The Oxidation and Assimilation of C₂ Compounds by *Hyphomicrobium* sp.

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SUMMARY

Hyphomicrobium sp. were grown on ethanol, acetate, 3-hydroxybutyrate and methanol. The specific activities of the following enzymes were measured in cell-free extracts of *Hyphomicrobium* strain x: ethanol dehydrogenase, acetaldehyde dehydrogenase, acetoxykinase, 3-hydroxybutyrate dehydrogenase, β -keto thiolase, citrate synthase, aconitate hydratase, isocitrate dehydrogenase, α -oxoglutarate dehydrogenase, succinate dehydrogenase, fumarate hydratase, malate dehydrogenase, isocitrate lyase, malate synthase, malate dehydrogenase (decarboxylating), phosphoenolpyruvate carboxylase, pyruvate kinase, phosphoenolpyruvate carboxykinase, phosphopyruvate hydratase and phosphoglycerate mutase. From a comparison of the specific activities of these enzymes in the different cell-free extracts, it was concluded that during growth of *Hyphomicrobium* sp. on either ethanol, acetate or 3-hydroxybutyrate, energy and reducing power is generated mainly by the tricarboxylic acid (TCA) cycle. Carbon is assimilated via the glyoxylate cycle with phosphoenolpyruvate carboxykinase functioning as the main gluconeogenic enzyme. The conversion of ethanol, acetate and 3-hydroxybutyrate to acetyl-CoA occurs by pathways previously reported in other micro-organisms.

INTRODUCTION

Strains of the genus *Hyphomicrobium* have been isolated and purified from a wide range of natural sources (Attwood & Harder, 1972). When the strains were screened for their ability to grow in mineral media supplemented with different carbon compounds as the sole source of carbon and energy, only a limited number of compounds could be utilized (Attwood & Harder, 1973). The C₁ compounds methanol, methylamine and formate, the C₂ compounds ethanol and acetate, and the C₄ compound 3-hydroxybutyrate could support growth. On the basis of these results we decided to investigate the dissimilation and assimilation pathways of (i) C₁ compounds (Harder, Attwood & Quayle, 1973), and (ii) C₂ compounds and 3-hydroxybutyrate. The present paper reports the results obtained when the organisms were grown on ethanol, acetate and 3-hydroxybutyrate.

METHODS

Growth of the organism. *Hyphomicrobium* strains x and G were used throughout the investigation. Both strains were maintained as previously reported (Harder *et al.* 1973).

Growth in liquid media was in 3 l flasks containing 750 ml of an inorganic salts medium

supplemented with the appropriate carbon and energy source (0.5 %) (Attwood & Harder, 1972). A 2 % inoculum of organisms previously grown on the same carbon source was added to the flask and incubated on a shaker at 30 °C (Harder *et al.* 1973). When the organisms reached the mid-exponential phase of growth they were harvested by centrifuging at 6500 *g* for 20 min, washed with 50 mM-sodium-potassium phosphate buffer pH 7.0, and either resuspended in the same buffer containing 5 mM-MgCl₂ and 2 mM-2-mercaptoethanol or stored at -20 °C. Determinations of the specific growth rate, μ [$\mu = 1/x (dx/dt)$ where x is a function of organism mass], were made by following the change in extinction at 430 nm using conical flasks (250 ml) fitted with optically matched side arms.

Preparation of cell-free extracts. Bacteria were disrupted in an ultrasonic disintegrator by treating a suspension of 1 g wt of organisms in 4 ml sodium-potassium phosphate buffer (50 mM, pH 7.0) containing 2-mercaptoethanol (2 mM) for 3×1 min at 0 °C, and the supernatant obtained after high speed centrifuging was used as the crude extract (Harder *et al.* 1973).

Protein determination. Protein was assayed by the Folin-Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as standard.

Chemicals. Acetyl-CoA was prepared as described by Stadtman (1957) and acetoacetyl-CoA as described by Senior & Dawes (1973). Purified enzymes, nicotinamide nucleotides, nucleotides and coenzyme A were obtained from Boehringer Corp. (London) Ltd, and bovine serum albumin from Armour Pharmaceutical Co., Eastbourne, Sussex.

Enzyme assays. All spectrophotometric assays were performed on a Unicam SP1800 recording spectrophotometer at 30 °C. The amounts of enzyme assayed were such that the rates were linear with respect to time for at least 3 min and were proportional to the amount of extract added. α -Oxoglutarate dehydrogenase was assayed as the complex by the method of Amarasingham & Davis (1965), and assayed spectrophotometrically at 420 nm and pH 6.0 with ferricyanide as the electron acceptor according to Hager & Kornberg (1961). The following enzymes were assayed by published procedures: ethanol dehydrogenase (NAD), EC. 1.1.1.1 (Biochemica catalogue, 1968; C. G. Boehringer und Soehne GmbH, Mannheim, Germany); acetaldehyde dehydrogenase (NAD), EC. 1.2.1.3 (Racker, 1955); acetaldehyde dehydrogenase (CoA) (Dawes & Foster, 1956); acetothio kinase, EC. 6.2.1.1 (Jones & Lipmann, 1955); acetokinase, EC. 2.7.2.1 (Rose, 1955); phosphotransacetylase, EC. 2.3.1.7 (Stadtman, 1955); 3-hydroxybutyrate dehydrogenase (NAD), EC. 1.1.1.30 (Senior & Dawes, 1973); β -keto thiolase reductase (cleavage), EC. 2.3.1.16 (Senior & Dawes, 1973); acetyl CoA reductase, EC. 1.1.1.36 (Senior & Dawes, 1973); citrate synthase, EC. 4.1.3.7 (Srere, 1969); aconitate hydratase, EC. 4.2.13 (Fansler & Lowenstein, 1969); isocitrate dehydrogenase, EC. 1.1.1.41 and EC. 1.1.1.42 (Cleland, Thompson & Barden, 1969); succinate dehydrogenase, EC. 1.3.99.2 (Arrigoni & Singer, 1962); fumarate hydratase, EC. 4.2.12 (Hill & Bradshaw, 1969); malate dehydrogenase, EC. 1.1.3.7 (Harder *et al.* 1973); isocitrate lyase, EC. 4.1.3.1 (Dixon & Kornberg, 1959); malate synthase, EC. 4.1.3.2 (Dixon & Kornberg, 1969); phosphoenolpyruvate (PEP) carboxylase, EC. 4.1.1.31 (Canovas & Kornberg, 1969); pyruvate carboxylase, EC. 6.4.1.1 (Seubert & Weicker, 1969); PEP carboxykinase, EC. 4.1.1.32 (Salem, Wagner, Hacking & Quayle, 1973); PEP synthase (Cooper & Kornberg, 1969); pyruvate kinase, EC. 2.7.1.40 (Biochemica Catalogue, 1968, Boehringer); malate dehydrogenase (decarboxylating), EC. 1.1.1.38 and EC. 1.1.1.40 (Hsu & Lardy, 1969); lactate dehydrogenase, EC. 1.1.1.27 (Biochemica Catalogue, 1968, Boehringer); pyruvate dehydrogenase, EC. 6.4.1.1 (Amarasingham & Davis, 1965).

Table 1. *Specific activities of enzymes involved in the oxidation of ethanol in extracts of ethanol- and methanol-grown Hyphomicrobium x*

Assay methods were as described in Methods. The values in parentheses refer to measurements made on a second batch of organisms grown separately. n.d., Not detected.

Enzyme	Specific activity ($\mu\text{mol/min/mg protein}$)	
	Ethanol-grown	Methanol-grown
Ethanol dehydrogenase (NAD)	0.13 (0.14)	n.d.
Acetaldehyde dehydrogenase (NAD)	0.01 (0.01)	n.d.
Acetaldehyde dehydrogenase (NADP)	n.d.	n.d.
Acetaldehyde dehydrogenase (CoA)	0.01 (0.01)	n.d.
Acetothiokinase	0.01 (0.01)	0.002 (0.003)
Acetokinase	n.d.	n.d.
Phosphotransacetylase	n.d.	n.d.

RESULTS AND DISCUSSION

The results obtained for *Hyphomicrobium* strains x and G were essentially similar and in the following Tables only data for strain x are presented. The doubling time for this strain at 30 °C in a mineral medium with methanol (0.5 %, v/v) as the carbon and energy source was 6.9 h; with ethanol (0.5 %, v/v) 9.8 h; with acetate (0.5 %, v/v) 19 h and with 3-hydroxybutyrate (0.5 %, v/v) 35 h.

The enzymes of ethanol oxidation

Large quantities of organisms (10 g) were obtained which had been grown in liquid culture on either ethanol or methanol. The latter organisms were used in control experiments.

Crude extracts were prepared, and using the procedures outlined above the extracts were assayed for activities of enzymes probably associated with growth on ethanol. Table 1 shows the specific activities of enzymes associated with ethanol oxidation. Ethanol-grown cells contain an active NAD-dependent ethanol dehydrogenase together with CoA- and NAD-dependent acetaldehyde dehydrogenases. The NAD-dependent ethanol dehydrogenase and the CoA-dependent acetaldehyde dehydrogenase were reversible. To show the reversibility of the CoA-dependent acetaldehyde dehydrogenase, hydroxylamine (20 μM final concentration) had to be added to the reaction mixture to inhibit the activity of the alcohol dehydrogenase which was interfering with the assay in the reverse direction. No attempts were made to show the reversibility of the NAD-dependent acetaldehyde dehydrogenase, since any acetate formed can be activated by acetothiokinase activity. Thus the oxidation of ethanol appears to occur by its conversion to acetaldehyde in the presence of NAD. The acetaldehyde produced can either be converted to acetate which is then activated by acetothiokinase to acetyl-CoA, or be converted directly in a reaction dependent upon CoA (Fig. 1a). If this is true one would expect that the major part of the energy and reducing power required for growth would be generated from acetyl-CoA by its oxidation in a complete TCA cycle. Table 2 shows the specific activities of the TCA cycle enzymes in ethanol-grown cells. As expected, these cells can generate energy and reducing power through a complete TCA cycle and, together with a glyoxylate cycle, can assimilate carbon from acetyl-CoA to C_4 compounds.

Fig. 1. Pathways for the conversion of (a) ethanol, (b) acetate and (c) 3-hydroxy butyrate to acetyl-CoA in *Hyphomicrobium x*.

The enzymes of carbon assimilation in ethanol-grown cells

To complete the carbon assimilation pathway, possible routes for gluconeogenesis were investigated (Table 3). Ethanol-grown cells contain a malate dehydrogenase (decarboxylating) (NADP) enzyme. However, this is unlikely to have a physiological role in gluconeogenesis since enzyme kinetics favour the reaction converting pyruvate into malate. Furthermore, the cells do not have an active PEP synthase; thus any pyruvate formed cannot be converted into phosphoenolpyruvate. Neither ethanol- nor methanol-grown cells had a detectable pyruvate dehydrogenase, pyruvate carboxylase or lactate dehydrogenase. Thus the organism is unable to metabolize pyruvate except, presumably, to alanine. Unlike methanol-grown cells which appear not to be dependent upon an active phosphoenolpyruvate carboxykinase for gluconeogenesis (Harder *et al.* 1973), ethanol-grown *Hyphomicrobium* possess an active phosphoenolpyruvate carboxykinase (Table 3). Thus oxaloacetic acid can be converted directly into phosphoenolpyruvate by an ATP-dependent carboxylation reaction. The phosphoenolpyruvate formed can be metabolized by a reversal of the glycolytic pathway through phosphopyruvate hydratase and phosphoglycerate mutase. Thus ethanol is metabolized to acetyl-CoA and the energy and reducing power is

Table 2. *Specific activities of enzymes of the TCA and glyoxylate cycles in extracts of ethanol-, acetate-, 3-hydroxybutyrate- and methanol-grown Hyphomicrobium x*

Assay methods were as described in Methods. The values in parentheses refer to measurements made on a second batch of organisms grown separately. —, Not assayed; n.d., not detected.

Enzyme	Specific activity ($\mu\text{mol/min/mg protein}$)			
	Ethanol-grown	Acetate-grown	3-Hydroxybutyrate-grown	Methanol-grown
Citrate synthase	0.65 (0.60)	0.7	0.62 (0.65)	0.15 (0.18)
Aconitate hydratase	0.6 (0.7)	0.73	0.82	0.17 (0.2)
Isocitrate dehydrogenase (NADP)	0.35 (0.45)	0.15	0.15	0.09 (0.11)
α -Oxoglutarate dehydrogenase	0.02 (0.02)	—	—	Trace
Succinate dehydrogenase	0.005 (0.004)	0.002	0.001	—
Fumarate hydrolase	0.32 (0.37)	0.24	0.38	—
Malate dehydrogenase	8.8 (9.9)	5.1	8.3	1.7 (1.9)
Isocitrate lyase	0.04 (0.05)	0.04	0.03	0.02 (0.02)
Malate synthase	0.08 (0.08)	0.01	0.01	n.d.

Table 3. *Specific activities of enzymes involved in possible gluconeogenic pathways in extracts of ethanol-, acetate-, 3-hydroxybutyrate- and methanol-grown Hyphomicrobium x*

Assay methods were as described in Methods. The values in parentheses refer to measurements made on a second batch of organisms grown separately. —, Not assayed; n.d., not detected.

Enzyme	Specific activity ($\mu\text{mol/min/mg protein}$)			
	Ethanol-grown	Acetate-grown	3-Hydroxybutyrate-grown	Methanol-grown
Phosphoenolpyruvate synthase	n.d.	n.d.	n.d.	n.d.
Pyruvate kinase	0.02 (0.02)	0.01	0.01	0.02 (0.02)
Pyruvate dehydrogenase	n.d.	n.d.	—	n.d.
Pyruvate carboxylase	n.d.	n.d.	n.d.	n.d.
Phosphoenolpyruvate carboxylase (acetyl CoA and CO_2 dependent)	0.02 (0.02)	0.01	0.01	0.25 (0.25)
Phosphoenolpyruvate carboxykinase	0.01	0.001	0.001	Trace
Malate dehydrogenase (decarboxylating) (NADP)	0.04 (0.05)	0.01	—	n.d.
Malate dehydrogenase (decarboxylating) (NAD)	n.d.	n.d.	n.d.	n.d.
Phosphopyruvate hydratase	0.35 (0.34)	0.05	0.03	0.2 (0.2)
Phosphoglycerate mutase	0.02 (0.03)	0.02	0.02	0.04 (0.04)

generated from the acetyl-CoA by its oxidation in a complete TCA cycle. The synthesis of cell material occurs by the assimilation of acetyl-CoA through the glyoxylate cycle with PEP carboxykinase functioning as the main gluconeogenic enzyme.

The enzymes of acetate oxidation

Crude extracts of acetate-grown and ethanol-grown cells were prepared and assayed for enzymes which would result in a conversion of acetate into acetyl-CoA. The ethanol-grown cells were used in control experiments. The results showed that acetate was directly activated by acetothio kinase ($0.05 \mu\text{mol/min/mg protein}$) rather than converted to acetyl phosphate by acetokinase and then to acetyl-CoA by phosphotransacetylase. Again, energy and

reducing power would be expected to be generated via a complete TCA cycle. This is shown to occur (see Table 2). Thus acetate is activated to acetyl-CoA and then the acetyl-CoA is oxidized via the TCA cycle and assimilated by the glyoxylate cycle together with gluconeogenesis via phosphoenolpyruvate carboxykinase in a manner similar to that found in ethanol-grown cells (Fig. 1*b*).

The enzymes of 3-hydroxybutyrate oxidation

When a wide range of C_3 , C_4 and C_6 compounds (pyruvate, lactate, serine, propanol, succinate, malate, citrate, butanol, 3-hydroxybutyrate, fructose and glucose) were tested as possible growth substrates for *Hyphomicrobium* sp., growth only occurred with 3-hydroxybutyrate. It was suggested that a possible explanation for the inability to grow on substrates containing more than two carbon atoms might be a lack of pyruvate dehydrogenase activity (Attwood & Harder, 1973) (Table 3). If this supposition is correct, the organism is unable to generate acetyl-CoA from such substrates and therefore cannot obtain energy from the oxidation of these compounds. Although 3-hydroxybutyrate is a C_4 compound it was considered likely that the initial metabolism would result in its conversion into acetyl-CoA (Senior & Dawes, 1973). Crude extracts of 3-hydroxybutyrate-grown cells and ethanol-grown cells were prepared and assayed for the enzymes of 3-hydroxybutyrate oxidation. The extract of ethanol-grown cells was used as a control. The results indicate that 3-hydroxybutyrate is converted to acetyl-CoA by enzyme reactions reported in *Azotobacter beijerinckii* (Senior & Dawes, 1973) and *Hydrogenomonas eutropha* (Oeding & Schlegel, 1973). 3-Hydroxybutyrate is oxidized to acetoacetate by D-3-hydroxybutyrate dehydrogenase (NAD) ($0.09 \mu\text{mol/min/mg}$ protein) which is then activated by acetoacetate:succinate CoA transferase to give acetoacetyl-CoA. The acetoacetyl-CoA is cleaved by β -keto thiolase ($0.05 \mu\text{mol/min/mg}$ protein) to give acetyl-CoA. No attempts were made to assay acetoacetate:succinate CoA transferase in the present study. Reduction of the acetoacetyl-CoA to D(-) 3-hydroxybutyryl-CoA could not be detected (Fig. 1*c*). Again, energy and reducing power are generated in the TCA cycle (see Table 2) and carbon assimilation is via the glyoxylate cycle with phosphoenolpyruvate carboxykinase probably the main gluconeogenic enzyme (Table 3).

Thus, *Hyphomicrobium* sp. grow on ethanol, acetate and 3-hydroxybutyrate in essentially the same manner. The compounds are converted to acetyl-CoA and completely oxidized in a TCA cycle to produce energy and reducing power. Carbon is assimilated in the glyoxylate cycle and gluconeogenesis via phosphoenolpyruvate carboxykinase. The conversion of ethanol, acetate and 3-hydroxybutyrate to acetyl-CoA occurs via well-documented pathways (Kornberg, 1970; Senior & Dawes, 1973).

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