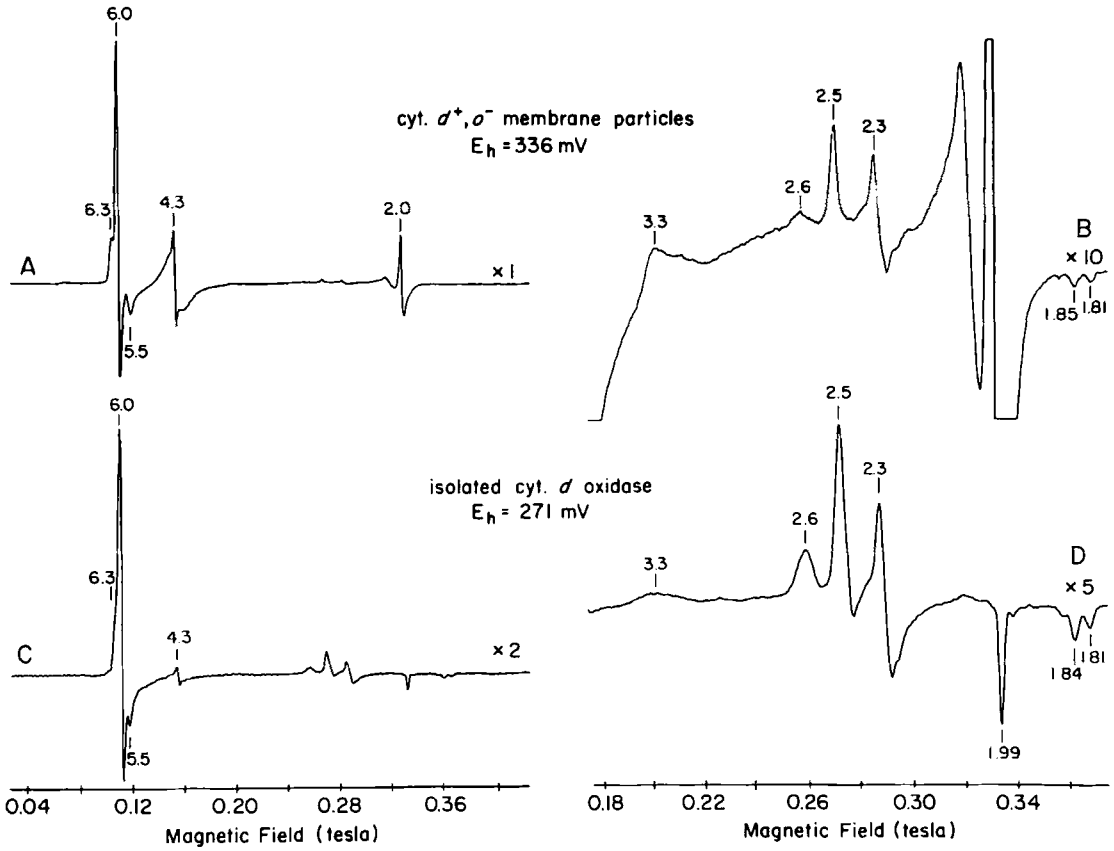

Fig. 1. EPR spectra of *E. coli* RG127[pNG2] membrane particles and the isolated cytochrome *d* complex. *E. coli* particles were suspended in pH 8.0 buffer and poised at the redox potential shown in the presence of the mediators as described in the Materials and Methods section. The isolated cytochrome *d* complex was suspended in 100 mM Hepps (pH 8.0), 20 mM EDTA and 10 mM cholate, to a concentration of 75 μM and poised as above. EPR conditions for traces A and C were: microwave power, 1 mW; modulation amplitude, $1.25 \cdot 10^{-3}$ tesla; scan rate, 0.1 tesla/min; time constant, 0.064 s; temperature 7 K; microwave frequency, 9.32 GHz. For traces B and D the EPR conditions were the same except for the scan rate, $5 \cdot 10^{-2}$ tesla/min; the modulation amplitude, $1.6 \cdot 10^{-3}$ tesla; and the time constant, 0.128 s.

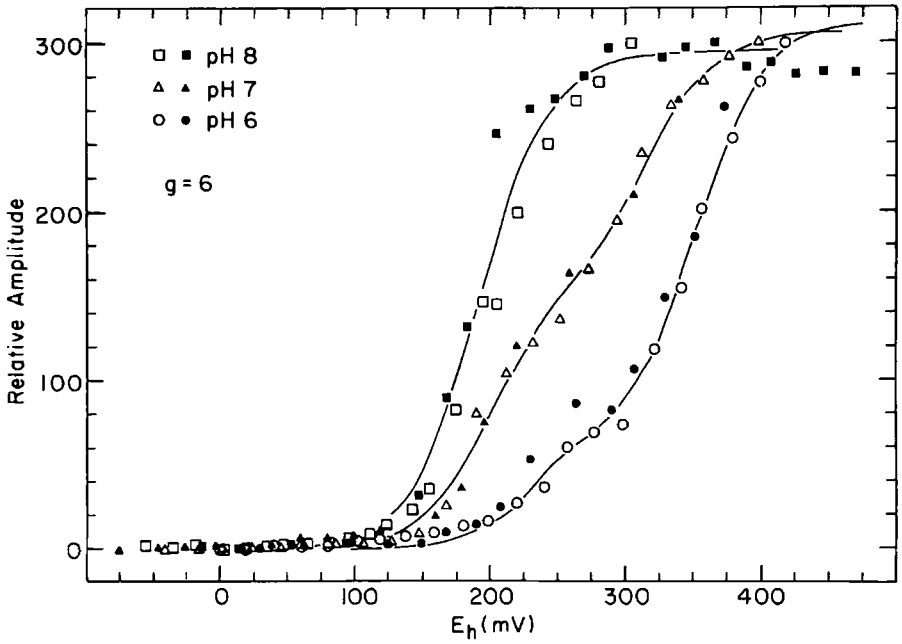

Fig. 2. Redox titration of the $g = 6.0$ signal at pH 6.0, 7.0 and 8.0. *E. coli* membrane particles were suspended in the appropriate pH buffer at a concentration of approx. 90 μM cytochrome *d*, as described in Fig. 1, except that 20 μM DAD was added. Through the data are drawn theoretical redox titration curves consisting of two one-electron components with midpoints of 230 mV (22%) and 353 mV (78%); and 200 mV (50%) and 315 mV (50%) for pH 6.0 and 7.0, respectively. Through the pH 8.0 data is drawn a single-component one-electron redox-titration curve with a midpoint of 195 mV. Open and closed symbols represent different preparations.

assumed that these signals are from different forms of the same species, i.e., cytochrome *d*. The separation of these signals into individual components will be discussed later. The final signal at $g = 3.3$ arises from another low-spin species and is typical of a low-spin cytochrome. All of the signals seen in these spectra, with the exception of the $g = 3.3$ signal, have been reported previously and were assigned to various cytochromes within the cytochrome-*d* complex.

Assignment of cytochrome d

We have investigated the redox properties of the high-spin signals seen in membranes from the strain RG127[pNG2] which overproduced the cytochrome *d*

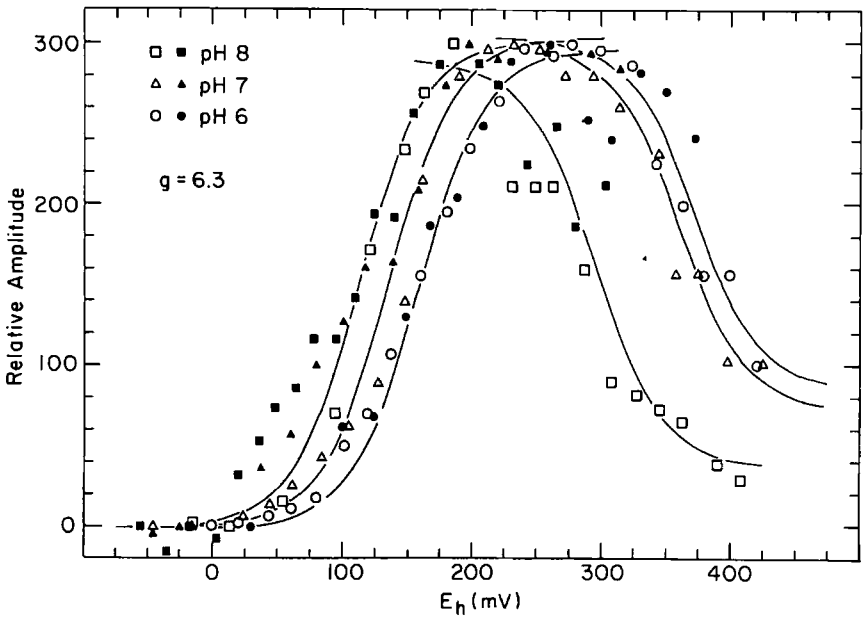

Fig. 3. Redox titration of the $g = 6.3$ signal at pH 6.0, 7.0 and 8.0. The samples were identical to those used in Fig. 2. Through the data are drawn single-component redox-titration curves with midpoints of 159, 136 and 112 mV for the appearance of the signal and 371, 361 and 295 mV for its disappearance at pH 6, 7 and 8, respectively.

complex but does not contain the cytochrome *o* complex. Fig. 2 shows several redox titrations of the $g = 6.0$ axial signal at pH 6, 7 and 8. The $g = 6.0$ signal titrated as a single species with redox midpoint potential of 200 mV at pH 8 (squares). The same signal titrates as two species with midpoints of 190 and 315 mV at pH 7 (triangles), and 230 and 350 mV and pH 6 (circles). The amounts of each redox species in the titration varies with the pH. If the data from these titrations are fitted to a single redox species at each pH, then the E_{m7} of the $g = 6.0$ signal is 260 mV and follows a -60 mV per pH unit dependence. The midpoint potential and its pH dependence, assuming a single species, are very similar to those reported for cytochrome *d* based on optical titrations [13]. Given the similarities of the redox properties of this signal and optically identified cytochrome *d*, we have assigned the high spin axial species at $g = 6.0$ to cytochrome *d*. The resolution of cytochrome *d* into two redox species at pH 6 and 7 was not seen in the optical experiments performed on similar preparations. The difference between the EPR and optical experiments may arise from a freezing artifact.

Previously, only the low-spin species at $g_{y,z} = 2.3$, 2.5 had been assigned to cytochrome *d* by Hata et al. [17]. We have reinvestigated the redox and pH properties of the $g = 2.5$ species seen in Fig. 1. In our experiments the $g = 2.5$ signal exhibited the same redox properties as the high-spin axial $g = 6.0$ signal including the resolution into two redox species at pH 7 (data not shown). Therefore we have also assigned the low-spin rhombic signals of the $g_{x,y,z} = 1.81$, 2.35, 2.48 species to cytochrome *d*. However, as described later, measurements of the relative spin concentrations of the high- and low-spin forms of cytochrome *d* indicate that the low-spin form of cytochrome *d* is only a minor component of the complex.

Assignment of cytochrome b-595

We have assigned cytochrome *b*-595 to the rhombic high spin species based on the same criteria as we have used for cytochrome *d*. Fig. 3 shows redox titrations of the $g_z = 6.3$ signal heights, measured from the low field baseline, at the same three pH values as in Fig. 2. Here the signal titrates as a single species with an $E_{m7.0}$ of 136 mV and follows a -30 mV/pH unit dependence, analogous to the optical titrations of cytochrome *b*-595, within experimental error [13]. This signal also appears to diminish in amplitude when the redox potential is raised through the midpoint of cytochrome *d*, as has been reported previously [19]. Recently, Poole and Williams [20] have presented optical evidence to support the notion that cytochrome *b*-595 is a direct electron donor to cytochrome *d*. Combining this with previous EPR data [18], these investigators have suggested that cytochrome *b*-595 is the rhombic high-spin species at $g_{y,z} = 5.5$ and 6.3. Also Hata-Tanaka et al. [21] have made the same assignment based on more recent carbon monoxide-binding studies. Their results are consistent with our assignment of the $g_{y,z} = 5.5$, 6.3 species to cytochrome *b*-595. The optical spectrum of cytochrome *b*-595 is very similar to that of the high-spin *b* cytochrome of cytochrome-*c* peroxidase [14]. Quantitation of this signal relative to that of the high-spin axial signal indicates a 1 : 1 ratio. Given the very low concentration of the low-spin cytochrome-*d* signal, these results suggest that there is only one *d* heme per complex rather than two as indicated by carbon monoxide binding and coulometry [14]. This will be discussed further below.

Assignment of cytochrome b-558

In order to identify the EPR signal of cytochrome *b*-558 we have utilized a mutant of *E. coli*, GR48N[pNG10], that overproduces subunit I of the cytochrome-*d* complex which contains only cytochrome *b*-558 [22]. Neither cytochrome *d* nor cytochrome *b*-595 of the cytochrome-*d* complex are present, although the cytochrome-*o* complex is present as the terminal oxidase. In Fig. 4 we present EPR spectra of membrane particles from this mutant which were poised at 181 mV, pH 8, (traces A and A′), 91 mV (traces B and B′), and their difference. Under the conditions of the experiment, the major portion of cytochrome *b*-558 is oxidized at 181 mV and is mostly reduced at 91 mV. Although in this mutant the cytochrome-*o* oxidase represents less than 10% of the total cytochrome content, a change due to the cytochrome-*o* complex occurs in this redox potential range. To ensure the proper assignment we have also examined the isolated cytochrome-*b*-558 protein (traces C and C′).

In the previous studies of membranes enriched in the cytochrome-*o* complex, signals at $g = 6.0$ and 3.0 were reported and assigned to the cytochrome-*o* complex [17]. In membranes poised at 181 mV, pH 8, prominent signals at $g = 6.0$, 4.3, 3.3 are seen. The 4.3 signal is associated with non-specific iron in the membranes. When the potential is lowered to 91 mV, the intensities of the $g = 6$ and 3.3 signals are diminished. A species at $g = 3.1$ has now become more clearly discernible. In our study the $g = 3.1$ species, which is not well resolved, may correspond to the $g_z = 3.0$ species of the cytochrome-*o* complex (cytochrome *b*-562). In the difference spectra signals at $g = 6.0$ and 3.3 are seen. The $g = 6.0$ signal most likely comes from the cytochrome-*o* complex. The only signal present in these membranes that has not been previously assigned to the cytochrome-*o* complex is at $g_z = 3.3$. Since this signal is present in both the isolated cytochrome-*d* complex and in membranes which contain amplified cytochrome *b*-558, and cannot be assigned to a component of the cytochrome-*o* oxidase, we have assigned this signal to cytochrome *b*-558.

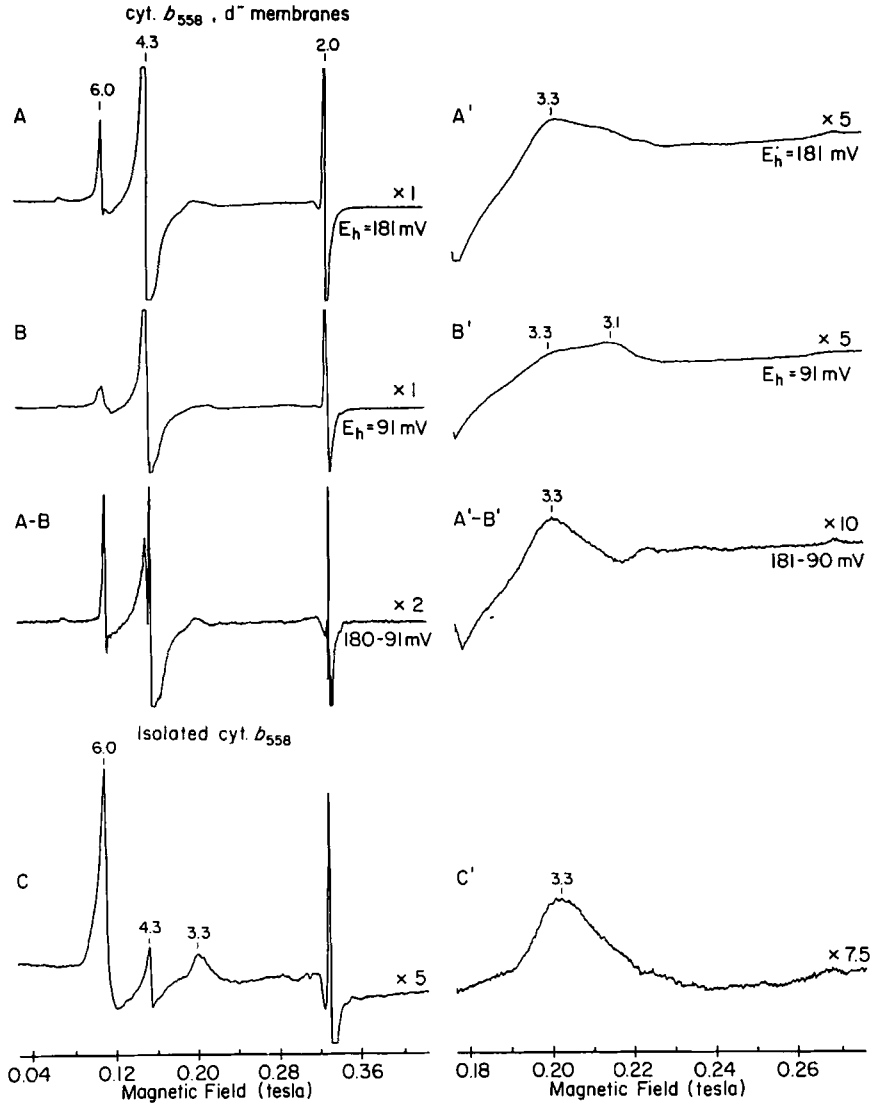

Fig. 4. EPR spectrum of cytochrome *b*-558. *E. coli* GR84N[pNG10] membrane particles were suspended in pH 8.0 buffer with 10 mM EDTA and were poised at the redox potentials indicated. The isolated cytochrome *b*-558 was suspended in the same buffer with 0.1% Tween 80 present. EPR conditions for traces A and B: microwave power, 1 mW; modulation amplitude, $1.25 \cdot 10^{-3}$ tesla; scan rate, 0.1 tesla/min; time constant, 0.1278 s; temperature, 7 K. The EPR conditions for trace C were the same as in traces A and B, except for the modulation amplitude, $2 \cdot 10^{-3}$ tesla. EPR conditions for traces A', B' and C': microwave power, 1 mW; modulation amplitude, $2.5 \cdot 10^{-3}$ tesla; scan rate, $12.5 \cdot 10^{-3}$ tesla/min; time constant, 0.5 s; temperature, 7 K.

This assignment is further supported by EPR spectra obtained from subunit I of the cytochrome *d* complex which contains only cytochrome *b*-558 (Fig. 4, traces C and C'). In these spectra, signals are seen at $g = 6.0$ and 3.3. The $g = 6.0$ species was quantitated relative to a ferrimyoglobin standard and represented approximately one-third of the total cytochrome-*b* concentration. Therefore the major portion of cytochrome *b*-558 is considered to be represented by the $g_z = 3.3$ signal. Since there are no other cytochromes present, the $g = 6.0$ signal in this case most likely comes from modification of the low-spin form of cytochrome *b*-558 which may have occurred during isolation. This $g = 6.0$ signal seen in the isolated cytochrome-*b*-558 preparation should not be confused with signals seen at the same g value in membrane particles which contain the complete cytochrome-*d* or cytochrome-*o* complexes. The assignment of cytochrome *b*-558 to a low spin form is also in agreement with optical studies which show this cytochrome to have the spectrum typical of a low spin heme [14]. This assignment is also supported by the redox midpoint potential of the $g = 3.3$ signal, $E_{m7} = 195$ mV, and the pH dependence of the midpoint potential, -40 mV from pH 6 to 10.

pH dependence of the redox midpoint potential

A summary of the redox titration data of each species is presented in Fig. 5. The $g_z = 6.3$ signal of cytochrome *b*-595 (△) follows a -30 mV/pH unit dependence over the entire pH range measured (pH 6–10). The $g_z = 3.3$

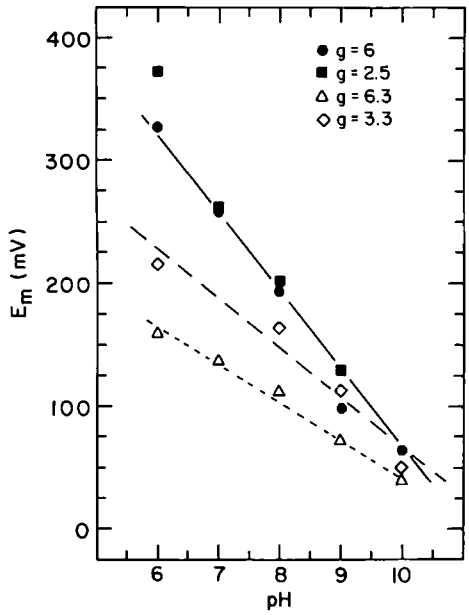

Fig. 5. pH dependence of the cytochrome *d* oxidase components in membrane particles. Redox titrations of the $g = 6$ (○), $g = 2.5$ (□), $g = 6.3$ (△) and $g = 3.3$ (◇) signals were analyzed for a single redox component and the midpoint potentials plotted. The dotted line represents a −30 mV/pH unit change, the dashed line represents a −40 mV/pH unit change, and the solid line represents a −60 mV/pH unit change.

signal of cytochrome *b*-558 (◇) exhibits a −40 mV/pH unit dependence from pH 6 to 10. Both cytochromes *b*-558 and *b*-595 have been previously reported to follow a −40 mV/pH unit dependence, based on the spectrophotometric redox titration data [13]. In their study, Lorence et al. performed a considerably more detailed redox titration but over a narrower pH range (pH 6–8) than what is presented here. The difference in our observations may be explained by the difference in the experimental accuracy and pH range of the two data sets. Cytochrome *d*, which is represented by both the high-spin axial species, $g = 6.0$, and the low-spin rhombic species, $g_z = 2.5$, follows a −60 mV/pH unit dependence with an E_{m7} of 260 mV in agreement with optical data [13]. At pH 10 no measurements could be made on the $g_z = 2.5$ signal, since it did not appear in the spectra and the $g = 6.0$ axial species showed considerably reduced amplitude relative to the $g_z = 6.3$ species. It appears that at pH 10, cytochrome *d* is unstable and is being denatured. When the $g = 6.0$ or the $g = 2.5$ signals of cytochrome *d* are considered as a single redox species,the midpoint potential follows a −60 mV/pH unit dependence from pH 6 to 10.

Spectral resolution and quantitation of cytochrome b-595 and d

As we have shown above, cytochrome *d* appears to be represented by both a high-spin and a low-spin form. Fig. 6 presents spectra of the low-spin cytochrome *d* and the portion of the high-spin cytochrome *d* seen in lower magnetic fields. Also shown is the portion of the cytochrome *b*-595 spectrum seen in the same region. A simulation of each spectrum is presented as a dotted

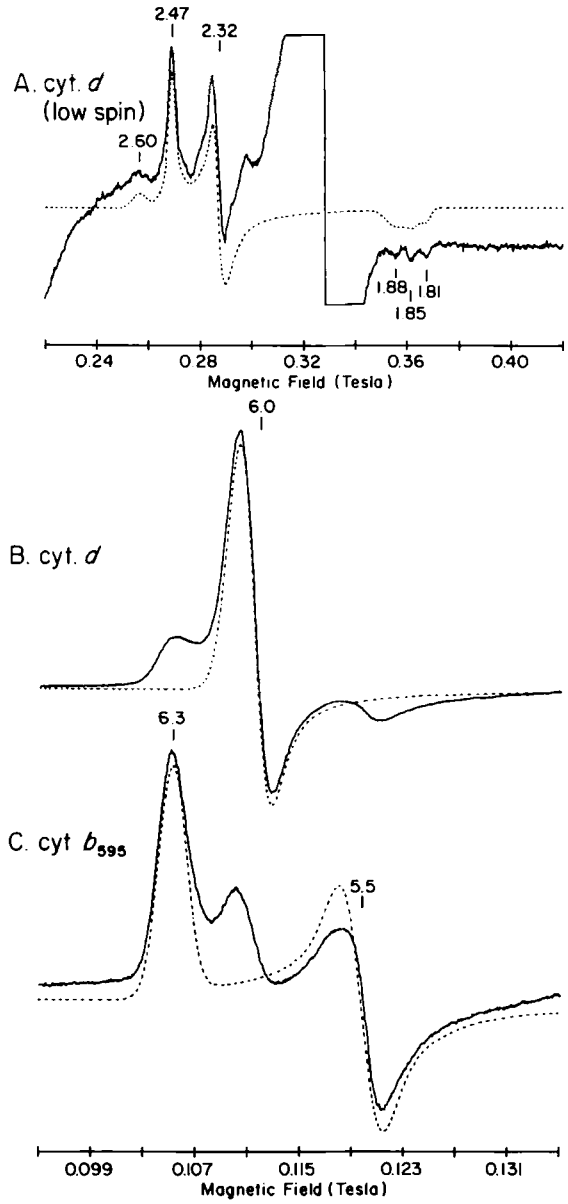

Fig. 6. EPR spectra and simulations of cytochromes *d* and *b*-595. Shown as solid lines are the spectra of cytochrome *d* (low spin) [A], cytochrome *d* (high spin) [B], and cytochrome *b*-595 [C]. The simulation of each species is shown as a dotted line. The parameters used in the simulations are presented in Table I. (A) Membrane particles were poised at 200 mV (pH 8.0), where a sample was taken and frozen until the spectrum was recorded. The EPR conditions were: microwave power, 0.5 mW; microwave frequency, 9.321 GHz; scan rate, $5 \cdot 10^{-2}$ tesla/min; time constant, 0.128 s; modulation amplitude, $1.6 \cdot 10^{-3}$ tesla; temperature, 11 K. (B) *E. coli* membrane particles were suspended in pH 6.0 buffer, mediators added, and poised at 381 mV. A sample was then taken and frozen until the EPR spectrum was recorded. The EPR conditions were as follows: microwave power, 1 mW; microwave frequency, 9.321 GHz; scan rate, $2 \cdot 10^{-2}$ tesla/min; time constant, 0.064 s; modulation amplitude, $5 \cdot 10^{-4}$ tesla; temperature, 7 K. (C) The membrane particles were then poised at 160 mV and a sample taken. The EPR conditions were the same as in spectrum B.

line. The spectrum of the low-spin cytochrome *d* presented here was simulated by the combination of three different components. It is important to point out here that as the complex ages, the number of the low-spin components and the amounts of them appear to increase. This is obvious when the spectra in Fig. 1 are compared with the present spectrum. Previously in Fig. 1 signals were apparent at $g = 2.61$, 2.48, 2.35, 1.85 and 1.81. These signals are still seen in the present spectrum with an additional trough around $g = 1.88$ and a slight trough on the high field side of the $g = 2.47$ peak. The first simulated species has g values of 2.4765, 2.327 and 1.845. The signals are seen in all preparations, even in freshly prepared membranes. The second species, simulated with g values of 2.60, 2.31 and 1.82, also appears in freshly prepared membranes but with a very low $g = 2.60$ amplitude. As the sample is mistreated or aged this species appears to increase. Finally, a third species is apparent in this sample and was simulated with g values of 2.477, 2.326 and 1.88. The g values of the three species presented here may not be completely correct, since the assignments of the g_x peaks for the first two species were made by assuming that the $g = 2.60$ species was a more rhombic form of the first $g = 2.48$ species. The summation of these simulations though does approximate the observed spectrum quite well and allows for the quantitation of these signals.

The high spin cytochrome *d* is simulated as a single species with $g_{x,y,z}$ values of 1.99, 5.965, 6.017. The $g_x = 1.99$ value was assumed, in accordance with other high-spin species previously reported. A $g = 1.99$ signal is clearly seen in the isolated complex, Fig. 1, but due to overlap of other signals in the membrane it could not be conclusively assigned to either cytochrome *d* or cytochrome *b*-595. Cytochrome *b*-595 is also simulated as a single species with $g_{x,y,z}$ values of 1.99, 5.54, 6.30. Again the $g_x = 1.99$ signal is assumed to be correct. The simulation of cytochrome *b*-595 fits quite well in the g_z peak region but the g_y peak does not fit the simulation as well because of a considerable spectral asymmetry. The cause of this asymmetry may be g strain induced by the protein or may indicate some interaction of cytochrome *b*-595 with another species along the g_y axis.

These simulations were used to quantitate the EPR signals of the cytochromes in several samples. The high-spin cytochrome *d* was found to be present in a 5 : 1 ratio over the low-spin cytochrome *d*. This suggests that the low-spin form is only a minor component of the complex and may be a denaturation product, since it is more readily seen in the aged complex. The high-spin signals of cytochrome *d* and *b*-595 were found to be present at a concentration of nearly 1 : 1 in fresh preparations and at an average concentration of 0.7 : 1 in aged preparations. In Table I is presented a summary of the g values and line widths used in the simulations. Also presented is a comparison of the concentration of the components measured by EPR and optical spectroscopy for a single sample. The concentration of cytochrome *b*-595 when measured by either EPR or optical spectroscopy gave very similar results. On the other hand, the concentration of cytochrome *d* measured by optical spectroscopy is more than twice that observed by EPR. Even with assuming a 20% error in the EPR measurement, and taking into account that this was a somewhat aged sample, the total cytochrome *d* would be at most equal to that of cytochrome *b*-595 as

TABLE I

Physicochemical properties of cytochromes b-558, b-595 and d

Component	Simulation parameters						Relative contribution (%)	Total concentration (μM)	
	g_x, g_y, g_z			l_x, l_y, l_z ($\times 10^{-4}$ tesla)				EPR	optically
Cytochrome *d* (low spin)									
species 1	1.88,	2.326,	2.477	40,	40,	40	43.5 (6.8) [a]	5.0± 1.0	–
species 2	1.845,	2.327,	2.465	32,	15,	14	37.1 (5.7)	4.2± 0.8	–
species 3	1.82,	2.31,	2.6	20,	16,	32	19.4 (3.0)	2.2± 0.4	–
Cytochrome *d* (high spin)	1.99 [b],	5.965,	6.017	10,	15,	13 [c]	100 (84.5)	61.7±12.3	162 [d]
Cytochrome *b*-595	1.99 [b],	5.54,	6.3	10,	15.5,	12.5 [c]	100	110 ±22	115 [e]
Cytochrome *b*-558	3.3,	–,	–	–,	–,	–	100	–	115 [e]

[a] The numbers shown in parentheses represent the percent of the total cytochrome *d* present in the sample (high spin + low spin).

[b] The $g = 1.99$ value was obtained from the spectrum of the isolated complex and assumed to be the same for both high-spin species.

[c] The l_z value is estimated from the isolated complex signal at $g = 1.99$.

[d] The concentration of cytochrome *d* was measured from an air-oxidized-minus-dithionite-reduced sample at 628–607 nm using the extinct coefficient of 7.4 $mM^{-1} \cdot cm^{-1}$.

[e] The concentration of the total *b* cytochrome was measured at 561–570 nm using an extinction coefficient of 10.4 $mM^{-1} \cdot cm^{-1}$. It was then assumed that half of the total cytochrome *b* was repreented by cytochrome *b*-595.

[f] The EPR spectrum of cytochrome *b*-558 was not simulated. The measured g_z value is given.

seen in the freshly prepared samples. This observation suggest that there is only one cytochrome-*d* heme per complex rather than the two as previously suggested [14].

Discussion

We have assigned the high spin axial $g = 6.0$ signal to cytochrome *d* based on the thermodynamic properties of this signal. The low spin rhombic $g_{x,y,z} = 1.85$, 2.4, 2.5 had also been assigned to cytochrome *d* but appears to represent only a minor component. Room temperature optical spectra and the propensity of this cytochrome to ligate to compounds such as cyanide suggest that cytochrome *d* exists as a high spin species in agreement with our results. The presence of this cytochrome in both a high-spin and low-spin form within a single complex is somewhat unusual but has also been reported for the *b* cytochrome of cytochrome-*c* peroxidase [29]. However, in cytochrome *c* peroxidase the low-spin form of the *b* cytochrome appears to be generated by the freezing process and is not seen if 60% glycerol is added prior to freezing [29]. In order to determine if the low spin rhombic species of cytochrome *d* comes from a freezing artifact, we have frozen samples in the presence of up to 60% glycerol. There was no change in the relative peak amplitudes of the $g = 6$ and $g = 2.5$ peaks in the presence or absence of glycerol. Hata et al. [17] have assinged only the low-spin species to cytochrome *d*. They have assigned the high-spin axial species to cytochrome *b*-558. These assignments were based on the redox midpoint potentials obtained at a single pH in which the high-spin axial and low-spin rhombic signals titrated with different redox midpoint potentials. In our experiments we have found that both the high-spin axial and the low-spin rhombic species titrate with the same redox midpoint potential, and have the same pH dependence, in disagreement with Hata et al. [17].

The rhombic high-spin species at $g_{y,z} = 5.5$ and 6.3 has been assigned to cytochrome *b*-595 again based on the thermodynamic properties. Since the thermodynamic properties of cytochrome *b*-595 and *b*-558 are very similar, we have used the isolated cytochrome-*b*-558 protein to differentiate it from cytochrome *b*-595. The assignment of a high-spin species to cytochrome *b*-595 is in agreement with the observation that the optical spectrum of cytochrome *b*-595 is very similar to that of the high-spin *b* cytochrome found in cytochrome *c* peroxidase [14]. Based on carbon monoxide photolysis experiments Hata-Tanaka et al. [21] have also assigned the high spin rhombic $g_{y,z} = 5.5$, 6.3 signal to cytochrome *b*-595. In addition, low-temperature optical experiments by Poole and Williams [20] have led them to assign the $g_{y,z} = 5.5$, 6.3 rhombic high-spin signal to cytochrome *b*-595. At present this is the only component whose assignment has been generally agreed upon.

The low spin $g_z = 3.3$ species has been assigned to cytochrome *b*-558 based on the thermodynamic properties of this signal measured in the membrane preparations and on the EPR spectrum of the isolated cytochrome-*b*-558 protein. Until the other *g* values of this cytochrome are determined quantitation of this signal is not possible. Attempts to determine the other *g* values of cytochrome *b*-558 with the isolated protein, 50 μM cytochrome *b*, have not been successful. This suggests that cytochrome *b*-558 has very small $g_{x,y}$ signals making them difficult to detect. Presumably it is the low EPR amplitude of this cytochrome that has prevented this signal from being reported previously. Typically in our experiments we have used concentrations of 50–100 μM complex which is 2–4 times that used by other researchers. This has only been possible due to the amplification of the complex within the membranes and shows the usefulness of this approach.

Quantitation of the signals from cytochrome *d* and *b*-595 indicate a ratio of approx. 1 : 1 in this complex. Previously, it has been proposed that there are two cytochrome-*d* hemes per complex and one heme each of cytochrome *b*-595 and *b*-558 [14]. Coulometric titrations by Lorence et al. [14] gave values of 2.1 ± 0.3 total *b* hemes per complex. Pyridine hemochromogen measurements by the same authors resulted in 1.9 ± 0.3 *b* hemes per complex. Finally, measurements of the concentration of cytochrome *b*-558 in the isolated complex, which can be obtained from the β band, resulted in 0.9 ± 0.1 cytochrome *b*-558 per complex. These results are all self-consistent with one *b*-558 and one *b*-595 per complex. These results also agree with our EPR measurements in which we obtain 110 μM cytochrome *b*-595 spins in a preparation with 230 μM total cytochrome *b* (115 μM cytochrome *b*-595 and 115 μM cytochrome *b*-558). The total cytochrome-*b* concentration was estimated using the extinction coefficient of 10.4 $cm^{-1} \cdot mM^{-1}$ (561 nm minus 570 nm) reported by Lorence et al. [14]. On the other hand, measurements of the total cytochrome *d* have not been as consistent. Coulometric titration resulted in 1.4 ± 0.3 *d* hemes per complex. Carbon monoxide-binding studies indicated 1.6 ± 0.3 *d* hemes per complex. These results were used to obtain an extinction coefficient of 7.4 $cm^{-1} \cdot mM^{-1}$ (628 nm minus 607 nm, reduced minus air oxidized). In the sample that was used to measure the total cytochrome *b*, the cytochrome-*d* concentration was measured to be 162 μM by optical spectroscopy and a total of 73.1 ± 14.6 μM by EPR spectroscopy (the sum of both the high-spin and low-spin signals). These results can be explained in two ways. One is that, in spite of our efforts to obtain an anaerobic system, enough oxygen leaked into the system to result in oxygen binding to approx. half of the complexes giving an EPR silent species for half of the cytochrome *d*. We have tried to eliminate this possibility by using several sys-

tems in series to remove oxygen from the argon used to flow over the sample during the redox titration and a positive gas pressure inside the titration vessel to ensure no gas flow into the vessel from outside. All of our experiments have resulted in similar numbers. Although we cannot completely rule out the possibility of an oxygen leak, we believe this to be at most a small source of error in the measurements. Another possible explanation is that the extinction coefficient measured by coulometry and carbon-monoxide binding is in error. Carbon monoxide binds to cytochrome *b*-595 at high carbon-monoxide concentrations [14] but no apparent optical change is seen in the spectrum of cytochrome *b*-595 at the low concentrations used in the binding titrations. Rothery et al. [30] have shown that NO binds to cytochrome *b*-595 and that this binding is pH dependent. From this result they have suggested that carbon monoxide also binds to cytochrome *b*-595 and this could result in an overestimation of the number of cytochrome *d* hemes (W.J. Ingledew, personal communication). The number obtained from the coulometric titration, 1.4 ± 0.3, is low enough to be consistent with a single *d* heme if it is assumed that there is some contamination of the sample by other redox-active species. It should also be noted that measurements of the total iron content of the isolated complex by the methods of atomic absorption and colorimetric analysis gave values close to three iron atoms per complex while neutron activation gave numbers closer to four iron atoms per complex [10]. It therefore seems quite probable that there is only one *d* heme per complex. Nevertheless, at this time the stoichiometry of the *d* hemes per complex still remains as an unresolved question.

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