

The purification, characterization and role of the *d*-type cytochrome oxidase of *Klebsiella pneumoniae* during nitrogen fixation

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Klebsiella pneumoniae synthesized only *b*-type and *d*-type cytochromes under the wide range of growth conditions tested, and reaction with CO revealed two potential oxidases. The *o*-type oxidase was produced only in the presence of O₂ and appeared to be repressed by glucose. The *d*-type oxidase was, by contrast, produced only in the absence of measurable O₂ (<1 µM), and was the only oxidase expressed in nitrogen-fixing conditions. It was extracted from the membrane, purified and shown to be similar to that from *E. coli* in being a heterodimer (subunits of *M*_r 52 000 and 35 000), in containing two distinguishable *b* haems and haem *d* (one or two molecules per molecule of oxidase), and in being able to react with O₂ to give a stable oxygenated intermediate. The purified *d*-type cytochrome oxidase had a very high affinity for O₂ (*K*_m 20 nM; measured by the spectral properties of leghaemoglobin). It is proposed that this provides a role for this oxidase in lowering the O₂ concentration to allow nitrogenase synthesis and function, and to provide a terminal oxidase to permit electron-transport-coupled ATP synthesis which supports the increase in efficiency of nitrogen fixation observed under microaerobic conditions.

Introduction

Nitrogen fixation in the facultative anaerobe *Klebsiella pneumoniae* occurs only in anaerobic or microaerobic conditions. Low O₂ concentrations (optimum about 30 nM) enhance nitrogenase synthesis, activity and efficiency (mols N₂ fixed per mol of carbon substrate consumed) compared with that occurring during anaerobiosis (Hill, 1976, 1988; Hill *et al.*, 1984). This is presumably due to provision of ATP for nitrogenase activity by way of O₂-dependent respiration. Higher O₂ concentrations inhibit both synthesis and activity of nitrogenase, with 50% inhibition occurring at about 100 nM-O₂ which is close to the estimated *K*_s of the dominant terminal oxidase (about 80 nM, estimated from O₂-consumption measurements with whole bacteria) (Bergersen & Turner, 1979; Bergersen *et al.*, 1982; Hill *et al.*, 1984). This raises the important question of the nature of the oxidase(s) responsible for respiration at the very low O₂ (microaerobic) concentrations that support more efficient nitrogen fixation, and which is involved in creating these conditions suitable for derepression of nitrogenase synthesis.

Very little information is available on the cytochromes of *K. pneumoniae* and none at all is available for nitrogen-fixing conditions (Harrison, 1972) but, because *K. pneumoniae* is related to *E. coli*, it might be expected that their cytochrome complements would be similar. *E. coli* has two terminal oxidases, an *o*-type oxidase and a *d*-type oxidase (Anraku & Gennis, 1987; Ingledew & Poole, 1984). They are similar in oxidizing ubiquinol but they differ in their O₂-affinity and regulation of their synthesis. The cytochrome *o* complex has a lower affinity for O₂ (*K*_m = 2.9 µM) and is predominant during growth at high O₂ concentrations, whereas cytochrome *d* has a higher affinity for O₂ (*K*_m = 0.38 µM) and is predominant at lower O₂ concentrations (Kita *et al.*, 1984*a, b*; Rice & Hempfling, 1978). Observations using antibodies to cytochrome *d* of *E. coli* suggested that the membranes of *K. pneumoniae* are similar to those of *E. coli* in containing a *d*-type oxidase (Kranz *et al.*, 1984; Kranz & Gennis, 1985).

The present paper shows that the sole oxidase detectable during microaerobic nitrogen fixation is a *d*-type oxidase. This has been purified and characterized, and its exceptionally high affinity for O₂ (measured with leghaemoglobin) shown to be compatible with a critical role in supporting microaerobic nitrogen fixation.

Abbreviation: TMPD, 2,3,5,6-tetramethyl *p*-phenylenediamine dihydrochloride.

Methods

Growth of bacteria in continuous culture and preparation of membranes for determination of cytochrome and oxidase content. *K. pneumoniae* (oxytoca) (NCIB 12204) was maintained as described for *E. coli* by Smith *et al.* (1988). The growth medium contained Na_2HPO_4 (73 mM), KH_2PO_4 (18 mM), nitroacetic acid (300 μM) and the following (final concentration in mg l^{-1}): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (60); $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (4.4); Na_2SO_4 (345); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (340); $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (7.6) and ferric citrate $\cdot 5\text{H}_2\text{O}$ (49). The medium also contained 50 ml l^{-1} of the trace element solution of Poole *et al.* (1979) (but omitting the cations listed above). The carbon sources were glucose (0.5–2%, w/v), glycerol (1%, w/v) or sodium succinate (50 mM). The nitrogen source was ammonium sulphate (6 mM), tryptone (0.7–1.0 g l^{-1}) or 'white spot' N_2 gas. All anaerobic cultures were sparged with a stream (100 ml min^{-1}) of 'white spot' N_2 . When necessary the O_2 transfer rate was adjusted by diluting inflowing air with N_2 or by altering the stirrer speed.

The bacteria were grown in continuous culture at 30 °C in an 800 ml working-volume vessel at a dilution rate of 0.15 h^{-1} . The pH was controlled to 6.7 \pm 0.2 by automatic addition of 2 M-NaOH or 1 M-HCl. The dissolved O_2 concentration in the culture was measured with a lead/silver oxygen electrode (LH Engineering) calibrated at 0% and 20% air-saturation. The lowest recordable O_2 concentration was about 1 μM . Nutrient concentrations are given in Table 1.

Bacteria were considered to be in steady-state conditions when the cell density was constant and there was no measurable limiting nutrient. The time allowed for a new steady-state to be established after a change in growth conditions was six culture volume replacements. Culture samples were tested for purity and for the *Nif*⁺ phenotype by plating on minimal agar medium plus appropriate carbon source, on nutrient agar and on nitrogen-free solid medium (incubated anaerobically) as described by Cannon (1980). A given nutrient was considered to be growth-limiting when a decrease in its concentration led to a proportional decrease in cell density, when it was not detectable in the culture, and when an increase in concentration of non-limiting nutrients (nitrogen, oxygen or carbon) led to no change in cell density.

Bacteria for preparation of membranes were collected from 'overspill' culture (10 litres) (at 4 °C) over a few days. Bacterial suspensions were concentrated (5-fold) using a Pellicon membrane system (Millipore) and then harvested, and membrane fractions prepared exactly as described by Smith *et al.* (1988).

The same medium as that described above was used for growth on a large scale for preparation of the *d*-type oxidase except that glucose was replaced by 0.1% glycerol plus 50 mM-sodium succinate, the tryptone concentration was increased to 4 g l^{-1} , ammonium sulphate (20 mM) and yeast extract (1 g l^{-1}) added and the phosphate concentration doubled (to 192 mM). Bacteria were grown in batch culture in a 20 litre fermenter at 30 °C and harvested well-into the stationary phase (at 4 d). The culture was sparged with 4 litres of air min^{-1} with an agitation rate of 150 r.p.m. After visible growth had occurred dissolved O_2 was not detectable.

Spectrophotometric characterization of haems, cytochromes and oxidases. All spectra were recorded on a Shimadzu UV-3000 dual-beam/dual-wavelength spectrophotometer fitted with an attachment for measurements at 77 K when required. A spectral bandwidth of 2 nm, a lightpath of 10 mm (or 2 mm, when stated) and a scan speed of 100 nm min^{-1} were used. For characterization of the haems present in purified oxidase or in membrane preparations and whole bacteria the alkaline pyridine haemochrome method of Fuhrop & Smith (1975) was used. Immediately after production of the haemochrome, the dithionite-reduced-minus-hydrogen-peroxide-oxidized difference spectrum was recorded. For determination of cytochromes in membranes, samples in 20 mM-Tris/HCl (pH 8.0) containing 1 mM-EDTA were oxidized by air (cytochrome *d*) or by addition of a crystal of ammonium

persulphate or potassium ferricyanide. They were reduced by addition of sodium dithionite or appropriate respiratory substrate (20 mM). The extinction coefficients for reduced-minus-oxidized spectra were those used for determination of cytochromes in *E. coli*: total cytochrome *b*, 17.5 $\text{cm}^{-1} \text{mm}^{-1}$ (558–575 nm) (Kita *et al.*, 1984a); cytochrome *d*, 7.4 $\text{cm}^{-1} \text{mm}^{-1}$ (628–607 nm) (Lorence *et al.*, 1986). Determination of cytochrome *o* was from (reduced plus CO)-minus-reduced spectra recorded after sparging with CO for 2 min. The extinction coefficient was that used for studies of *E. coli* cytochrome *o*: 145 $\text{cm}^{-1} \text{mm}^{-1}$ (416–430 nm) (Kita *et al.*, 1984a).

Purification of the d-type cytochrome oxidase. The method was based on that of Miller & Gennis (1983), the main difference being the replacement of chromatography on hydroxyapatite with anion-exchange chromatography on Pharmacia Mono-Q. Membranes from 30 g wet wt of bacteria were suspended in 20 mM-Tris/HCl (pH 8.0) containing 1 mM-EDTA and 0.5 M-KCl and centrifuged at 150 000 *g* for 90 min. They were resuspended in the same buffer (lacking KCl) and sodium deoxycholate added to give a final concentration of 0.2% and final protein concentration of 5 mg ml^{-1} . The suspension was stirred for 15 min on ice, the membranes sedimented (as above), and suspended in 75 mM-potassium phosphate (pH 6.3), containing 150 mM-KCl and 1 mM-EDTA (buffer A). The oxidase was solubilized from the membranes by addition of 120 mM-Zwittergent 3-12 (Calbiochem) to give a final detergent concentration of 60 mM and a membrane protein concentration of 12 mg ml^{-1} . The mixture was stirred on ice for 30 min and centrifuged at 150 000 *g* for 2 h. Chromatography of the supernatant on DEAE-Sepharose FF (Pharmacia) was done essentially as described by Miller & Gennis (1983). Pooled active fractions were concentrated about 3-fold by ultrafiltration (Amicon YM-50 membrane) to give a solution of 1 $\text{mg protein ml}^{-1}$, which was desalted on a Pharmacia PD10 column equilibrated with buffer A containing 4 mM-Zwittergent 3-12 and chromatographed on a 1 ml Mono-Q column equilibrated in the same buffer, using a 33 ml linear NaCl gradient (150–400 mM). Peak fractions were concentrated to 1 $\text{mg protein ml}^{-1}$ and glycerol added to a final concentration of 10% (v/v) before storage in liquid nitrogen.

Analytical gel filtration. This was done on a Pharmacia Superose 12 column equilibrated with buffer A containing 4 mM-Zwittergent 3-12 and run at a flow rate of 0.3 ml min^{-1} .

Determination of cytochrome d during purification. This was based on the oxidation of 2,3,5,6-tetramethyl *p*-phenylenediamine dihydrochloride (TMPD). This was dissolved (1 mg ml^{-1}) in 20 mM-Tris/HCl (pH 7.9) containing 0.05% Triton X-100. Samples of column fractions (10 μl) were mixed with 200 μl of fresh TMPD reagent in wells of microtitre plates and absorbance at 630 nm recorded within 2 min using a microtitre plate reader (Dynatech).

Protein determination and amino acid composition. Protein was measured by the bicinchoninic acid method adapted for use on microtitre plates as described by Smith *et al.* (1988). For determination of its amino acid composition 100 μg of purified oxidase was precipitated from solution with an equal volume of ice-cold acetone, washed twice in acetone, suspended in 100 μl of AnalR water (BDH) and transferred to a hydrolysis tube. HCl plus phenol (0.5 ml of 5 M-HCl containing 0.1% phenol) were added and the tube evacuated before hydrolysis at 110 °C for 24 h. Amino acids were determined on a Perkin Elmer amino acid analyser.

SDS-PAGE and assay for Nif polypeptides. SDS-PAGE was done as described by Laemmli (1970) except that samples contained a final concentration of 4% (w/v) SDS and were not heated unless specifically stated. Gels were stained with Coomassie Brilliant Blue or with silver stain (Bio-Rad). *Nif* polypeptides were determined by immunoblot analysis as described by Smith *et al.* (1988).

Determination of oxidase activity in the oxygen electrode. O_2 consumption was measured in a Rank oxygen electrode at 30 °C in a 1 ml reaction mixture containing 50 mM-Tris/HCl (pH 7.0 or 7.9), BSA (1 mg ml⁻¹) and 0.05% Tween 20 (Sigma) or Triton X-100 (BDH). Electron donors were either 100 μ M-ubiquinol-1 (half its K_m) (a gift from Hoffman La Roche), 1 mM-duroquinol (Sigma) or 1.0 mM-TMPD (Sigma). Dithiothreitol (8 mM) was used to maintain the quinols in the reduced state, and 5.0 mM-ascorbate to keep TMPD in its reduced state. Assays using duroquinol were done at pH 7.9.

Determination of the O_2 -affinity of the *d*-type cytochrome oxidase. This was measured by following the deoxygenation of leghaemoglobin. Ferric azide leghaemoglobin was a kind gift of F. J. Bergersen (CSIRO, Canberra, Australia); it was converted for use to the ferrous oxygenated form by the method of Bergersen & Turner (1979) and oxygen affinity measured as described by Appleby & Bergersen (1980). A glass cuvette contained 50 μ M-oxyleghaemoglobin, 0.05% Tween 20, 55 μ M-ubiquinol-1 and 8 mM-dithiothreitol in 50 mM-sodium phosphate buffer (pH 7.4), to give a total volume of 1.9 ml completely filling the cuvette which was sealed with a Suba-seal so as to exclude all air. The spectrum of ferrous oxyleghaemoglobin was recorded in the dual-wavelength mode between 500 nm and 600 nm (reference wavelength 587 nm) using a buffer baseline. Purified oxidase (5 μ g) was added and the change in fractional saturation of oxyhaemoglobin against time was recorded at 575 nm (against a reference of 561 nm). The free dissolved O_2 concentration and total O_2 in the cuvette were calculated at 6 s intervals during deoxygenation by the method of Bergersen & Turner (1979) using the published binding constant of ferrous leghaemoglobin for oxygen of 43.5 nM. Steady-state kinetic data were fitted to the Michaelis-Menten equation by computer-aided iterative regression (Biodata Handling with Microcomputers by R. B. Barlow, Elsevier-Biosoft).

Reconstitution of the *d*-type oxidase with phospholipid. Phospholipid stocks (Sigma) were stored in chloroform/methanol (2:1, v/v) at -20 °C. Samples were dried under N_2 and solubilized by sonication until clear in 100 mM-Tris/HCl (pH 7.9) containing 50 mM-sodium cholate (recrystallized from methanol) to give a final concentration of 5–20 mg phospholipid ml⁻¹. Total bacterial phospholipid was prepared by solvent extraction of 50 g wet wt *K. pneumoniae* by the method of Bligh & Dyer (1959) as applied by Ames (1968). This was solubilized in the same way as the pure phospholipids. Equal volumes of purified oxidase (1.1 mg ml⁻¹) and solubilized phospholipid were incubated together for 1 h at room-temperature and a 10 μ l sample of the incubation mixture diluted 100-fold into the assay mixture used for measurement of activity in the oxygen electrode as described above (the neutral detergent was omitted from the reaction mixture).

Results

Cytochromes of *K. pneumoniae*

Spectra of pyridine haemochromes prepared from membrane fractions were characteristic of haem *b*, haem *d* or both (see Barrett, 1956); no haem *c* or haem *a* was detected (data not shown). Very low concentrations of cytochrome *b* were detected in soluble fractions, but these were not investigated further.

The spectra presented in Figs 1–3 confirm that the only cytochromes in membranes of *K. pneumoniae* were *b*- and *d*-type cytochromes. Fig. 1 shows the spectra of membranes of bacteria grown in microaerobic condi-

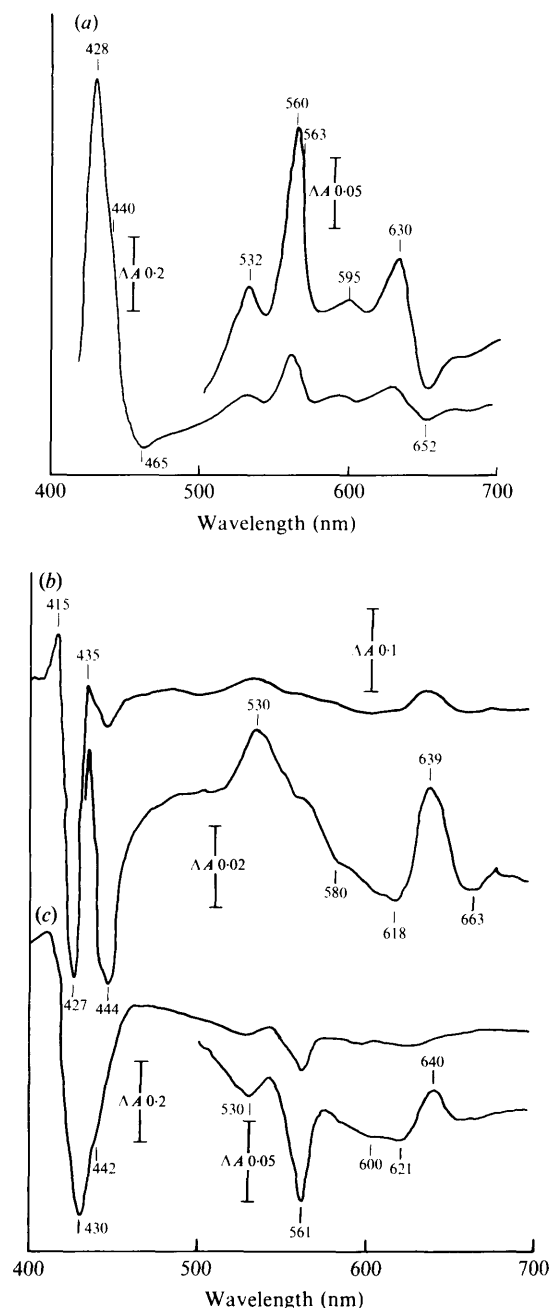


Fig. 1. Absorption spectra of membranes from bacteria grown on succinate with tryptone as nitrogen source in O_2 -limited, microaerobic conditions. The protein concentration was 11.3 mg ml⁻¹. (a) Dithionite-reduced-minus-persulphate-oxidized difference spectrum; (b) (reduced plus CO)-minus-reduced difference spectrum (after sparging with CO for 30 s); (c) after sparging with CO for 2 min.

tions (O_2 -limited) with succinate, and tryptone as the nitrogen source. In these conditions *b*-type cytochromes (adsorbing at 557–562 nm) and cytochrome *d* (peak at 630 nm) were present. In the absence of any haem *a* the peak at 595 nm almost certainly corresponds to the

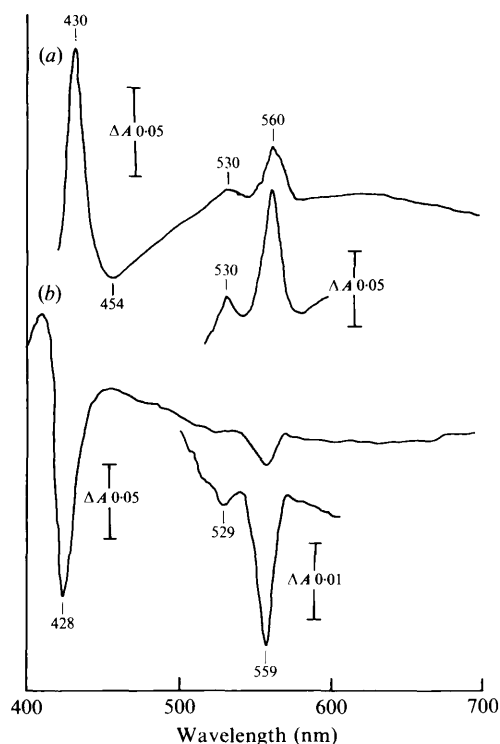


Fig. 2. Absorption spectra of membranes from bacteria grown on glycerol with tryptone as nitrogen source in nitrogen-limited conditions (O_2 concentration 10–20 μM). The protein concentration was 6.2 mg ml⁻¹. (a) Dithionite-reduced-minus-persulphate-oxidized difference spectrum; (b) (reduced plus CO)-minus-reduced difference spectrum after sparging with CO for 2 min.

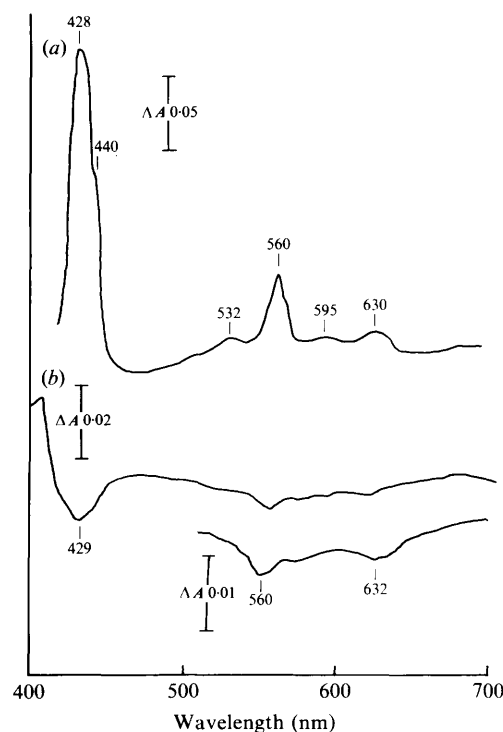


Fig. 3. Absorption spectra of membranes from nitrogen-fixing bacteria grown on glucose in O_2 -limited, microaerobic conditions (O_2 concentration <1 μM). The protein concentration was 18 mg ml⁻¹. (a) Dithionite-reduced-minus-persulphate-oxidized difference spectrum; (b) (reduced plus CO)-minus-reduced difference spectrum after sparging with CO for 2 min.

cytochrome *b*-595 component of the *d*-type cytochrome oxidase of *E. coli* (Lorence *et al.*, 1986; Poole, 1988); this component was also seen in the spectrum of the purified oxidase (see below). Fig. 1 (*b*, *c*) shows that some of the *b*-type cytochrome (troughs at about 430 nm and 560 nm) and the cytochrome *d* (trough at about 620 nm) were able to react with CO, indicating the presence of some cytochrome *o* (an oxidase containing haem *b*), and confirming the identification of the component absorbing at 630 nm as part of a *d*-type oxidase (see Poole, 1988 for a review of the spectra of these potential oxidases).

The spectra shown in Fig. 2 demonstrate that cytochrome *d* was absent during growth in aerobic conditions (O_2 excess) on glycerol with tryptone as the limiting nutrient. In these growth conditions the only CO-reactive species was a *b*-type cytochrome, presumably cytochrome *o*.

Fig. 3 shows the spectra of membranes from bacteria grown on glucose with N_2 as nitrogen source and O_2 as the growth-limiting nutrient, which indicate that in these microaerobic conditions cytochromes *b* and *d* were present. The spectrum shown in Fig. 3(*b*) is probably due to the CO reactivity of some of the haem *b* of the *d*-type

oxidase, as shown in spectra of the pure protein (Figs 5 and 6). If the spectrum were assumed to be due to the presence of cytochrome *o* then its content would be 6 nmol (mg protein)⁻¹, which is about 10% of the cytochrome *d* content and less than 2% of the cytochrome *o* present in any other growth conditions.

These results indicate that *K. pneumoniae* is very similar to *E. coli* in having two potential oxidases, cytochrome *o* and cytochrome *d*, the relative amounts being determined by the growth conditions.

Table 1 summarizes the results of a broader survey of the effect of growth conditions on synthesis of the cytochromes of *K. pneumoniae*. It is evident from these results that in all anaerobic conditions the major potential oxidase was *d*-type oxidase; this was regardless of the nitrogen source (N_2 , ammonia or tryptone). It is possible that the cytochrome *o* is repressed by glucose as is the case in *E. coli* for other respiratory enzymes such as succinate dehydrogenase (Ingledew & Poole, 1984). By contrast, cytochrome *d* synthesis was completely repressed in the presence of excess O_2 , as shown previously with the oxidase of '*Klebsiella aerogenes*' (Moss, 1956; Harrison, 1972) and of *E. coli* (Ingledew & Poole, 1984).

Table 1. *Effect of growth conditions on synthesis of cytochromes in continuous culture*

K. pneumoniae was grown as described in Methods with a dilution rate (*D*) of 0.15 h⁻¹. The glucose concentration was 0.5% except for growth conditions 2 and 11, when it was 2%. The concentration of ammonium chloride in 3 and 7 was 6 mM. The concentration of fumarate in 4 was 30 mM. The concentration of tryptone was 0.5 g l⁻¹ in condition 5, 0.7 g l⁻¹ in 9 and 11, and 1 g l⁻¹ in 8 and 10. Cytochrome concentrations in membranes were measured from difference spectra of the sort illustrated in Figs 1–3; the values presented for cytochrome *b* are total values including the cytochrome *o*. Although the results presented here are from single steady-state cultures, each growth state was repeated at least once and the results were always within 10% of those recorded here. The lowest concentration of the cytochromes that could be determined with confidence was 10 pmol (mg membrane protein)⁻¹. In microaerobic conditions (O₂-limited) the O₂ concentration was below the limit of detection by the oxygen electrode (1 µM). In aerobic conditions the O₂ concentrations were 10–200 µM. All cultures grown with N₂ as sole nitrogen source were shown to contain Nif polypeptides as detected by immunoassay. In addition to these cultures, microaerobic cultures with glycerol as carbon source and tryptone as nitrogen source also had low levels (about 5% of nitrogen-fixing cultures) of Nif polypeptides; this is indicated by +/– in the Table. ND, Not detected [<10 pmol cytochrome (mg membrane protein)⁻¹].

Carbon source	Oxygen status	Nif polypeptides	Growth limitation	Nitrogen source	Cytochrome concn [pmol (mg protein) ⁻¹]		
					Cyt. <i>b</i>	Cyt. <i>o</i>	Cyt. <i>d</i>
1. Glucose	Anaerobic	+	Carbon	N ₂	120	ND	60
2. Glucose	Anaerobic	+	Nitrogen	N ₂	110	ND	40
3. Glucose	Anaerobic	–	Carbon	Ammonia	100	ND	40
4. Glucose + fumarate	Anaerobic	–	Carbon	N ₂	160	ND	70
5. Glucose	Anaerobic	–	Carbon	Tryptone	110	ND	50
6. Glucose	Microaerobic	+	Oxygen	N ₂	130	ND	60
7. Glucose	Microaerobic	–	Oxygen	Ammonia	150	ND	60
8. Glycerol	Microaerobic	+/–	Oxygen	Tryptone	560	180	170
9. Succinate	Microaerobic	–	Oxygen	Tryptone	640	430	390
10. Glycerol	Aerobic	–	Nitrogen	Tryptone	360	220	ND
11. Succinate	Aerobic	–	Nitrogen	Tryptone	500	290	ND

In the present work with *K. pneumoniae*, both oxidases were only present together during respiratory growth in O₂-limited conditions with glycerol or succinate, when the total cytochrome content was also at a maximum.

The only oxidase detected during nitrogen fixation was the *d*-type oxidase. This was true for all nutrient limitations tested (nitrogen, carbon or oxygen) and for growth in which the main energy source was fermentation (anaerobic) or respiration (microaerobic).

These results suggest that the nitrogen fixation occurring under microaerobic conditions may be supported by way of a respiratory chain involving cytochrome *d*. In order to investigate further this possibility, the cytochrome *d* complex was purified and characterized with respect to its O₂-affinity.

Purification of the d-type oxidase

When grown on succinate under O₂-deficient conditions this oxidase constituted about 15% of the membrane protein. This was extracted and purified as described in Methods. Washing the membranes with 0.5 M-KCl removed about 20% of the total cytochrome *b* while leaving all the cytochrome *d* in the membranes. Extraction of these washed membranes with 0.2% deoxycholate removed little of the cytochrome *d* but

increased its specific content by 1.5-fold. This step was particularly useful because it led to selective release of D-lactate dehydrogenase and cytochrome *b*-556. Solubilization of the cytochrome *d* from the resulting enriched membranes was then achieved with the zwitterionic sulphobetaine detergent Zwittergent 3-12 as described by Miller & Gennis (1983), whose procedure forms the basis of this purification method. The results of the purification procedure are summarized in Table 2. The yield was about 10% and the increase in purity about 10-fold on the basis of specific cytochrome *d* content. The oxidase could not be purified further by any methods attempted and it eluted as a single symmetrical peak during FPLC gel-filtration on Pharmacia Superose 12. On the basis of this, and of SDS-PAGE, and spectral analysis ($A_{412}/A_{280} = 0.92$) it was concluded that the oxidase was at least 95% pure.

M_r of components of the purified oxidase

SDS-PAGE showed that the oxidase consisted of two polypeptides, designated I and II. The larger subunit (I) had an apparent *M_r* of 52000. As found with the enzyme from *E. coli* (Miller & Gennis, 1983), the *M_r* of subunit II depended on the gel density during electrophoresis. To resolve this problem the *M_r* was measured for gels with a

Table 2. Purification of the *d*-type cytochrome oxidase

The purification procedure is fully described in Methods. The cytochrome *b*-558 component was determined from the reduced-minus-oxidized difference spectrum, using an extinction coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$ (560–580 nm) (Green *et al.*, 1986); the cytochrome *d* component was determined from the reduced-minus-oxygenated difference spectrum, using an extinction coefficient of $7.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (628–607 nm) (Lorence *et al.*, 1986). The values given for the membranes are prior to detergent washing. The purification factor presented is based on specific cytochrome *d* content. When based on activities of the oxidase measured in 0.05% Triton X-100 using ubiquinol-1 as substrate, the purification factor was 5.6 overall, presumably due to loss of some activity during purification.

Purification step	Protein (mg)	Cytochrome concn [nmol (mg protein) ⁻¹]		A_{412}/A_{280}	Purification (-fold)	Yield (%)
		Cyt. <i>b</i> -558	Cyt. <i>d</i>			
Membranes	638	1.7	2.0	—	1	100
Solubilized membranes	224	3.7	4.8	0.12	2.5	87
DEAE-Sepharose	27	10.4	13.3	0.54	6.8	29
Mono-Q	6	15.6	19.3	0.92	9.9	9

range of cross-linking (10–15%, w/v, acrylamide) and the M_r determined by Ferguson analysis (Hendrich & Smith, 1978). This gave M_r values for the subunits of 52000 and 36000. The aggregation state of the dissociated subunits was very temperature-sensitive, 2 min heating (at 70 °C) during sample preparation for SDS-PAGE resulting in complete aggregation of subunit I and its retention at the gel boundary. Boiling samples for 2 min prevented either subunit from entering the gels. Often a larger polypeptide (M_r 80000) was seen as a faint band. This was absent when the subunits were separated by preparative SDS-PAGE and then run separately. When the subunits were mixed this band was once more seen, suggesting that it was due to association of the subunits during electrophoresis. During gel-filtration on Pharmacia Superose 12 in 4 mM-Zwittergent 3-12 the oxidase eluted as a single peak corresponding to an apparent M_r of about 70000, suggesting that it is a heterodimer with subunits of 52000 and 36000. When this was repeated in 10 mM-deoxycholate the oxidase was seen to be very much more highly aggregated, the apparent M_r being about 240000.

Cytochrome composition of the purified oxidase

The absorption spectra presented in Fig. 4 show two cytochrome *b* components (absorbing at about 560 nm and 595 nm) and the cytochrome *d* (absorbing at about 630 nm) in the reduced form. Fig. 5 shows the characteristic absorption of the oxygenated form at

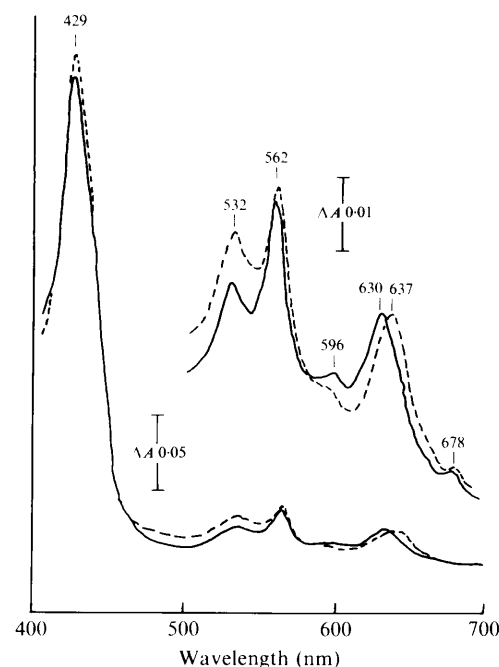


Fig. 4. Absorption spectra (room-temperature) of dithionite-reduced *d*-type cytochrome oxidase. The sample contained 65 μg purified oxidase ml^{-1} in 75 mM-potassium phosphate (pH 6.3) containing 150 mM-KCl, 1 mM-EDTA and 4 mM-Zwittergent 3-12. —, Dithionite-reduced oxidase; ----, dithionite-reduced oxidase after sparging with CO for 30 s.

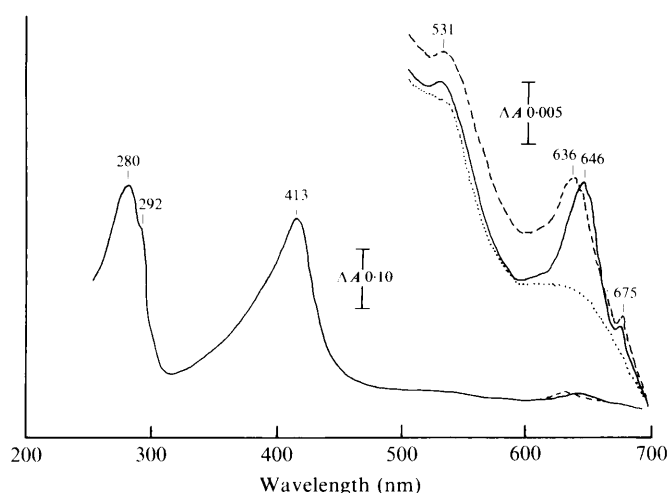


Fig. 5. Absorption spectra (room-temperature) of the air-oxidized form of the *d*-type cytochrome oxidase. Conditions are as described in Fig. 4. Spectra are as follows: —, air-oxidized; ----, air-oxidized after sparging with CO for 30 s; ····, spectrum after removal of all O_2 from a sealed cuvette by addition of 5 mM-ubiquinol-1 and 16 mM-dithiothreitol followed by oxidation with a trace of hydrogen peroxide (6 μl 2%, v/v, H_2O_2).

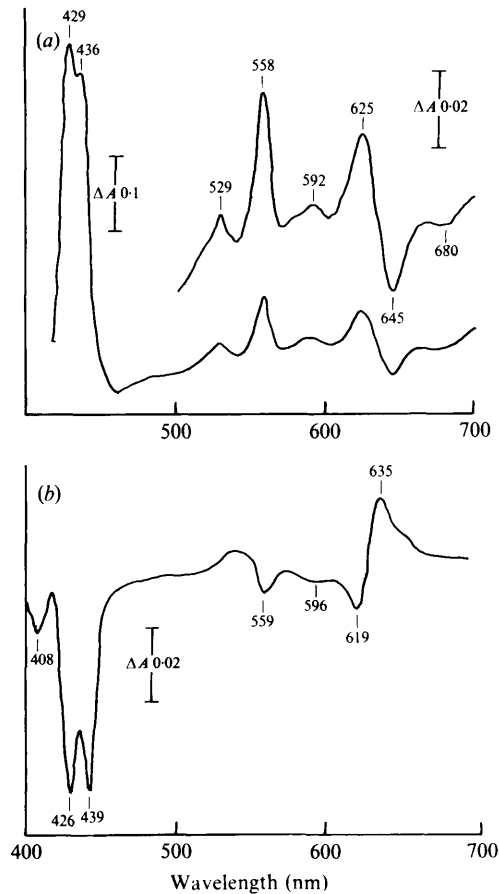


Fig. 6. Low-temperature difference spectra (77 K) of the *d*-type cytochrome oxidase. Conditions are as described in Fig. 4 except that the protein concentration was doubled ($130 \mu\text{g ml}^{-1}$) and the lightpath was 2 mm. (a) Dithionite-reduced-minus-air-oxidized (oxygenated) difference spectrum; (b) (dithionite-reduced plus CO)-minus-dithionite-reduced difference spectrum (CO was sparged for 30 s before freezing).

about 650 nm, which is due to formation of a stable oxygenated intermediate (Poole *et al.*, 1983). The spectrum of the presumed oxidized form (prepared by addition of hydrogen peroxide in the absence of O_2) is also shown. The low-temperature spectrum (Fig. 6a) shows that the peak associated with cytochrome *b*-558 was not split and so probably represents a single component. Fig. 6(b) shows that, over a period of 2 min, CO reacted with both components, cytochrome *b* and cytochrome *d*. Formation of pyridine haemochromes showed that no haem *a* was present confirming that the peak at 595 nm (Fig. 6a) was not due to cytochrome *a*₁ but was due to a component containing haem *b* as demonstrated previously for the *E. coli* enzyme (Lorence *et al.*, 1986). The absorption spectra shown in Figs 4–6 confirm that the purified oxidase is almost identical to that of *E. coli* (Miller & Gennis, 1983; Kita *et al.*, 1984b).

The spectra also indicate that the cytochrome *b*-558, cytochrome *b*-595 and cytochrome *d* are present in the ratio 1.08:1.00:1.4. These ratios are very similar to those determined for the *E. coli* enzyme by Lorence *et al.* (1986), who concluded that there are 2 mols of cytochrome *d* per mol of oxidase complex. It should be noted, however, that more recent estimates based on EPR spectroscopy indicate that there is only 1 mol each of cytochrome *b*-595 and cytochrome *d* per mol of the *E. coli* oxidase (Rothery & Ingledew, 1989).

Amino acid composition of the oxidase

The amino acid composition was similar to that published by Miller & Gennis (1983) for the *E. coli* enzyme whose values are presented in the following list (percentage of each amino acid per mol): Asx, 7.8 (8.2); Thr, 4.2 (6.2); Ser, 3.2 (4.9); Glx, 7.6 (7.2); Gly, 11.7 (9.3); Ala, 10.6 (11.5); Val, 8.4 (8.8); Met, 4.4 (3.5); Pro, 4.0 (4.1); Ile, 5.5 (7.1); Leu, 13.0 (12.8); Tyr, 3.7 (3.4); Phe, 8.0 (6.9); His, 2.3 (1.5); Lys, 2.8 (4.1); Arg, 1.6 (1.4).

Antisera raised to the complete oxidase of *K. pneumoniae* reacted with a polypeptide on Western blots of SDS extracts of *E. coli* membranes that comigrated with subunit I of the *K. pneumoniae*. Subunit II failed to transfer efficiently during blotting as noted previously by Kranz & Gennis (1985).

Substrate specificity and kinetic studies of the oxidase

Table 3 presents the steady-state kinetic parameters of the purified oxidase which demonstrate that the preparation is an active oxidase. The pH optimum with ubiquinol-1 as substrate was 7.9 (± 0.1). The specific activity was about 30% of that of the oxidase isolated from *E. coli* (Miller & Gennis, 1983). The turnover number (k_{cat}) of the oxidase (in terms of cytochrome *d*

Table 3. Steady-state kinetic parameters of the *d*-type cytochrome oxidase

All reaction mixtures contained 0.05% Triton X-100 and all rates were corrected for any auto-oxidation of substrates. ND, Not determined.

Substrate	pH	K_m (mM)	V [$\mu\text{mol O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$]
Ubiquinol-1	7.0	0.22	50
Ubiquinol-1	7.9	0.21	117
Duroquinol	7.0	0.19	10
Duroquinol	7.9	ND	18
TMPD	7.0	2.8	32

content) was 400 s^{-1} at pH 7.9 with ubiquinol-1 as substrate and with 0.05% Triton X-100 in the reaction mixture. This compares with an estimated turnover number *in vivo* (for a range of growth states) of 300–700 s^{-1} . The natural substrate for the oxidase is likely to be ubiquinol-8, the predominant form of quinone in enteric bacteria. When using ubiquinol-1 as substrate the K_i for KCN was 0.33 mM; this compares with the value of 2 mM-KCN required for 50% inhibition of the enzyme from *E. coli* (Anraku & Gennis, 1987).

Requirement for activity of the oxidase for detergent or neutral lipid

The oxidase was similar to mitochondrial cytochrome aa_3 in that it was largely inactive after isolation and purification when assayed in the absence of phospholipid or neutral detergents (Table 4). There was no activity when the charged detergent (cholate) or the zwitterionic detergent (Zwittergent 3-12) that were used in the solubilization and purification of the enzyme replaced the neutral detergents Tween or Triton as shown in preliminary studies of the *E. coli* oxidase (Lorence *et al.*, 1986). Exchanging Zwittergent 3-12 with cholate by gel-filtration prior to assay had no effect on the measured rates. The optimum concentration of Tween 20 in the assay mixture was about 0.05%. Above

0.2% this detergent was inhibitory. The results in Table 4 also show that the neutral detergent was not required when the oxidase was first reconstituted with phospholipid prior to assay. The highest activity was obtained with phospholipids extracted from *K. pneumoniae*, but rates were not much lower when phosphatidylglycerol or phosphatidylethanolamine were used; these are the main phospholipids of membranes of *E. coli*, and probably also of *K. pneumoniae* (Ames, 1968).

Affinity of pure *d*-type oxidase for O_2

Estimates of the O_2 -affinity of the cytochrome *d* of other bacteria have been published but these have been based on measurements with oxygen electrodes which are not suitable for accurate determinations at low O_2 concentrations (Hill, 1988). The purified *d*-type oxidase from *K. pneumoniae* was able to catalyse the removal of O_2 from a solution containing soyabean oxyleghaemoglobin (K_s 43.5 nM) resulting in its complete deoxygenation. The rate of the later phase of deoxygenation was used to calculate the steady-state O_2 -consumption kinetics at concentrations of free O_2 below 200 nM. The results (not shown) demonstrated that the oxidase has a single high-affinity site for binding O_2 with an apparent K_m value of 20 nM. This value is much lower than that published for any other purified bacterial oxidase and, in particular, it

Table 4. Dependence of *d*-type cytochrome oxidase activity on detergents or phospholipids

Activity was measured in the standard oxygen electrode assay system with duroquinol (1 mM) as substrate using oxidase isolated in Zwittergent 3-12. The values in parentheses are for oxidase which was first transferred to 5 mM-cholate by passage down a PD10 gel-filtration column. To measure the effect of phospholipid on activity of the oxidase equal volumes of purified oxidase were mixed with phospholipid (previously solubilized in Tris/HCl buffer (pH 7.9) containing 50 mM-cholate as described in Methods. The final concentrations of phospholipid in the 'reconstitution' mixtures were as follows (mg ml^{-1}): phosphatidylcholine (10); phosphatidylglycerol (9.5); phosphatidylethanolamine (5.0); and total phospholipid from *K. pneumoniae* (5.0). Activity of the oxidase was then determined by using 0.01 ml of this mixture in a 1 ml reaction mixture in an oxygen electrode. All the 'reconstitution' assays recorded below therefore contain 0.5 mM-cholate. For the control recorded in the Table, cholate was omitted from the 'reconstitution' mixture. The recorded values are means of three assays, all of which gave values within 5% of those recorded. Although no systematic examination of the effect of phospholipid concentration was undertaken, doubling of the phospholipid concentrations did not increase the activities above the values recorded below. It should be noted that the incubation period in Tris buffer, which was part of the 'reconstitution' process, led to a decrease to 48% of the specific activity of the oxidase (assayed in the presence of Tween 20); the activities recorded after 'reconstitution' should therefore be compared with a control value of $5.7 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Activity in standard assay		Activity after 'reconstitution'	
Detergent	Specific activity [$\mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$]	Phospholipid	Specific activity [$\mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$]
None	0.02 (0.02)	None	0.70
Triton X-100 (0.05%)	4.93 (5.22)	Phosphatidylcholine	1.04
Tween 20 (0.05%)	11.94 (13.00)	Phosphatidylglycerol	7.46
Cholate (0.05%)	0.07 (0.14)	Phosphatidylethanolamine	7.02
Zwittergent (0.5%)	0.02 (0.02)	Total phospholipid from	8.50
Cholate (0.05%) plus Tween 20 (0.05%)	7.31	<i>K. pneumoniae</i>	

is much lower than that measured for the purified oxidase of *E. coli* (380 nM) (Kita *et al.*, 1984*b*). However, the value for the *E. coli* enzyme was obtained from measurements with an oxygen electrode and hence is likely to be subject to error for measurements at low O₂ concentrations. The value assigned to the oxidase in whole cells of *E. coli* (Rice & Hempfling, 1978) was closer to the value measured for the pure oxidase from *K. pneumoniae* in the present work.

It has been previously shown that 50% inhibition of activity and synthesis of nitrogenase occurred at about 100 nM-O₂ which was close to the *K_s* of the dominant terminal oxidase; this was estimated from O₂-consumption measurements with whole bacteria using the same system for O₂ determination as used in the present work for measurement of the *K_m* value for the purified oxidase (Bergersen & Turner, 1979; Bergersen *et al.*, 1982; Hill *et al.*, 1984). The estimated value using whole bacteria was about 80 nM. The apparent discrepancy between this value and that for the oxidase purified in the present work (20 nM) must presumably be due to O₂-gradients between the growth medium and the site of binding of O₂ to the oxidase.

Discussion

The results described above confirm that *K. pneumoniae* contains a *d*-type oxidase very similar to that previously described in *E. coli* (Miller & Gennis, 1983; Lorence *et al.*, 1986; Miller *et al.*, 1988; Kita *et al.*, 1984*b*; Finlayson & Ingledew, 1985). The exceptionally high affinity for O₂ may be a unique property of the *K. pneumoniae* *d*-type oxidase.

In *E. coli* no specific role for the oxidases containing cytochrome *d* or cytochrome *o* has been proposed, mutants lacking either enzyme being able to grow normally under laboratory conditions (Au *et al.*, 1985; Green & Gennis, 1983). Georgiou *et al.* (1988) have shown that the regulation of synthesis of cytochrome *d* in *E. coli* is at the level of transcription, the *cyd* operon being transcriptionally inactive at high O₂ concentrations. They concluded that there was no obvious need for the oxidase, except perhaps as an O₂-scavenger, for which there has been no demonstrated need in *E. coli*.

The main feature that distinguishes *K. pneumoniae* from *E. coli* is that *K. pneumoniae* is able to fix nitrogen. In this organism the *d*-type oxidase was always produced in nitrogen-fixing conditions, when it was the sole oxidase. Its most important property in this context is its extremely high affinity for O₂, the highest affinity for any oxidase previously described (*K_m* 20 nM). It is proposed that this ensures that it is able to lower the free O₂ concentration in solution so that nitrogenase synthe-

sis may be derepressed and so that it may be protected from the inhibitory effects of O₂ (respiratory protection) as previously proposed for *Azotobacter* (Yates, 1988). The affinity of the alternative oxidase, cytochrome *o* (*K_m* 200 nM), measured in whole cells of *E. coli*, is about 10% of that of cytochrome *d* and hence usually unable to maintain the low O₂ concentration essential for derepression of nitrogenase (less than about 100 nM). It is also proposed that the *d*-type oxidase is able to function as the terminal oxidase in an energy-generating electron transport chain in order to support an enhanced efficiency of nitrogen fixation at low O₂ concentrations. The presence of this oxidase after anaerobic growth on glucose presumably explains why, after glucose depletion, nitrogen fixation only occurs in the presence of added O₂ (Hill, 1976). We presume that the glucose fermentation products are providing electrons for this electron transport to O₂ by way of the *d*-type oxidase described in this work. To test these proposals it will be necessary to characterize *cyd* mutants with respect to microaerobic nitrogen fixation (it should not occur) and to identify those fermentation substrates which are able to support electron transport by way of cytochrome *d*.

It is clear that this proposed role for the *d*-type oxidase is not directly relevant to its function in *E. coli* as that organism does not fix nitrogen. It is possible that this high-affinity *d*-type oxidase may have developed (evolutionarily) in nitrogen-fixing *Klebsiella* and that its presence in *E. coli* might merely reflect its retention rather than a specific function in this organism. Alternatively, the present work directs attention to the possibility that in *E. coli* there may be some, as yet unidentified, oxygen-sensitive enzymes whose activities provide some benefit to the organism in microaerobic growth conditions.

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