The 650 nm Chromophore in Escherichia coli is an ‘Oxy-’ or Oxygenated Compound, Not the Oxidized Form of Cytochrome Oxidase d: An Hypothesis

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The form of cytochrome d in Escherichia coli and Azotobacter vinelandii that shows an absorption maximum at 648 to 652 nm (‘cytochrome d$_{650}$’) is generally regarded as the oxidized form of this terminal oxidase. Membranes from E. coli grown under oxygen-limited conditions, when treated with ferricyanide, do not reveal cytochrome d$_{650}$, whereas a sharp symmetrical band at 652 nm results from the reaction of the reduced enzyme with O$_2$ at either room temperature or after flash photolysis of the CO-ligated form at $-130$ °C. Electron paramagnetic resonance spectroscopy of cytochrome d$_{650}$ trapped at $-130$ °C shows that its spectrum is indistinguishable from the CO-ligated form and does not reveal resonances of high spin ferric haem previously attributed to cytochrome d. An hypothesis is proposed in which cytochrome d$_{650}$ is an early intermediate in the reaction of reduced cytochrome d with oxygen and is not the fully-oxidized (ferric) species. An analogy between cytochrome d$_{650}$ and oxyhaemoglobin is presented and the hypothesis discussed in relation to earlier work, in which the indirect interconversions of reduced cytochrome d and d$_{650}$ have been explained by proposing the existence of an ‘invisible’ form. It is suggested that this form could be the oxidized enzyme.

INTRODUCTION

Cytochrome d (formerly cytochrome a$_2$) has been identified as a terminal cytochrome oxidase in Escherichia coli, on the basis of the appearance of a band at about 637 nm in action spectra for the relief of CO-inhibited respiration (Castor & Chance, 1959). The reduced form shows an absorption maximum at about 630 nm, whilst a further, sharp band at even longer wavelengths (648 to 652 nm) appears when cells or membranes containing cytochrome d are aerated. This phenomenon was first described by Keilin (1934) and has been amply confirmed since (Lemberg & Barrett, 1973). The absorbance of this form is generally seen as a trough at about 650 nm in reduced minus aerated difference spectra and attributed to the oxidized form of the enzyme in both E. coli and Azotobacter chroococcum (Keilin, 1966). This form will be referred to hereafter as cytochrome d$_{650}$, although the position of the band has been variously reported to lie between about 647 and 653 nm. It may be relevant that the position of the absorbance of the Pseudomonas cytochrome d$_{650}$ is very sensitive to pH (Yamanaka & Okunuki, 1963).

Cytochrome d$_{650}$ exhibits most unusual behaviour in potentiometric redox titrations (Pudek & Bragg, 1976a; Hendler & Shrager, 1979). Furthermore, in both E. coli and Azotobacter vinelandii, it does not appear to be a direct oxidation–reduction product of the reduced cytochrome. To explain these results, it has been proposed (Pudek & Bragg, 1974, 1976b) that there
exists a further form of cytochrome d (termed \( d^* \) for the \( E. coli \) enzyme), intermediate between the oxidized and reduced forms, and which has no characteristic absorbance in the near-infrared region of the spectrum. This form also reacts with cyanide:

\[
\begin{align*}
&\text{Oxidized} \quad d_{680}^{\text{ox}} \quad \text{Reduction} \quad d^* \quad \text{Reduction} \quad d_{638}^{\text{red}} \\
&\text{d}^* \cdot \text{HCN}
\end{align*}
\]

where subscripts denote maxima (if any) and superscripts indicate the proposed redox state. The latter property of \( d^* \) has not been specified. A similar intermediate has been proposed in \( A. vinelandii \) by Kauffman & van Gelder (1973a, b).

In this paper, we present an optical and electron paramagnetic resonance (EPR) characterization of cytochrome \( d_{50} \) trapped at sub-zero temperatures. We also propose that this form of the cytochrome is an early intermediate in the reaction of reduced cytochrome \( d \) with oxygen, whereas \( d^* \) (i.e. the 'invisible form') may be equated with the oxidized form. This hypothesis is consistent with previously published data on cytochrome \( d \) and explains in part the curious ligand binding and potentiometric properties of this oxidase. Parts of this work have been presented before in abstract form (Poole, 1982; Poole \textit{et al.}, 1982a).

**METHODS**

**Organism, growth conditions and preparation of cells.** \( E. coli \) K12 (strain A1002) was grown as described by Poole \& Chance (1981), except that the concentration of sodium succinate was reduced to 20 mM and 6 litre batch cultures were grown in a New Brunswick fermenter. Sparging with sterile air was at 0.6 l air min\(^{-1}\) and the stirring speed was sufficient only to prevent sedimentation of cells. The \( O_2 \) transfer rate to the medium was 8.4 mmol l\(^{-1}\) h\(^{-1}\). A growth curve for similar oxygen-limited conditions is shown in Scott \& Poole (1982). Cells were harvested 19 to 23 h after inoculation with a stationary phase starter culture, when \( A_{660} \) (undiluted, 1 cm cuvettes) was 0.4 to 0.8. Harvesting by centrifugation and washing, and resuspension of cells in buffer containing 30\% (v/v) ethylene glycol as cryosolvent, was as described previously (Poole \textit{et al.}, 1979), except that the cell concentration was 20\% (wet wt/v). Protein was determined in such suspensions by Lowry's method, using BSA as standard, with appropriate controls for the presence of ethylene glycol. Cells to be used for preparing membrane particles were resuspended in the same buffer but lacking cryosolvent.

**Further treatment of suspensions for low-temperature spectral studies.** The procedure used was that described by Poole \& Chance (1981), except for minor modifications. The cell suspension was reduced by incubation with 10 mm-succinate for 5 min (although 1 min incubation gave >95\% of the reduction level of the cytochromes obtained with \( Na_2S_2O_4 \)). Oxygen was introduced to the CO-ligated, reduced cell suspension in a cuvette (2 mm path length) by vigorously stirring with a closely-fitting steel wire at 22 to 25 °C, giving 200 to 400 \( \mu \)M-\( O_2 \) (Chance \textit{et al.}, 1975a). The oxygen-supplemented sample was trapped by freezing at \(-78 \) °C in an ethanol-dry ice bath and then equilibrated at lower temperatures in the dual-wavelength spectrophotometer; during this period the sample was protected from measuring and reference beams with a shutter. Photoysis of the CO compound was achieved with a xenon lamp.

**Scanning spectrophotometry.** The Johnson Foundation dual-wavelength scanning spectrophotometer (DBS-3) was used (Poole \& Chance, 1981). Transmitted light was monitored with a S-10 type photomultiplier (EM1 9592B) in the 390 to 700 nm range, with 500 nm as a reference wavelength.

**Preparation of membrane particles.** Washed cells, previously stored at \(-20 \) °C, were thawed, washed again with a medium that contained 50 mm-Tris, 2 mm-MgCl\(_2\), 6H\(_2\)O and 1 mm-EGTA (pH 7.4), and then resuspended in the same at about 0.33 g (wet wt cells) ml\(^{-1}\). The cells were disrupted in a Sonifier Cell Disrupter model W185 (Heat Systems-Ultrasonics Inc., Plainview, N.Y., U.S.A.), fitted with a probe having an end diameter of 13 mm and operating at 40\% of maximum power. Five 30 s periods of treatment were interspersed with 15 s periods of cooling, which was aided by maintaining the sample in a salt-ice-water slurry. After adding a few grains of DNAase (Sigma), the sonicate was centrifuged at 10000 \( \times \) g (wet wt cells) ml\(^{-1}\), and the resulting supernatant pooled with the first. This was centrifuged for 40 min at 340000 \( \times \) g (integrated force time = \( 8.8 \times 10^{10} \) rad\(^3\) s\(^{-1}\)) in a type 55-2 Ti rotor of a Beckman L8-55 centrifuge. The supernatant was discarded and the pellet resuspended and homogenized in buffer and recentrifuged to yield the membrane particle fraction.

**EPR studies.** Membranes, suspended in the above buffer, supplemented with 30\% (v/v) ethylene glycol, were reduced in an EPR tube with 6 mm-succinate and CO was bubbled into the suspension at 0 °C for 15 min. The preparation was cooled to \(-23.5 \) °C in an ethanol-dry ice bath for 10 min, supplemented with \( O_2 \) in the tube by stirring with five strokes of a closely-fitting stirrer and immediately frozen at \(-78 \) °C. EPR spectra were obtained.
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on the sample after transfer in the dark to the spectrometer and again after photolysis in a cryostat at −135 °C (±2 °C) using 10 flashes of a xenon flash lamp. Further spectra were obtained after incubation of the photolysed sample, as detailed in Results. Spectra were run on a Varian 109 X-band spectrometer equipped with a Nicolet computer for data accumulation and handling, and an Air Products helium cryostat. The temperature at which spectra were run was 10 K, the microwave power 2 mW (frequency 9.13 GHz), modulation frequency 100 kHz and modulation amplitude 2 mT.

RESULTS

Absolute and difference spectra of defined valence and ligand-bound states

As an aid to interpretation of the spectral shape seen after flash photolysis, Fig. 1 includes spectra of variously-treated suspensions of whole cells from O_{2}-limited cultures. The absolute spectrum (that is a sample spectrum minus the spectrum of buttermilk, which has no characteristic absorbances in this wavelength range) of a ferricyanide-oxidized suspension is shown in Fig. 1(a). The spectrum shows no distinctive features, except for a peak at 434 nm (not shown), which probably has contributions from cytochromes, flavins and oxidized ferricyanide (Hendler & Shrager, 1979). Note that, under these conditions, the absorbance centred at about 650 to 652 nm, (see below) and widely interpreted (see Discussion) as the signal from the oxidized form of cytochrome d, is very weak or absent.

Spectrum (b) in Fig. 1 was obtained after vigorously aerating a cell suspension (with no added ferricyanide) by stirring with a coiled wire. The prominent features are a sharp band at 652 nm, a broad absorbance centred at 535 nm and a shoulder at 560 nm, which may arise from slight reduction of cytochrome(s) b in the steady state. Spectra (c) to (e) are difference spectra in which the reference is the CO-liganded, reduced form. They may, therefore, be compared directly with spectra obtained after photolysis of the CO-liganded, reduced form (see below) in which the pre-photolysis spectrum serves as the reference. In Fig. 1(e) the ‘sample’ spectrum is that of a ferricyanide-treated suspension, and, since the spectrum of this is featureless in the 520 to 700 nm region (Fig. 1a), the major troughs in Fig. 1(c) (561 and approx. 640 nm) arise from the absorbances of a b-type cytochrome and the CO-liganded oxidase, respectively, in the reference spectrum. In Fig. 1(d) the sample spectrum was of an aerated suspension. Oxidized b-type cytochromes are largely responsible for the troughs at 532 and 561 nm. Cytochrome d absorbances are seen at 637 and 654 nm whilst, centred at about 680 nm, there is a very broad but distinct signal whose identity is uncertain; it is also seen in the absolute spectrum (Fig. 1b). In the fully reduced minus CO difference spectrum (Fig. 1e) the major CO-binding pigments identified are a-type cytochrome(s) (largely cytochrome a_{1}; Poole et al., 1981; Poole et al., 1982b) at 444 nm (not shown), cytochrome o (562 nm), cytochrome a_{1} (broad peak between about 595 and 600 nm) and cytochrome d. The ‘derivative’ shape of the spectrum of the last component in the near-infrared region is a result of the CO-induced red shift of the reduced form.

These spectra may be compared with the form (Fig. 1f) seen in the first scan after photolysis in the presence of O_{2} and at the lowest temperatures readily available with the current dual-wavelength scanning spectrophotometer. Maximal absorbance changes in the near-infrared were only obtained with 20 to 30 xenon lamp flashes; the inhibition by CO of cytochrome d-supported respiration was regarded as ‘light-insensitive’ by Keilin & Harpley (1941). The difference spectrum (with the CO + reduced form as reference) has a symmetrical, intense absorbance at 653 nm and a trough at 636 nm. The baseline (CO + reduced minus CO-reduced; Fig. 1g) is flat. This first detectable product of photolysis is clearly not the reduced form (compare with Fig. 1e). In contrast, under these experimental conditions, other cytochrome oxidases studied to date (Chance et al., 1975b; Poole et al., 1979) reveal a spectrum after photolysis at this temperature that is characteristic of the reduced form. Since the recombination of CO after photolysis would give a flat spectrum resembling the baseline (Fig. 1g), the compound in Fig. 1(f) must represent the product of the reaction with O_{2}.

The spectra in the 520 to 700 nm region of Figs 1(d) and 1(f) each show a prominent peak in the 652 to 654 nm region, but differ in three respects. First, the post-photolysis spectrum (Fig. 1f) does not exhibit the 532 and 561 nm troughs attributable to oxidized cytochrome(s) b.
Fig. 1. Low temperature absolute and difference spectra of oxygen-limited E. coli and comparison with that obtained after laser photolysis of a reduced + CO sample in the presence of O₂. All spectra were recorded with the dual-wavelength spectrophotometer (reference wavelength 500 nm) and each is the difference between a stored reference spectrum and the sample. In (a) and (b) the reference spectrum is that of buttermilk; in (c) to (g) it is a reduced + CO sample. Spectrum (a) is the 'absolute' spectrum of a suspension oxidized with a few grains of K₃Fe(CN)₆, while (b) is a sample 'oxidized' with vigorous aeration. Spectra (c), (d) and (e) are the difference spectra of K₃Fe(CN)₆-oxidized, aerated and succinate-reduced samples respectively, minus the reduced + CO form. Baseline (g) is a reduced + CO (pre-flash) minus reduced + CO difference spectrum. (f) is the same sample after photolysis (in the presence of 400 μM-O₂) minus the same reduced + CO form. The temperature was −120 °C, except in (f) and (g) (−113 °C) and (c) (−80 °C). The scan speed was 1.4 nm s⁻¹ and the protein concentration approx. 14 mg ml⁻¹. The vertical bar represents ΔA of 0.04, 0.04, 0.04, 0.08, 0.008 and 0.008 in (a) to (g), respectively.

Secondly, Fig. 1(f) does not show the broad shoulder to the red side of the 652 nm peak. Thirdly, the intensity of the 652 nm band is greater than that of the 636 nm trough in the post-photolysis spectrum, whilst in the difference spectra of Fig. 1(d) and Fig. 2 of Poole & Chance (1980), the signal magnitudes are similar. It seems appropriate, therefore, to identify the compound in Fig. 1(f) as an early intermediate in the reaction of cytochrome d with O₂.

Attempts to detect an earlier intermediate in the reaction with O₂ have been unsuccessful using the scanning apparatus (results not shown). Using fast scan rates over a more restricted wavelength range, such that the 630 to 650 nm region is scanned within about 4 s of the xenon flash at −132 °C, the spectrum still resembled that in Fig. 1(f).

**EPR signals from the compound trapped at low temperatures**

Oxidized membrane particles from A. vinelandii exhibit intense EPR signals at around g = 6, which have been attributed to the high-spin ferric haem resonance of cytochrome d (Dervartanian et al., 1973; Kauffman et al., 1975). A detailed study of the analogous EPR signals in E. coli membranes will be presented elsewhere (C. Kumar, R. K. Poole, I. Salmon & B. Chance, unpublished results). Although our interpretation of these signals is different from that of the above authors, the pertinent point, shown in Fig. 2, is that photolysis of the CO-liganded...
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Fig. 2. Low temperature EPR spectra of cytochrome \(d\) in E. coli membranes. The absolute spectrum of a CO-liganded, reduced sample (before photolysis) is shown in (a). After photolysis with a xenon lamp at \(-130^\circ\mathrm{C}\), and 10 min further incubation at this temperature, the absolute spectrum (b) was recorded. Spectrum (c) is the difference, (b) minus (a). The same sample was warmed to, and incubated for 5 min at, \(-40^\circ\mathrm{C}\) in the dark, and a further spectrum recorded. The sensitivity was \(5 \times 10^3\) for (a), (b) and (c) and \(2.5 \times 10^3\) for (d). EPR conditions are given in Methods.

cytochrome \(d\), under conditions that elicit the distinctive optical spectra described above, gives no EPR signals that can be attributed to oxidized cytochrome \(d\). Fig. 2(a) shows that the absolute spectrum of the pre-photolysis sample shows a minor signal around \(g = 6\) that we attribute to a small amount of haem oxidized during introduction of \(O_2\). By comparison with the signal strength of a fully-oxidized sample (not shown), we estimate the proportion of the total haem that is oxidized to be about 15%. Spectra 2(b) and 2(c) show that photolysis of such a sample at \(-130^\circ\mathrm{C}\) and incubation at this temperature for 10 min (thus mimicking the conditions for the optical spectrum of Fig. 1(f)) produces no EPR-detectable changes in the preparation. To confirm that oxygenation of the sample and photolysis were sufficient to allow eventual oxidation, the same sample was held at \(-40^\circ\mathrm{C}\) for 5 min. A subsequent spectrum showed extensive oxidation of high-spin haems in the \(g = 6\) region. Note that spectrum (d) is at one-half of the amplification of (a) to (c).

DISCUSSION

In this section, we (i) review the unusual behaviour described by others of cytochrome \(d\), particularly the form absorbing at about 650 nm, (ii) advance an alternative hypothesis for the identities of \(d_{650}\) and the ‘invisible form’, based on previous and present results, and (iii) re-interpret existing data in the context of the hypothesis.

The pertinent, striking observations on cytochrome \(d\) in E. coli fall into three categories.

1. Potentiometric redox titrations

Two studies have been made in which the disappearance of the reduced form (628 nm) was monitored under anoxic conditions at successively higher potentials. Midpoints \((E_{m,70})\) of +260 mV (one electron transfer, i.e. \(n = 1\)) and +280 mV have been reported by Pudek & Bragg (1976a) and Reid & Ingledew (1979), respectively. However, there was no corresponding appearance of the 648 nm absorbance even at potentials of over +400 mV; ‘only following addition of pulses of water saturated with oxygen or of \(H_2O_2\) did the 648 nm band reappear’ (Pudek & Bragg, 1976a).

The only potentiometric data on the 648 to 650 nm peak comes from a different technique (automated electrode potentiometry) used by Hendler & Schrager (1979) in which, prior to
titration, the system was cycled through a preliminary phase of air oxidation followed by endogenous substrate reduction. Under these conditions (air oxidation), the spectra revealed two major components at 630 to 634 nm and at 650 nm. The absorbance difference (630 minus 650 nm) and the individual 634 nm and 650 nm components revealed by Gaussian analysis showed very unusual and complex behaviour during titration. To accommodate the data, the reduced (634 nm) component had to be fitted by two components \( n = 2 \), \( n = 4 \) and the 650 nm peak by four (transferring 1, 2, 4 and 4 electrons, respectively). Changes of the latter peak with voltage showed a dip (where the peak was undetectable) at about \(+ 326 \text{ mV}\). Increasing the voltage elicited a small increase in the 650 nm peak whilst decreasing the voltage caused a simultaneous and extensive increase in both the 634 nm (reduced) and 650 nm components (compare Figs J and 12 in Hendler & Shrager, 1979).

It is difficult to reconcile these potentiometric experiments with cytochrome \( d_{650} \) being the oxidized \( (\text{Fe}^{3+}) \) form.

2. Indirect interconversions of the reduced and \( d_{650} \) forms

Further lines of evidence suggest that the components responsible for the reduced and 650 nm bands, respectively, do not appear to be direct oxidation-reduction products of one another.

Firstly, when \( \text{AgNO}_3 \) was added to succinate-reduced membrane particles, oxidation of cytochrome \( d \) was observed (Bragg & Rainnie, 1974). In successive reduced (plus \( \text{Ag}^+ \)) minus oxidized (with \( \text{H}_2\text{O}_2 \)) difference spectra, the 628 nm peak disappeared more rapidly than the trough, which was assumed to arise from the presence of the oxidized form in the reference cuvette. If the reduced cytochrome had been converted directly to the 650 nm form in the ‘front’ cuvette, this disparity would not be expected. The results were interpreted by assuming that cytochrome \( d \) oxidation (giving the 650 nm form) proceeded via the formation of a species with little absorbance in the near-infrared region.

Secondly, the addition of substrate to membranes in an anaerobic suspension caused the 648 nm band to disappear well in advance of the appearance of the 628 nm band, again suggesting the involvement of an ‘invisible’ intermediate (Pudek & Bragg, 1974). In the same paper, cyanide was reported to cause the 648 nm absorption to disappear slowly without the appearance of a new band. The disappearance occurred with a second-order rate constant \( (K = 0.011 \text{ M}^{-1} \text{s}^{-1}) \) that was too low to account for the inhibition of NADH oxidase by cyanide \( (K = 0.26 \text{ M}^{-1} \text{s}^{-1}) \). However, under turnover conditions (i.e. in the presence of NADH and \( \text{O}_2 \)), the 648 nm band disappeared very rapidly \( (K = 0.58 \text{ M}^{-1} \text{s}^{-1}) \). It was suggested that the turnover rate of the respiratory chain influenced the steady-state level of an invisible intermediate species \( (d^*) \) that bound cyanide (see Introduction).

3. Trapping of the invisible species

Difference spectra of particles in the steady state, oxidizing ascorbate in the presence of tetramethyl phenylenediamine, have shown an increased trough at 648 nm as the temperature was lowered (Pudek & Bragg, 1976b). This was interpreted as the result of disappearance of the 648 nm ('oxidized') form in the sample cuvette. However, under aerobic steady-state conditions, a terminal oxidase would be expected to be extensively oxidized (Chance & Williams, 1956). In addition, electron transfer reactions between redox components of lower potential appear to have higher energies of activation and thus be more temperature sensitive (Erecińska & Chance, 1972; Douzou, 1977), and so it seems unlikely that the oxidized form would become less prominent as the temperature was lowered. An alternative explanation is that the steady-state level of an intermediate form \( (d_{650}) \) increased in the reference cuvette (no substrate) at lower temperatures.

The above observations on the \( E. \text{coli} \) cytochrome \( d \) have been largely confirmed and extended in analogous work with \( A. \text{vinelandii} \). The main points of similarity follow.

(i) The 648 nm band is not a direct oxidation product of reduced cytochrome \( d \) (Kauffman & van Gelder, 1973a).
Identity of cytochrome $d_{650}$ in *E. coli*

![Diagram of cytochrome interconversions](image)

Fig. 3. Proposed interconversions of the forms of cytochrome $d$ and some of their reactions with ligands. Reduced cytochrome $d$ reacts with CO to give a spectrally distinct species (reaction 1). The reaction is reversible by light (2). In the presence of $O_2$, photolysis of the CO complex (3) yields the form that is the subject of this paper, an oxygenated compound that is analogous to oxyhaemoglobin. The same compound can be formed (4) by aerating the reduced oxidase. The reverse reaction (5) can be observed on exhaustion of $O_2$ (e.g. Pudek & Bragg, 1974). In *A. vinelandii* (Kauffman & van Gelder, 1980) addition of CO to $d_{650}$ (6) forms a species that is not spectrally identical with the form that results from adding CO, then NADH to $d_{650}$ (7). The $E_{m,1}$ for oxidoreduction (8) of cytochrome $d$ shown (Reid & Ingledew, 1979) is typical of reported values. The oxidized form (previously $d^*$) reacts with cyanide. Treatment of cyanocytochrome $d$ with reductant (10) regenerates the reduced form in *A. vinelandii* (Kauffman & van Gelder, 1973b). The formation of the oxidized $d$ from $d_{650}$ (11) proceeds via the formation of optically- and EPR-detectable intermediates. The reactions of cytochrome $d$ with low molecular weight compounds of nitrogen in *A. vinelandii* are not shown (Kauffman et al., 1980).

(ii) A further oxidized conformation of the enzyme, scarcely absorbing in the red region, is proposed (Kauffman & van Gelder, 1973a). This is cytochrome $d_v$ and is analogous to $d^*$.

(iii) Cyanide causes a slow disappearance of the 648 nm band and inhibits formation of the reduced band (Kauffman & van Gelder, 1973b).

(iv) The rate of disappearance of the 648 nm band in particles oxidizing NADH, in the presence of cyanide, can be related to the rate of inhibition of oxygen uptake (Kauffman & van Gelder, 1974).

In addition, *A. vinelandii* cytochrome $d$ binds several other ligands (Kauffman et al., 1980). Of particular interest is the finding that the 648 nm component, like the reduced form, binds CO and reacts with NH$_2$OH.

**Hypothesis**

We suggest that the fully-oxidized form of cytochrome $d$ has no characteristic absorbance in the red region of the spectrum and is thus equivalent to forms $d_v$ or $d^*$ proposed by others, whereas cytochrome $d_{650}$ is an early intermediate in the reaction of the reduced oxidase with oxygen. The hypothesis is the converse of previous proposals, which invoke the existence of a hypothetical, invisible intermediate and an oxidized configuration with unusual optical properties. Based on the necessity of oxygen for the formation of cytochrome $d_{650}$, Pudek & Bragg (1976b) originally proposed that it ‘might be analogous to the “oxygenated” cytochrome oxidase of mitochondria (Okunuki, 1966).

Figure 3 summarizes the interconversions of the recognized forms of cytochrome $d$ in *E. coli* and *A. vinelandii*. The reduced form absorbs maximally at 628 to 631 nm; its peak is shifted approximately 4 to 5 nm to the red on binding CO, a reaction that is reversible by high light intensities but readily observable only at temperatures close to that of liquid He (Poole et al.,...
Photolysis of the CO complex in the presence of O₂ yields cytochrome d₆₅₀. The evidence for an ‘invisible’ intermediate between d₆₅₀ and the reduced form is summarized above. Optical studies of the transition between cytochrome d₆₅₀ and the oxidized form are presented in the accompanying paper (Poole et al., 1983). The transition from d₆₅₀ to the reduced form may be rapid in the absence of oxygen (Pudek & Bragg, 1974). Also shown in Fig. 3 at the periphery of these interconversions are the reactions of these forms with other ligands. Not all of these reactions have yet been demonstrated in E. coli, however.

The main attractions of the hypothesis are as follows.

1. It eliminates the proposal that, in binding to cytochrome d₆₅₀, CO binds to an oxidized haemoprotein. Other cytochrome oxidases bind CO only in the reduced form (for references see Yoshikawa & Caughey, 1982).

2. Similarly, NH₂OH reacts with d₆₅₀ to give a compound with a spectrum very similar to that produced by adding NO₂⁻ or NO to the ferrous form (Kauffman et al., 1980). It has been suggested that the compound formed with NH₂OH is the NO-cytochrome d complex resulting from oxidation of NH₂OH to NO.

3. The ‘invisible’ form of the oxidase has been proposed as the cyanide-binding form. Ferric cytochrome a₃ in mitochondria also reacts rapidly with cyanide under turnover conditions (Wikström et al., 1981).

4. A partial explanation of the curious potentiometric behaviour of cytochrome d₆₅₀, in which both this form and the reduced form appear at potentials lower than about 325 mV (Hendler & Shrager, 1979), may be offered. The inability to detect any form of the enzyme at this potential may be due to the presence of the oxidized form (‘invisible’ in the present hypothesis), so that reducing the potential in the presence of O₂ leads to the formation of either the fully-reduced form or the d₆₅₀ intermediate. The haem(s) in both species are proposed to be in the ferrous state, but if cytochrome d₆₅₀ is indeed analogous to oxyhaemoglobin, some electron transfer to the ligand would be expected (Thomson, 1977).

5. The appearance of the reduced form of cytochrome d on adding dithionite to cyanocytocrome d is slow. Similarly, cytochrome a₃ is not easily reduced when complexed with cyanide (Yonetani, 1960).

The main distinguishing feature of cytochrome d₆₅₀, if the hypothesis is correct, is its extreme stability at physiological temperatures, whereas the A, B and C-type intermediate species postulated for mitochondrial cytochrome c oxidase (Chance et al., 1975b) and oxy-cytochrome o (Poole et al., 1979) are elusive, transient and can be observed at high occupancy only at sub-zero temperatures. Stable reaction intermediates in bacterial cytochrome oxidases are not unprecedented, however. In the well-characterized cytochrome o of the aerobic myxobacterium *Vitreoscilla*, at least two intermediates have been observed (for a review, see Gonzales-Prevatt & Webster, 1979). Compound D may be trapped at 0 °C and has features expected of an oxy-compound. It is converted by catalytic amounts of O₂ to the so-called ‘oxygenated’ compound, which has distinctive spectral properties and is stable for minutes or hours at room temperature, suggesting that the rate-limiting step occurs subsequent to its formation. Secondly, supplementing the cytochrome oxidase of *Pseudomonas aeruginosa* with a reductant and oxygen resulted in the appearance of an ‘oxygenated’ intermediate at the haem d₁ moiety, which persisted while oxygen was present (Shimada & Orii, 1976).

Chance et al. (1975b) have emphasized the importance of identifying oxygen compounds of cytochrome oxidase: ‘kinetic and spectroscopic studies of the enzyme could well be confused by unrecognized intermediate compounds . . . which would obscure the validity of kinetic analysis on the one hand and confound spectroscopic analysis on the other.’ For example, erroneous assignment of cytochrome d₆₅₀ to the oxidized (Fe³⁺) form would render the use by Smith et al. (1970) and Haddock et al. (1976) of the wavelength pairs (630 minus 655 nm) inappropriate for measurement of the oxidation and reduction of cytochrome d.

Finally, we have recently examined the solubilized and aerated cytochrome d by resonance Raman spectroscopy (excitation at 647·1 nm). Absorptions at 1078 to 1105 cm⁻¹ have been detected and attributed to the oxygen–oxygen stretching frequency of the oxidase–oxygen
adduct (Poole et al., 1982c). These findings suggest a further analogy between cytochrome d650 on the one hand and oxymyoglobin and oxyhaemoglobin on the other, and fully support the present hypothesis.

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