Periplasmic location of *p*-cresol methylhydroxylase in *Pseudomonas putida*

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The cellular location of the flavocytochrome *c*, *p*-cresol methylhydroxylase was investigated in two strains of *Pseudomonas putida*. In both cases the enzymes were shown to be located in the periplasmic fraction by their release during treatment of the bacteria with EDTA and lysozyme in a solution containing a high concentration of sucrose. For strain NCIB 9869 the finding is in accord with the suggestion that the physiological acceptor for the enyme is azurin as this too was shown to be located mostly in the periplasm.

Flavocytochrome c *Azurin* *Periplasmic protein*

1. INTRODUCTION

A number of enzymes have now been shown to be located in the periplasmic space in bacteria [1-6]. Evidence for this usually comes for their release by osmotic shock or by treatment of the bacteria with EDTA and lysozyme which breaks down the cell walls, but in isotonic medium leaves intact spheroplasts containing the cytoplasmic enzymes, although other approaches have been used [6]. Using the EDTA-lysozyme procedure we show here that the enzyme *p*-cresol methylhydroxylase in *Pseudomonas putida* is also found in the periplasm.

This enzyme catalyses the initial reaction in the metabolism of *p*-cresol by a number of bacterial species. It converts *p*-cresol into *p*-hydroxybenzyl alcohol by a dehydrogenation reaction followed by hydration and can also oxidize the product to *p*-hydroxybenzaldehyde in a second dehydrogenation step [7]. The enzyme from *P. putida* NCIB 9866 [7] and the two enzymes, hydroxylase A and hydroxylase B, that are produced by *P. putida* NCIB 9869 under different growth conditions [8] have been studied in considerable detail. They range in size from 100 to 115 kDa and consist of two equal-sized subunits, one a flavoprotein in which the flavin is convalently attached by a 8α-*O*-tyrosyl linkage [9] and the other a cytochrome *c*. It has been shown that an azurin, a small copper-containing protein, is produced by *P. putida* NCIB 9869 and that this will accept electrons from the *p*-cresol methylhydroxylase and will also link electron flow to membranes resulting in O_2 uptake [10]. For this to be the natural acceptor for the enzyme it must be in the same cell compartment and it too is shown here to be located in the periplasm.

2. MATERIALS AND METHODS

Bacteria were maintained and grown as described by Hopper and Taylor [7]. For investigation of hydroxylase A in *P. putida* NCIB 9869 the organism was grown in medium containing 0.4% (w/v) succinate as carbon source after prior growth on 3,5-xylenol. To study the distribution of hydroxylase B a strain of this organism that had lost the ability to synthesize hydroxylase A was grown in medium containing 0.03% (w/v) *p*-cresol as carbon source. For *P. putida* NCIB 9866 0.03% (w/v) *p*-cresol was the carbon source.

The bacteria were harvested at 25°C and were fractionated essentially by the procedure described by Wood [1]. The bacteria harvested from 1 l of

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culture were resuspended in 40 ml of 40 mM Tris-HCl (pH 8.0), containing 4 mM Na_2 EDTA and 0.5 M sucrose at 30°C and 10 mg of lysozyme were added. After 2 min, $MgCl_2$ was added to 10 mM and incubation continued at 30°C for 30 min. The suspension was then centrifuged at 15 000 × *g* for 20 min at 25°C to give the supernatant periplasmic fraction and a pellet of spheroplasts. The pellet was resuspended in 40 ml of 10 mM Tris-HCl and 2 mM Na_2EDTA (pH 8.0), at 30°C; this resulted in release of cytoplasmic proteins and DNA to give a very viscous solution. After 15 min $MgCl_2$ was added to 10 mM together with a small amount of DNase. After a further 15 min incubation the solution was centrifuged at 15 000 × *g* for 30 min at 4°C to give a supernatant solution containing the cytoplasmic proteins and a pellet of membrane fragments. The pellet was resuspended in 10 ml of Tris-HCl (pH 8.0).

To assess the distribution of azurin, 400 ml of periplasmic fraction and 200 ml of cytoplasmic fraction from 4 g wet wt of bacteria were each passed by pressure filtration through an Amicon YM30 membrane to remove proteins larger than about 30 kDa and then concentrated to a few ml by filtration through a YM5 membrane. The difference in absorbance at 620 nm between ferricyanide oxidized and dithionite reduced samples was taken as a measure of the amount of azurin in each fraction.

p-Cresol methylhydroxylase was assayed as described by McIntire and Singer [11] with 2.2 mM phenazine methosulphate and including 1 mM KCN. Isocitrate dehydrogenase was assayed by following the increase in absorbance at 340 nm in a 1 cm path-length cuvette containing in 3 ml of 50 mM triethanolamine buffer (pH 7.4), 4 mM $MnSO_4$, 0.33 mM $NADP^+$, 6 mM sodium isocitrate and enzyme at 25°C. Malic enzyme (EC 1.1.1.40) and malate dehydrogenase were assayed at 25°C as described by Hopper et al. [12].

3. RESULTS

Incubation of freshly grown bacteria with lysozyme and EDTA in buffer containing a high concentration of sucrose produces spheroplasts with the release of periplasmic proteins [1]. The spheroplasts can be removed by centrifuging and then burst by resuspension in more dilute solution followed by separation into the cytoplasmic and membrane fractions by centrifuging. *P. putida* NCIB 9866 grown on *p*-cresol was fractionated in this way and the distribution of *p*-cresol methylhydroxylase among these fractions is shown in table 1. As a measure of the efficiency of the fractionation malic enzyme and isocitrate dehydrogenase were assayed as cytoplasmic markers and malate dehydrogenase as a marker for membranes [12].

Hydroxylase A in *P. putida* NCIB 9869 is plasmid encoded and is produced constitutively as long as that part of the plasmid is maintained by growth on 3,5-xylenol [13]. The high levels of enzymes for 3,5-xylenol metabolism and hydroxylase A are maintained for several generations after growth on 3,5-xylenol but then decline with loss of part of the plasmid. Thus for studies on the distribution of hydroxylase A bacteria were grown on succinate after initial growth on 3,5-xylenol. The results are given in table 2. Hydroxylase B in *P. putida* 9869, however, is induced by growth on *p*-cresol and for studies of its distribution a strain of the organism that had lost the ability to grow on

Table 1

Distribution of enzymes in *P. putida* NCIB 9866

Fraction	Enzyme			
	Malic enzyme	Isocitrate dehydrogenase	Malate dehydrogenase	*p*-Cresol methyl-hydroxylase
	(% total recovery)			
Periplasm	0	0	8.3	88.9
Cytoplasm	100	100	0	9.2
Membrane	0	0	91.7	1.9

Table 2

Distribution of *p*-cresol methylhydroxylase A and marker enzymes in *P. putida* 9869

Fraction	Enzyme			
	Malic enzyme	Isocitrate dehydrogenase	Malate dehydrogenase	*p*-Cresol methylhydroxylase A
	(% total recovery)			
Periplasm	21.0	14.2	3.7	73
Cytoplasm	79.0	85.8	10.1	23.5
Membrane	0	0	86.2	3.5

3,5-xylenol and also the constitutive hydroxylase A was grown on *p*-cresol. The results are given in table 3.

Azurin was more difficult to assay and fractions from a large-scale treatment of bacteria had to be concentrated in order to estimate the azurin from its absorbance at 620 nm in the oxidized form. A blue colour could be seen in the concentrated periplasmic fraction from *P. putida* 9869 and from the A_{620} this contained 88% of the azurin compared with 12% in the cytoplasm. Thus, in this organism, the azurin was found in the same fraction as the *p*-cresol methylhydroxylase.

4. DISCUSSION

The results in tables 1–3 show that all 3 *p*-cresol methylhydroxylases were released by treatment of the bacteria with EDTA and lysozyme and are, therefore, located in the periplasm. Only when *P. putida* 9869 was grown under conditions to produce hydroxylase A (table 2) did any significant breakage of the spheroplasts occur during the treatment as indicated by the presence of some malic enzyme and isocitrate dehydrogenase, the cytoplasmic markers, in the periplasmic fraction. In the other two cases (tables 1,3) the spheroplasts remained intact with no cytoplasmic markers detected in the periplasm. The conditions used did not give 100% release of the *p*-cresol methylhydroxylases but the small amount centrifuging with the spheroplasts probably reflects incomplete breakdown of the cell walls.

The periplasmic location of the *p*-cresol methylhydroxylases is in accord with the proposal by Wood [14] that different classes of *c*-type cytochrome originated as proteins positioned in the periplasmic space or on the periplasmic side of the membrane, the covalent binding of the haem preventing its loss to the surrounding medium. Not only do these hydroxylases contain a *c*-type cytochrome subunit but the flavin too is covalently bound to a second subunit, possibly for the same reasons, and our findings raise many interesting questions about the transport and assembly of the enzymes.

It has been suggested that the periplasmic location of methanol dehydrogenase in *Paracoccus*

Table 3

Distribution of *p*-cresol methylhydroxylase B and marker enzymes in *P. putida* 9869

Fraction	Enzyme			
	Malic enzyme	Isocitrate dehydrogenase	Malate dehydrogenase	*p*-Cresol methylhydroxylase B
	(% total recovery)			
Periplasm	0	0	0	90.5
Cytoplasm	100	100	2	8.6
Membrane	0	0	98	0.9

denitrificans limits damage to the cell from the reaction product, formaldehyde [3]. Cresols too are potentially damaging to bacteria and there may be advantages in their modication before entry into the cell, though it should be remembered that in other pathways for cresol metabolism by Pseudomonads the first step is a hydroxylation probably catalysed by an NADH requiring monooxygenase which is unlikely to be periplasmic [15,16].

Reduced *p*-cresol methylhydroxylase is not reoxidised directly by O_2 or by membranes which contain a cytochrome oxidase and in most experiments the artificial electron acceptor, phenazine methosulphate, has been used. In a search for the natural electron acceptor an azurin was isolated from *P. putida* 9869 [10]. This protein is readily reduced by the enzyme and will link electron flow to a membrane component resulting in O_2 uptake. Wood [1] showed that the azuring in *P. aeruginosa* is largely periplasmic and, as this is also the case for *P. putida* 9869 azurin, it lends support to the suggestion that it is the natural acceptor for *p*-cresol methylhydroxylase.

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