Cleavage of Malyl-Coenzyme A into Acetyl-Coenzyme A and Glyoxylate by *Pseudomonas* AM1 and other C₁-Unit-Utilizing Bacteria

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1. Malyl-CoA lyase was found in high activity in extracts of *Pseudomonas* AM1, *Pseudomonas* MA, *Pseudomonas* MS, *Hyphomicrobium* X and *Methylosinus trichosporium*. 2. The enzyme cleaves (2*S*)-malyl-CoA into equimolar amounts of acetyl-CoA and glyoxylate in the presence of Mg²⁺. 3. The specific activity of malyl-CoA lyase was several-fold higher in *Pseudomonas* AM1 when grown on C₁ compounds than when grown on C₂, C₃ or C₄ compounds. This suggests that the enzyme plays a specially important role in C₁ metabolism. 4. It is suggested that its role in C₁ metabolism, in organisms utilizing the serine pathway, is to provide the glyoxylate necessary to sustain operation of this pathway. 5. The activity of malyl-CoA lyase in extracts of *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X is 27–50 times higher than the activity of ATP- and CoA-dependent cleavage of malate, suggesting that the latter activity may be due to coupling of two enzymes, malate thiokinase and malyl-CoA lyase. 6. Methane-grown *Pseudomonas methanica* and *Methylococcus capsulatus*, which are not known to use the serine pathway, possess appreciable amounts of malyl-CoA lyase. Instead of being used primarily for carbon assimilation, the enzyme may here serve as a route to glycine during biosynthesis of purines and proteins.

Synthesis of cell constituents by *Pseudomonas* AM1 growing on C₁ compounds is thought to involve reactions of the serine pathway (see Quayle, 1972), summarized as follows:

has been found in extracts of malate-plus-glutamate-grown *Rhodopseudomonas spheroides* (Tuboi & Kikuchi, 1962, 1963), methylamine-grown *Pseudomonas* MA (Hersh & Bellion, 1972; Bellion & Hersh,

Double arrows indicate more than one step.

The serine pathway uses one molecule of glyoxylate (in reaction 3) to produce each molecule of phosphoenolpyruvate or oxaloacetate. Radioisotopic evidence from experiments with whole organisms indicates that the glyoxylate arises from cleavage of a C₄

1972), and trimethylamine-grown *Bacterium* 5H2 (Cox & Zatman, 1973). Mue *et al.* (1964) and Tuboi & Kikuchi (1965) obtained evidence that the ATP- and CoA-dependent malate lyase activity in extracts of *R. spheroides* is due to two enzymes catalysing, respectively:

compound derived from succinate (Salem *et al.*, 1972), but direct enzymic evidence for this transformation has been lacking.

An ATP- and CoA-dependent malate lyase catalysing the reaction:

No enzyme system catalysing the overall reaction (6) has been detected in extracts of methanol-grown *Pseudomonas* AM1 by Bellion & Hersh (1972) or ourselves. The present paper records the finding that extracts of *Pseudomonas* AM1 and other C₁-utilizing

bacteria do, however, contain, in high activity, a malyl-CoA lyase catalysing reaction (8). The possibility is discussed that this enzyme provides the glyoxylate necessary for the serine pathway.

A preliminary account of this work has been presented (Salem *et al.*, 1973a).

Materials and Methods

Chemicals

(S) - (β -Hydroxysuccinyl) - *N* - octanoylcysteamine was a gift from Professor H. Eggerer (Universität Regensburg, Germany). (2*S*)-4-Malyl-CoA was prepared from (S)-(β -hydroxysuccinyl)-*N*-octanoylcysteamine by a modification of the ester interchange method described by Eggerer & Grünwälder (1964); (S) - (β - hydroxysuccinyl) - *N* - octanoylcysteamine (16mg; 50 μ mol) and CoA (20mg; 26 μ mol) were dissolved in 4ml of 0.2M-KHCO₃, 1ml of diethyl ether (peroxide-free) was added and the mixture shaken at room temperature for 1h. The mixture was then extracted with ether in a continuous-extraction apparatus for 1h, with that part of the extraction apparatus containing the aqueous phase kept at 0°C in an ice-bath. The aqueous phase was then withdrawn and acidified to pH4 with 5M-HCl at 0°C. The acidified mixture was again continuously extracted with ether for 3h, the aqueous layer being maintained at 0°C. At the end of this time, the aqueous phase was withdrawn and the dissolved ether was removed from it with a stream of N₂. The resulting solution of (2*S*)-4-malyl-CoA (approx. 3.5mm) was stored either at 0°C or at -15°C, and was stable under these conditions for several weeks.

Acetyl-CoA and 3-carboxypropionyl-CoA (succinyl-CoA) were prepared by reaction of their respective acid anhydrides with CoA, as described by Stadtman (1957). Acetyl-CoA, 3-carboxypropionyl-CoA and 4-malyl-CoA were assayed chemically from the decrease in absorbance at 232nm (acetyl-CoA and 3-carboxypropionyl-CoA) or 234nm (4-malyl-CoA) associated with their hydrolysis in 0.1M-NaOH at 30°C for 10min. The differences in molar extinction coefficients of the acyl-CoA derivatives and their hydrolysis products were taken to be 4.5×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for acetyl-CoA and 3-carboxypropionyl-CoA (Stadtman, 1957) and 4.8×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for 4-malyl-CoA (Eggerer & Grünwälder, 1964). Acetyl-CoA was assayed enzymically by arsenolysis in the presence of phosphate acetyltransferase (acetyl-CoA-orthophosphate acetyltransferase, EC 2.3.1.8) and arsenate (Stadtman, 1957) or conversion into citrate and CoA in the presence of oxaloacetate and citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] followed by assay of CoA with 5,5'-dithiobis-(2-nitrobenzoate) (Tubbs & Garland, 1969).

Acetylhydroxamate and 4-malylmonohydroxamate

were prepared from acetic anhydride and 4-malyl-CoA respectively by reaction with neutral hydroxylamine (Stadtman, 1957).

Purified enzymes, nucleotides and CoA were purchased from Boehringer Corp. (London) Ltd, London W5 2TZ, U.K.

Maintenance and growth of the organisms

Pseudomonas AM1 (N.C.I.B. 9133) was maintained in slope culture on an inorganic-salts medium (Jayasuriya, 1955) containing methylamine hydrochloride (50mm) and agar (1.5%, w/v). The organism was subcultured monthly and stored at 4°C after incubation for 3-4 days at 30°C. Liquid cultures were prepared as described by Heptinstall & Quayle (1970) with methanol (0.5%, v/v) as carbon source. Other alternative carbon sources were: ethanol (0.1%, v/v), sodium DL-malate (100mm), sodium lactate (30mm), sodium succinate (50mm), sodium 3-hydroxybutyrate (50mm), sodium oxalate (50mm), sodium glyoxylate (10mm) and methylamine hydrochloride (50mm).

Pseudomonas MA, Shaw strain, from Dr. E. Bellion, University of Texas Southwestern Medical School, Dallas, Tex., U.S.A., was cultured on a salts medium (Shaw *et al.*, 1966) with methylamine (100mm) as carbon and nitrogen source, or with succinate (100mm)+NH₄Cl (100mm).

Pseudomonas MS, from Dr. C. Wagner, Veterans Administration Hospital, Department of Microbiology, Nashville, Tenn., U.S.A., was maintained and grown on methanol or succinate in the media used for *Pseudomonas* AM1.

Hyphomicrobium X, from Dr. M. M. Attwood, Department of Microbiology, University of Sheffield, Sheffield, U.K., was grown on methanol (0.5%, v/v) or ethanol (0.5%, v/v) in the salts medium of Attwood & Harder (1972).

Pseudomonas methanica was grown on methane as described by Leadbetter & Foster (1958).

Methylosinus trichosporium, strain OB3b, from Professor R. Whittenbury, Department of Biological Sciences, University of Warwick, Coventry, U.K., was maintained and grown on methane as described by Lawrence & Quayle (1970).

Methylococcus capsulatus was grown on methane at 37°C in a salts medium containing (per litre): Na₂HPO₄ (0.6g), KH₂PO₄ (0.4g), NH₄Cl (0.4g), MgSO₄ $\cdot$ 7H₂O (0.2g), FeCl₃ $\cdot$ 6H₂O (16.7mg), CaCl₂ $\cdot$ 2H₂O (0.66mg), ZnSO₄ $\cdot$ 7H₂O (0.18mg), CuSO₄ $\cdot$ 5H₂O (0.16mg), MnSO₄ $\cdot$ 4H₂O (0.15mg), CoCl₂ $\cdot$ 6H₂O (0.18mg), H₃BO₃ (0.1mg), Na₂MoO₄ $\cdot$ 2H₂O (0.3mg).

Preparation of cell-free extracts

Ultrasonic extracts were prepared as described by Harder & Quayle (1971). The supernatant fluids after

centrifuging at 20000g for 30 min at 0°C were used for enzyme assays. For some purposes, the enzyme extract obtained from methanol-grown *Pseudomonas* AM1 was treated with protamine sulphate and (NH₄)₂SO₄ as follows. To the crude ultrasonic extract at 0°C was added protamine sulphate (1 mg/mg of protein) in 20 mM-potassium phosphate buffer, pH 7.0, and the supernatant fluid after centrifuging (20000g; 10 min) was retained for precipitation of protein by (NH₄)₂SO₄. The protein precipitated between 50 and 70% saturation of (NH₄)₂SO₄ was collected by centrifugation (20000g; 10 min) and redissolved in 20 mM-potassium phosphate buffer, pH 7.0, and dialysed for 18 h at 2°C against the same buffer. About half the total of 4-malyl-CoA lyase activity in the original crude extract was recovered in this fraction. The extract obtained by this procedure retained more than 58% of its 4-malyl-CoA lyase activity during storage at -15°C for 1 month.

Enzyme assays

Spectrophotometric results were obtained with a Unicam SP.1800 recording spectrophotometer. All enzyme assays were conducted at 30°C.

Malate lyase (CoA-acetylating, ATP-cleaving). This was assayed spectrophotometrically at 324 nm by measuring the cleavage of L-malate to acetyl-CoA and glyoxylate with added phenylhydrazine, as described by Bellion & Hersh (1972).

Malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2]. This enzyme was assayed by the method of Dixon & Kornberg (1962).

Protein determinations

Protein was measured by the Folin-Ciocalteu method as described by Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Chromatography of dinitrophenylhydrazones

The method used to isolate acidic dinitrophenylhydrazones was modified from that described by Kun & Garcia-Hernandez (1957). Dinitrophenylhydrazones in aqueous solution were first partitioned into 2 ml of ethyl acetate and acidic derivatives were then extracted by shaking with 1 ml of aq. Na₂CO₃ (10%, w/v). The carbonate extract was rapidly removed, acidified to pH 1.0 with HCl and re-extracted with ethyl acetate (2 × 2 ml). This extract was concentrated by evaporation in a stream of N₂ and chromatographed by t.l.c. on cellulose (100 μm thickness) with a solvent containing butan-1-ol-ethanol-aq. NH₃ (sp.gr. 0.88)-water (140:20:1:39, by vol.) (El Hawary & Thompson, 1953).

Chromatography of acyl thiol esters and acyl hydroxamates

Acyl thiol esters (0.25 μmol) were chromatographed on thin-layer plates of cellulose (100 μm thickness) with as solvent, ethanol-0.1 M-sodium acetate, pH 4.5 (1:1, v/v) (Stadtman, 1957). CoA, either free or present in an acyl thiol ester, was located as a zone of absorption under u.v. light. Thiols and thiol esters were located as red spots after chromatograms were sprayed with nitroprusside reagent and then with methanolic NaOH (Stadtman, 1957). Acyl hydroxamates (0.1 μmol) were chromatographed on thin-layer plates of cellulose (100 μm thickness) in a solvent consisting of propan-2-ol-pyridine-water (1:1:1, by vol.) (Vagelos, 1960) and located by spraying with 5% (w/v) FeCl₃·6H₂O in ethanol (0.1 M with respect to HCl) (Stadtman & Barker, 1950).

Results

An enzyme system catalysing reaction (6) has been found in extracts of *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X when grown on C₁ substrates (Table 1); no activity was found in extracts

Table 1. Activity of ATP- and CoA-dependent malate lyase in extracts of bacteria able to grow on C₁ compounds
ATP- and CoA-dependent malate lyase was measured by the method of Bellion & Hersh (1972). *, Not detectable.

Organism	Carbon source for growth	Specific activity of malate lyase (μmol/h per mg of protein)
<i>Pseudomonas</i> AM1	Methanol	0*
<i>Pseudomonas</i> MA	Methylamine	2.47
	Succinate	0*
<i>Pseudomonas</i> MS	Methanol	1.65
<i>Hyphomicrobium</i> X	Methanol	0.76
	Ethanol	0*
<i>P. methanica</i>	Methane	0*
<i>Mtc. capsulatus</i>	Methane	0*
<i>Mts. trichosporium</i>	Methane	0*

of *Pseudomonas* AM1 or three methane-utilizing bacteria. When 0.15 mM 4-malyl-CoA was substituted in the reaction mixture in place of malate, CoA and ATP, there was a rapid increase in E_{324} equivalent to the formation of 90–100 μ mol of glyoxylate phenylhydrazone/h per mg of protein. The requirements for this reaction were then studied.

Requirements for the cleavage of 4-malyl-CoA by Pseudomonas AM1 extract

The basal reaction mixture for the study and routine assay of 4-malyl-CoA lyase contained in a final volume of 1 ml: potassium phosphate buffer, pH 7.4 (100 μ mol), $MgCl_2$ (10 μ mol), phenylhydrazine-HCl (5 μ mol) and enzymic extract (2–100 μ g of protein). The reaction was initiated by the addition of 0.2 μ mol of 4-malyl-CoA and the E_{324} was recorded at 30°C by using a reference mixture lacking 4-malyl-CoA. Under these conditions, with extracts of the methanol-grown organism, there was an increase in E_{324} corresponding to the formation of 80–100 μ mol of glyoxylate phenylhydrazone/h per mg of protein.

The requirements of the reaction were studied by using the enzyme fraction precipitating between 50 and 70% of saturation with $(NH_4)_2SO_4$. No activity was detected when $MgCl_2$ was omitted or when 4-malyl-CoA was replaced by sodium L-malate, CoA or (S)-(β-hydroxysuccinyl)-N-octanoylcysteamine (0.25 μ mol in 0.2 M- K_2CO_3). Replacement of Mg^{2+} by Mn^{2+} decreased the activity by more than 50%, but addition of 2-mercaptoethanol (5 μ mol) to the basal mixture did not affect the rate of reaction. When measured in 0.1 M-Tris-HCl buffer, pH 7.4, the reaction proceeded at about half the rate observed in phosphate buffer. No change in extinction was observed in a control tube containing boiled extract or when phenylhydrazine was omitted from the mixture.

Identification of the products of 4-malyl-CoA cleavage

The following experiments showed that glyoxylate and acetyl-CoA were the products formed from cleavage of 4-malyl-CoA.

The absorption spectrum of the reaction product formed in the presence of phenylhydrazine in the basal reaction mixture described above displayed a maximum at 323 nm and was identical with that obtained with authentic glyoxylate phenylhydrazone.

Glyoxylate was also identified as a cleavage product by chromatography of its 2,4-dinitrophenylhydrazone. A reaction mixture (3 ml) containing potassium phosphate buffer, pH 7.4 (300 μ mol), $MgCl_2$ (30 μ mol), 4-malyl-CoA (1.5 μ mol) and ultrasonic extract of methanol-grown *Pseudomonas* AM1 (1 mg of protein) was incubated for 15 min at 30°C and the reaction was stopped by adding conc. HCl (0.1 ml). To this solution 1 ml of 2,4-dinitrophenylhydrazine (0.1%, w/v, in 2 M-HCl) was added, and

after standing for 10 min at 30°C the dinitrophenylhydrazones were extracted into 2 ml of ethyl acetate and the acidic dinitrophenylhydrazones chromatographed by t.l.c. on cellulose. Two major chromatographic spots were detected, with R_F values of 0.39 and 0.57, identical with those obtained with an authentic glyoxylate dinitrophenylhydrazone preparation. No acidic dinitrophenylhydrazones were detected in ethyl acetate extracts of control reaction mixtures lacking 4-malyl-CoA or complete reaction mixtures stopped with conc. HCl at zero time.

Evidence for acetyl-CoA as a cleavage product was inferred from its hydrolysis catalysed by phosphate acetyltransferase in arsenate buffer (Stadtman, 1957). The ability of ultrasonic extracts of methanol-grown *Pseudomonas* AM1 to cleave 4-malyl-CoA was not significantly impaired when potassium phosphate in the basal assay mixture was replaced by potassium arsenate buffer, pH 7.0 (50 mM). To demonstrate the production of acetyl-CoA, the reaction mixture contained in a final volume of 1 ml: potassium arsenate buffer, pH 7.0 (50 μ mol), $MgCl_2$ (10 μ mol), 4-malyl-CoA (0.2 μ mol) and ultrasonic extract of methanol-grown *Pseudomonas* AM1 (10 μ g of protein). The change in E_{232} was measured against a reference solution lacking 4-malyl-CoA. A slow decrease in E_{232} (less than 0.01 unit/min) was observed, presumably representing any difference in extinction between 4-malyl-CoA and acyl-CoA product or reflecting a slow spontaneous or enzymic hydrolysis of an acyl-CoA. On addition of phosphate acetyltransferase (5 EC units) to each cuvette, a rapid decrease in extinction occurred (0.20 unit/min), indicating the hydrolysis of an acyl thiol ester by this enzyme. No such rapid reaction occurred in the absence of bacterial extract, showing that 4-malyl-CoA was not hydrolysed by phosphate acetyltransferase. Instead, the latter enzyme catalysed hydrolysis of a thiol ester product of the reaction between the bacterial extract and 4-malyl-CoA. Although this product was likely to be acetyl-CoA, phosphate acetyltransferase may also hydrolyse propionyl and butyryl thiol esters under these conditions (Stadtman, 1957).

Further evidence for acetyl-CoA was obtained by t.l.c. on cellulose of the thiol ester products of the cleavage reaction and also their acyl hydroxamate derivatives. The reaction mixture for this purpose contained in a volume of 1 ml: potassium phosphate buffer, pH 7.4 (100 μ mol), $MgCl_2$ (10 μ mol), phenylhydrazine (5 μ mol), crude ultrasonic extract of methanol-grown *Pseudomonas* AM1 (100 μ g of protein) and 4-malyl-CoA (0.5 μ mol). A control tube containing boiled bacterial extract and all other constituents except enzymic extract was treated in parallel. The reaction mixtures were incubated at 30°C for 5 min and samples (50 μ l) were withdrawn without further treatment for t.l.c. of acyl thiol esters. The remaining mixtures were incubated for a further 10 min with

20 μ l of neutral 2M-hydroxylamine. Ethanol (10ml) was then added to each tube and the insoluble material was removed by centrifugation (5000g; 20min). The supernatant fluids were evaporated to dryness under reduced pressure, the residue was redissolved in 0.2ml of ethanol, and samples (25 μ l) were taken for t.l.c. of acyl hydroxamates.

Free authentic thiol esters were of limited value as chromatographic markers, since other components in the reaction mixture considerably decreased the R_F values of the thiol esters. Consequently, authentic 4-malyl-CoA and acetyl-CoA were added to tubes containing all the components of the reaction mixture (except for the malyl-CoA substrate) with enzymic extract replaced by a boiled extract. In this way, spots corresponding exactly to both 4-malyl-CoA (R_F 0.24) and acetyl-CoA (R_F 0.43) were identified in the complete reaction mixture incubated with active enzymic extract, but acetyl-CoA was not formed from 4-malyl-CoA in the control tube incubated with boiled extract.

Chromatography of the hydroxamates formed from the reaction mixture containing enzymic extract showed two spots: a minor one (R_F 0.33) which co-chromatographed with authentic 4-malylmono-hydroxamate, and a major spot (R_F 0.84) which co-chromatographed with authentic acetyl hydroxamate. The latter spot was not detected in the control mixture containing boiled extract.

Stoichiometry of the malyl-CoA lyase reaction

The stoichiometry of acetyl-CoA and glyoxylate formation from 4-malyl-CoA was determined by taking advantage of the activity of 4-malyl-CoA lyase in arsenate buffer. In this buffer, the cleavage reaction was pulled to completion by arsenolysis of acetyl-

CoA by added phosphate acetyltransferase and was monitored by recording E_{232} (ϵ_{232} for acetyl-CoA = 4.5×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹). Phenylhydrazine, which absorbs strongly at this wavelength, was not present in the reaction mixture at this stage. When no further change of absorbance occurred, the wavelength was altered to 324nm and the total change in absorbance on addition of phenylhydrazine was measured (ϵ^{324} for glyoxylate phenylhydrazone = 1.7×10^4 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹).

In a typical experiment, the reaction mixture contained in a volume of 1 ml: potassium arsenate buffer, pH 7.0 (50 μ mol), MgCl₂ (10 μ mol), 4-malyl-CoA (0.08 μ mol), phosphate acetyltransferase (5 EC units) and the partially purified ultrasonic extract of methanol-grown *Pseudomonas* AM1 (16 μ g of protein). The reference mixture lacked 4-malyl-CoA. The change in E_{232} was 0.365 unit. After addition of phenylhydrazine (5 μ mol), the total change in E_{324} was 1.210 units. These results show that the formation of 0.081 μ mol of acetyl-CoA was accompanied by the formation of 0.071 μ mol of glyoxylate; this approximates to that expected for reaction (8).

Activity of malyl-CoA lyase in Pseudomonas AM1 grown on different carbon sources

The activity of malyl-CoA lyase was measured in ultrasonic extracts of *Pseudomonas* AM1 grown on a variety of carbon sources (Table 2). The specific activity during growth on C₁ compounds was several-fold higher than on any of the C₂, C₃ and C₄ substrates tested, suggesting that the enzyme plays a specially important role in C₁ metabolism. The presence of glyoxylate in a methanol growth medium did not significantly depress the specific activity of the enzyme.

Table 2. Activity of malyl-CoA lyase and malate synthase in ultrasonic extracts of *Pseudomonas* AM1 grown on various carbon sources

The basal reaction mixture described in the text was used to measure malyl-CoA lyase. Malate synthase was measured by the method of Dixon & Kornberg (1962). —, Not tested.

Carbon source for growth	Specific activity (μ mol/h per mg of protein)		Ratio of activities
	Malyl-CoA lyase	Malate synthase	
Methanol	99.0	1.3	76
Methylamine	87.0	1.5	58
Ethanol	12.2	1.2	10
Oxalate	31.3	1.3	24
Lactate	20.5	0.6	34
Succinate	18.0	0.6	30
Malate	14.5	0.5	29
3-Hydroxybutyrate	19.4	1.1	18
Methanol + glyoxylate	98.0	—	

Since the products of 4-malyl-CoA cleavage are identical with the substrates for malate synthase, the possibility is raised that 4-malyl-CoA cleavage may be due to a partial reaction catalysed by malate synthase. Accordingly, the activity of malate synthase was measured by the glyoxylate-dependent disappearance of acetyl-CoA (Dixon & Kornberg, 1962) in the same series of extracts of *Pseudomonas* AM1 grown on different carbon sources (Table 2). Malate synthase was present at low activities under all growth conditions tested, but the varying ratios of malyl-CoA lyase to malate synthase indicate that the two activities relate to different enzymes.

Malyl-CoA lyase in other bacteria which utilize C₁ compounds

Malyl-CoA lyase was found in extracts of a variety of bacteria grown on C₁ compounds (Table 3). The enzyme activity was considerably diminished or, in some cases, was undetectable during growth on multi-carbon substrates, emphasizing its probable physiological role in assimilation of C₁ compounds.

Of considerable interest are results with the methane-utilizing bacteria. Like *Pseudomonas* AM1, these organisms contain no detectable ATP- and CoA-dependent malate lyase (Table 1), yet their extracts catalyse the cleavage of 4-malyl-CoA (Table 3). The cleavage of a C₄ compound in this manner plays no known part in the pathways of net C₁ assimilation in *P. methanica* and *Mtc. capsulatus* (Kemp & Quayle, 1967; Lawrence *et al.*, 1970). *Mts. trichosporium*, however, uses the serine pathway for growth on C₁ compounds (Lawrence & Quayle, 1970).

It is further apparent from the results in Tables 1 and 3 that the extracts of C₁-grown *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X, which contain ATP- and CoA-dependent malate

lyase, cleaved 4-malyl-CoA 27–50 times faster than they catalysed the ATP- and CoA-dependent cleavage of malate. These results are discussed below in relation to the possible resolution of the ATP- and CoA-dependent malate lyase (reaction 6) into two component reactions (7) and (8).

Discussion

The present work has shown the existence of an enzyme in *Pseudomonas* AM1 which cleaves 4-malyl-CoA into acetyl-CoA and glyoxylate. It seems unlikely that this activity could be due to a partial reaction catalysed by enzymes such as citrate synthase or malate synthase. Although citrate synthase can hydrolyse 4-malyl-CoA to malate and CoA (Eggerer *et al.*, 1964), no cleavage reaction to acetyl-CoA and glyoxylate catalysed by this enzyme has been observed. Further, the reaction catalysed by the citrate synthase from *Pseudomonas* AM1, as with other citrate synthases, does not depend on a metal ion; this contrasts with the strict dependence of malyl-CoA lyase on Mg²⁺ ions. The independent variation of the specific activities of malyl-CoA lyase and malate synthase in *Pseudomonas* AM1 grown on different substrates argues against these two activities relating to a single enzyme. This would also correlate with the behaviour of the malate synthases from baker's yeast and *Pseudomonas ovalis* (Chester), which catalyse neither hydrolysis of 4-malyl-CoA nor its cleavage to acetyl-CoA and glyoxylate (Dixon *et al.*, 1960; Dixon & Kornberg, 1962; Eggerer *et al.*, 1964). Further work is now needed to clarify the significance of the relatively low specific activities of 'malate synthase' recorded in crude extracts of those organisms (e.g. *Pseudomonas* AM1 itself, *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X) which also possess high activities of malyl-CoA lyase. It is clear that reversal of reaction (8) coupled with a malyl-CoA

Table 3. Activity of malyl-CoA lyase in extracts of C₁-utilizing bacteria

Enzymic activity was measured in samples of each extract (2–100 µg of protein) by using the basal reaction mixture described in the text. *, Not detectable.

Organism	Carbon source for growth	Specific activity of malyl-CoA lyase (µmol/h per mg of protein)
<i>Pseudomonas</i> AM1	Methanol	99.0
	Succinate	18.0
<i>Pseudomonas</i> MA	Methylamine	66.5
	Succinate	0*
<i>Hyphomicrobium</i> X	Methanol	38.0
	Ethanol	0.7
<i>Pseudomonas</i> MS	Methanol	69.2
<i>Mtc. capsulatus</i>	Methane	6.0
<i>P. methanica</i>	Methane	3.7
<i>Mts. trichosporium</i>	Methane	11.1

deacylase or acyl-CoA transferase could be wrongly attributed to the activity of malate synthase.

Growth of *Pseudomonas* AM1 on C₁ compounds involves entry of a molecule of glyoxylate into the serine pathway; evidence from isotopic studies indicates that this glyoxylate arises from cleavage of a C₄ compound derived from succinate (Salem *et al.*, 1972). The finding that the specific activity of maly-CoA lyase in *Pseudomonas* AM1 is severalfold higher when the organism is grown on C₁ substrates than on C₂, C₃ or C₄ substrates points to maly-CoA lyase supplying this glyoxylate *in vivo*. The validity of this should be rigorously established by mutant studies. If maly-CoA lyase does indeed provide the necessary glyoxylate, then two problems are raised for which, in the case of *Pseudomonas* AM1, solutions are not apparent: (a) the formation of 4-maly-CoA, (b) the further metabolism of acetyl-CoA. In regard to the first question, some other organisms which are known to possess maly-CoA lyase activity (*Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X) can also effect the overall reaction (6) (Hersh & Bellion, 1972; Bellion & Hersh, 1972; Harder *et al.*, 1973) i.e. they possess malate thiokinase activity, either as part of one enzyme complex catalysing reaction (6) or as a separate enzyme catalysing reaction (7). Attempts to detect malate thiokinase by standard enzymic assay in extracts of *Pseudomonas* AM1 have so far been unsuccessful, nor has acyl-CoA transfer to malate from donors such as acetyl-CoA, 3-carboxypropionyl-CoA or oxalyl-CoA been observed in such extracts.

Assuming that the oxaloacetate skeleton can be converted into 4-maly-CoA, cleavage of this by reaction (8) can furnish glyoxylate for recycling into the serine pathway. This results in a cycle that can effect the net synthesis of acetyl-CoA from a C₁ unit and a molecule of CO₂. To replenish this cycle with glyoxylate when intermediates such as glycine, serine, phosphoglycerate or oxaloacetate are withdrawn for biosynthetic purposes it is necessary to convert some acetyl-CoA into glyoxylate. A sequence which can, in effect, accomplish the required oxidation of the methyl group of acetate involves the enzymes citrate synthase, aconitase and isocitrate lyase:

This reaction sequence can operate in methylamine-grown *Pseudomonas* MA (Bellion & Hersh, 1972), methanol-grown *Hyphomicrobium* X (Harder *et al.*, 1973) and may also occur in trimethylamine-grown *Bacterium* 5H2 (Cox & Zatman, 1973). Appreciable activities of isocitrate lyase have not, however, been detected in *Pseudomonas* AM1 (Large & Quayle, 1963; Dunstan *et al.*, 1972a). Therefore some other route should exist in *Pseudomonas* AM1 which effects the oxidation of acetate to glyoxylate. Such a

route has already been inferred to explain growth of the organism on 3-hydroxybutyrate, malonate and ethanol (Anthony & Dunstan, 1972; Dunstan & Anthony, 1972; Dunstan *et al.*, 1972a,b) and lactate and pyruvate (Salem *et al.*, 1973b). It has been suggested that growth on all these substrates proceeds by initial conversion of them into acetate (or a derivative), followed by oxidation of part of the acetate to glyoxylate and condensation of the acetate and glyoxylate skeletons to a C₄ compound, possibly by means of malate synthase. The nature of the conversion of acetate into glyoxylate is not known but it may involve glycollate as an intermediate (Dunstan *et al.*, 1972b; Salem & Quayle, 1971).

Comparison of the specific activity of maly-CoA lyase shows that unlike *Pseudomonas* MA and *Hyphomicrobium* X, *Pseudomonas* AM1 retains appreciable activity of maly-CoA lyase during growth on substrates such as succinate, where a primary assimilatory role for the enzyme is not expected. This residual maly-CoA lyase activity may be related to the mode of regulation of the serine pathway enzymes in *Pseudomonas* AM1. Dunstan *et al.* (1972b) suggested, from a statistical analysis of the specific activities of serine-pathway enzymes in *Pseudomonas* AM1 grown on different substrates, that hydroxypyruvate reductase, serine-glyoxylateam inotransferase and glycerate kinase are regulated co-ordinately. The ratio of the specific activity of hydroxypyruvate reductase in the methanol-grown and succinate-grown organism has been recorded as 4.5 (Large & Quayle, 1963) and 5.7 (Dunstan *et al.*, 1972b); the corresponding ratio for maly-CoA lyase is 5.5. It would clearly be worthwhile to establish statistically whether this similar ratio means that maly-CoA lyase is regulated co-ordinately with the other three enzymes.

The presence of maly-CoA lyase in methane-utilizing bacteria is of special interest. *Mts. trichosporium* appears to use the serine pathway for assimilation of C₁ compounds (Lawrence & Quayle, 1970). *P. methanica* and *Mtc. capsulatus*, on the other hand, use the hexose phosphate route for C₁ assimilation (Kemp & Quayle, 1967; Lawrence *et al.*, 1970), which does not require substrate quantities of glyoxylate to sustain it. Here, maly-CoA lyase may, at the relatively low activity present, serve to supply glyoxylate for the synthesis of glycine for protein synthesis and possibly for porphyrin synthesis, as suggested by Tuboi & Kikuchi (1963) for growth of *R. spheroides* on malate plus glutamate.

Comparison of the specific activities of maly-CoA lyase and the ATP- and CoA-dependent malate lyase in *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X grown on C₁ compounds (Tables 1 and 3) shows that the former activity is 27–50-fold greater than the latter. This strongly suggests that in all three organisms, as in *R. spheroides* (Mue *et al.*, 1964;

Tuboi & Kikuchi, 1965), the overall reaction (6) is due to the presence of two enzymes catalysing reactions (7) and (8), the malate thiokinase being present in rate-limiting amounts. This would also correlate with our consistent observation with extracts of *Pseudomonas* MA, *Pseudomonas* MS and *Hyphomicrobium* X of a lag of 1–2 min before a constant rate of reaction (6) was reached, in contrast with reaction (8), which showed no lag.

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References

- Anthony, C. & Dunstan, P. M. (1972) *Biochem. J.* **130**, 30P–31P
- Attwood, M. M. & Harder, W. (1972) *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **38**, 369–378
- Bellion, E. & Hersh, L. B. (1972) *Arch. Biochem. Biophys.* **153**, 368–374
- Cox, R. B. & Zatman, L. J. (1973) *Biochem. Soc. Trans.* **1**, 669–671
- Dixon, G. H. & Kornberg, H. L. (1962) *Methods Enzymol.* **5**, 633–637
- Dixon, G. H., Kornberg, H. L. & Lund, P. (1960) *Biochim. Biophys. Acta* **41**, 217–233
- Dunstan, P. M. & Anthony, C. (1972) *Biochem. J.* **130**, 31P
- 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
- Eggerer, H. & Grünewälder, C. (1964) *Justus Liebigs Ann. Chem.* **677**, 200–208
- Eggerer, H., Remberger, U. & Grünewälder, C. (1964) *Biochem. Z.* **339**, 436–453
- El Hawary, M. F. S. & Thompson, R. H. S. (1953) *Biochem. J.* **53**, 340–347
- Harder, W. & Quayle, J. R. (1971) *Biochem. J.* **121**, 753–762
- Harder, W., Attwood, M. M. & Quayle, J. R. (1973) *J. Gen. Microbiol.* in the press
- Heptinstall, J. & Quayle, J. R. (1970) *Biochem. J.* **117**, 563–572
- Hersh, L. B. & Bellion, E. (1972) *Biochem. Biophys. Res. Commun.* **48**, 712–719
- Jayasuriya, G. C. N. (1955) *J. Gen. Microbiol.* **12**, 419–428
- Kemp, M. B. & Quayle, J. R. (1967) *Biochem. J.* **102**, 94–102
- Kun, E. & Garcia-Hernandez, M. (1957) *Biochim. Biophys. Acta* **23**, 181–186
- Large, P. J. & Quayle, J. R. (1963) *Biochem. J.* **87**, 386–396
- Lawrence, A. J. & Quayle, J. R. (1970) *J. Gen. Microbiol.* **63**, 371–374
- Lawrence, A. J., Kemp, M. B. & Quayle, J. R. (1970) *Biochem. J.* **116**, 631–639
- Leadbetter, E. R. & Foster, J. F. (1958) *Arch. Mikrobiol.* **30**, 91–118
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mue, S., Tuboi, S. & Kikuchi, G. (1964) *J. Biochem. (Tokyo)* **56**, 545–551
- Quayle, J. R. (1972) *Advan. Microbial Physiol.* **7**, 119–203
- Salem, A. R. & Quayle, J. R. (1971) *Biochem. J.* **124**, 74P
- Salem, A. R., Large, P. J. & Quayle, J. R. (1972) *Biochem. J.* **124**, 1203–1211
- Salem, A. R., Hacking, A. J. & Quayle, J. R. (1973a) *J. Gen. Microbiol.* in the press
- Salem, A. R., Wagner, C., Hacking, A. J. & Quayle, J. R. (1973b) *J. Gen. Microbiol.* **76**, 375–388
- Shaw, W. V., Tsai, L. & Stadtman, E. R. (1966) *J. Biol. Chem.* **241**, 935–945
- Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931–941
- Stadtman, E. R. & Barker, H. A. (1950) *J. Biol. Chem.* **184**, 769–793
- Tubbs, P. K. & Garland, P. B. (1969) *Methods Enzymol.* **13**, 535–551
- Tuboi, S. & Kikuchi, G. (1962) *Biochim. Biophys. Acta* **62**, 188–190
- Tuboi, S. & Kikuchi, G. (1963) *J. Biochem. (Tokyo)* **53**, 364–373
- Tuboi, S. & Kikuchi, G. (1965) *Biochim. Biophys. Acta* **96**, 148–153
- Vagelos, P. R. (1960) *J. Biol. Chem.* **235**, 346–350