The occurrence and function of a-type cytochromes in the aerobic respiratory chain of *Comamonas percolans* NCTC 1937 grown under O₂-sufficient and O₂-limited conditions

HUW D. WILLIAMS,*† HWE B. TAN and ROBERT K. POOLE

Microbial Physiology Research Group, Biosphere Sciences Division, King's College London, Campden Hill Road, London W8 7AH, UK

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Intact cells of *Comamonas percolans* (NCTC 1937) after growth under O₂-limited conditions in batch culture exhibit, in addition to b- and c-type cytochromes, an unusual pigment absorbing variably at 588 to 594 nm in reduced minus oxidized difference spectra at room temperature. CO difference spectra suggest ligand binding by this pigment and also reveal a prominent 448 nm absorption minimum and CO-binding b- and/or c-type cytochromes. Although the 588 to 594 nm-absorbing component is reminiscent of 'cytochrome a₁', claimed to be a terminal oxidase in some bacteria, O₂-limited cells of *C. percolans* contain no detectable haem A. In contrast, cells grown under O₂-sufficient conditions exhibit a membrane-bound cytochrome aa₃ and contain haem A. Low-temperature photodissociation studies of O₂-sufficient cells show cytochrome aa₃ to be functional in CO and O₂ binding and suggest that aa₃ is the terminal oxidase of a cytochrome b- and c-containing aerobic respiratory chain. Analogous studies of O₂-limited cells reveal a component absorbing, in its unliganded state, at 448 nm. Exposure of such CO-ligated cells to white actinic light is followed by oxidation of b- and/or c-type cytochromes and, although the functional oxidase has not been identified, we conclude that *C. percolans* does not utilize a cytochrome oxidase of the a₁ type.

Introduction

Cytochromes of the a-type, i.e. haemoproteins with haem A as the prosthetic group, are important constituents of bacterial and eukaryotic respiratory chains. The best-studied example is cytochrome aa₃ or cytochrome c oxidase (EC 1.9.3.1), which is the terminal oxidase of the mitochondrial electron transport chain (see Wikström *et al.*, 1981, for a review). Certain bacteria also possess aa₃-type cytochromes which are similar to the mitochondrial enzyme in the complement of redox centres (1 haem a₁:1 haem a₃:2 or 3 Cu) and in their spectral characteristics, but which are much simpler in subunit composition, having only two or three constituent polypeptides (for reviews, see Ludwig, 1987; Poole, 1983, 1988).

The early studies of Keilin & Warburg (for a review, see Keilin, 1966) suggested the presence in certain bacteria of an apparently related oxidase but with an α-

maximum sufficiently removed (to shorter wavelengths) from the corresponding band of mitochondrial and *Bacillus subtilis* cytochromes aa₃ to warrant a distinguishing subscript. This 'cytochrome a₁' was confirmed as a competent oxidase in an *Acetobacter* strain by Castor & Chance (1955, 1959). However, recent studies on haemoproteins resembling 'cytochrome a₁' in various bacteria have highlighted the functional diversity of these pigments and cast doubt on the assumption that haemoproteins with an α-maximum at 585 to 596 nm (in reduced minus oxidized difference spectra) should be called 'cytochrome a₁'. Poole *et al.* (1985) proposed a classification of a₁-like haemoproteins and suggested that true cytochromes a₁ should be shown to contain haem A. Few a₁-like haemoproteins satisfy this criterion; thus, both the soluble (Poole *et al.*, 1984, 1986) and membrane-bound pigments (Lorence *et al.*, 1986) in *Escherichia coli* are high-spin b-type haemoproteins and neither is an oxidase per se. The soluble species is a hydroperoxidase (Poole *et al.*, 1986) as is a spectrally similar pigment in *Bradyrhizobium japonicum* (C. A. Appleby & R. K. Poole, unpublished results), while the membrane-bound pigment is a constituent of the
cytochrome bd oxidase complex (Lorence et al., 1986) and functions in direct electron donation to cytochrome d (Poole & Williams, 1987). A similar function has been ascribed to the cytochrome a-like pigment of Acetobacter pasteurianus NCIB 6428 (Williams & Poole, 1987).

We have investigated the occurrence and role of an a-like cytochrome in Comamonas percolans (NCTC 1937), a Gram-negative bacterium of uncertain taxonomic affiliation. It was originally considered to be a Vibrio sp. but later work proposed this strain of C. percolans to be the type species of the genus Comamonas (Davies & Park, 1962), whereas Stanier et al. (1966) included Comamonas among the aerobic pseudomonads. This study on the cytochromes of C. percolans was prompted by a taxonomic survey (Spicher, 1974) of numerous genera, which included a number of representatives of Comamonas. Cytochrome a-like pigments were evident in each, although the \( \alpha \)-absorption maxima (reduced minus oxidized) varied from 595 to 603 nm. In view of the few bacteria now thought to contain true cytochromes a, strain NCTC 1937, which exhibited a peak at 595 nm, was selected for study. Here we report the occurrence of cytochrome aa3 in aerobically grown, oxygen-sufficient cells and demonstrate its oxidase role using low-temperature ligand-exchange techniques. A cytochrome a-like haemoprotein, of uncertain function, was found in cells from oxygen-limited cultures.

**Methods**

**Organism, growth and preparation of subcellular fractions.** Comamonas percolans NCTC 1937 was obtained from the National Collection of Type Cultures (Central Public Health Laboratories, London, UK) and grown on nutrient agar (NA) and nutrient broth (NB) made up as recommended by the manufacturers (Oxoid). The organism was grown on NA slopes and incubated 24 h at 30°C. A starter culture for 0,-sufficient growth was prepared by washing cells from a NA slope into 25 ml of NB in a 250 ml conical flask and centrifuged at 15000 g for 10 min. The cells were harvested after 20 to 24 h when the OD \( \text{OD}_{600} \) was 0.4 to 0.5.

Cells were harvested using an Alpha-Laval continuous-action centrifuge, resuspended in 50 mM-potassium phosphate buffer (pH 7.0) and centrifuged at 15000 g for 15 min. The cells were then washed and resuspended in the same buffer and recentrifuged. If necessary, cells were stored at \(-20\)°C until needed.

To prepare subcellular fractions, harvested and washed cells were resuspended in 50 mM-potassium phosphate buffer (pH 7.0) at 1 g wet weight ml\(^{-1}\) and a few grains of DNAase added. The cells were disrupted by ultrasonication using an MSE 150W sonicator (probe end diameter 9.5 mm) for five periods of 30 s each, separated by 15 s intervals. The sample in the sonicator vessel was surrounded by an ice–salt slurry to aid cooling. The suspension was centrifuged at 12000 g for 15 min to leave a cell-free extract (S\(_c\)), which was further fractionated (Poole & Haddock, 1974) by centrifugation at 225000 g for 60 min to yield a ‘high-speed supernatant’ (S\(_h\)) and membrane pellet (P\(_h\)). The latter was homogenized in buffer and centrifuged at 225000 g for 60 min to give a washed pellet (P\(_w\)) and second supernatant (S\(_d\)). Fractions were stored at \(-20\)°C until needed.

**Spectrophotometry.** Washed cells were resuspended in buffer to a protein concentration of approximately 15 mg ml\(^{-1}\). Room-temperature difference spectra (reduced minus oxidized and reduced + CO minus reduced) were recorded using a Johnson Foundation DBS-3 dual-wavelength scanning spectrophotometer as described previously (Williams & Poole, 1987). Samples were reduced with a few grains (<5 mg) of sodium dithionite and oxidized in the presence of a small amount (<5 mg) of ammonium persulphate.

For low-temperature ligand-exchange studies the procedure of Williams & Poole (1987) was followed, but photolysis of the CO-ligated, reduced oxidases was achieved by 30 s exposure to ‘white’ light from a 150 W projector lamp, focused by a lens on to the afferent limb of a bifurcated light-guide leading to the cuvette surface.

**Haem extraction and pyridine haemochrome spectra of extracted haems.** Haem extraction of membranes prepared from O\(_2\)-sufficient and O\(_2\)-limited cells was based on the method of Poole et al. (1984). Membranes prepared from a minimum of 18 g of cells were resuspended in 15 ml of ice-cold acetone/water (4:1, v/v) by hand homogenization and stirred vigorously for 15 min. The membranes were recovered by filtering the suspension through 0.25 μm nylon filters (Ulpipore N66) under vacuum. The membranes were next stirred in 25 ml of chloroform for 20 min at room temperature and the chloroform decanted. Then, 15 ml of chloroform/pyridine (2:1, v/v) was added and the mixture stirred for 90 min in the dark. The resulting haem extract was decanted and filtered as above to remove any lipid. Haem extracts from membranes of O\(_2\)-sufficient cells were yellow in colour, while those from membranes of O\(_2\)-limited cells were pink. These extracts were used directly for spectral analysis after adding an equal volume of 0.15 M-NaOH. Samples were reduced with sodium dithionite (<3 mg ml\(^{-1}\)) and oxidized with a few grains (<5 mg) of ammonium persulphate.

**Measurement of O\(_2\) uptake and KNC titrations.** These were determined polarographically with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) as described by Poole (1977). Samples (3 ml) of membranes resuspended in 50 mM-potassium phosphate buffer (pH 7.0) (protein concentration approximately 1 mg ml\(^{-1}\)), were transferred to the electrode vessel at \(30\)°C, stirred vigorously for 15 min and photolysed for 10 min to yield a ‘high-speed supernatant’ (S\(_h\)) and membrane pellet (P\(_h\)). The latter was homogenized in buffer and centrifuged at 225000 g for 60 min to give a washed pellet (P\(_w\)) and second supernatant (S\(_d\)). Fractions were stored at \(-20\)°C until needed.

**Protein.** This was assayed by the method of Markwell et al. (1978).

**Chemicals.** General reagents were from BDH, Fisons or Sigma, and were of AnalR grade wherever possible. CO was from BOC Special Gases.
Results

Cytochrome composition of C. percolans NCTC 1937 following O₂-sufficient and O₂-limited growth

The reduced minus oxidized difference spectrum of intact cells of C. percolans grown with vigorous aeration [11 air min⁻¹ (1 of culture)⁻¹] is shown in Fig. 1(a). There was a broad α-maximum at 556 nm, probably due mainly to b-type cytochrome(s), and a peak at 606 nm, suggesting the presence of cytochrome aa₃. A weak shoulder at about 550 nm might indicate a low concentration of c-type cytochrome(s). Additionally, there was a broad absorbance centred at 670 nm due to an unknown chromophore. The presence of cytochrome aa₃ was confirmed by the CO-difference spectrum (Fig. 1b), which showed a maximum at 592 nm, due to the CO-complex of haem a₃, and troughs at 444 and 612 nm, resulting from the loss of the reduced a₃ on binding CO. The maxima at 420, 543 and 580 nm, together with the asymmetric trough at 561 nm in the CO-difference spectrum, indicated one or more CO-binding cytochromes b to be present.

Cells grown under O₂-limited conditions [0.11 air min⁻¹ (1 of medium)⁻¹] were a deep red colour compared to the light brown colour of cells grown with vigorous aeration. The most striking spectral difference was the presence of an α-maximum at 588 to 594 nm in reduced minus oxidized difference spectra (Fig. 1c, d). However, there was variation in the position of the α-maximum and in the spectral shape of the signal even in two separately scanned samples from the same cell suspension and apparently treated in an identical fashion (Fig. 1c, d). This α-maximum was always observed, in whole cells, in the region expected for a 'classical' cytochrome a₁. There was also a prominent trough at 620 to 624 nm. The CO-difference spectrum of O₂-limited cells (Fig. 1e) had a minimum at 448 nm and a maximum at 420 nm,
probably attributable to the cytochrome $a_1$-like component. The $a$-region differed from that observed in $O_2$-sufficient cells (Fig. 1b), having a minimum at 590 nm and a broad maximum at 640 nm. The spectrum also indicated the presence of at least one CO-binding cytochrome $b$ (minimum at 555 to 560 nm). $O_2$-limited cells had about twofold greater levels of cytochromes $b$ and $c$ on a protein basis, compared to $O_2$-sufficient cells, possibly explaining the deep red appearance of the former.

Subcellular distribution of cytochromes in C. perolans grown under $O_2$-sufficient and $O_2$-limited conditions

Cell-free extracts ($S_0$) of $O_2$-sufficient C. perolans had a similar cytochrome composition to whole cells (data not shown). Spectral analysis of high-speed supernatant ($S_1$) and washed membrane fractions ($P_2$) showed that the majority (73 to 89%) of cytochrome $aa_3$ was present in the sedimentable membrane fraction ($P_2$). Also present in this fraction was a CO-binding cytochrome $b$ ($x$-trough at 562 nm) whose spectrum suggested it was a low-spin cytochrome $b$ (Wood, 1984). However, in the high-speed supernatant ($S_1$) weak signals from a CO-binding cytochrome $b$ ($x$-maximum at 554 nm) were observed, which spectrally resembled a high-spin cytochrome $b$ (data not shown).

A similar fractionation of $O_2$-limited cells showed that the cytochrome $a_1$-like component was membrane-associated, as >80% of it, when quantified using the $x$-maximum in reduced minus oxidized spectra, was found in the sedimentable membrane fraction ($P_2$). The $x$-maximum varied between 595 and 600 nm even in spectra from the same batch of membranes, although the 592 nm peak of an $a_1$-CO complex was never observed. CO-difference spectra of the membrane fraction indicated a high-spin CO-binding cytochrome $b$ to be present (minimum at 560 nm). A second, soluble, high-spin CO-binding cytochrome $b$ was detected in the high-speed supernatant fraction (minimum at 556 nm) and spectrally resembled that found (in smaller amounts) in $O_2$-sufficient cells (data not shown).

Spectral changes in the 580 to 610 nm region of $O_2$-limited cells

As described above, the position of the $x$-maximum of the $a_1$-like component of $O_2$-limited cells varied from 590 to 600 nm in spectra of the same batch of cells or membranes. This was studied further in the reduced minus oxidized spectra shown in Fig. 2. Since the reduced spectrum was the same in each of the difference spectra plotted, any changes seen must have occurred in the oxidized suspension. In the first spectrum scanned after oxidizing the membrane suspension (Fig. 2a) there were maxima at 527, 557 and 604 nm. After 3 min the suspension was rescanned and a second reduced minus oxidized spectrum (b) again plotted. This was repeated after a further 5 min to yield spectrum (c). Note that the same original reduced spectrum was used for all the replotted difference spectra. The unlabelled spectrum is a reduced minus reduced baseline. Spectra were recorded at 5.7 nm s$^{-1}$, a bandwidth of 8 nm and a reference wavelength of 500 nm. The vertical bar represents a $\Delta A$ of 0.04.
branes from O$_2$-sufficient cells exhibited a monophasic inhibition curve, indicating only one site of inhibition, with 50% inhibition occurring at about 500 μM-KCN. In contrast, the KCN inhibition curve of succinate oxidase activity in membranes from O$_2$-limited cells was biphasic, indicating two inhibition sites, with 50% inhibition occurring at about 2.5 μM and about 3.5 mM, respectively. This suggests that there are two KCN-sensitive oxidases functioning under O$_2$-limiting conditions, which are distinct from the single oxidase present in membranes from O$_2$-sufficient cells.

### Reaction with CO and O$_2$ of cytochromes in O$_2$-sufficient cells

The reaction of potential cytochrome oxidases, in O$_2$-sufficient cells, with CO and O$_2$ was investigated using low-temperature photolysis procedures (Chance et al., 1975; Williams & Poole, 1987). When an endogenously reduced, CO-saturated cell suspension was photolysed in the absence of O$_2$ with the focused beam from a 150 W projector lamp for 30 s at −128°C, the resulting (post-photolysis minus pre-photolysis) photodissociation spectrum showed the features of a pure cytochrome a$_3$ photodissociation spectrum (Fig. 4a). There were absorbance maxima at 448 and 615 nm, due to the appearance of reduced cytochrome a$_3$ in the sample, and minima at 430 and 592 nm, due to the loss of CO-ligated cytochrome a$_3$ from the sample (Poole et al., 1979a; Wikström et al., 1981; Sone et al., 1984). No changes occurred upon repetitive scanning at this temperature. There were no signals, in the Soret or a-regions, of the spectrum attributable to photodissociation of cytochrome o (Poole et al., 1979b; De Maio et al., 1983; Williams & Poole, 1988) despite the presence in these cells of CO-binding cytochrome o (Fig. 1b). When photolysis was performed at higher temperatures (for example, −87°C: Fig. 4b), recombination of CO to haem a$_3$ occurred (Fig. 4c). Rephotolysis of the sample to which CO had recombined reformd the original spectrum (data not shown). The kinetics of CO-recombination to haem a$_3$ were biphasic when analysed as semi-logarithmic plots (not shown) but measurements of the pseudo-first-order velocity constant for the initial fast phase, over the temperature range −103 to −78°C, allowed construction of an Arrhenius plot from which an activation energy of 36-4 kJ mol$^{-1}$ was obtained. This value is similar to that for cytochrome a$_3$ in bovine heart (35.1 kJ mol$^{-1}$; Erecinska & Chance, 1972), Schizosaccharomyces pombe (28.9 kJ mol$^{-1}$; Poole et al., 1979a) and the thermophilic bacterium PS3 (29.3 kJ mol$^{-1}$; Sone et al., 1984).

The reaction with O$_2$ was studied after supplementing the cell sample with O$_2$ prior to freezing. Spectral
changes after photodissociation of CO from the cytochrome $a_3$–CO complex in the presence of $O_2$ at $-128^\circ C$ are shown in Fig. 5(a). The initial spectrum after photolysis was similar to that observed in the absence of $O_2$ (Fig. 4a) except that the Soret trough at 433 nm was deeper, the $\alpha$-signals had a lower extinction and the $\alpha$-maximum due to $a_3$ was at a shorter wavelength (610 nm). However, upon repetitive scanning, the Soret features approached the baseline (the spectrum of the CO-liganded enzyme), indicative of ligand-binding. The non-photodissociability of the complex thus formed (data not shown) suggested that the ligand was $O_2$, not CO. The velocity of ligand binding was greater in the presence of $O_2$; for example, at $-103^\circ C$ the pseudo-first-order velocity constant was 0.135 min$^{-1}$ in the presence of $O_2$ and 0.019 min$^{-1}$ in its absence. When the kinetics of oxygen binding were analysed in semi-logarithmic plots (not shown) a biphasic pattern was obtained; measurements of the rates of the initial fast phase of $O_2$-binding, over the temperature range $-128^\circ C$ to $-92^\circ C$, gave an activation energy of 4.76 kJ mol$^{-1}$ for the $O_2$-binding reaction. The initial change in the $\alpha$-region at $-128^\circ C$ following photolysis
was an increase in absorbance at 592 nm and a small decrease at 610 nm. No changes were observed after the third scan. At $-103 \, ^\circ C$, further reactions occurred (not shown). $O_2$-binding was faster and minima started to form at 530 nm and at 554 nm, shifting in later scans to 568 nm. These changes suggested the further reaction of cytochrome $aa_3$ and the oxidation of $b$/$c$-type cytochromes. At $-92 \, ^\circ C$, the 448 nm maximum had disappeared by the first scan and this was accompanied by oxidation of a $b$-type cytochrome at 567 nm, while at $-43 \, ^\circ C$ (Fig. 5b) oxidation of a third cytochrome $b$, at 555 nm, was seen, concomitant with decreases in absorbance in the Soret region. Little change was seen in the $\alpha$-signals of cytochrome $aa_3$ at these higher temperatures except that the prominent 610 nm maximum disappeared and this was accompanied by a decrease in absorbance at 594 nm. The changes indicated that electron-transfer to cytochrome $aa_3$ had occurred with oxidation of $b$/$c$-type cytochromes.

Reactions with CO and $O_2$ of haemoproteins in $O_2$-limited cells

The unusual and variable cytochrome spectra of cells from $O_2$-limited cultures (see Fig. 1c to e and Fig. 2) prompted an attempt to identify a ligand-binding component that could be identified as a putative terminal oxidase on low-temperature photodissociation of intact cells. Such low-temperature spectra, however, like room-temperature spectra, were variable in form. In the absence of $O_2$ in the cuvette the simplest photodissociation difference spectrum recorded (Fig. 6a) exhibited a peak at 448 nm and a weak trough at 590 nm. These features might conventionally be attributed to photolysis of the CO compound of an $\alpha$-type cytochrome (e.g. Fig. 4a, b) but alkaline pyridine haemochrome spectra of extracted haems failed to show haem A. An alternative candidate is a $b$-596-like haemoprotein, i.e. a high-spin, haem-B-containing protein and, indeed, CO difference spectra (Fig. 1e) showed the presence of such a component. At $-83 \, ^\circ C$ (not shown), light-reversible ligand recombination was suggested by diminution of the 448 nm peak, indicative of reaction with CO. The kinetics of CO recombination were biphasic; the pseudo-first-order velocity constant for the initial fast phase was $0.074 \, \text{min}^{-1}$, approximately fivefold slower than that of CO recombination to cytochrome $a_3$ at this temperature (see above). In other photodissociation spectra at $-102 \, ^\circ C$ in the absence of $O_2$ (not shown), additional absorption maxima at 421, 524 and 552 nm, of unknown origin, were observed, perhaps arising from photodissociable CO compounds of $b$- and/or $c$-type cytochromes.

The corresponding photodissociation spectrum at $-102 \, ^\circ C$ of cells in the presence of $O_2$ (Fig. 6b) revealed again a prominent 448 nm peak, only partially light sensitive at both $-102$ and $-69 \, ^\circ C$. This suggests that the recombining ligand was CO, not $O_2$. Following photolysis in the presence of $O_2$ at $-69 \, ^\circ C$ (Fig. 6c), the small 449 nm peak disappeared within two scans, concomitant with large decreases in absorbance at 432, 525 and 555 nm, indicative of oxidation of $b$- and/or $c$-type cytochromes.
**Discussion**

The aim of this work was to investigate the occurrence of cytochrome $a_1$ in *C. perolans* and to study its role in the respiratory chain. Spectral analysis of cells from highly aerated cultures demonstrated the presence of cytochrome $aa_3$ ($\alpha$-maximum at $\sim 606$ nm in reduced minus oxidized spectra) but not cytochrome $a_1$. However, when grown under $O_2$-limited conditions, *C. perolans* lacked cytochrome $aa_3$ but had an $\alpha$-maximum at approximately 595 nm, indicating the presence of a cytochrome $a_1$. CO-difference spectra demonstrated its ability to bind CO and justified its consideration as a putative terminal oxidase. The variable position of the $\alpha$-absorption band, and recent information on the cytochrome $a_1$-like haemoproteins of *E. coli* (Poole et al., 1986; Lorence et al., 1986), *Acetobacter pasteurianus* (Williams & Poole, 1987) and *Halobacterium halobium* (Fukomori et al., 1985) that demonstrate some so-called cytochrome $a_1$-like haemoproteins to contain high-spin haem $b$, led us to study this pigment further.

Cytochrome $aa_3$ of $O_2$-sufficient cells was mainly membrane-associated. Some $a_1$-like haemoproteins have been shown to be soluble hydroperoxidases (Poole et al., 1986; Fukomori et al., 1985). However, most (>80%) of the cytochrome $a_1$-like pigment of *C. perolans* was found in the membrane fraction.

Photochemical action spectroscopy of some strains of *A. pasteurianus* suggested that cytochrome $a_1$ was the sole cytochrome oxidase (Castor & Chance, 1959). However, recent studies, albeit on a strain containing cytochrome oxidases $o$ and $d$ and a cytochrome $a_1$-like pigment, demonstrated the role of the latter in direct electron transfer to cytochrome $d$ in its reaction with $O_2$ (Williams & Poole, 1986, 1987). Spectral analysis of pyridine haemochromes formed from haems extracted from membranes of $O_2$-limited *C. perolans* failed to demonstrate the presence of haem $A$, precluding description of the $a_1$-like pigment as a true cytochrome $a_1$ (Poole et al., 1985). The same method showed haem $A$ in the cytochrome $aa_3$-containing membranes of $O_2$-sufficient cells. The $a_1$-like cytochrome present in membranes of $O_2$-limited *C. perolans* is probably a high-spin haem-$b$-containing pigment and can tentatively be called a cytochrome $b-595$. We obtained no evidence for the presence of cytochrome $d$, with which cytochrome $b-595$ is associated in an oxidase complex in *E. coli* (Poole, 1988).

The simplest interpretation of the inhibition by KCN in membranes from $O_2$-sufficient cells of *C. perolans* is that a single KCN-sensitive oxidase is responsible for all the succinate-dependent $O_2$ uptake. However, the presence of several oxidases with similar KCN sensitivities cannot be excluded. In contrast, membranes from $O_2$-limited cells showed a biphasic KCN-titration curve, indicating the presence of at least two KCN-sensitive oxidases. We detected no cyanide-resistant respiration such as is found in *Pseudomonas aeruginosa* (Matsushita et al., 1983).

Photolysis at low temperature of $O_2$-limited cells was inconclusive in determining whether or not the presumptive cytochrome $b-595$ was functioning as a terminal oxidase. Nevertheless, following photodissociation in the absence of $O_2$, a component was consistently observed with a maximum at about 448 nm and a trough at 590 nm which is probably due to photodissociation of cytochrome $b-595$. The variable appearance of additional signals possibly attributable to photodissociation of $b$- or $c$-type cytochromes is not understood. Photodissociation in the presence of $O_2$ did not show conclusively $O_2$-binding to cytochrome $b-595$ as it was possible to, at least partially, reverse ligand binding by rephotolysis in the presence of $O_2$, indicating that the major recombining ligand to cytochrome $b-595$ is CO and not $O_2$. However, there was no direct evidence for $O_2$-binding to any other components in $O_2$-limited cells over the temperature range studied (down to $-150 \, ^\circ C$), despite the presence of a number of $CO$-binding cytochromes in the cells, and so the pigment with an absorbance band at 449 nm remains a likely candidate for one of the two oxidases suggested by the KCN titrations. At higher temperatures ($-69 \, ^\circ C$) photolysis-dependent oxidation of $b$- and $c$-type cytochromes was seen, indicating that electron-transfer to $O_2$ was occurring via a $CO$-inhibited terminal oxidase. A possible reason for the inability to detect a second oxidase is that upon photolysis the presumptive oxidase(s) bind(s) ligands (CO and $O_2$) too rapidly to be observed and that the $O_2$-liganded form is spectrally indistinguishable from the CO-complex. Cytochrome $d$ provides a precedent for this behaviour; ligand binding after photolysis of the CO form is observed only at liquid helium temperatures (Poole et al., 1982).

The results presented in this paper provide another example of a bacterium modifying its respiratory chain composition, and in particular its cytochrome oxidases, in response to the available $O_2$ (Poole, 1983). However this change is mediated, it must involve the coordinate expression and synthesis of two, or probably three, terminal oxidases. The control must affect the haem $A$ biosynthetic pathway as well as oxidase structural polypeptides, as haem $A$ is not found under $O_2$-limited conditions.

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**References**


