REQUIREMENT OF TWO PROTEIN FRACTIONS FOR O-DEMETHYLASE ACTIVITY IN *PSEUDOMONAS TESTOSTERONI*

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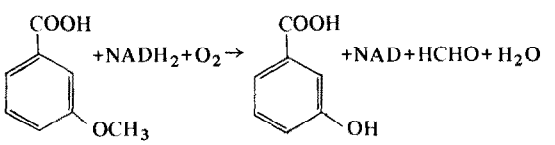
1. Introduction

Our previous studies of the metabolism of aryl-methyl ethers by *Pseudomonas aeruginosa* showed that after growth on vanillate, extracts of cells could *O*-demethylate several 3-methoxybenzoates, in addition to vanillate itself [1]. The stoicheiometry of these reactions was that expected for a mono-oxygenase reaction:



Unlike other pseudomonads, *Ps. aeruginosa* T1 was unable to grow on, or demethylate 4-methoxybenzoate (*p*-anisate) or its derivatives [2–4].

We have now investigated the enzymic activities of *Pseudomonas testosteroni* after growth on vanillate and find that both 3- and 4-methoxybenzoates are demethylated. As was reported previously for other pseudomonads, the ether cleavage activities are extremely sensitive to dilution and to oxidation by air [1–4]. These properties have made it difficult to purify the demethylase activity, as has been observed also by Bernhardt, Staudinger and Ullrich [4]. Only by using anaerobic techniques we have been able to show that the demethylase activity of *Ps. testosteroni is* a complex of at least two protein fractions, both of which appear to be sensitive to oxygen.

2. Materials and methods

Ps. testosteroni and *Ps. aeruginosa* T1 were grown as described previously [1]. Preparation of extracts and assays of demethylase activity have also been described [1]. Details of individual experiments appear in the legends to the figures and the tables.

3. Results

The total amount of oxygen consumed during oxidation of veratrate (3,4-dimethoxybenzoate) by whole cell suspensions of *Ps. testosteroni* first suggested that this species had a broader spectrum of demethylase activities than *Ps. aeruginosa.*

Thus, approximately 4 moles of O_2 were consumed, per mole of veratrate supplied, by suspensions of *Ps. testosteroni* (table 1). This is far in excess of that required for a single demethylation reaction, as occurs when suspensions of *Ps. aeruginosa* are employed [1]. This suggested that both the 3- and 4-methoxyl groups were oxidized by *Ps. testosteroni,* followed by cleavage of the ring of the product, protocatechuate. This was confirmed by the ability of extracts of *Ps. testosteroni* to oxidize *p*-methoxybenzoate, as well as veratrate and *m*-methoxybenzoate. Further, α-hydroxy-γ-car-

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Table 1
Comparison of *O*-demethylase activities *Ps. aeruginosa* T1 of *Ps. testosteroni*

Substrate	Oxygen consumed (nmoles O_2/nmole ether supplied)	
	Ps. aeruginosa	*Ps. testosteroni*
Vanillate	2.5–3.2	2.5–2.9
Veratrate	Not oxidized*	3.4–4.2
m-Methoxybenzoate	Not oxidized*	1.5
p-Methoxybenzoate	Not oxidized	1.5
Protocatechuate	1.7–2.2	0.8–1.3

* Not oxidized by whole cell suspensions but are good substrates in cell-free preparations.

The data were taken from polarographic traces in which washed suspensions of cells were allowed to oxidize limiting amounts of the ether supplied. Endogenous respiratory values were assumed to continue during substrate oxidation and were subtracted. Reaction mixtures contained: 50 mM tris/HCl buffer, pH 7.6 (3 ml); cell suspension (40 μl, approx. 1 mg dry weight cells); and 25 mM substrate (2–10 μl as required).

boxymuconic semialdehyde, the known ring fission product of protocatechuate [5, 6] was produced from veratrate, with the release of more than 1 mole of formaldehyde per mole of veratrate. Table 2 shows the substrate specificity for the *O*-demethylase activity in extracts of *Ps. testosteroni*. Both 3- and 4-methoxyl groups of benzoates are demethylated and methyl substituents are also hydroxylated. Bernhardt et al. also observed that *p*-toluate was a substrate for their 4-*O*-demethylase [4].

Fig. 1 shows the effect of enzyme concentration on NADH oxidase and vanillate demethylase activities. As observed by others [2, 4] and ourselves [1] demethylase activity is proportional to protein concentration of extracts only above a minimum threshold. This suggested that some limiting factor was diluted out in the assay mixture. However, we find that dialysis or ultrafiltration of extracts under N_2 does not affect demethylase activity, which is contrary to the data of Cartwright and Smith for another pseudomonad [2]. Furthermore, dilution of the cell-free extracts under N_2 (ten or twenty-fold for 24 hr) does not result in loss of demethylase activity provided the minimum protein concentration is supplied in the assay mixture. Any operation such as exposure of extracts to air, $(NH_4)_2SO_4$ precipitation, or column chromatography on gels or ion-exchangers resulted in almost complete loss of activity, which we have not been able to recover.

3.1. *Gel filtration of crude extracts*

Small amounts of *O*-demethylase activity can be recovered from Sephadex G-50 or G-25 filtrations. However, this activity shows distinctly different kinetics in the standard assays (fig. 2). Lag periods of varying length occur; their duration is related to the final activity of the fraction. Thus, longer lags are exhibited by fractions of low activity.

When crude extracts of *Ps. testosteroni* (or *Ps. aeruginosa*) are chromatographed on Sephadex G-75 or G-150 columns, almost negligible demethylase activity is recovered, even when all fractions are combined in the assay mixture. These results and our previous demonstration of the lability of the system to air led us to chromatograph the extracts under N_2 and collect all fractions manually under N_2. Approximately 10 percent of the activity could be recovered when single fractions were assayed. Recombination of the fractions in the assay mixture (described in legend to fig. 3) which had been kept anaerobic, revealed the presence of two distinct protein bands both of which are required for vanillate stimulated oxidation of NADH by O_2 (fig. 3). These have subsequently been completely separated from each other.

4. Discussion

Although all mono-oxygenases catalyze the incorporation of one atom of molecular oxygen into a substrate molecule and reduce the other atom of oxygen to water, there is a wide variety of electron

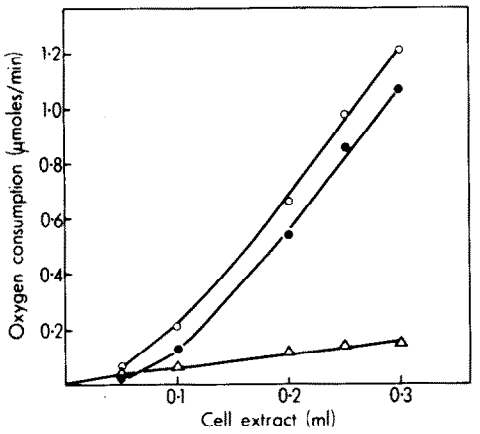

Fig. 1. Effect of enzyme concentration on vanillate demethylase activity. Assay mixtures contained 50 mM tris/HCl, pH 7.2 (2 ml); cell free extract as indicated; 25 mM NADH (40 μl); 25 mM vanillate (20 μl); and water to 3 ml. Temperature, 30°. (△—△) NADH oxidase; (○—○) vanillate stimulated oxidations of NADH; (●—●) vanillate stimulated respiration minus NADH respiration. Oxygen consumption was measured polarographically.

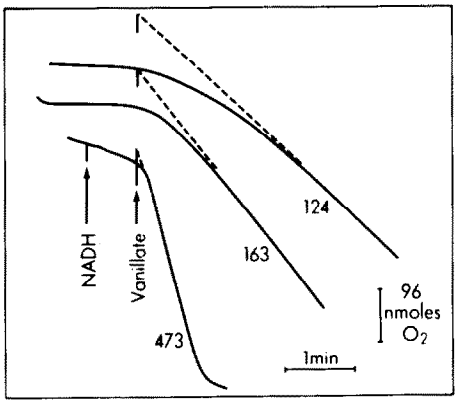

Fig. 2. Appearance of lag periods in assays before full *O*-demethylase activity is displayed in fractions from columns. The reaction mixture contained: 50 mM tris/HCl, pH 7.6 (0.5 ml); Sephadex G-50 column fractions 10 and 11 (2 ml, top two curves) or crude extract (0.4 ml, bottom curve) and 20 mM tris/HCl, pH 7.6 to 2.5 ml. 25 mM NADH (20 μl) and 25 mM vanillate (20μl) were added as indicated. Numbers under the lines represent nmoles O_2/min. Lag periods are 2.5, 1.3 and 0.15 min for fractions 10, 11 and crude extract respectively.

Table 2
Substrate specificity shown by extracts of *Ps. testosteroni* after growth on vanillate

Substrate	Quantity supplied (nmoles)	Oxidation rate (nmoles/min)	Total O_2 consumed (nmoles O_2/nmole substrate)
None	—	113	—
Vanillate	125	284	2.2
m-Methoxybenzoate	250	173	1.1
p-Methoxybenzoate	250	210	0.83
Veratrate	87.5	480	3.2
Syringate	75	265	3.2
m-Toluate	625	174	N.D.
2,3,4-Trimethoxybenzoate	625	115	N.D.
3,4,5-Trimethoxybenzoate	625	118	N.D.
3-Hydroxy-4-methylbenzoate	625	176	N.D.
2,4-Dimethoxybenzoate	625	206	N.D.

N.D. not determined
Reaction mixture contained: 50 mM tris/HCl, pH 8.0 (2.5 ml); dialyzed 100,000 *g* supernatant of French press extracts of cells (0.75 ml approximately 12 mg of protein/ml); 25 mM NADH (40 μl) and 25 mM substrate solutions to give the final concentrations shown. Oxygen was measured polarographically. Temperature, 30°.

carriers and terminal oxidases that participate in these reactions. Thus, hydroxylation of methyne carbon atoms as on aromatic rings invariably involves flavoproteins that catalyze both the electron transport from nicotinamide nucleotides and the activation of oxygen to yield hydroxylated substrate and water, e.g., *p*-hy-

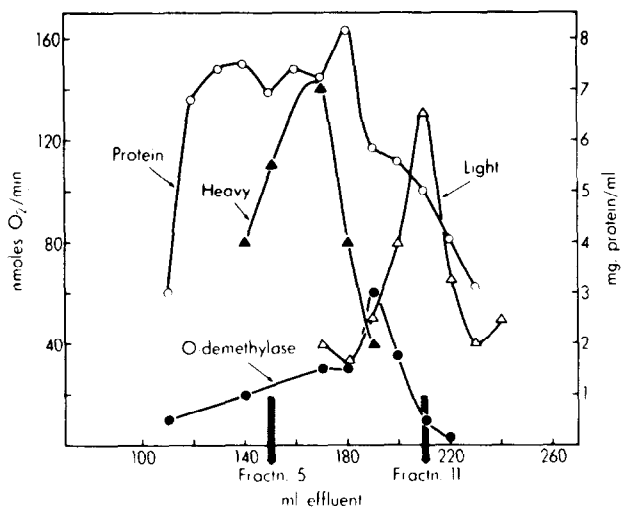

Fig. 3. Chromatography of *O*-demethylase activity on Sephadex G-150. Supernatants of crude extracts of *Ps. testosteroni* from 100,000 *g* sedimentations (28 ml) were loaded onto a Sephadex G-150 column (25 X 820 mm) in 20 mM tris/HCl previously equilibrated with N_2 saturated buffer. Fractions (9–12 ml) were collected manually into McCartney bottles (serum caps) with continuous flushing of N_2, and stored at 0°, stoppered under N_2. Vanillate *O*-demethylase activity was assayed in each fraction (●—●, *O*-demethylase) and in combination with other fractions (▲—▲, heavy components assayed in combination with fraction no. 11) and (△—△, light components assayed in combination with fraction no. 5). Assays utilized 0.5 ml of each fraction and air-saturated 20 mM tris/HCl buffer, pH 7.6 was used to bring the volume to 3.0 ml. 25 mM NADH (20 μl) and 25 mM vanillate (20 μl) were added to complete the assay mixture and O_2 consumption was measured polarographically. Temperature, 30°.

droxybenzoate hydroxylase [7, 8], salicylate hydroxylase [9, 10] and orcinol hydroxylase [11]. Those enzyme systems that catalyze hydroxylation of methylene or methyl carbon atoms appear to utilize a more complex pathway of electron transport involving two or three protein fraction, one of which is responsible for the oxygenation of the substrate. Thus, the camphor methylene hydroxylase complex of *Ps. putida* utilizes the non-haem iron protein, putidaredoxin, to pass electrons to a cytochrome *P*-450, the protein which also catalyzes the hydroxylation [12]. Cytochrome *P*-450 has also been implicated in the hydroxylation of alkanes in Corynbacteria [13]. ω-Hydroxylation of alkanes (or fatty acids) by *Ps. oleovorans,* however, does not involve cytochrome *P*-450; instead at least three proteins are necessary two of which are involved in electron transport [14, 15].

The *O*-demethylase activities of *Ps. testosteroni* further document the extreme lability of these ether cleavage enzymes to oxygen [1–4]. The dependence of the *O*-demethylase on a minimum protein concentration in crude extracts for activity was also described earlier [1–4] and it seemed likely that some dialysable cofactor was required [2, 3]. However, the appearance of lag phases in the progress curves (fig. 2) suggested that a partially inactive species of the enzyme system was being reactivated in the presence of substrates. The lag phase was shown also by Bernhardt, Staudinger and Ullrich [4] but they did not comment on it. Since we have been able to resolve the demethylase activity into two protein fractions, both of which are required for activity, the lag phase seen in some assays may represent a reassembly or coupling of these fractions. We have not yet examined the two protein fractions in detail for other enzymic activities, but one of them catalyzes electron transport from $NADH_2$ to certain dyes. Bernhardt et al. [4] have successfully obtained a six-fold purification of 4-*O*-demethylase activity by $(NH_4)_2SO_4$ fractionation and gel filtration under N_2, but separation into two or more components was not observed.

It is not at all clear yet whether the *O*-demethylase activity for the 3- or 4-methoxyl substituents of benzoate is due to the same enzyme or due to the presence of different enzymes, induced during growth of *Ps. testosteroni* on vanillate. The substrate specificities exhibited by the demethylase in other strains of pseudomonads [1–4] appear to be limited to either the 3- or the 4- substituents of benzoate. As Bernhardt et al. [4] have shown for their pseudomonad, methyl substituents also appear to be substrates yielding hydroxymethyl derivatives (table 2), lending further support to the hypothesis that the methyl carbon of the methoxyl group is the atom that is hydroxylated.

Acknowledgement

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