The Oxidation of Nicotinic Acid by *Pseudomonas ovalis* Chester

THE TERMINAL OXIDASE

By M. V. JONES and D. E. HUGHES

Department of Microbiology, University College, Cardiff CF2 1TA, U.K.

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In cell-free extracts of *Pseudomonas ovalis* nicotinic acid oxidase is confined to the wall-membrane fraction. It is associated with an electron-transport chain comprising *b*- and *c*-type cytochromes only, differing proportions of which are reduced by nicotinate and NADH. CO difference-spectra show two CO-binding pigments, cytochrome *o* (absorption maximum at 417 nm) and another component absorbing maximally at 425 nm. Cytochrome *o* is not reduced by NADH or by succinate but is by nicotinate, which can also reduce the '425' CO-binding pigment. The effects of inhibitors of terminal oxidation support the idea of two terminal oxidases and a scheme involving the '425' CO-binding pigment and the other components of the electron-transport chain is proposed.

Many *Pseudomonas* species are able to grow on a wide range of compounds as sole carbon source. This is possible because they have a variety of inducible catabolic pathways (see review by Ornston, 1971). Some of the enzymes involved in these inducible pathways are membrane-bound and associated with an electron-transport chain. By examining the changes that take place during the induction process it is hoped that much more might be learnt about the structural and functional organization of the bacterial membrane. With this aim a study of nicotinic acid oxidation in *Pseudomonas ovalis* Chester was undertaken.

Earlier work on *Pseudomonas fluorescens* KB1 had shown that the first step in nicotinic acid degradation was a hydroxylation (Hughes, 1955) and that this involved the addition of a hydroxyl group derived from water rather than oxygen (Hunt *et al.*, 1958). The enzyme involved has been partially purified by Hunt (1959) who showed it to be particulate and associated with an electron-transport chain involving cytochromes *b* and *c*. In the absence of either cytochrome *a* or *o* it was suggested that cytochrome *c* peroxidase might be functioning as the terminal oxidase.

In this paper we report the presence of cytochrome *o* in *Ps. ovalis*, when grown on nicotinic acid, and examine the roles of this and cytochrome *c* peroxidase in terminal oxidation.

Experimental

Methods

Maintenance and growth of organism. *Pseudomonas ovalis* Chester was maintained on agar slopes of nicotinate medium (Hunt, 1959). For the growth of

larger quantities of cells one loopful of cells was inoculated into a 1-litre conical flask containing 500 ml of liquid medium and incubated on an orbital shaker at 30°C for 18 h. This was used to inoculate a 10-litre glass fermenter (L.H. Engineering, Stoke Poges, Bucks., U.K.) containing 7.5 litres of the same medium. Silicone M/S Antifoam Emulsion, 0.5 ml (Hopkin and Williams, Chadwell Heath, Essex, U.K.), was added to prevent foaming. The culture was aerated (5 litres/min) at 30°C. After 7–8 h of growth the cells were harvested in late-exponential phase of growth in an MSE 6L Mistral centrifuge, and washed once in cold 50 mM-potassium phosphate buffer (pH 7.4) containing 5 mM-MgCl₂. The sedimented cells, after recentrifugation, were used immediately or stored at –20°C.

Preparation of the wall-membrane fraction. The frozen cell paste was crushed in a Hughes (1951) press at –25°C. The crushed cells were homogenized with 2 vol. of cold 50 mM-potassium phosphate buffer (pH 7.4) containing 5 mM-MgCl₂ in a glass Kontes (Vineland, N.J., U.S.A.) homogenizer. Approx. 0.1 mg of crystalline deoxyribonuclease (DNAase) was added to decrease the viscosity. The mixture was left on ice for 15 min, before centrifuging for 90 min at 100 000 *g* in a MSE 65 High Speed centrifuge (*r*_{av.} = 7.38 cm). The yellow supernatant was carefully decanted and the upper red gelatinous layer of the pellet resuspended in 25 ml of phosphate buffer. This constituted the wall-membrane fraction as previously described (Francis *et al.*, 1963).

Spectrophotometry. Spectra were traced on a Cary 14 split-beam recording spectrophotometer at room temperature in cuvettes of 1 cm light-path. Cytochrome and flavin concentrations were measured from the reduced-minus-oxidized difference spectra by using the following wavelength pairs (nm) and

values of ϵ (litre·mol⁻¹·cm⁻¹): cytochrome *b* (559–575) = 20×10^3 ; cytochrome *c* (552–540) = 19×10^3 ; flavin and non-haem iron (465–510) = 11×10^3 (Chance & Williams, 1955). Cytochrome *o* was estimated from the reduced-plus-carbon monoxide-minus-reduced difference spectrum. CO gas was bubbled through the dithionite-reduced sample until the peak at 417 nm became constant in height, usually after 2–3 min. The value of ϵ recommended by Daniel (1970), of 170×10^3 litre·mol⁻¹·cm⁻¹ for the peak–trough difference (417–432 nm), was used in preference to the peak–plateau extinction coefficient, as this gave more reproducible results.

Oxygen uptake. The O₂ uptake of the wall-membrane fraction and supernatant was measured polarographically with an O₂ electrode (Lloyd & Brookman, 1967). Substrates and inhibitors were added from a microsyringe after the insertion of the electrode.

Enzyme assays. Nicotinic acid hydroxylase was measured by the method of Hunt (1959). Cytochrome *c* oxidase and cytochrome *c* peroxidase were assayed by using mammalian cytochrome *c* by the method of Ellfolk & Soininen (1970). All enzyme assays were done at room temperature.

Other analytical methods. Pyridine haemochromogens were prepared by the method of Falk (1964). The 6-hydroxynicotinic acid was measured from its absorption at 295 nm ($\epsilon = 5.5 \times 10^3$ litre·mol⁻¹·cm⁻¹; Behrman & Stonier, 1958). Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin as the standard.

Chemicals

Deoxyribonuclease, NADH and cytochrome *c* (horse heart type III) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., bovine plasma albumin (fraction V) was obtained from Armour Pharmaceuticals, Eastbourne, Sussex, U.K., 6-hydroxynicotinic acid (2-hydroxypyridine-5-carboxylic acid) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and all other

chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Composition and distribution of the respiratory system

The amounts of cytochrome and flavin present in whole homogenate, cell wall-membrane fraction and supernatant after centrifugation for 9×10^6 g·min are shown in Table 1. Clearly, the cytochromes are largely associated with the wall-membrane fraction and the supernatant contains only flavin and a small amount of cytochrome *c*. In the difference spectrum of the wall-membrane fraction (Fig. 1, curve *a*) there is no evidence of *a*-type cytochrome. Extraction of this fraction with acidified acetone and formation of alkaline pyridine haemochromogens also failed to show the presence of any haem *a*. The wall-membrane fraction oxidized nicotinate rapidly (Table 2) and 0.5 mol of O₂/mol of nicotinate was consumed. The nicotinate was quantitatively converted into 6-hydroxynicotinate which, after removal of the wall-membrane material by centrifugation, could be measured by its characteristic u.v. spectrum (Hughes, 1955). The 6-hydroxynicotinate was not oxidized further by the wall-membrane fraction but was slowly oxidized by the supernatant (Table 2). The cells were also found to grow readily on 6-hydroxynicotinate as sole carbon source. The nicotinate oxidase system in *Ps. ovalis* Chester therefore closely resembles those previously described in *Ps. fluorescens* KB1 (Hughes, 1955) and *Ps. fluorescens* N9 (Behrman & Stanier, 1958).

The wall-membrane fraction also showed NADH and succinate oxidase activities and high ascorbate+NNN'N'-tetramethylphenylenediamine oxidase activity. The supernatant did not oxidize succinate although some oxidation of NADH and ascorbate+NNN'N'-tetramethylphenylenediamine was observed (Table 2).

Table 1. *Distribution of the respiratory components in Pseudomonas ovalis* Chester

Cytochrome and flavin contents were estimated from the reduced-minus-oxidized difference spectra. Fractionation of the cells is described in the Experimental section. Concentrations, in nmol/mg of protein, are of a typical preparation.

Fraction ...	Content of electron-transport components		
	Whole homogenate	Wall-membrane fraction	Supernatant fraction
Cytochrome <i>c</i>	0.23	0.34	0.03
Cytochrome <i>b</i>	0.19	0.35	0.0
Cytochrome <i>o</i>	0.05	0.10	0.0
Flavin	1.70	0.51	1.37

Substrate reduction of the cytochrome chain

Nicotinate, succinate, NADH and ascorbate + *NNN'*-tetramethylphenylenediamine reduced the cytochromes present in the wall-membrane fraction. Total cytochrome was estimated from the dithionite-

Fig. 1. Difference spectra of the wall-membrane fraction

Both cuvettes contained the wall-membrane fraction (18.5 mg of total protein) in 2.5 ml of 50 mM-potassium phosphate buffer (pH 7.4). Curve (a) shows the reduced (dithionite)-minus-oxidized (ferricyanide) spectrum. Curve (b) shows the wall-membrane fraction reduced (by the addition of 0.1 ml of 0.2 M-sodium nicotinate)-minus-oxidized suspension. Curve (c) shows the wall-membrane fraction reduced (with 1 mg of NADH)-minus-oxidized suspension.

reduced minus ferricyanide-oxidized difference spectrum (Fig. 1, curve a).

When nicotinate replaces dithionite as the reductant only a part of total cytochrome is reduced. The absorption maximum in the α -region is at 551 nm but a shoulder at 558 nm can also be seen (Fig. 1, curve b). The percentage of *c* and *b* cytochrome reduced varies slightly in different preparations but is usually within the limits of 60–65% of cytochrome *c* and 40–45% of cytochrome *b*. This represents the total cytochrome reducible by nicotinate and not that measured from a steady-state spectrum. Fig. 1, curve c, shows the spectrum obtained when NADH is the substrate. The absorption band is more symmetrical in the α -region, having its maximum at 557 nm, suggesting that similar amounts of cytochrome *b* and *c* are reduced. Succinate-reduced preparations also have their α -region absorption maxima at 557 nm (not shown) but in some cases a slight shoulder at 553 nm has been recorded. If this was caused by the same cytochrome *c* that is reduced by nicotinate this shoulder might have been expected to occur at 551 nm. It is therefore possible that there are two cytochromes *c* in this organism. Hunt (1959) recorded both cytochrome *c*₅₅₁ and *c*₅₅₃ as being present in his particulate preparations from nicotinate-grown cells but both appeared reducible by nicotinate. Ascorbate + *NNN'*-tetramethylphenylenediamine as substrate gives a spectrum identical with that produced by nicotinate except that there is less bleaching in the flavin (450–470 nm) region of the spectrum. That nicotinate and ascorbate + *NNN'*-tetramethylphenylenediamine produce different spectra from NADH and succinate when acting as reductants might suggest that there are two possible pathways from substrate to oxygen.

The ability of the different substrates to reduce the

Table 2. Oxidase activity of the wall-membrane and supernatant fractions of *Pseudomonas ovalis* Chester

O₂ uptake was measured at 30°C in an O₂ electrode vessel which contained 2.5 ml of 50 mM-potassium phosphate buffer, pH 7.4, and 2–3 mg of protein. The substrate was added, after the insertion of the electrode, by means of a 50 μ l microsyringe. Substrates were sodium nicotinate (0.2 M), sodium 6-hydroxynicotinate (0.2 M), sodium succinate (0.2 M), NADH (10 mg/ml), tris-ascorbate (0.1 M) plus *NNN'*-tetramethylphenylenediamine (10 mM).

Substrate	Oxygen uptake (nmol of O ₂ /min per mg of protein)	
	Wall-membrane fraction	Supernatant
Nicotinate	121	0
6-Hydroxynicotinate	0	2
Succinate	23	0
NADH	39	7
Ascorbate + <i>NNN'</i> -tetramethyl- phenylenediamine	229	4

cytochrome *o* present in the particulate fraction was examined. CO was bubbled in a steady stream through a portion of the particulate fraction for 2 min, substrate was then added and the difference spectrum between this and non-CO-treated particulate fraction plus substrate was recorded after 2–3 min. After reduction with dithionite a typical cytochrome *o* spectrum was obtained (Fig. 2, curve *a*) with an absorption maximum at 417 nm and minimum at 432 nm. A similar spectrum was obtained when ascorbate + *NNN'*-tetramethylphenylenediamine was the reductant (Fig. 2, curve *b*). With NADH or succinate the spectrum is markedly different, with the peak now occurring at 425 nm and a trough at 405 nm (Fig. 2, curve *c*). Fig. 2, curve *d*, shows the spectrum obtained if nicotinate is the reductant. There is apparently little reduction of either the cytochrome *o* or the '425' component. This spectrum could also be the result of the reduction of both components, and the resulting combined spectrum being that shown in Fig. 2, curve *d*.

That the latter explanation is correct was shown by the following test. The difference spectrum between CO-treated particulate fraction reduced with nicotinate and CO-treated fraction reduced with ascorbate + *NNN'*-tetramethylphenylenediamine was found to be similar to that obtained by NADH (Fig. 2, curve *c*). Conversely the difference spectrum obtained

between CO-treated fraction reduced with nicotinate and CO-treated fraction reduced with NADH was similar to that obtained by dithionite reduction (Fig. 2, curve *a*). These results indicate that nicotinate is able to reduce both cytochrome *o* and the '425' component.

Nature of the '425' CO-binding component

A pigment having a peak at 425 nm in its CO-difference spectrum has not been described in a *Pseudomonas* species before. Compounds that react with CO usually also react with O₂ and therefore it is possible that this compound is acting as a terminal oxidase. Cytochrome *c* peroxidase has been implicated in the oxidation of nicotinate (Hunt, 1959) although no CO spectrum has been described for the cytochrome *c* peroxidase purified from *Ps. fluorescens* by Ellfolk & Soininen (1970). According to Lenhoff & Kaplan (1956) substrate oxidation generates H₂O₂ which is used by the peroxidase in the re-oxidation of the cytochrome chain. Artificial electron donors, such as ascorbate + *NNN'*-tetramethylphenylenediamine, would not produce any H₂O₂ and therefore not react with the peroxidase. It might be possible to form the CO complex if exogenous H₂O₂ is added with the artificial electron-donating system. Fig. 3, curve *a*, shows that

Fig. 2. Substrate-reducible CO-binding pigments in the wall-membrane fraction

The sample cuvette contained 2.5 ml of the wall-membrane fraction in 50 mM-phosphate buffer, pH 7.4, through which CO had been bubbled for 2 min. The reference cuvette contained the same wall-membrane preparation untreated with CO. Reductant was added to each cuvette and the difference spectrum recorded after 3 min. Reductants were: (a) 1 mg of Na₂S₂O₄; (b) 0.1 ml of ascorbate (0.1 M) + *NNN'*-tetramethylphenylenediamine (10 mM), (c) 0.1 ml of NADH (10 mg/ml); (d) 0.1 ml of sodium nicotinate (0.2 M).

the 425 nm absorption maximum can be demonstrated in this way. The spectrum between 380–600 nm is also shown (Fig. 3, curve *b*) for the NADH-reduced CO-complex. In both cases another small absorption maximum at 558 nm is formed.

Fig. 3. Reduction of the '425' component by ascorbate + *NNN'*-tetramethylphenylenediamine in the presence of H_2O_2 .

A suitably diluted portion of the wall-membrane fraction was treated with CO as described before. Ascorbate + *NNN'*-tetramethylphenylenediamine (0.1 ml) was added to both cuvettes and then 25 μ l of H_2O_2 (10 mM) was added to the sample cuvette. The spectrum was recorded after 3 min (curve *a*). Curve (*b*) is the NADH-reduced spectrum obtained as described in Fig. 2.

Inhibitors of terminal oxidation

The effects of three known inhibitors of terminal oxidation, CN^- , N_3^- and CO, on the oxidation of NADH, nicotinate and ascorbate + *NNN'*-tetramethylphenylenediamine by the wall-membrane fraction are shown in Table 3.

Cyanide. This considerably inhibited the oxidation of all three substrates at 0.5 mM and was completely inhibitory at higher concentrations. There appeared to be no cyanide-insensitive respiration.

Azide. At pH 7.4 NaN_3 (1 mM) did not inhibit the oxidation of NADH though it did inhibit nicotinate oxidation slightly and ascorbate + *NNN'*-tetramethylphenylenediamine oxidation to a significant extent. The inhibition was increased at 5 mM- NaN_3 , and NADH oxidation was also slightly affected. At pH 5.3 the inhibitions caused by both 1 and 5 mM- NaN_3 were increased. The relative insensitivity of NADH oxidation to low concentrations of azide at pH 7.4 suggests that a peroxidase as described by Lenhof & Kaplan (1956) is operating. The azide-sensitivity of the ascorbate + *NNN'*-tetramethylphenylenediamine oxidation at pH 7.4 might imply that the peroxidase is not involved as its terminal oxidase. The effects of N_3^- and H_2O_2 on the oxidation of reduced mammalian cytochrome *c* by the wall-membrane fraction was also examined. At pH 7.4 the fraction would reoxidize cytochrome *c* (5.15 nmol of cytochrome *c*/min per mg of protein) and this reaction was inhibited by 78% by 1 mM- NaN_3 . In the presence of H_2O_2 the rate of oxidation of reduced cytochrome *c* was increased to 14.9 nmol of cytochrome *c*/min per mg of protein. The increased rate was not sensitive to 1 mM- NaN_3 . At lower pH values the reoxidation of cytochrome *c* in the absence of H_2O_2 was 95% inhibited by 1 mM- NaN_3 and in the presence of H_2O_2 , by 25%. These results suggest

Table 3. Effect of inhibitors of terminal oxidation on the O_2 uptake of the wall-membrane fraction of *Ps. ovalis* Chester

O_2 uptake was measured with an O_2 electrode as described in the Experimental section. Inhibitors were added by means of a microsyringe after the rate caused by the addition of substrate had been established. The effect of N_3^- was also measured at pH 5.3 by using 0.05 M-potassium citrate-phosphate buffer. CO inhibition was measured in a mixture of air- and CO-saturated buffer (ratio 1:6). Results are expressed as % inhibition of the initial rate of substrate oxidation.

Inhibitor	Concentration (mM)	% inhibition		
		Nicotinate	NADH	Ascorbate + <i>NNN'</i> -tetramethylphenylenediamine
N_3^- (pH 7.4)	1	17	0	30
	5	61	28	69
N_3^- (pH 5.3)	1	44	26	59
	5	75	53	88
CN^-	0.5	82	93	86
CO		55	51	48

that there are two terminal oxidases that differ in their sensitivity to azide.

Carbon monoxide. Inhibition by CO was measured by using mixtures of air- and CO-saturated buffer. A ratio of 1:6 for air-saturated:CO-saturated buffer was used and the wall-membrane fraction was incubated in this for 30s before the addition of the substrate. The oxidation of substrates was linear down to low concentrations of dissolved O_2 (13 nmol of O_2 /ml). The oxidation of all three substrates was inhibited to a similar extent (Table 3). If the wall-membrane fraction was treated with CO for up to 5 min and a small volume (0.05–0.1 ml) of the inhibited suspension added to air-saturated buffer in the electrode vessel, the inhibition of NADH oxidation was increased (up to 70%) but no inhibition of nicotinate oxidation was found. This might indicate that the CO complex formed by one of the oxidases is unstable. CO did not have any inhibitory effect on nicotinic acid hydroxylase when assayed by the reduction of ferricyanide by the method of Hunt (1959).

Discussion

The respiratory system of *Ps. ovalis* grown on nicotinate is similar to that described by Hunt (1959) in *Ps. fluorescens* KB1. The inducible nicotinate oxidase system is associated with the wall-membrane fraction and is several times more active than NADH of succinate oxidases.

Hunt (1959) failed to show any cytochrome *o* in *Ps. fluorescens* KB1 and suggested that cytochrome *c* peroxidase was functioning as the terminal oxidase. Cytochrome *o* has now been found to be present in the wall-membrane fraction in *Ps. ovalis* and evidence is presented to show that it can be reduced by nicotin-

ate but not by NADH or succinate. Another CO-binding pigment is also present and its reaction with ascorbate + *NNN'*-tetramethylphenylenediamine in the presence of H_2O_2 suggests that this might be a peroxidase.

The effects of N_3^- again suggest that two terminal oxidases are present, one of which is relatively insensitive to N_3^- at pH 7.4 and which is involved in NADH oxidation. Since NADH is unable to reduce cytochrome *o* it is probable that the azide-'insensitive' oxidase is the compound forming the 425 nm peak in the CO difference-spectrum. Cytochrome *c* peroxidase is present as shown by the reoxidation of mammalian cytochrome *c* in the presence of H_2O_2 . In the absence of evidence to the contrary it is proposed that the '425' component is cytochrome *c* peroxidase. No evidence is available on the reaction of CO with the cytochrome *c* peroxidase that has been purified from *Ps. fluorescens* (Ellfolk & Soininen, 1970) but peroxidases associated with plant mitochondria have been shown to have CO spectra similar to cytochrome *o* (Plesnicar *et al.*, 1967).

Nicotinate, which can reduce both CO-binding pigments, is less sensitive to N_3^- than ascorbate + *NNN'*-tetramethylphenylenediamine, which reduces only cytochrome *o*. CO seems to inhibit the oxidation of all three substrates equally, although there is some suggestion that the stability of the CO complexes formed by the two oxidases may differ. The CO complex formed by cytochrome *o* is easily dissociated in a number of bacterial systems (Oka & Arima, 1965; Jones & Redfearn, 1967). Eady *et al.* (1971) have described a CO-binding component resembling cytochrome *o* in *Ps. aminovorans* grown on methylamine as sole carbon source. They found that the secondary amine oxidase was very sensitive to inhibition by CO and suggested that this CO-binding pigment is involved in the secondary amine

Scheme 1. Proposed electron-transport system of *Pseudomonas ovalis* Chester grown on nicotinic acid as sole carbon source

oxidase, in much the same way that the CO-binding pigment 'P450' has been found to be involved in the hydroxylation of camphor by *Ps. putida* (Katagiri *et al.*, 1968). No inhibition of the nicotinic acid hydroxylase by CO in *Ps. ovalis* could be detected. It therefore seems that the only role of cytochrome *o* is as a terminal oxidase. Peterson (1970) found that the content of cytochrome *o* in *Pseudomonas oleovorans* was much higher when cells were grown on hexane than when grown on glucose. He could not find any role for cytochrome *o* in the CO-hydroxylation reaction. It might be that cytochrome functions as an alternative terminal oxidase in this system, as it seems to do in *Ps. ovalis*.

The reaction of the cytochromes with ascorbate + *NNN'*-tetramethylphenylenediamine suggests that only cytochromes *c* and *o* are reduced. Since cytochrome *o* is apparently the terminal oxidase the reaction sequence must be ascorbate $\rightarrow$ cytochrome *c* $\rightarrow$ cytochrome *o* $\rightarrow$ O₂.

By comparison, nicotinate reduces cytochromes *c* and *o* and also cytochrome *c* peroxidase. NADH and succinate are unable to reduce cytochrome *o* but are able to reduce the peroxidase and some cytochromes *c* and *b*. There is some suggestion that the cytochrome *c* reduced is different from that reduced by nicotinate or ascorbate + *NNN'*-tetramethylphenylenediamine. The probable organization of these components is shown in Scheme 1. This is the simplest scheme possible and involvement of other respiratory carriers such as non-haem iron or quinones is not excluded. Both quinone and non-haem iron have been found in wall-membrane fractions (M. Jones, unpublished work).

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