The Cytochromes of *Acetobacter pasteurianus* NCIB 6428. Evidence of a Role for a Cytochrome a_1 -like Haemoprotein in Electron Transfer to Cytochrome Oxidase d

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Cells from oxygen-limited cultures of *Acetobacter pasteurianus* NCIB 6428 contained cytochrome d , a 596 nm absorbance (in reduced minus oxidized difference spectra at room temperature) similar to cytochrome a_1 , and b -type cytochromes, including a cytochrome o -like pigment. Oxygen-sufficient cells lacked the d and a_1 -like components. Pyridine haemochrome spectra suggested the presence of haem a . Photolysis with white light at -126°C of an anoxic suspension of reduced oxygen-limited cells, into which CO had been bubbled, elicited a photodissociation spectrum that revealed the cytochrome o -like species, but not cytochromes d or a_1 . When a similar suspension, but to which O_2 had been added at -23°C , was photolysed at -126 to -132°C , using white light or irradiation with a He–Ne laser, an additional, intense absorbance at 648 nm (relative to the CO-liganded, reduced form) was observed, attributable to an oxygenated (Fe^{2+}O_2 or $\text{Fe}^{3+}\text{O}_2^-$) complex of cytochrome oxidase d . Photolysis at progressively warmer temperatures, or successive scans of a sample photolysed at -91°C , revealed (i) increasing depth of a trough (relative to the CO-liganded, reduced form) at 598 nm attributed to the a_1 -like haemoprotein and (ii) cytochrome b oxidation. Laser photolysis of the cytochrome d -CO complex at -81°C in the presence of oxygen suggested that oxidation of cytochrome a_1 preceded that of cytochrome(s) b . It is proposed that, in this strain of *Acetobacter*, a cytochrome a_1 -like pigment mediates electron transfer from cytochrome(s) b to cytochrome oxidase d , but that cytochrome a_1 is not itself an oxidase.

INTRODUCTION

Cytochrome a_1 has remained the least well understood of putative cytochrome oxidases. The early studies on alcohol-containing suspensions of *Acetobacter pasteurianus* led Warburg *et al.* (1933) to conclude that the absorption bands at 550, 553 and 560 nm were of reduced cytochromes whilst the band at 589 nm belonged to 'der Atmungsferment' or CO-sensitive 'oxygen-transporting ferment'. Keilin's largely unpublished findings (see Keilin, 1966) differed markedly from Warburg's. In *A. pasteurianus*, *A. peroxydans* and *Gluconobacter* (formerly *Acetobacter*) *suboxydans*, Keilin found not only a 590 nm band (cytochrome a_1) but also cytochrome d . There was considerable variation of the cytochrome complement with strain and growth conditions but the cytochrome a_1 band was always weak. CO difference spectra (Chance, 1953*a*) and photodissociation spectra (Chance, 1953*b*) of an *Acetobacter* strain provided by Dr Lucille Smith agreed closely in showing features attributable to the CO compound of cytochrome a_1 (427 and 590 nm) as well as the reduced, unliganded form at 442 nm. Photochemical action spectroscopy (Castor & Chance, 1955) confirmed Warburg's earlier spectra and demonstrated an oxidase role for cytochrome a_1 in *A. pasteurianus* as well as in *A. peroxydans*, *Azotobacter vinelandii* and possibly *Proteus vulgaris* (Castor & Chance, 1959). Another strain, *Acetobacter* T-71, was reported to have only cytochrome a_1 as an oxidase, which displayed an unusually high affinity for oxygen and a fast second order rate constant for the

oxidase reaction (Meyer & Jones, 1973). In retrospect, the choice of *Acetobacter* species and strains was fortunate because, in many bacteria, cytochrome a_1 coexists with cytochrome d , itself an oxidase (Poole, 1983). In only a very few species is there good evidence that cytochrome a_1 is an oxidase (Poole *et al.*, 1985*a*). For example, despite one report to the contrary (Edwards *et al.*, 1981), it now seems that both the soluble (Poole *et al.*, 1986) and the membrane-bound (Lorence *et al.*, 1986) a_1 -like haemoproteins in *Escherichia coli* are high-spin b -type haemoproteins. The former is a hydroperoxidase, but the function of the latter, which copurifies in an oxidase complex with cytochrome d , is unclear.

In this paper, we report the application to *A. pasteurianus* of low-temperature photolysis and ligand-exchange techniques, which have proved useful in studying oxidases of the aa_3 -, o - and d -types in other bacteria (Poole, 1983; Poole *et al.*, 1985*b*). We report that cytochrome a_1 is unlikely to be an oxidase in *A. pasteurianus* NCIB 6428, but may be involved in electron transfer to cytochrome oxidase d . Parts of this work have been published in abstract form (Williams & Poole, 1986).

METHODS

Organism and growth. *Acetobacter pasteurianus* NCIB 6428 was obtained from the National Collection of Industrial Bacteria (Torry Research Station, Aberdeen, UK) and grown on a medium, pH 7, containing (g l⁻¹) yeast extract (5), peptone (3) and mannitol (10). The organism was maintained as samples supplemented with 12.5% (v/v) glycerol and stored at -20 °C or on medium solidified with agar (15 g l⁻¹) and stored at 4 °C.

Starter cultures for oxygen-sufficient growths were prepared by inoculating a 4 ml frozen glycerol stock culture into 25 ml of medium in a 250 ml conical flask and shaking (200 r.p.m.) for about 10 h at 30 °C. This culture was then used to inoculate 10 l of medium in a 12 l Biostat V fermenter (FT Scientific), sparged with air at 10 l 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 400 min, when the OD₆₀₀ was 2.0 to 3.0, measured (after diluting 1:10 with water) against a water blank in 1 cm pathlength cuvettes using a Pye Unicam SP6-450 spectrophotometer.

For aerobic, but oxygen-limited, growth a 4 ml frozen stock 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. A portion was used to inoculate (1% inoculum) either 10 l or 20 l of medium, stirred slowly. Air was sparged into the culture at 0.1 l min⁻¹ (1 medium)⁻¹. The culture was harvested after 20 to 24 h, when the OD₆₀₀ was 0.6 to 0.7 measured as above.

Estimates of dissolved oxygen tension and respiration rates during growth were made by immediately transferring 3 ml of culture to the unstirred vessel of a calibrated oxygen electrode apparatus (Rank). After reading the oxygen tension, stirring was started and respiration rates measured.

Cells were harvested using an Alpha-Laval continuous action centrifuge. The cells were resuspended in 46 mM-potassium phosphate buffer (pH 7.0) and centrifuged at 15000 *g* for 10 to 15 min. The cells were then washed by resuspension and hand homogenization in the same buffer and recentrifuged. If necessary, cells were stored at -20 °C until needed.

Spectrophotometry. Washed cells were resuspended in buffer by homogenization. Difference spectra (reduced minus oxidized) were recorded at 77 K using a Pye Unicam SP1700 split-beam spectrophotometer equipped with a custom-built cryogenic accessory (Jones & Poole, 1985). Difference spectra at room temperature were recorded using a Johnson Foundation DBS-3 dual-wavelength scanning spectrophotometer similar to that described by Poole & Chance (1981). Light from a 60 W tungsten halogen lamp was focussed on the entrance slits of two concave holographic grating monochromators (J-Y type H-20 visible, SA Instruments). The output of the monochromators (one fixed to provide a reference wavelength, the other driven by a stepping motor) was modulated by a resonant tuning fork vibrating mirror and focussed on the cuvette. Transmitted light was measured using a Hamamatsu type R928 side window photomultiplier tube. A microprocessor interrogated the chosen scan conditions and dictated the operation of the stepping motor as well as providing digital memories for up to four spectra. Difference spectra were plotted directly on a Hewlett Packard HP7010B X-Y recorder.

For conventional, 'static' spectra, samples were reduced with a few grains of fresh sodium dithionite and oxidized by (a) shaking for 30 s, or adding a few grains of (b) potassium ferricyanide or (c) ammonium persulphate. CO was used without further treatment by bubbling the reduced sample in a cuvette for 5 min; the top of the cuvette was smeared with silicone antifoam to prevent frothing.

For low-temperature, ligand-exchange studies (Chance, 1978), cells from oxygen-limited cultures were washed and resuspended to about 33% (packed v/v) in 46 mM-potassium phosphate buffer (pH 7.0) and ethylene glycol was added to give a final concentration of 30% (v/v). Cells were allowed to become anoxic by endogenous respiration at room temperature in cuvettes of about 1 ml capacity and 2 mm path length, and then bubbled with CO for 5 min. The cuvette was cooled in an ethanol/solid CO₂ bath to -23 °C and allowed to equilibrate for 5 min in the dark. Where indicated, O₂ was added by vigorously stirring the sample with vertical strokes of a closely

fitting coiled wire for 30 s. The anoxic or O_2 -supplemented sample was then quickly frozen in an ethanol/dry CO_2 bath at $-78^\circ C$ where it was maintained for at least 5 min in the dark before further equilibration (10 to 20 min) at the temperature of the experiment in the sample compartment of the dual-wavelength spectrophotometer. Temperature control ($\pm 1^\circ C$) was achieved by blowing a stream of N_2 (cooled by circulation through liquid N_2) over the base of the cuvette coupled with a small heater and temperature regulator circuit. Illumination by the measuring and reference beams was avoided. The sample was then scanned twice generating a reduced + CO minus reduced + CO baseline and then photolysed. Photolysis by white light was achieved using 30 s exposure to the light from a 150 W projector lamp which was focussed by a lens on to one afferent limb of a bifurcated light guide leading to the cuvette, the other limb transmitting reference and measuring beams. Alternatively, photolysis was with a 60 s exposure via the bifurcated light guide to the beam from a He-Ne laser (maximum output 30 mW; 632.8 nm; Hughes Aircraft Co.), powered by a model 4030 power supply. A model 3970-H collimator expanded by ten diameters the laser beam at the point of exit. After photolysis, the sample was either repetitively scanned using the prephotolysis spectrum as baseline or monitored in a conventional dual-wavelength mode, plotting the absorbance difference between fixed measuring and reference wavelengths as a function of time on an Oxford series 3000 recorder.

Haem extraction and pyridine haemochrome spectra of extracted haems. Haem extraction of *A. pasteurianus* oxygen-limited cells was achieved using the method of Smith & Caughey (1978) with the modifications of Poole *et al.* (1984). The resulting haem-containing extracts were dissolved in 12.4 M-pyridine; immediately before recording the spectrum an equal volume of 0.15 M-NaOH was added. Sample reduction was with sodium dithionite ($<3\text{ mg ml}^{-1}$) and oxidation was with a few grains of ammonium persulphate.

Protein. This was assayed by the Lowry method after dilution of cell suspensions to a level where cell dissolution was accomplished by the alkaline reagent.

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

RESULTS

Cytochrome composition of various A. pasteurianus strains

The strains of *A. pasteurianus* previously reported by Chance and his collaborators to possess cytochrome a_1 as sole oxidase (see Introduction) or the strain (T-71) used by Meyer & Jones (1973), which contained cytochrome a_1 as its major CO-binding pigment, appear to have been lost. Therefore, four strains (NCIB 6428, 6930, 8163 and 8148) were examined; after oxygen-sufficient growth, all strains were similar in exhibiting cytochrome o , but none showed evidence of cytochrome a_1 . One strain (NCIB 6428) was selected for study of the effect of growth conditions on cytochrome composition.

Cytochrome composition of A. pasteurianus NCIB 6428 after oxygen-sufficient and -limited growth

Aerobic growth in a vigorously stirred and sparged fermenter caused the dissolved oxygen concentration to fall gradually during growth to about $185\text{ nmol } O_2\text{ ml}^{-1}$ in stationary phase, when the OD_{600} was 8 and the respiration rate was $120\text{ nmol } O_2\text{ min}^{-1}(\text{ml culture})^{-1}$. Culture pH rose from 7 to 9.5 (results not shown). Cells grown under oxygen-sufficient conditions contained *b*-type cytochromes with α -absorption maxima at 561 nm at room temperature (Fig. 1*a*) and 554 and 563 nm at 77 K (Fig. 1*b*). No *c*-type cytochromes were evident. There was merely a hint of haemoprotein absorbance near 595 nm. The CO-difference spectrum (Fig. 1*c*) showed features attributable to *b*-type cytochrome(s).

When the air supply rate to a fermenter was decreased 10-fold, growth soon became oxygen-limited. The dissolved O_2 concentration fell within 150 min of inoculation to about 30 nmol ml^{-1} and then declined to undetectable levels. Because of the method adopted for measuring respiration rates (Methods), their monitoring was precluded for most of the culture period. The final OD_{600} was about 0.8 and the pH remained near 7 (results not shown). Oxygen-limited cells contained high levels of cytochrome *d*, as indicated by the peak at 628 nm (room temperature; Fig. 1*d*) and 627 nm (77 K; Fig. 1*e*). The trough at 648 to 651 nm is presumably due to the presence of the oxygenated form in the reference cuvette (Poole *et al.*, 1983*a*; see also Discussion). The 596 nm absorbance in Fig. 1(*d*) is indicative of a cytochrome- a_1 -like component and is shifted to an unusually large extent (to 586 nm) at 77 K (Fig. 1*e*). The *b*-type

Fig. 1. Difference spectra of *A. pasteurianus* grown under O_2 -sufficient (a-c) or -limited (d-f) conditions. Spectra (a) and (d) are room temperature reduced (dithionite) *minus* oxidized (persulphate) spectra, (b) and (e) 77 K reduced *minus* oxidized spectra and (c) and (f) room temperature reduced + CO *minus* reduced difference spectra. Unlabelled spectra are baselines, oxidized *minus* oxidized or reduced *minus* reduced as appropriate. 77 K spectra were recorded at 4 nm s^{-1} and a spectral band width of 1 nm, while room temperature spectra were recorded at 5.7 nm s^{-1} , a spectral band width of 8 nm and a reference wavelength of 500 nm (in the dual wavelength mode). The vertical bar represents, from 380 to 500 nm or 570 nm, ΔA 0.04 (a, d), 0.02 (b, e) and from above 500 or 570 nm, 0.02, 0.019, 0.01, 0.004, 0.008 and 0.004 in (a) to (f) respectively. Cells were suspended to a protein concentration of 40 to 50 mg ml^{-1} .

cytochromes appeared similar to those of oxygen-sufficient cells. The CO difference spectrum (Fig. 1f) showed cytochrome *d* (622 and 642 nm features) and CO-binding cytochrome *b*(s) (417, 436 and the features between 530 and 570 nm). The 440 and 594 nm troughs are probably attributable to the cytochrome-*a*₁-like pigment. Thus, oxygen-limited cells were selected for further studies of this component.

Pyridine haemochrome spectra of haems extracted from oxygen-limited A. pasteurianus

Alkaline pyridine haemochrome spectra were recorded of extracts prepared from oxygen-limited *A. pasteurianus*. The spectrum of the native pyridine haemochrome showed maxima at 420, 590 and 650 nm and a very weak signal at 556 nm. In the reduced pyridine haemochrome spectrum, there were enhanced absorbances at 420 and 556 nm, but the signal at 590 nm was smaller, whereas that at 650 nm had disappeared. The peak at 590 nm in the native spectrum suggested the presence of haem *a* (Fuhrhop & Smith, 1975); the decrease in signals following reduction with dithionite is also consistent with previous observations of pyridine haemochromogens of haem *a* from cytochrome *c* oxidase (Morrison & Horie, 1965). The 556 nm band in the reduced spectrum suggested the presence of haem *b*. There was no evidence for the presence of haem *c*.

Fig. 2. Difference spectra of oxygen-limited cells with a stored spectrum of an endogenously reduced + CO sample as the reference. Spectra shown are (a) endogenously reduced, (b) 'oxidized' with vigorous aeration, and (c) oxidized with a few grains of ferricyanide. The dashed spectra are reduced *minus* reduced, aerated *minus* aerated, and ferricyanide oxidized *minus* oxidized baselines for (a), (b) and (c) respectively. The temperature was -80°C , the scan speed 2.86 nm s^{-1} , the spectral band width 8 nm , and the reference wavelength 500 nm . The vertical bar represents a ΔA below 500 nm , of 0.02 , 0.04 and 0.04 in (a) to (c) respectively and above 500 nm a ΔA of 0.01 .

Spectra to aid interpretation of low-temperature ligand-binding experiments

The spectra shown in Fig. 2 were recorded at -80°C . The reference spectrum was that of an endogenously reduced plus CO spectrum as would be the case in experiments initiated by photolysis. Fig. 2(a) shows an endogenously reduced *minus* reduced + CO spectrum, whereas Fig. 2(b) shows an air-oxidized *minus* reduced + CO spectrum. In the latter, the maximum at 654 nm suggests that some cytochrome *d* is present as '*d*-650' (oxygenated cytochrome *d*; see later). Cytochrome a_1 appears oxidized in this spectrum (trough at 593 nm). Fig. 2(c) shows a ferricyanide-oxidized *minus* reduced + CO spectrum. Cytochrome *d* is oxidized in this spectrum. However, the lack of a trough at 593 nm and the presence of a peak at 445 nm suggest that cytochrome a_1 was not oxidized by ferricyanide.

Fig. 3. Photolysis and wavelength scanning of CO-liganded *A. pasteurianus* in the absence of O_2 at $-126^\circ C$ (a, b) or in the presence of oxygen at $-127^\circ C$ (c, d). The spectrum of a suspension of CO-liganded, endogenously reduced cells was scanned and stored in the digital memory of the dual-wavelength spectrophotometer. Subsequent scans were difference spectra, the stored spectrum being subtracted. The first scan, before photolysis, yielded a reduced + CO *minus* reduced + CO baseline (a, c). Spectra (b) and (d) show the first spectra scanned following a 30 s exposure to white light. In (c) and (d), O_2 was introduced by stirring before freezing. Spectra were scanned at 2.86 nm s^{-1} with 500 nm as reference wavelength and a spectral band width of 8 nm. The vertical bar represents a ΔA of 0.01 up to 500 nm and 0.004 above 500 nm. Wavelengths (in nm) of distinctive features of the spectrum are shown.

Photolysis of reduced, CO-liganded cytochromes in intact cells of oxygen-limited A. pasteurianus

As a prerequisite to studying the reactions of cytochrome oxidase(s) with O_2 , attempts were made to photodissociate CO from cytochromes a_1 , d and o . An endogenously reduced, CO-saturated sample was photolysed for 30 s with white light at $-126^\circ C$. The resulting (post-photolysis *minus* pre-photolysis) photodissociation spectrum showed the features of a pure cytochrome o photodissociation spectrum (Fig. 3b; see Poole *et al.*, 1979). There were absorption maxima at 431, 552 and 580 nm, due to the appearance of reduced cytochrome o in the sample cuvette, and minima at 414, 532 and 566 nm, due to the loss of CO-liganded cytochrome o from the sample cuvette. Repetitive scanning of this sample for 17 min after photolysis showed no change in the spectrum, suggesting a lack of CO-recombination (data not

Fig. 4. Photolysis and rapid wavelength scanning of CO-liganded *A. pasteurianus* cells in the presence of O_2 at $-151^\circ C$. The spectrum of a suspension of CO-liganded, endogenously reduced cells, to which O_2 had been added at $-23^\circ C$ by stirring, was scanned and stored in the digital memory of the dual-wavelength spectrophotometer. Subsequent scans were difference spectra, the stored spectrum being subtracted. The dashed line represents the first scan recorded before photolysis. Following 10 s photolysis by white light the post-photolysis spectrum was scanned 12 s later at a scan speed of 5.7 nm s^{-1} . The scan was complete 29.5 s after the start of photolysis. The reference wavelength was 608 nm and the spectral band width 8 nm. The vertical bar represents a ΔA of 0.001.

shown). There were no signals in the Soret or α -regions of the spectrum, that could have corresponded to a photodissociation spectrum of cytochrome a_1 or cytochrome d . When a similar experiment was done in the presence of O_2 , the difference spectrum had an intense maximum at 648 nm and a minimum at 632 nm (Fig. 3*d*). This spectrum was not of the reduced form of cytochrome d , which, as shown in Fig. 2(*a*) (relative to the reduced + CO form) had a weak peak at 620 nm and a trough at 638 nm. The form of cytochrome d in Fig. 3(*d*) is thought to be an early intermediate (' d -650') in the reaction of cytochrome d with O_2 (see later). The trough at 632 nm resulted from the disappearance of the CO-liganded form following photolysis. This result indicates that the cytochrome d -CO complex is photolysed under the conditions used but that, in the absence of oxygen, CO-recombination is so fast as to be complete before the first scan. However, no signals were observed in the Soret region (around 440 nm) or the α -region (around 590 nm), which could be attributed to cytochrome a_1 reacting with O_2 . The Soret signals from cytochrome d are weak (Poole, 1983; Poole *et al.*, 1982*a*).

Identity of the cytochrome d -650 intermediate

Fig. 4 shows photolysis of a reduced, CO-saturated sample in the presence of O_2 at $-151^\circ C$, the lowest temperature attainable with the liquid nitrogen cryostat used. The spectrum was scanned from 600 to 700 nm at the fastest available scan speed (5.71 nm s^{-1}), the scan being completed 29.5 s after commencing a 10 s period of photolysis. In the first scan after photolysis, the cytochrome d -650 intermediate appeared. Therefore, within the limits of our experimental capabilities, the d -650 compound is the first detected intermediate in the reaction between cytochrome d and O_2 .

Use of a He-Ne laser for photolysis allowed specific photolysis of the cytochrome d -CO complex, since the energy line (632.8 nm) is very close to the absorption maximum of the ferrous d -CO compound at 634 nm and well-removed from the CO compounds of o - and a -type oxidases. Therefore, subsequent reactions are assumed to be a consequence of the reaction of cytochrome d with O_2 or CO. Laser photolysis of a reduced, CO-saturated sample in the presence of O_2 at $-132^\circ C$ (results not shown) resulted in the formation of the d -650 compound. A small signal seen in the Soret region (at 432 nm) was presumably due to non-specific photolysis of cytochrome o , but accounted for less than 5% of the signal seen following white light photolysis. The spectrum was virtually featureless in the cytochrome(s) b region, indicating that there had been no oxidation of b -type cytochromes or cytochrome a_1 . Therefore, presumably no electron-transfer had occurred to cytochrome d . The initial step in the reduction

of O_2 must involve its binding to the reduced haem and, as the *d*-650 compound is the first observed intermediate, it is suggested that it is oxygenated, reduced cytochrome *d* ($Fe^{2+}O_2$ or $Fe^{3+}O_2^-$).

Subsequent reactions of cytochrome d-650

Fig. 5 shows the first spectra, and their respective baselines, following photolysis, with white light, of reduced, CO-saturated intact cells in the presence of O_2 at successively warmer temperatures. After photolysis at $-100^\circ C$ (Fig. 5*a*) the difference spectrum showed the intense peak at 648 nm attributed to the formation of *d*-650 and a trough at 632 nm resulting from the disappearance of the CO-liganded form upon photolysis. At $-88^\circ C$ (Fig. 5*b*) the major peak and trough remained but a broad shoulder appeared at around 675 to 680 nm. Further warming to $-80^\circ C$ (Fig. 5*c*) caused this shoulder to intensify with concomitant loss of the peak, now shifted to 655 nm, until at $-71^\circ C$ (Fig. 5*d*) and $-62^\circ C$ (Fig. 5*e*) the 648 nm peak was no longer seen, but replaced by a broad absorbance from 660 to 680 nm. Over this temperature range, changes in the 632 to 637 nm trough were small. At $-88^\circ C$ (Fig. 5*b*) and warmer temperatures, troughs near 532 and 563 nm deepened, indicating oxidation of *b*-type cytochromes presumably due to electron-transfer to cytochrome *d* and/or cytochrome *o*. Changes in the Soret region reflected the same events. At $-88^\circ C$ (Fig. 5*b*) a trough was seen at 598 nm, suggesting that cytochrome a_1 was partially oxidized (see Fig. 2*b*). A similar trough appeared at all warmer temperatures. This suggested that cytochrome a_1 has a role in electron-transfer to cytochrome *d* and/or *o* in their reduction of O_2 . An alternative explanation is that cytochrome a_1 has itself reacted with O_2 , forming intermediate species with no distinct optical signals.

The same reactions were observed if the reaction was followed, after photolysis, by repetitive scanning at a constant temperature. At $-91^\circ C$ (results not shown) there was loss of *d*-650 and a concomitant increase in absorbance at 670 to 680 nm; the 635 nm trough was relatively constant and there was a decrease in absorbances at 563 and 537 nm due to oxidation of *b*-type cytochromes.

Kinetics of the loss of d-650 and the formation of the 670 to 680 nm component

The disappearance of *d*-650 and the appearance of the absorbance at 670 to 680 nm may represent the loss and formation, respectively, of sequential intermediates in the reaction of *A. pasteurianus* cytochrome *d* with O_2 . To investigate this possibility, the kinetics of each reaction were followed at $-87^\circ C$ after photolysis of reduced, CO-saturated samples in the presence of O_2 . Fig. 6(*a*) shows the loss of *d*-650 and Fig. 6(*b*) the formation of the 670 to 680 nm absorbing species. The kinetics of these two reactions were very different, suggesting that the loss of *d*-650 and the formation of the higher wavelength species did not represent sequential stages in the reaction of cytochrome *d* with O_2 , unless the 675 nm component was being slowly converted to a third component not having a distinct optical spectrum.

*Reactions of cytochromes a_1 , *b* and *d* studied by laser photolysis and temperature-recycling*

To investigate the possible role of cytochrome a_1 in electron-transfer to cytochrome *d*, a temperature-recycling experiment was done (Fig. 7). A reduced, CO-saturated sample was photolysed, using a He-Ne laser, at $-81^\circ C$ in the presence of O_2 . The first spectrum after laser photolysis (Fig. 7*b*) showed *d*-650 with a shoulder already forming at about 675 nm. Troughs were present at 595 and 440 nm corresponding to the oxidation of cytochrome a_1 (see Fig. 2*b*). Assignment of the latter band to the a_1 -like component seems justified in view of (i) the weak absorbance of cytochrome *d* in this region (Poole *et al.*, 1982*a*) and (ii) the fact that both *a*-type cytochromes and the high-spin *b*-type haemoproteins, with which they may be confused, have prominent absorbances near 440 nm (e.g. Poole *et al.*, 1986). The spectrum scanned after transient warming cycles to $-69^\circ C$ and then to $-60^\circ C$ showed a deepening of the trough at 563 nm and a shift in the Soret trough of 5 nm to 435 nm due to oxidation of a cytochrome *b* (*b*-435). This indicated successive oxidation of at least two components, firstly cytochrome a_1 and then cytochrome *b*-435, providing evidence that cytochrome a_1 is involved in electron-transfer to cytochrome *d*.

Fig. 5. Wavelength scanning following photolysis of *A. pasteurianus* cells in the presence of O_2 over a range of temperatures. The spectrum of a suspension of CO-liganded endogenously reduced cells, to which O_2 had been added at $-23^\circ C$ by stirring, was scanned at $-100^\circ C$ and stored in the digital memory of the dual-wavelength spectrophotometer. Subtraction of this from the subsequent scan of the unphotolysed sample gave the reduced + CO minus reduced + CO baseline (dashed line). The sample was photolysed at $-100^\circ C$ by a 30 s exposure to white light and spectrum (a) recorded using the stored spectrum as reference. In (b) to (e) the procedure was identical but the spectra were recorded at $-88^\circ C$ (b), $-81^\circ C$ (c), $-71^\circ C$ (d) and $-62^\circ C$ (e). In each case the reference wavelength was 500 nm, and the spectral band width 8 nm; the scan speed was 2.86 nm s^{-1} . The vertical bar represents a ΔA of 0.01 below 500 nm and 0.002 above 500 nm.

Fig. 6

Fig. 7

Fig. 6. Kinetics of the reaction of cytochrome *d* with oxygen at -87°C . The absorbance changes measured at 650 minus 635 nm and 675 minus 710 nm in the dual-wavelength spectrophotometer were followed after photolysis by white light of endogenously reduced, CO-liganded *A. pasteurianus* cells, to which O_2 had been added at -23°C by stirring. The vertical arrow indicates when the samples were photolysed, by exposure to white light for 30 s. The vertical bar represents a ΔA of 0.003.

Fig. 7. Oxidation of cytochromes following photolysis of CO-liganded *A. pasteurianus* cells in the presence of O_2 by monochromatic light. The spectrum of an endogenously reduced, CO-liganded cell suspension, to which O_2 had been added by stirring, at -23°C , was scanned at -80°C , stored in the digital memory of the dual-wavelength spectrophotometer and subtracted from subsequent spectra. The reduced + CO minus reduced + CO baseline is shown by (a). Photolysis was by a 60 s exposure to the beam of a He-Ne laser and was followed 60 s later by another scan (b; solid line). The sample was scanned again after 15 min (data not shown) and was then warmed to -69°C for 5 min, returned to -81°C (total cycle time was 9 min) and then warmed again, to -60°C for 7.5 min [spectrum (c); dashed line; total cycle time was 10.5 min]. Spectra were scanned at 2.86 nm s^{-1} with 500 nm as reference wavelength. The spectral band width was 8 nm. The vertical bar represents a ΔA of 0.01 up to 500 nm and 0.004 above 500 nm.

DISCUSSION

It is unfortunate that the strains of *Acetobacter* spp. used by earlier investigators appear to be unavailable, especially as photochemical action spectra of Castor & Chance (1955) indicated a strain having cytochrome a_1 as a sole oxidase. The apparent discrepancies in cytochrome composition evident in the literature on the acetic acid bacteria may in part be due to the extreme lability of the biochemical characteristics used in classification (see Shimwell & Carr, 1964, and references therein). It is of interest that, although cytochromes were not included in Shimwell's surveys, catalase activity was variable.

In the absence of evidence to the contrary, and in view of the apparent presence of haem *a*, we tentatively assign the band near 596 nm seen in reduced *minus* oxidized difference spectra of *A. pasteurianus* NCIB 6428 to 'cytochrome a_1 ', although we recognize that spectrally similar haemoproteins have recently been identified as high-spin *b*-type haemoproteins (Poole *et al.*, 1986; Lorence *et al.*, 1986) and that 'true' cytochromes a_1 (Class 1 in the classification of Poole *et al.*, 1985*a*) have been defined on the basis of their roles as oxidases. There is no evidence to date that the a_1 -like component in the present strain of *A. pasteurianus* is an oxidase. Other spectral features of this component are difficult to identify since it has not been purified, but by analogy with *a*-type cytochromes (e.g. in *aa_3*) and also with haemoprotein *b*-590 (Poole *et al.*, 1986) we suppose it to have a prominent Soret absorbance in the reduced form near 440 nm. The peak at 610 nm in CO difference spectra (e.g. Fig. 1*f*) may be due to the CO-liganded ferrous form, as has been suggested in *Acinetobacter* (Ensley & Finnerty, 1980) but this would distinguish this a_1 -like component from cytochrome a_3 (590 nm; Poole, 1983) and haemoprotein *b*-590 (574 nm; Poole *et al.*, 1986). The most characteristic feature remains the absorbance of the reduced form near 596 nm.

Cytochrome d in *A. pasteurianus* NCIB 6428 appears to react with CO and O_2 at sub-zero temperatures in a manner very similar to that observed for *E. coli* (Poole *et al.*, 1983*a, b*). In particular, photolysis of the CO compound in the absence of oxygen is followed by a rapid recombination with CO that is unmeasurable even at -130°C to -150°C using conventional scanning apparatus but observable at liquid helium temperatures (Poole *et al.*, 1982*a*). Oxygen also binds rapidly to give a form with a distinctive optical signal near 650 nm (Poole *et al.*, 1983*a*) which has been assigned to an oxygenated intermediate (Poole *et al.*, 1982*b*, 1983*a, b*; Hata *et al.*, 1985). In neither organism are the subsequent reactions, occurring on repetitive scanning or at higher temperatures, fully understood. Thus, the common appearance of the species with a broad absorbance maximum at 675 to 680 nm is unexplained. The kinetics of its appearance suggest that it may not be a direct product of further reaction of the oxygenated species but it is also possible that it is rapidly turned over to give a third species with unrecognized or indistinct optical features. Indeed, this third species may be the fully oxidized enzyme which, according to the hypothesis of Poole *et al.* (1983*a*), does not absorb significantly in the red region of the spectrum and is equivalent to the invisible ' d^* ' or ' d_x ' forms reported by others.

Diminution of the 648 nm form and increased absorbance near 680 nm are accompanied by oxidation of 'cytochrome a_1 ' and then of a cytochrome b , providing evidence of electron transfer from b to ' a_1 ' to cytochrome d . Such a scheme was proposed in earlier studies in *E. coli* (Hendler & Burgess, 1974), but has remained unsupported by experimental evidence until very recently, when we showed that in *E. coli* membranes, the loss of absorbance due to the oxygenated form is accompanied by oxidation of a low-spin cytochrome b and an a_1 -like component (Poole & Williams, 1987). An alternative explanation of the present results with *A. pasteurianus* is that the He-Ne laser also significantly photolysis the a_1 -CO complex, resulting possibly in O_2 /CO exchange and subsequent oxidation of the a_1 -like species. However, (i) the signal attributed to oxidized cytochrome a_1 was no more prominent after white light photolysis than after laser photolysis, and (ii) the a_1 -like component may not bind CO.

The identity of the component absorbing at 680 nm is uncertain, but it would be of interest to investigate whether it can be formed from the ferric form and peroxide as has been demonstrated in *E. coli* (R. K. Poole and H. D. Williams, unpublished) and thus be identified as a peroxy intermediate.

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REFERENCES

- CASTOR, L. N. & CHANCE, B. (1955). Photochemical action spectra of carbon-monoxide-inhibited respiration. *Journal of Biological Chemistry* **217**, 453-465.
- CASTOR, L. N. & CHANCE, B. (1959). Photochemical determination of the oxidases of bacteria. *Journal of Biological Chemistry* **234**, 1587-1592.
- CHANCE, B. (1953*a*). The carbon monoxide compounds of the cytochrome oxidases. I. Difference spectra. *Journal of Biological Chemistry* **202**, 383-396.
- CHANCE, B. (1953*b*). The carbon monoxide compounds of the cytochrome oxidases. II. Photodissociation spectra. *Journal of Biological Chemistry* **202**, 397-404.
- CHANCE, B. (1978). Cytochrome kinetics at low temperatures: trapping and ligand exchange. *Methods in Enzymology* **54**, 102-111.
- EDWARDS, C., BEER, S., SIVIRAM, A. & CHANCE, B. (1981). Photochemical action spectra of bacterial a - and o -type oxidases using a dye laser. *FEBS Letters* **128**, 205-207.
- ENSLEY, B. D. & FINNERTY, W. R. (1980). Influences of growth substrates and oxygen on the electron transport system in *Acinetobacter* sp. HO1-N. *Journal of Bacteriology* **142**, 859-868.
- FUHRHOP, J. H. & SMITH, M. K. (1975). Laboratory methods. In *Porphyrins and Metalloporphyrins*, pp. 757-869. Edited by K. M. Smith. Amsterdam: Elsevier Scientific.
- HATA, A., KIRINO, Y., MATSUURA, K., ITOH, S., HIYAMA, T., KONISHI, K., KITA, K. & ANRAKU, Y. (1985). Assignment of ESR signals of *Escherichia coli* terminal oxidase complexes. *Biochimica et biophysica acta* **810**, 62-72.
- HENDLER, R. W. & BURGESS, A. H. (1974). Fractionation of the electron transport chain of *Escherichia coli*. *Biochimica et biophysica acta* **357**, 215-230.
- JONES, C. W. & POOLE, R. K. (1985). The analysis of cytochromes. *Methods in Microbiology* **18**, 285-328.
- KEILIN, D. (1966). *The History of Cell Respiration and Cytochrome*. Cambridge: Cambridge University Press.
- LORENCE, R. M., KOLAND, J. G. & GENNIS, R. B. (1986). Coulometric and spectroscopic analysis of

- the purified cytochrome *d* complex of *Escherichia coli*: evidence for the identification of 'cytochrome *a*₁' as cytochrome *b*₅₉₅. *Biochemistry* **25**, 2314–2321.
- MEYER, D. J. & JONES, C. W. (1973). Oxidative phosphorylation in bacteria which contain different cytochrome oxidases. *European Journal of Biochemistry* **36**, 144–151.
- MORRISON, M. & HORIE, S. (1965). Determination of heme *a* concentration in cytochrome preparations by haemochromogen method. *Analytical Biochemistry* **12**, 77–82.
- POOLE, R. K. (1983). Bacterial cytochrome oxidases. A structurally and functionally diverse group of electron-transfer proteins. *Biochimica et biophysica acta* **726**, 205–243.
- POOLE, R. K. & CHANCE, B. (1981). The reaction of cytochrome *o* in *Escherichia coli* K12 with oxygen. Evidence for a spectrally and kinetically distinct cytochrome *o* in cells from oxygen-limited cultures. *Journal of General Microbiology* **126**, 277–287.
- POOLE, R. K. & WILLIAMS, H. D. (1987). Proposal that the function of the membrane-bound cytochrome *a*₁-like haemoprotein ('cytochrome *b*₅₉₅') in *Escherichia coli* is direct electron donation to cytochrome *d*. *FEBS Letters* (in the Press).
- POOLE, R. K., WARING, A. J. & CHANCE, B. (1979). The reaction of cytochrome *o* in *Escherichia coli* with oxygen. Low temperature kinetics and spectral studies. *Biochemical Journal* **184**, 379–389.
- POOLE, R. K., SIVARAM, A., SALMON, I. & CHANCE, B. (1982*a*). Photolysis at very low temperatures of CO-liganded cytochrome oxidase (cytochrome *d*) in oxygen-limited *Escherichia coli*. *FEBS Letters* **141**, 237–241.
- POOLE, R. K., BAINES, B. S., HUBBARD, J. A. M., HUGHES, M. N. & CAMPBELL, N. J. (1982*b*). Resonance Raman spectroscopy of an oxygenated intermediate species of cytochrome oxidase *d* from *Escherichia coli*. *FEBS Letters* **150**, 147–150.
- POOLE, R. K., KUMAR, C., SALMON, I. & CHANCE, B. (1983*a*). The 650 nm chromophore in *Escherichia coli* is an oxygenated compound, not the oxidized form of cytochrome oxidase *d*: an hypothesis. *Journal of General Microbiology* **129**, 1335–1344.
- POOLE, R. K., SALMON, I. & CHANCE, B. (1983*b*). The reaction with oxygen of cytochrome oxidase (cytochrome *d*) in *Escherichia coli* K12: optical studies of intermediate species and cytochrome *b* oxidation at sub-zero temperatures. *Journal of General Microbiology* **129**, 1345–1355.
- POOLE, R. K., BAINES, B. S., CURTIS, S. J., WILLIAMS, H. D. & WOOD, P. M. (1984). Haemoprotein b-590 (*Escherichia coli*); redesignation of a bacterial 'cytochrome *a*₁'. *Journal of General Microbiology* **130**, 3055–3058.
- (1985*a*). Sensor sensationalism? Alternative views on the nature and role of 'cytochrome *a*₁' in bacteria. *Microbiological Sciences* **2**, 21–24.
- POOLE, R. K., BAINES, B. S., HUBBARD, J. A. M. & WILLIAMS, H. D. (1985*b*). Microbial metabolism of oxygen. The binding and reduction of oxygen by bacterial cytochrome oxidases. In *Microbial Gas Metabolism: Mechanistic, Metabolic and Biotechnological Aspects*, pp. 31–62. Edited by R. K. Poole & C. S. Dow. London: Academic Press.
- POOLE, R. K., BAINES, B. S. & APPLEBY, C. A. (1986). Haemoprotein b-590 (*Escherichia coli*), a reducible catalase and peroxidase: evidence for its close relationship to hydroperoxidase I and a 'cytochrome *a*₁*b*' preparation. *Journal of General Microbiology* **132**, 1525–1539.
- SHIMWELL, J. L. & CARR, J. G. (1964). Mutant frequency in *Acetobacter*. *Nature, London* **201**, 1051–1052.
- SMITH, M. L. & CAUGHEY, W. S. (1978). New methods for isolation and characterization of hemes. *Methods in Enzymology* **52**, 421–438.
- WARBURG, O., NEGELEIN, E. & HAAS, E. (1933). Spektroskopischer Nachweis des sauerstoffübertragenden Ferments neben Cytochrom. *Biochemische Zeitschrift* **266**, 1–8.
- WILLIAMS, H. D. & POOLE, R. K. (1986). Low temperature spectrophotometric studies of cytochromes *a*₁ and *d* in *Acetobacter pasteurianus* (NCIB 6428). *Biochemical Society Transactions* **14**, 1215.