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