The Purification and Properties of Cyclohexanone Oxygenase from *Nocardia globerula* CL1

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Nocardia globerula CL1 grows with cyclohexanol as sole source of carbon and oxidizes it by the induced pathway: cyclohexanol $\rightarrow$ cyclohexanone $\rightarrow$ hexanoic ϵ -lactone $\rightarrow$ 6-hydroxyhexanoate $\rightarrow$ adipate (Norris & Trudgill, 1971). Formation of hexanoic ϵ -lactone from cyclohexanone is catalysed by a mixed-function oxygenase that requires NADPH as an electron donor and a supply of molecular O_2 .

This oxygenase has been purified 15-fold by protamine sulphate treatment and DEAE-cellulose, hydroxyapatite and Sephadex G-200 chromatography. Such preparations are electrophoretically homogeneous, give a single symmetrical sedimentation peak in the ultracentrifuge ($s_{20,w}^0$ 3.9S) and a molecular weight of 53000.

The pure enzyme is bright yellow and displays absorption maxima at 278, 376 and 435nm and minima at 248 and 395nm. Addition of NADPH causes a loss of the absorption peaks at 376 and 435nm, which are regained on the addition of excess of cyclohexanone. Dissociation of the prosthetic group by treatment with aq. 95% (v/v) ethanol at 50°C followed by chromatography (Maslowski, 1965) revealed it to be FAD. Assay of the FAD content of the holoenzyme showed the presence of 1 mol/53000g of enzyme.

At the optimum pH of 8.4 and 30°C the enzyme has K_m (cyclohexanone) 1.5 μ M, K_m (NADPH) 31 μ M and a catalytic-centre activity of 1018, on the basis of an assumed single catalytic centre.

Transition-metal-ion chelating agents are without effect on catalytic activity, but thiol-active agents cause severe or complete inhibition. Titration of enzyme activity with *p*-hydroxymercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoate) revealed 1 mol of susceptible SH group/53000g. Attempts to determine whether the thiol group is functional at the catalytic site or merely vicinal, made by exchanging the bulky thionitrobenzoate group donated by 5,5'-dithiobis-(2-nitrobenzoate) for the compact cyanide residue (Chung *et al.*, 1971), failed to restore significant activity. This is interpreted as indicating that the thiol group is a functional one at the catalytic centre.

This enzyme contrasts markedly with the (+)-camphor oxygenase of *Pseudomonas putida* C1B that catalyses a parallel insertion of oxygen into (+)-camphor in the presence of NADH and is a loose complex of three proteins, two of which weakly bind

FMN. This latter enzyme has also been reported at various stages in purification to require Fe^{2+} for activity (Conrad *et al.*, 1965; Trudgill *et al.*, 1966a,b; Yu & Gunsalus, 1969).

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A Novel Pathway for Bacterial Growth on Malonate and β -Hydroxybutyrate

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In a survey of potential growth substrates it was found that the pink methanol-utilizing bacterium *Pseudomonas* AM1 grows well on malonate and β -hydroxybutyrate, the growth rate on β -hydroxybutyrate being nearly equal to that on methanol. The usual pathway for metabolism of ethanol, acetate, malonate and β -hydroxybutyrate involves conversion into acetyl-CoA, which is then assimilated by way of the glyoxylate by-pass. However, there are negligible amounts of isocitrate lyase in *Pseudomonas* AM1, and there is considerable evidence that ethanol is metabolized to malate by a novel route involving acetate, glycollate and glyoxylate (Dunstan *et al.*, 1972a). The results in the present communication demonstrate that this alternative pathway is also essential for the metabolism of malonate and β -hydroxybutyrate by *Pseudomonas* AM1.

Mutant PCT48, unable to convert acetate into glycollate (the first step of this pathway) (Dunstan *et al.*, 1972b), was unable to grow on ethanol, acetate, malonate or β -hydroxybutyrate unless glycollate or glyoxylate was also provided in the medium.

The next step in the pathway is the oxidation of glycollate to glyoxylate, a reaction probably mediated by hydroxypyruvate reductase. It is possible that the low hydroxypyruvate reductase activity in ethanol-grown organisms (specific activity 28 μ mol/h per mg of protein) is responsible for the low growth rate on this substrate. *Pseudomonas* AM1 grows on β -hydroxybutyrate more rapidly than on ethanol (mean generation times 7-8h and 10h respectively). It might therefore be expected that the hydroxypyruvate

reductase activity would be greater in β -hydroxybutyrate-grown cells than in ethanol-grown cells. Because hydroxypyruvate reductase and serine-glyoxylate aminotransferase are co-ordinately regulated (Dunstan *et al.*, 1972b) the activities of both enzymes should be higher, although only the first is likely to be essential for growth on β -hydroxybutyrate. Measurement of the two enzyme activities has confirmed this prediction; the specific activities ($\mu\text{mol/h}$ per mg of protein) of hydroxypyruvate reductase and serine-glyoxylate aminotransferase were 39 and 1.8 respectively in β -hydroxybutyrate-grown organisms.

The present results indicate that malonate and β -hydroxybutyrate are assimilated in *Pseudomonas* AM1 by the novel pathway previously shown to operate during growth on ethanol. This route presumably also provides a means of generating precursors for biosynthesis from the endogenous reserves of poly- β -hydroxybutyrate, which constitute up to 8% of the dry weight of these organisms.

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Dunstan, P. M., Anthony, C. & Drabble, W. T. (1972a) *Biochem. J.* **128**, 99–106

Dunstan, P. M., Anthony, C. & Drabble, W. T. (1972b) *Biochem. J.* **128**, 107–115

Microbial Growth on C_1 and C_2 Compounds: the Metabolism of Acetate to Glycine in *Pseudomonas* AM1

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Pseudomonas AM1 is a pink bacterium capable of growth on C_1 compounds. These substrates are assimilated by condensation of 5,10-methylenetetrahydrofolate with glycine to give serine, which is converted into glycolytic intermediates (Large & Quayle, 1963). To maintain the operation of this pathway glyoxylate must be synthesized from C_1 units and then transaminated to glycine (Harder & Quayle, 1971). Evidence has been presented that acetate and glycollate are the precursors of glyoxylate (Dunstan *et al.*, 1972b). The enzymes converting acetate into glyoxylate also operate during growth on C_2 compounds. In this case glyoxylate is not transaminated to glycine but condenses with acetyl-CoA to give malate (Dunstan *et al.*, 1972b).

Consideration of the observations presented above indicates that glycine should be an early product of [^{14}C]acetate metabolism by methanol-grown cells containing induced activities of the specific serine-glyoxylate aminotransferase. This prediction was

tested by incubating methanol-grown organisms with [^{14}C]acetate; samples were removed at various time-intervals and radioactive compounds were separated and identified as described by Dunstan *et al.* (1972a). From these results the proportions of radioactivity in non-volatile metabolic intermediates at each sampling time were determined. The proportion of radioactivity in glutamate was 4.5% at the first sample time (7s) and increased during the course of the experiment. The proportion of radioactivity in malate increased from 15 to 23% (at 30s), then decreased. A similar pattern of incorporation was measured for succinate+fumarate (which were not distinguished), but the proportion of radioactivity in these compounds was about one-quarter of that detected in malate. The only compounds that appeared to be early intermediates (i.e. those for which the proportion of radioactivity was constant or decreased from the earliest times) were glycine (6% in the first sample) and an unknown substance (compound 'B') (15% in the first sample). This compound 'B' has been previously implicated in the metabolism of acetate by *Pseudomonas* AM1. These labelling patterns contrast with those obtained with ethanol-grown organisms, where the early intermediates in the metabolism of acetate were glycollate, malate and compound 'B'.

In summary, the results presented here confirm that enzymes capable of rapid metabolism of acetate to glycine are present during growth of *Pseudomonas* AM1 on methanol.

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The Microbial Metabolism of Nitrilotriacetate

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An organism that is capable of utilizing nitrilotriacetate as sole source of carbon and nitrogen for growth was isolated by elective culture and tentatively identified as a pseudomonad. The generation time on this substrate was 6h. Unlike a previously described pseudomonad (Focht & Joseph, 1971), the present organism grew more rapidly on nitrilotriacetate than on its possible metabolites iminodiacetate and glycine, and washed suspensions of nitrilotriacetate-grown cells showed very little ability