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

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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 *a*₁', 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 *aa*₃ and contain haem A. Low-temperature photodissociation studies of O₂-sufficient cells show cytochrome *aa*₃ to be functional in CO and O₂ binding and suggest that *aa*₃ 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-liganded 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 *a*₁ 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 *aa*₃ 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 *aa*₃-type cytochromes which are similar to the mitochondrial enzyme in the complement of redox centres (1 haem *a*:1 haem *a*₃:2 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 *aa*₃ to warrant a distinguishing subscript. This 'cytochrome *a*₁' was confirmed as a competent oxidase in an *Acetobacter* strain by Castor & Chance (1955, 1959). However, recent studies on haemoproteins resembling 'cytochrome *a*₁' 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 *a*₁'. Poole *et al.* (1985) proposed a classification of *a*₁-like haemoproteins and suggested that true cytochromes *a*₁ should be shown to contain haem A. Few *a*₁-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

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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*₁-like pigment of *Acetobacter pasteurianus* NCIB 6428 (Williams & Poole, 1987).

We have investigated the occurrence and role of an *a*₁-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 α -absorption maxima (reduced *minus* oxidized) varied from 595 to 603 nm. In view of the few bacteria now thought to contain true cytochromes *a*₁, strain NCTC 1937, which exhibited a peak at 595 nm, was selected for study. Here we report the occurrence of cytochrome *aa*₃ in aerobically grown, oxygen-sufficient cells and demonstrate its oxidase role using low-temperature ligand-exchange techniques. A cytochrome *a*₁-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 maintained on NA slopes and subcultured every few weeks.

Starter cultures for O₂-sufficient growth were prepared by washing cells from a NA slope into 25 ml of NB in a 250 ml conical flask and shaking (200 r.p.m.) for 8 to 10 h at 30 °C. This culture was then used to inoculate 10 litres of NB medium in a 12 litre Biostat V fermenter (FT Scientific), sparged with air at 10 litres min⁻¹, and stirred vigorously. A small amount of antifoam (about 5 ml) was added to prevent excessive foaming. The culture was harvested after about 450 min, when the OD₆₀₀ was about 1.0, measured (after diluting 1:10 with water) against a water blank in 1 cm pathlength cuvettes using a Pye-Unicam SP-450 spectrophotometer.

For O₂-limited growth, a slope culture was used to inoculate 200 ml of medium in a 250 ml conical flask and the culture was grown at 30 °C without shaking for 20 to 24 h. This was used to inoculate 20 litres of medium, and the culture was stirred slowly and sparged with air at 2 litres min⁻¹. The culture was harvested after 20 to 24 h when the OD₆₀₀ 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 10 to 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 (S₀), which was further fractionated (Poole & Haddock, 1974) by centrifugation at 225000 *g* for 60 min to yield a 'high-speed supernatant' (S₁) and membrane pellet (P₁). The latter was homogenized in buffer and recentrifuged at 225000 *g* for 60 min to give a washed pellet (P₂) and second supernatant (S₂). 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-liganded, 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 O₂-sufficient and O₂-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 μ m nylon filters (Ultipore 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 O₂-sufficient cells were yellow in colour, while those from membranes of O₂-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 O₂ uptake and KCN 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, the lid of the vessel was replaced, substrate was added to a final concentration of 1 mM, and a recording of O₂-tension was obtained. The electrode was calibrated using O₂-saturated water at 30 °C (240 nmol O₂ ml⁻¹) and then making it anoxic using a few grains (≤ 5 mg) of sodium dithionite. The effect of KCN on O₂ uptake was investigated by adding suitable amounts of freshly prepared KCN solution (pH 7.5 to 8.5) 3 to 5 min after addition of sodium succinate, when a linear rate of respiration had been attained. The effect of cyanide was virtually instantaneous. Inhibition is expressed as a percentage of the uninhibited rate in the same trace.

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

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

Fig. 1. Difference spectra, recorded at room temperature, of intact cells of *C. percolans* grown under O_2 -sufficient (a, b) or O_2 -limited (c, d, e) conditions. (a), (c) and (d) are reduced (dithionite) minus oxidized (persulphate) spectra; (b) and (e) are reduced + CO minus reduced difference spectra. Unlabelled spectra are baselines, oxidized minus oxidized or reduced minus reduced as appropriate. Spectra were recorded at 5.7 nm s^{-1} , a spectral bandwidth of 8 nm and a reference wavelength of 500 nm (in the dual-wavelength mode). The vertical bar represents, from 380 to 500 nm, ΔA 0.04 (a, d) or 0.02 (b, c, e) and, from above 500 nm, 0.01 (a, b) or 0.02 (c, d, e).

Results

Cytochrome composition of *C. percolans* NCTC 1937 following O_2 -sufficient and O_2 -limited growth

The reduced minus oxidized difference spectrum of intact cells of *C. percolans* grown with vigorous aeration [1 l air min^{-1} ($1 \text{ l of culture}^{-1}$)] 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 *aa*₃. 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 *aa*₃ was confirmed by the CO-difference spectrum (Fig. 1b), which showed a maximum at 592 nm, due to the CO-complex of haem *a*₃, and troughs at 444 and 612 nm, resulting from the loss of the reduced *a*₃ 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 O_2 -limited conditions [$0.11 \text{ air min}^{-1}$ (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 *a*₁. There was also a prominent trough at 620 to 624 nm. The CO-difference spectrum of O_2 -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 α -region differed from that observed in O_2 -sufficient cells (Fig. 1*b*), 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. percolans* grown under O_2 -sufficient and O_2 -limited conditions

Cell-free extracts (S_0) of O_2 -sufficient *C. percolans* 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 (α -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 (α -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 α -maximum in reduced *minus* oxidized spectra, was found in the sedimentable membrane fraction (P_2). The α -maximum varied between 595 and 600 nm even in spectra from the same batch of membranes, although the 592 nm peak of an a_3 -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 α -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

Fig. 2. Spectral changes in air-oxidized washed membranes (P_2) from O_2 -limited cells of *C. percolans*. A reduced (dithionite) suspension was scanned and stored in the digital memory of the dual-wavelength spectrophotometer. An air-oxidized sample was scanned and a reduced *minus* air-oxidized spectrum (a) was plotted. The air-oxidized sample was scanned again after 3 min and a 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 ΔA of 0.04.

oxidized suspension. In the first spectrum scanned after oxidizing the membrane suspension (Fig. 2*a*) there were maxima at 527, 557 and 604 nm. After 3 min the suspension was rescanned and a second reduced *minus* oxidized spectrum plotted (Fig. 2*b*). There was a small decrease in the height of the 557 nm peak but the α -maximum had shifted slightly to 602 nm and there was a small absorbance increase at 581 nm. When the sample was rescanned after a further 5 min, significant changes had occurred (Fig. 2*c*). The height of the α - and β -absorbance bands had decreased and shifted to 564 and 534 nm, respectively, suggesting that a cytochrome b absorbing at 557 nm had become reduced. The apparent absorbance maximum at 602 nm shifted to about 588 nm, accompanied by a dramatic change in the shape of the band. No such changes were observed in membrane fractions from O_2 -sufficient cells (data not shown). Similar changes were observed in experiments on whole cells; the nature of these changes is at present not understood (see Discussion).

Fig. 3. Inhibitory effect of KCN on succinate oxidase activities in membranes of *C. percolans* grown under O₂-sufficient (□) or O₂-limited (■) conditions. Oxidase activity was measured polarographically as described in Methods, with 1 mM-succinate as substrate.

Pyridine haemochrome spectra of haems extracted from O₂-sufficient and O₂-limited *C. percolans*

Alkaline pyridine haemochrome spectra were recorded of haem extracts prepared from membranes of O₂-sufficient and O₂-limited *C. percolans* (not shown). The spectrum of the reduced pyridine haemochrome of membranes from O₂-sufficient cells showed maxima at 422, 524, 556 and 588 nm; similar maxima were observed in a reduced *minus* oxidized spectrum. The maximum at 588 nm suggested the presence of haem A (Fuhrop & Smith, 1975), which presumably originated from the cytochrome *aa*₃ present in these cells. The 556 nm band indicates the presence of haem B. Haem C was not detected in these extracts.

Similar spectra of pyridine haemochromes of membranes from O₂-limited cells showed the 556 nm band, consistent with the presence of haem B, but there was no band at 588 nm, suggesting that haem A was absent from O₂-limited cells.

Effect of KCN on O₂ uptake by *C. percolans* membranes

Membranes from O₂-sufficient and O₂-limited *C. percolans* were able to oxidize a variety of substrates. The oxidation rates [nmol O₂ min⁻¹ (mg protein)⁻¹] for cells from O₂-sufficient and O₂-limited cultures, respectively, were: NADH, 79, 50; sodium succinate 98, 51; sodium lactate 20, 62. α -Glycerophosphate was oxidized, slowly [12 nmol O₂ min⁻¹ (mg protein)⁻¹], only by membranes prepared from O₂-sufficient cells. Fig. 3 shows the effects of KCN on the succinate oxidase activities of membranes from O₂-sufficient and O₂-limited cells. Mem-

branes from O₂-sufficient cells exhibited a monophasic inhibition curve, indicating only one site of inhibition, with 50% inhibition occurring at about 500 μ M-KCN. In contrast, the KCN inhibition curve of succinate oxidase activity in membranes from O₂-limited cells was biphasic, indicating two inhibition sites, with 50% inhibition occurring at about 2.5 μ M and about 3.5 mM, respectively. This suggests that there are two KCN-sensitive oxidases functioning under O₂-limiting conditions, which are distinct from the single oxidase present in membranes from O₂-sufficient cells.

Reaction with CO and O₂ of cytochromes in O₂-sufficient cells

The reaction of potential cytochrome oxidases, in O₂-sufficient cells, with CO and O₂ 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 O₂ 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 *aa*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-liganded 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 α -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 *b* (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 reformed 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; Erecksinska & 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 O₂ was studied after supplementing the cell sample with O₂ prior to freezing. Spectral

Fig. 4. Wavelength scanning following photolysis at -128°C (a) and -87°C (b, c), in the absence of O_2 , of intact cells from an O_2 -sufficient culture of *C. percolans*. The spectrum of CO-liganded, endogenously reduced cells was scanned and stored. In (a) and (b) the flat featureless spectrum represents the reduced + CO minus reduced + CO spectrum recorded before photolysis. Each sample was then photolysed at -128°C (a) or -87°C (b, c) by a 30 s exposure to white light from a 150 W lamp and scanned. Arrows at prominent wavelengths show the direction of changes in repetitive scans (b). The scan speed was 2.86 nm s^{-1} , the spectral bandwidth 8 nm, and the reference wavelength 500 nm. The vertical bar represents a ΔA of 0.01 up to 500 nm and 0.004 above 500 nm. The change in absorbance at 448 nm minus 470 nm following photolysis is shown in (c).

changes after photodissociation of CO from the cytochrome a_3 -CO complex in the presence of O_2 at -128°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 α -signals had a lower extinction and the α -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

Fig. 5. Wavelength scanning following photolysis at -128°C (a) and -43°C (b), in the presence of O_2 , of cells from an O_2 -sufficient culture of *C. percolans*. The spectrum of a suspension of CO-liganded, endogenously reduced cells, to which O_2 had been added at -23°C by stirring, was scanned at -128°C and stored. Subtraction of this from the subsequent scan of the unphotolysed sample gave the reduced + CO minus reduced + CO baseline. The sample was photolysed at -128°C by 30 s exposure to white light from a 150 W lamp and repetitively scanned (a). Scanning was initiated at 89 s intervals. In (b) the procedure was identical but the sample was photolysed and its spectrum recorded at -43°C . Other conditions were as in Fig. 4. The vertical bar represents, up to 500 nm, a ΔA of 0.01 in (a) and 0.02 in (b) and, above 500 nm, a ΔA of 0.002 (a, b).

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°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°C to -92°C , gave an activation energy of 4.76 kJ mol^{-1} for the O_2 -binding reaction. The initial change in the α -region at -128°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°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°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°C (Fig. 5*b*) 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 α -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. 1*c* 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. 6*a*) 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 a -type cytochrome (e.g. Fig. 4*a*, *b*) but alkaline pyridine haemochrome spectra of extracted haems failed to show haem A. An alternative candidate is a b -590-like haemoprotein, i.e. a high-spin, haem-B-containing protein and, indeed, CO difference spectra (Fig. 1*e*) showed the presence of such a component. At -83°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 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°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°C of cells in the presence of O_2 (Fig. 6*b*) revealed

Fig. 6. Photolysis and wavelength scanning of CO-treated cells from O_2 -limited cultures in the absence of O_2 at -102°C (*a*), and in the presence of O_2 at -102°C (*b*) and -69°C (*c*). The spectrum of a CO-liganded, endogenously reduced and either anoxic (*a*) or O_2 -supplemented (*b*, *c*) cell suspension was scanned and stored in the digital memory of the dual-wavelength spectrophotometer. In each case the flat featureless spectrum represents the reduced + CO minus reduced + CO spectrum recorded before photolysis. Each sample was then photolysed at -102°C (*a*, *b*) or -69°C (*c*) by a 30 s exposure to white light from a 150 W lamp, and scanned. The scan speed was 2.86 nm s^{-1} , the spectral bandwidth 8 nm and the reference wavelength 500 nm. In (*a*) the vertical bar represents a ΔA of 0.01 or 0.02 below and above 500 nm, respectively. In (*c*) spectral scanning was initiated at 80 s intervals.

again a prominent 448 nm peak, only partially light sensitive at both -102 and -69°C . This suggests that the recombining ligand was CO, not O_2 . Following photolysis in the presence of O_2 at -69°C (Fig. 6*c*), 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. percolans* and to study its role in the respiratory chain. Spectral analysis of cells from highly aerated cultures demonstrated the presence of cytochrome aa_3 (α -maximum at ~ 606 nm in reduced minus oxidized spectra) but not cytochrome a_1 . However, when grown under O_2 -limited conditions, *C. percolans* lacked cytochrome aa_3 but had an α -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 α -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* (Fukumori *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; Fukumori *et al.*, 1985). However, most ($> 80\%$) of the cytochrome a_1 -like pigment of *C. percolans* 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. percolans* 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. percolans* 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. percolans* 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°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°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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