Synthesis and Hydrolysis of Malyl-Coenzyme A by *Pseudomonas* AM1: an Apparent Malate Synthase Activity

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SUMMARY

The malate synthase activity detectable in crude extracts of *Pseudomonas* AM1 has been shown to be due to a coupling of a malyl-CoA hydrolase with malyl-CoA lyase and not due to a discrete malate synthase enzyme. The partial purification of this malyl-CoA hydrolase from *Pseudomonas* AM1 has shown that it is distinct from citrate synthase which also hydrolyses malyl-CoA. The malyl-CoA hydrolase has a low K_m for malyl-CoA ($7.0 \mu\text{M}$). A mutant of *Pseudomonas* AM1, 1CT51 (Taylor & Anthony, 1975), which is unable to grow on ethanol, malonate or 3-hydroxybutyrate, has been shown to have an altered malyl-CoA hydrolase with a K_m for malyl-CoA 30 times higher than that of the enzyme present in the wild-type organism. Two classes of revertants to growth on these substrates have been isolated: (i) those with a malyl-CoA hydrolase of similar K_m to the wild-type and (ii) those in which the malyl-CoA hydrolase activity remains the same as in the mutant 1CT51. The nature of the mutation leading to the latter class of revertants is unknown.

INTRODUCTION

Malate synthase activity (measured as the glyoxylate-dependent cleavage of the acyl-CoA bond in acetyl-CoA as in equation 3, below) has been reported in *Pseudomonas* AM1 at specific activities between 10 and 40 munits/mg protein in crude extracts of the organism grown on C_1 , C_2 , C_3 and C_4 compounds (Large & Quayle, 1963; Dunstan, Anthony & Drabble, 1972; Dunstan & Anthony, 1973; Salem, Hacking & Quayle, 1973*a, b*; Salem *et al.*, 1973*c*). A 'malate synthase pathway' has been proposed (see Anthony, 1975) as the route for carbon assimilation during the growth of *Pseudomonas* AM1 on ethanol, on malonate and on 3-hydroxybutyrate. This pathway is similar to the glyoxylate cycle (Kornberg, 1959), except in the way in which acetyl-CoA is converted to glyoxylate; *Pseudomonas* AM1 does not synthesize detectable amounts of isocitrate lyase under any growth conditions yet tested (Large & Quayle, 1963; Dunstan *et al.*, 1972) and the mechanism of this oxidation of acetate to glyoxylate is not known. Salem *et al.* (1973*b*) have pointed out that the malate synthase activity measured in crude extracts of *Pseudomonas* AM1 might be due to a coupling of malyl-CoA lyase which catalyses the reversible reaction (equation 1) (Hacking & Quayle, 1974) with a malyl-CoA hydrolase (equation 2) or acyl-CoA transferase, e.g.

Attempts to purify malate synthase from *Pseudomonas* AM1 by ammonium sulphate precipitation and ion-exchange chromatography have led only to an enrichment of malyl-CoA lyase activity (Quayle & Hancock, unpublished observations). However, a malate synthase

enzyme has been completely separated from malyl-CoA lyase in trimethylamine-grown bacterium 5H2 by Cox (1974); this is a facultative methylotroph which uses the isocitrate lyase-serine pathway for growth on C_1 compounds (Hampton & Zatman, 1973; Cox & Zatman, 1973). The molecular weights of malate synthase and malyl-CoA lyase in this organism were approximately 90000 and 290000 respectively and separation was achieved by gel-filtration on Sephadex G-200. This demonstration of a malate synthase in bacterium 5H2 suggested that it might be possible to determine, by gel-filtration, whether or not there is a similar enzyme present in *Pseudomonas* AM1. This paper reports the results of such an investigation in which a new enzyme catalysing the hydrolysis of malyl-CoA has been found.

METHODS

Chemicals. Coenzyme A, oxaloacetic acid, 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), catalase (EC. 1.11.1.6) (from beef liver), lactate dehydrogenase (EC. 1.1.1.27) (from rabbit muscle) and citrate synthase (EC. 4.1.3.7) (from pig heart) were obtained from Boehringer Corporation (London) Ltd, Lewes, East Sussex. Phenylhydrazine-HCl, glyoxylic acid and cytochrome *c*, type III (from horse heart) were from Sigma. Apoferritin was a gift from Dr Pauline M. Harrison, University of Sheffield. (2*S*)-4-Malyl-CoA, (2*R*)-4-malyl-CoA, acetyl-CoA and succinyl-CoA were prepared as described by Hacking & Quayle (1974); (*RS*)-3-hydroxybutyryl-CoA was prepared from β -butyrolactone (K & K Fine Chemicals, supplied by Kodak Ltd, Kirkby, Liverpool) and CoA by the method of Decker (1963) at 0 °C in 0.5 M-KHCO₃, pH 8.5. Acetyl-CoA was assayed enzymically using citrate synthase and excess oxaloacetate, and measuring the formation of free CoA with DTNB (Tubbs & Garland, 1969). Other CoA esters were assayed enzymically using malyl-CoA hydrolase prepared from succinate-grown *Pseudomonas* AM1 (see Results) or by hydrolysis in 0.1 M-NaOH at 30 °C for 10 min, followed by neutralization and measurement of free CoA with DTNB.

DEAE-cellulose (pre-swollen DE-52) was obtained from Whatman Biochemicals, and Sephadex G-200 from Pharmacia.

Maintenance and growth of organisms. The mineral medium of Jayasuriya (1955) supplemented with growth substrates at a concentration of 0.2 % was used as the growth medium. It was solidified for the preparation of agar slopes and plates by adding 1.5 % (w/v) Difco Bacto-agar. *Pseudomonas* AM1 (NCIB9133) was maintained on agar slopes containing methylamine hydrochloride. The organism was subcultured monthly and stored at 4 °C after incubation for 3 to 4 days at 30 °C.

The following mutants of *Pseudomonas* AM1 were generously supplied by Dr C. Anthony and Mr I. J. Taylor, University of Southampton: PCT57, ICT51 and ICT51R. PCT57R4 was isolated in this department by Salem, Hacking & Quayle (1974). PCT57, ICT51 and ICT51R were maintained on succinate-agar slopes and PCT57R4 was maintained on methylamine-agar slopes. Revertants of ICT51 isolated in this work, ICT51Ra1, a2, b1, b2 and c1, were maintained on hydroxybutyrate-agar slopes containing streptomycin (1 mg ml⁻¹).

For the preparation of crude extracts, cultures (1 l) were grown in 2 l conical flasks incubated at 30 °C on an orbital shaker (200 rev./min). The growth of liquid cultures was followed spectrophotometrically at 650 nm using a Pye Unicam SP600 spectrophotometer; readings were referred to an E_{650} -dry weight calibration curve for succinate-grown *Pseudomonas* AM1.

Isolation of revertants of ICT51. Streptomycin resistant (Sm^r) strains of ICT51 were prepared prior to isolation of revertants. ICT51 was grown in liquid succinate medium and

approximately 10^8 bacteria were spread on succinate-agar plates containing streptomycin (1 mg ml^{-1}). After incubation at 30°C for 7 days, several Sm^r colonies were picked off and streaked out on streptomycin-succinate-agar plates. Three of these Sm^r strains, designated ICT51a, b and c, which retained the growth characteristics of the original mutant, were grown in liquid succinate medium and approximately 10^8 bacteria plated out on hydroxybutyrate-agar plates containing streptomycin (1 mg ml^{-1}). Several spontaneous revertants were isolated from each ICT51 Sm^r strain and five of them, designated ICT51Ra1, a2, b1, b2 and c1 (from the three Sm^r strains) were streaked out and used in the experiments described.

Preparation of crude extracts. Cultures of organisms for the preparation of crude (bacteria-free) extracts were harvested in mid-exponential phase and washed once in ice-cold $0.02 \text{ M-KH}_2\text{PO}_4\text{-KOH}$ buffer pH 7.0. Pellets were either stored at -15°C or used immediately for the preparation of crude extracts. Thick suspensions of organisms in this phosphate buffer were disrupted by passing once through a French pressure cell (American Instrument Co., Silver Spring, Maryland, U.S.A.), pre-cooled to 0°C , at a pressure of $15000 \text{ lbf in}^{-2}$ (103 MPa); whole organisms and debris were removed by centrifuging at 35000 g for 30 min at 4°C . For one experiment (see text) organisms were washed and the extract prepared in 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.01 M-MgCl_2 .

Protein determination. Protein was measured with the Folin phenol reagent by the method of Kennedy & Fewson (1968), using crystalline bovine plasma albumin as the standard.

Enzyme assays. All enzyme assays were done at 30°C using a Pye Unicam SP1800 double-beam recording spectrophotometer.

Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC. 4.1.3.7] was assayed at pH 8.0 by the method of Srere (1969).

Malyl-CoA lyase [malyl-CoA glyoxylate-lyase, EC. 4.1.3.24] was assayed by the method of Salem *et al.* (1973b) but was detected in fractions of eluates from DEAE-cellulose and Sephadex G-200 columns using a lower concentration ($40 \mu\text{M}$) of malyl-CoA.

Malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC. 4.1.3.2] was assayed by the method of Dixon & Kornberg (1962). This activity was also assayed using a modification of the method in which the formation of free CoA was followed spectrophotometrically at 412 nm by including DTNB (0.2 mM) in the reaction mixture.

Malyl-CoA hydrolase was routinely assayed in a reaction mixture (total volume, 1 ml) containing $100 \mu\text{mol Tris-HCl}$ buffer, pH 8.0; $50 \mu\text{mol MgCl}_2$; $0.2 \mu\text{mol DTNB}$; $0.04 \mu\text{mol (2S)-4-malyl-CoA}$. (The DTNB was prepared as a 2 mM solution in $0.02 \text{ M-KH}_2\text{PO}_4\text{-KOH}$ buffer, pH 7.0.) Reactions were started by the addition of enzyme or malyl-CoA and followed spectrophotometrically at 412 nm . Mixtures lacking malyl-CoA or in which boiled extract was substituted for crude extract were used as controls. Specific activities were calculated assuming that ϵ_{412} for the mercaptide ion was $13.6 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ (Srere, 1969).

Enzyme units. One enzyme unit is the amount of enzyme that catalyses the transformation of $1 \mu\text{mol}$ of substrate/min (or the formation of $1 \mu\text{mol}$ of product/min) at 30°C under the assay conditions described.

Phosphate determination. Inorganic phosphate concentrations were determined using the Fiske & SubbaRow procedure (Leloir & Cardini, 1957).

Determination of K_m values. K_m values were determined by double reciprocal plots (Lineweaver & Burk, 1934).

Table 1. *Specific activities of malyl-CoA hydrolase, malyl-CoA lyase, citrate synthase and 'malate synthase' in crude extracts of Pseudomonas AM1 and mutants*

Enzymes were assayed as described in Methods; malyl-CoA hydrolase was assayed using 40 μ M-malyl-CoA.

Organism	Growth substrate	Mean doubling time (h)	Specific activities (munits/mg protein)			
			Malyl-CoA hydrolase	Malyl-CoA lyase	Citrate synthase	'Malate synthase'
<i>Pseudomonas</i> AM1 wild-type	Methanol	3.5	45	718	55	28
	Hydroxybutyrate	6.9	70	93	108	19
	Succinate	3.5	56	200	156	40
ICT51	Methanol	4.0	5	840	75	ND
	Succinate	4.5	14	195	181	ND
ICT51R	Hydroxybutyrate	9.7	7	112	42	ND
	Succinate	3.5	14	165	168	ND
ICT51Ra1	Hydroxybutyrate	14.5	11	270	71	ND
ICT51Ra2	Hydroxybutyrate	9.2	74	80	101	35
ICT51Rb1	Hydroxybutyrate	8.8	51	74	123	25
ICT51Rb2	Hydroxybutyrate	10.6	11	84	113	ND
ICT51RC1	Hydroxybutyrate	7.8	63	74	153	27
PCT57	Hydroxybutyrate	5.5	76	4	107	ND
PCT57R4	Methanol	5.9	43	730	75	38

ND, not detected.

RESULTS

Chromatography of crude extract on Sephadex G-200

An experiment similar to that described by Cox (1974) was performed in an attempt to resolve whether or not the malate synthase activity observed in crude extracts of *Pseudomonas* AM1 (Table 1) was due to the presence of a malate synthase enzyme. A crude extract (20 mg protein ml⁻¹) of succinate-grown *Pseudomonas* AM1 was prepared in 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.01 M-MgCl₂. Nucleic acids were removed at 0 °C by the dropwise addition of a solution of protamine sulphate (10 mg ml⁻¹ in the same Tris buffer) to a final concentration of 0.1 mg protamine sulphate per mg protein, followed by centrifuging (20000 g; 10 min). The supernatant (120 mg protein; 8 ml) was applied to a Sephadex G-200 column (85 × 2.5 cm) equilibrated at 4 °C with 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.01 M-MgCl₂. The column was eluted with this buffer and fractions (4.5 ml) were assayed for malate synthase, malyl-CoA lyase, malyl-CoA hydrolase and citrate synthase activities.

Apparent malate synthase activity (total 7.2 units) was detected in fractions 33 to 45 with maximum activity between fractions 38 and 40. No other peak of malate synthase activity was detected. However, coincident with this activity were peaks of malyl-CoA lyase activity (total 10.3 units) and a malyl-CoA hydrolase activity (total 7.4 units), both measured using 40 μ M-malyl-CoA. Citrate synthase was also present (total 12.6 units) but the peak of this activity lay between fractions 35 and 37. Thus the malate synthase activity detected in the eluate could have been due to a coupling of the malyl-CoA lyase and malyl-CoA hydrolase activities. The peak of this latter activity did not coincide with that of citrate synthase, indicating that the hydrolase activity was not due to citrate synthase, which is known to hydrolyse malyl-CoA (Eggerer, Remberger & Grünwälder, 1964). Fractions 36 to 42 were

Fig. 1. DEAE-cellulose chromatography of a crude extract of succinate-grown *Pseudomonas* AMI. Malyl-CoA hydrolase (○) and malyl-CoA lyase (□) were assayed with $40\ \mu\text{M}$ -malyl-CoA. Citrate synthase (●), and apparent malate synthase activity (■) were assayed as described in Methods. —, E_{280} of the column eluate, measured with a Uvicord u.v. monitor (light path 3 mm). ---, Phosphate concentration; the arrow shows where the phosphate gradient was started.

pooled and attempts were made to separate any malate synthase activity present from the malyl-CoA lyase and malyl-CoA hydrolase activities by fractionation with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ and also by chromatography on DEAE-cellulose, but without success. Both malyl-CoA lyase and malyl-CoA hydrolase were always present in those fractions which contained apparent malate synthase activity and at no time was more malate synthase activity detected than could be accounted for by the lyase and hydrolase acting together. Crude extracts of *Pseudomonas* AMI were then fractionated (see below) to separate the malyl-CoA hydrolase activity from malyl-CoA lyase and to characterize the former.

Chromatography of crude extract on DEAE-cellulose

A crude extract ($33\ \text{mg protein ml}^{-1}$) of succinate-grown *Pseudomonas* AMI was prepared in $0.02\ \text{M-KH}_2\text{PO}_4\text{-KOH}$ buffer, pH 7.0, and nucleic acids were removed by the addition of a protamine sulphate solution ($10\ \text{mg ml}^{-1}$) in this phosphate buffer to a final concentration of $0.1\ \text{mg protamine sulphate per mg protein}$. The supernatant ($130\ \text{mg protein}$; $7.7\ \text{ml}$) was applied to a column of DEAE-cellulose equilibrated at $4\ ^\circ\text{C}$ with $0.02\ \text{M-KH}_2\text{PO}_4\text{-KOH}$ buffer, pH 7.0, and the column was eluted with approx. $180\ \text{ml}$ of this buffer and then with a linear gradient of 0.02 to $0.5\ \text{M-KH}_2\text{PO}_4\text{-KOH}$ buffer, pH 7.0 ($500\ \text{ml}$ total) (Hacking & Quayle, 1974). Fractions ($4.5\ \text{ml}$) were collected and assayed for malyl-CoA hydrolase, malyl-CoA lyase, malate synthase and citrate synthase (Fig. 1). After individual fractions had been assayed for these enzyme activities and phosphate concentrations determined, fractions 70 to 78 and fractions 86 to 95 were combined to give two pools, I and II, respectively; these pools (approx. $40\ \text{ml}$ each) were concentrated to volumes of $3.9\ \text{ml}$ and $3.2\ \text{ml}$ respectively, by filtering under pressure (nitrogen gas at $30\ \text{lbf in}^{-2}$; $0.2\ \text{MPa}$) through a PM10 ultrafiltration membrane (Amicon Ltd, High Wycombe, Buckinghamshire). Pool I concentrate contained 8.9 units of malyl-CoA hydrolase; no malate synthase, citrate synthase or malyl-CoA lyase activities were detectable. Pool II concentrate contained 27.3 units of malyl-CoA lyase (measured with $200\ \mu\text{M}$ -malyl-CoA), 24.7 units of citrate synthase, but only 1.2 units of apparent malate synthase activity. The malyl-CoA hydrolase activity in this pool (1.4 units, measured with $40\ \mu\text{M}$ -malyl-CoA) was probably due in part to contamination by the hydrolase activity eluted earlier from the column (Fig. 1); but the

Table 2. K_m values for malyl-CoA: hydrolysis catalysed by crude extracts and partially purified preparations of *Pseudomonas* AM1 and mutants

Reaction mixtures (1 ml) contained: 100 μ mol Tris-HCl buffer, pH 8.0; 50 μ mol $MgCl_2$ (omitted from determinations using citrate synthase preparations); 0.2 μ mol DTNB; and one of a range of concentrations of malyl-CoA. Reactions were started by the addition of crude extract or enzyme preparation and the rate of hydrolysis at 30 °C was followed at 412 nm.

Organism	Growth substrate	K_m values (μ M)		
		Crude extract	DEAE-cellulose column eluate	
			Peak I (malyl-CoA hydrolase)	Peak II (citrate synthase)
<i>Pseudomonas</i> AM1 wild-type	Succinate	8.3	7.0	213 (190)*
	Hydroxybutyrate	9.8		
ICT5I	Succinate	220	210	154
	Methanol	250		
ICT5IR	Succinate	220	200	133
	Hydroxybutyrate	290		
ICT5IRa1	Hydroxybutyrate	140		
ICT5IRa2	Hydroxybutyrate	10.0		
ICT5IRb1	Hydroxybutyrate	10.5		
ICT5IRb2	Hydroxybutyrate	90		
ICT5IRc1	Hydroxybutyrate	7.9	7.1	96

* This value was determined using citrate synthase purified further on Sephadex G-200 (see text).

coincidence of this secondary peak of hydrolase activity with the citrate synthase peak suggested that the latter enzyme might also be contributing to the observed hydrolysis of malyl-CoA. The hydrolase activity in pool II was much increased when higher concentrations of malyl-CoA were used in the assay. A determination of the K_m values for malyl-CoA, hydrolysed by the enzymes present in pool I and pool II, showed that the K_m value for the hydrolase in pool I was much lower than that in pool II (Table 2). (The K_m value for the hydrolase activity of a more purified citrate synthase preparation was also determined, see below.) When equal volumes of pools I and II were mixed and assayed for malate synthase activity using either the standard or the modified assay (see Methods), a high apparent malate synthase activity was observed; the specific activity of the latter was similar to the observed specific activity of the malyl-CoA hydrolase in pool I.

Chromatography of pools I and II on Sephadex G-200: molecular weight determination

A portion (1 ml) of the concentrated pool I preparation containing 2.3 units of malyl-CoA hydrolase was mixed with 0.5 ml of the concentrated pool II preparation containing 4.3 units of malyl-CoA lyase. To this mixture, the following proteins were added as molecular-weight markers: 1.3 mg apoferritin, mol. wt 480000 (Harrison, 1963); 100 units of catalase, mol. wt 240000 (Samejima & Yang, 1963); 55 units of lactate dehydrogenase, mol. wt 132000 (Fromm, 1963); 3 mg of cytochrome *c*, mol. wt 12400 (Margoliash, 1962). The complete mixture was applied to a Sephadex G-200 column (90 $\times$ 2.5 cm) equilibrated at 4 °C with 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.01 M- $MgCl_2$. The column was eluted with the buffer used for equilibration and fractions (2.4 ml) were collected and assayed for malyl-CoA hydrolase and malyl-CoA lyase activities. Marker enzyme activities were assayed by the methods used by Andrews (1965). Apoferritin and cytochrome *c* were

Fig. 2. pH-dependence of malyl-CoA hydrolase. Activities were measured in 0.1 M-Tris-HCl (○), 0.1 M-triethanolamine hydrochloride-NaOH (●), 0.1 M-KH₂PO₄-KOH (□) and 0.1 M-diethanolamine-HCl (■) buffers. Reaction mixtures (1 ml) contained: 100 μ mol buffer; 50 μ mol MgCl₂; 0.2 μ mol DTNB; and 0.04 μ mol of malyl-CoA. Malyl-CoA hydrolase (pool I concentrate, 23 m units) prepared from succinate-grown *Pseudomonas* AM1 was added to start the reaction and rate of hydrolysis at 30 °C was followed at 412 nm.

detected by measurement of E_{230} and E_{412} respectively. Molecular weight values for malyl-CoA hydrolase and malyl-CoA lyase of 190000 and 220000 respectively were calculated using the method of Andrews (1965). The molecular weight of malyl-CoA lyase has been determined as 190000 by Hacking & Quayle (1974) using sedimentation equilibrium data for the purified enzyme. Citrate synthase activity was not detected in these fractions and a second experiment was performed in which 0.5 ml of the concentrated pool II preparation, containing 3.9 units of citrate synthase, was mixed with marker proteins and applied to a Sephadex G-200 column (66 $\times$ 2.6 cm) equilibrated at 4 °C with 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.1 M-KCl and 1 mM-EDTA (Weitzman, 1969). The column was eluted with this buffer and fractions (2.4 ml) collected. A total of 1.8 units of citrate synthase was recovered and a value of 300000 was calculated for the molecular weight of this enzyme. Fraction 47 which contained a total of 80 munits of citrate synthase and 19 munits of malyl-CoA lyase was used to determine the K_m for malyl-CoA in the malyl-CoA hydrolase activity of citrate synthase (Table 2).

Properties of malyl-CoA hydrolase

The following properties of the malyl-CoA hydrolase (pool I concentrate) prepared from succinate-grown *Pseudomonas* AM1 were examined. This preparation contained 0.40 units of malyl-CoA hydrolase per mg protein which represented about a 10-fold purification from the crude extract (Table 1).

Stability. A 50 % loss in specific activity was observed in a portion of the enzyme preparation stored for 3 months at -15 °C. Loss of activity was accelerated by repeated cycles of freezing and thawing.

pH optimum. The activity of malyl-CoA hydrolase was investigated as a function of pH over the range 6.4 to 9.8. The hydrolase was found to have a broad pH optimum with the peak of activity at pH 8.6 (Fig. 2).

Metal-ion requirement. Malyl-CoA hydrolase activity was increased by approx. 60 % in the presence of 5 mM-MgCl₂; 10 mM-MgCl₂ did not stimulate activity any further. No

Table 3. *Substrate specificity of malyl-CoA hydrolase*

Reaction mixtures (1 ml) contained: 100 μ mol Tris-HCl buffer, pH 8.0; 50 μ mol $MgCl_2$; 0.2 μ mol DTNB; and one of a range of concentrations of the thiol-ester substrates. Malyl-CoA hydrolase (pool I concentrate, 14 munits) prepared from succinate-grown *Pseudomonas* AM1 was added to start the reaction and the rate of hydrolysis at 30 °C was followed at 412 nm. K_m and V_{max} values for acetyl-CoA refer to the initial rate of hydrolysis (see text).

Substrate	$K_m(\mu M)$	V_{max} relative to malyl-CoA (%)
(2 <i>S</i>)-4-Malyl-CoA*	7.0	100
(2 <i>R</i>)-4-Malyl-CoA	not hydrolysed	
Succinyl-CoA	71	194
(<i>RS</i>)-3-Hydroxybutyryl-CoA	190	48
Acetyl-CoA	1100	143

* At malyl-CoA concentrations higher than 40 μM , substrate inhibition of hydrolysis was observed.

absolute requirement for Mg^{2+} was found, even in enzyme which was dialysed for 2 h at 4 °C against 2500 vols. of 0.02 M- KH_2PO_4 -KOH buffer, pH 7.0. The malyl-CoA hydrolase activity of citrate synthase (fraction 47, purified from pool II by Sephadex G-200 chromatography), was not stimulated by 5 mM- $MgCl_2$.

Substrate specificity. Malyl-CoA hydrolase completely hydrolysed (2*S*)-4-malyl-CoA. Some activity was observed when 0.2 μ mol of (2*R*)-4-malyl-CoA was substituted for (2*S*)-4-malyl-CoA in the standard reaction mixture, but the activity was low and the total substrate hydrolysed corresponded to only about 10 % of the substrate supplied (as estimated by alkaline hydrolysis of the thioester bond). This observation was similar to that made by Hacking & Quayle (1974) when examining the cleavage of a sample of the same batch of (2*R*)-4-malyl-CoA by malyl-CoA lyase, and is probably due to contamination of the 2*R* isomer by the 2*S* substrate. The inhibition of hydrolysis of (2*S*)-4-malyl-CoA by the 2*R* isomer was competitive with a value of 76 μM for the K_i of (2*R*)-4-malyl-CoA.

Succinyl-CoA and (*RS*)-3-hydroxybutyryl-CoA were hydrolysed by the enzyme with K_m values higher than the K_m with respect to malyl-CoA (Table 3). When acetyl-CoA was substituted for malyl-CoA, a rapid rate of hydrolysis was observed for a few seconds but this decreased to a very slow rate when only 5 % of the substrate had been hydrolysed. Further additions of acetyl-CoA did not increase this slow rate of hydrolysis significantly (although higher initial rates were observed with higher concentrations of acetyl-CoA).

The effect of acetyl-CoA on the hydrolysis of (2*S*)-4-malyl-CoA was investigated. The standard reaction mixture (1 ml) contained 4.5 munits enzyme and 0.068 μ mol acetyl-CoA. After 5 min, when the initial rapid rate of hydrolysis had slowed, malyl-CoA (0.04 μ mol) was added and the rate of hydrolysis recorded. This was repeated using a range of malyl-CoA concentrations. Increasing concentrations of acetyl-CoA caused a progressive decrease in the rate of hydrolysis of malyl-CoA, 50 % inhibition occurring with approx. 0.2 mM-acetyl-CoA. A double reciprocal plot of the data showed that the inhibition by acetyl-CoA was non-competitive and that the K_m for malyl-CoA was not significantly changed in the presence of acetyl-CoA. The nature of the inhibition by acetyl-CoA of its own hydrolysis and that of malyl-CoA was not further investigated.

Chromatography of the crude extract of mutant 1CT51 on DEAE-cellulose

1CT51, a mutant of *Pseudomonas* AM1 unable to grow on ethanol, malonate or 3-hydroxybutyrate, and lacking any apparent malate synthase activity (Taylor & Anthony, 1975), was grown in liquid succinate medium and a crude extract prepared as described in Methods.

Fig. 3. DEAE-cellulose chromatography of crude extract of succinate-grown mutant ICT51. Malylyl-CoA hydrolase (○) was assayed with $80\ \mu\text{M}$ -malylyl-CoA, and malylyl-CoA lyase (□) with $40\ \mu\text{M}$ -malylyl-CoA. Citrate synthase (●) was assayed as described in Methods. —, E_{280} of the column eluate, measured with a Uvicord u.v. monitor (light path 3 mm). ---, Phosphate concentration; the arrow shows where the phosphate gradient was started.

Table 4. Growth substrate specificity of *Pseudomonas* AM1 and mutants

Growth was tested by streaking each organism on agar plates containing potential growth substrates at a concentration of 0.2 %, except for the mixture of acetate and glyoxylate which were at 20 mM and 5 mM respectively. Growth was compared to the wild-type organism and to the growth of each strain on an agar plate containing no added growth substrate, after incubation at 30°C for 3 to 4 days and 7 to 10 days.

Substrate	<i>Ps.</i> AM1 wild- type	ICT51	ICT51 a, b, c	ICT51R	ICT51R a1	ICT51R a2, b1, b2, c1	PCT57	PCT57R4
Methanol	++	++	++	++	++	++	—	++
Ethanol	++	—	—	++	++	++	++	++
Acetate + glyoxylate	+	—	—	+	—	+	±	—
Hydroxybutyrate	++	±	±	++	++	++	++	++
Malonate	++	±	±	++	++	++	++	++
Lactate	++	++	++	++	++	++	++	++
Pyruvate	++	++	++	++	++	++	++	++
Succinate	++	++	++	++	++	++	++	++
Succinate + streptomycin (1 mg ml ⁻¹)	—	—	++	++	++	++	—	++

++, Good growth after 3 to 4 days incubation; +, good growth after 7 to 10 days incubation; ±, slight growth after 7 to 10 days incubation; —, no significant growth after 7 to 10 days incubation.

The extract ($39\ \text{mg protein ml}^{-1}$) was assayed for malylyl-CoA hydrolase, malylyl-CoA lyase, malate synthase and citrate synthase (Table 1); and then, after protamine sulphate treatment, the supernatant ($144\ \text{mg protein: 6 ml}$) was fractionated on a DEAE-cellulose column, in the same way as described for the extract from the wild-type organism (Fig. 3). In this experiment malylyl-CoA hydrolase was assayed in fractions using a higher concentration ($80\ \mu\text{M}$) of malylyl-CoA in the standard reaction mixture; this accounts for the higher activity of the second hydrolase peak (citrate synthase) in Fig. 3 compared with that shown in Fig. 1.

K_m values for both peaks of hydrolase activity were determined (Table 2). The K_m value with respect to malylyl-CoA for the citrate synthase in ICT51 was about 70 % of the value determined for the citrate synthase from the wild-type organism; but the malylyl-CoA hydrolase of ICT51 had a K_m value for malylyl-CoA about 30 times higher than that for the wild-type. The total activity recovered from ICT51 was only 5 % of that recovered from the same quantity of wild-type organism. This comparison is based on activity measured using the standard assay system for both ICT51 and wild-type, i.e. using $40\ \mu\text{M}$ malylyl-CoA in the

reaction mixture. Fractions 80 to 91 (Fig. 3), which were comparable with pool II from the wild-type organism, contained a total of 15.9 units of citrate synthase and 11.7 units of malyl-CoA lyase activity (approx. 65 % and 43 % respectively of the activities of these enzymes present in pool II).

Revertants of mutant ICT51

Taylor & Anthony (1976) have prepared a revertant of ICT51, named ICT51R, which can grow on a mixture of acetate (20 mM) and glyoxylate (5 mM). This revertant also regained the ability to grow on ethanol, malonate and 3-hydroxybutyrate (Table 4). However, Taylor & Anthony were unable to detect any apparent malate synthase activity in crude extracts of ICT51R. The malyl-CoA hydrolase activity found in extracts of ICT51R was similar to that found in ICT51 (Table 2) and the K_m for malyl-CoA hydrolysis in such extracts was high (220 μ M). Accordingly other revertants of ICT51 (to growth on 3-hydroxybutyrate) were prepared (see Methods) and crude extracts examined for malyl-CoA hydrolase to determine whether or not revertants similar to the wild-type organism could be isolated. Two classes of revertant were found: (i) those similar to ICT51R, i.e. having low malyl-CoA lyase activity in crude extracts and a high K_m for malyl-CoA and (ii) those similar to the wild-type organism, i.e. having high malyl-CoA hydrolase activity and a low K_m for malyl-CoA (Table 2). The growth substrate specificities of five of these revertants, ICT51RA1, a2, b1, b2 and c1 are included in Table 4. ICT51RA2, b1 and c1 have a low K_m for malyl-CoA hydrolysis; ICT51RA1 and b2 are similar to ICT51R in having a high K_m .

Chromatography of crude extracts of ICT51R and ICT51RC1 on DEAE-cellulose

ICT51R and ICT51RC1 were each grown in liquid 3-hydroxybutyrate medium and crude extracts fractionated on DEAE-cellulose columns as described for the fractionation of crude extracts of *Pseudomonas* AM1 and ICT51. With ICT51R, malyl-CoA lyase, citrate synthase and malyl-CoA hydrolase were eluted at the same phosphate concentrations and at similar activities to those at which these enzymes were eluted in the experiments with ICT51 (Fig. 3). With ICT51RC1, the results were very similar to those obtained for the chromatography of crude extract of the wild-type organism (Fig. 1).

DISCUSSION

Our results show that the 'malate synthase' activity observed in extracts of *Pseudomonas* AM1 can be explained in terms of the concerted action of malyl-CoA lyase and a novel malyl-CoA hydrolase, as indicated in equations 1 to 3 (see Introduction). No evidence could be found for a discrete malate synthase enzyme. Citrate synthase (from pig heart) is known to hydrolyse (2S)-4-malyl-CoA (Eggerer *et al.*, 1964) and the present work has shown that citrate synthase from *Pseudomonas* AM1 can do likewise. It might therefore be argued that citrate synthase is responsible for reaction 2 in the overall sequence (equations 1 to 3). The kinetic characteristics of the enzymes make this unlikely since the K_m value for malyl-CoA hydrolysed by citrate synthase is approximately 20 times higher than the K_m for malyl-CoA hydrolysed by the new hydrolase. Furthermore, the 30-fold increase in K_m for malyl-CoA hydrolase in mutant ICT51 results in the loss of significant malate synthase activity in crude extracts, despite the presence of wild-type levels of citrate synthase and malyl-CoA lyase. It therefore seems clear that the affinity of citrate synthase for malyl-CoA is too low to effect significant coupling in the reaction sequence (equations 1 to 3) in crude extracts. Further confirmation of the postulate that malate synthase activity is represented by the sum of equations 1 and 2 is given by mutant PCT57. This mutant lacks malyl-CoA lyase (Salem

et al., 1974) and has no apparent malate synthase activity (Table 1). Restoration of malyl-CoA lyase activity in revertant PCT57R4 results in restoration of malate synthase activity.

The evidence from mutants indicates that the reaction sequence (equations 1 to 3), probably functioning as a malate synthase, is involved in the metabolism of ethanol, malonate and 3-hydroxybutyrate by *Pseudomonas* AM1. Thus, 1CT51 possesses an altered malyl-CoA hydrolase and is unable to grow on these substrates, while some revertants with wild-type malyl-CoA hydrolase activity regain the ability to grow on them. The isolation of other revertants of 1CT51 which are able to grow on ethanol, malonate or 3-hydroxybutyrate but which still have the mutant (high K_m) hydrolase indicates that an alternative to the malate synthase pathway can exist or be brought into play by further mutation. This is not unexpected since such a pathway, not involving an overall malate synthase reaction, must exist to allow for the growth of mutant PCT57 on these substrates, despite its lack of malate synthase activity. Salem *et al.* (1973c) proposed that the metabolism of lactate and pyruvate by *Pseudomonas* AM1 differs fundamentally from that operating in the Enterobacteriaceae and that these substrates are catabolised to C_2 units from which C_4 compounds are formed via the malate synthase pathway. The observation that 1CT51 can grow well on pyruvate or lactate although it has no apparent malate synthase activity (Taylor & Anthony, 1975) indicates that although pyruvate and lactate may first be converted to C_2 units, an alternative pathway, not involving an overall malate synthase reaction, allows for the growth of 1CT51 (and possibly the wild-type organism) on these substrates. This alternative pathway may well be the same as that used for growth on ethanol, malonate or 3-hydroxybutyrate by those revertants of 1CT51 which still have the mutant (high K_m) malyl-CoA hydrolase. The nature of this alternative pathway is not known.

The specific activity of malyl-CoA hydrolase remained fairly constant in *Pseudomonas* AM1 grown on various substrates. Its presence in the C_1 -grown organism calls for a regulatory mechanism to prevent hydrolysis of malyl-CoA before it is cleaved to acetyl-CoA and glyoxylate. It will be recalled that in the operation of the serine pathway during growth on C_1 compounds, malyl-CoA lyase catalyses reaction (1) from right to left. The apparent inhibition of the hydrolase by acetyl-CoA suggests a possible means of regulation which may also prevent unwanted hydrolysis of acetyl-CoA itself.

Although a discrete malate synthase enzyme has not been found in *Pseudomonas* AM1, Cox (1974) has shown that such an enzyme is present in trimethylamine-grown bacterium 5H2. There is no obvious role for the enzyme during growth on C_1 -compounds by this organism which uses the isocitrate lyase-serine pathway. However, in contrast to *Pseudomonas* AM1, bacterium 5H2 is able to synthesize isocitrate lyase and thereby to grow on C_2 -compounds by the glyoxylate cycle. Hence the presence of a malate synthase enzyme in this organism may perhaps be correlated with its ability to synthesize isocitrate lyase, bearing in mind that these two glyoxylate cycle enzymes are associated in an operon in *Escherichia coli* (Vanderwinkel & de Vlieghere, 1968). Further work is needed to establish whether or not such a correlation holds amongst C_1 -utilizing organisms in general.

It is interesting to speculate whether there could be an evolutionary link between malate synthase, and malyl-CoA lyase and hydrolase. Thus malate synthase might have evolved from a fusion of primitive malyl-CoA lyase and hydrolase enzymes, or the latter two enzymes might have evolved by separation of two peptide sequences from a parent malate synthase protein.

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