Properties and Substrate Specificity of some Reactions Catalysed by a Short-Chain Fatty Acyl-Coenzyme A Synthetase from Seeds of *Pinus radiata*

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1. Crude extracts of seeds of *Pinus radiata* catalysed acetate-, propionate-, *n*-butyrate- and *n*-valerate-dependent PP_i -ATP exchange in the presence of MgCl_2 , which was apparently due to a single enzyme. Propionate was the preferred substrate. Crude extracts did not catalyse medium-chain or long-chain fatty acid-dependent exchange. 2. Ungerminated dry seeds contained short-chain fatty acyl-CoA synthetase activity. The activity per seed was approximately constant for 11 days after imbibition and then declined. The enzyme was located only in the female gametophyte tissue. 3. The synthetase was purified 70-fold. 4. Some properties of the enzyme were studied by $[^{32}\text{P}]\text{PP}_i$ -ATP exchange. K_m values for acetate, propionate, *n*-butyrate and *n*-valerate were 4.7, 0.21, 0.33 and 2.1 mM respectively. Competition experiments between acetate and propionate demonstrated that only one enzyme was involved and confirmed that the affinity of the enzyme for propionate was greater than that for acetate. CoA inhibited fatty acid-dependent PP_i -ATP exchange. The enzyme catalysed fatty acid-dependent $[^{32}\text{P}]\text{PP}_i$ -dATP exchange. 5. The enzyme also catalysed the fatty acyl-AMP-dependent synthesis of $[^{32}\text{P}]\text{ATP}$ from $[^{32}\text{P}]\text{PP}_i$. Apparent K_m (acetyl-AMP) and apparent K_m (propionyl-AMP) were $57\text{ }\mu\text{M}$ and $7.5\text{ }\mu\text{M}$ respectively. The reaction was inhibited by AMP and CoA. 6. Purified enzyme catalysed the synthesis of acetyl-CoA and propionyl-CoA. Apparent K_m (acetate) and apparent K_m (propionate) were 16 mM and 7.5 mM respectively. The rate of formation of acetyl-CoA was enhanced by pyrophosphatase. 7. It was concluded that fatty acyl adenylates are intermediates in the formation of the corresponding fatty acyl-CoA.

The conversion of storage lipid into sucrose and other sugars during germination of fatty seeds is now well understood (Hitchcock & Nichols, 1971), and consists of lipolysis, β -oxidation, the glyoxylate cycle and reversed glycolysis. The decrease in the content of storage lipid during germination is correlated with the activity of the glyoxylate-cycle enzymes isocitrate lyase and malate synthase (Beever, 1961). Seeds of many *Pinus* spp. are rich in lipid (Firenzuoli *et al.*, 1968a). Glyoxysomes, isocitrate lyase and malate synthase have been reported in germinating seeds of *Pinus* spp., and the activity of glyoxysomes is correlated with a decrease in lipid content and accumulation of sugars (Firenzuoli *et al.*, 1968a,b; Ching, 1970).

The β -oxidation pathway is initiated by fatty acyl-CoA synthetases. These enzymes have been demonstrated in the germinating fatty seeds of peanut (Rebeiz *et al.*, 1965a,b) and castor bean (Yamada & Stumpf, 1965; Takeuchi *et al.*, 1967; Cooper, 1971). The present paper reports a short-chain fatty acyl-CoA synthetase in the female gametophyte tissue of *Pinus radiata* seeds and describes the activity of the enzyme in relation to other biochemical parameters during germination. The partial purification of the enzyme, some properties and some reactions catalysed are also described.

Experimental

Chemicals

Hydroxylamine hydrochloride and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Fatty acids, fatty acid anhydrides, phenylhydrazine hydrochloride and GSH were from British Drug Houses, Poole, Dorset, U.K. AG 1-X2 anion-exchange resin (Cl^- form) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and reduced CoA (lithium salt) from Calbiochem, Los Angeles, Calif., U.S.A. Trisodium $\text{DL}(\pm)$ -isocitrate and protamine sulphate (ex salmon roe) were supplied by Koch-Light Laboratories, Colnbrook, Bucks., U.K.; protamine sulphate solution (2%, w/v, in 20 mM-Tris-HCl buffer, pH 8, containing 0.1 mM-EDTA) was stored at 2°C for 3 days and the clear supernatant solution was used. Sodium $[1\text{-}^{14}\text{C}]\text{acetate}$ and sodium $[1\text{-}^{14}\text{C}]\text{propionate}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; the specific radioactivities were adjusted to 0.1–4 Ci/mol with potassium acetate and potassium propionate respectively. Sepharose 4B and Sephadex G-50 (medium grade) were obtained from Pharmacia, Uppsala, Sweden. Other chemicals were obtained from the sources described by Shaw & Anderson (1972). Salt-free hydroxylamine was

prepared by neutralizing the hydrochloride with a suitable quantity of AG 1-X2 anion-exchange resin (OH⁻ form). The concentration of hydroxylamine was measured by the method of Bray *et al.* (1919). Acetohydroxamic acid was prepared by the method of Sladek *et al.* (1970) and propionohydroxamic acid was prepared by an analogous procedure. [³²P]PP_i was synthesized from [³²P]P_i by pyrolysis (Lee Peng, 1956).

Acetyl-AMP was synthesized by the method of Berg (1956b) and propionyl-AMP was synthesized by an analogous reaction. Adenylates were purified by anion-exchange chromatography (Berg, 1956b) with a column (6cm×2cm) of Bio-Rad AG 1-X2 (Cl⁻ form). Acetyl-AMP, propionyl-AMP and contaminating AMP were measured as described by Berg (1956b); since ferric acetohydroxamate and ferric octanohydroxamate have identical ϵ_{540} (Lee Peng, 1956), this coefficient was used when measuring propionyl-AMP concentration. After purification the pH of the pooled fractions was adjusted to 6.5 with KOH. The adenylates were stored at 2°C, but were unstable under these conditions; the concentrations of acetyl-AMP and propionyl-AMP decreased by 22 and 14% respectively after 13 days. Fatty acyl adenylates were heavily contaminated with AMP; immediately after purification only 31% of the total AMP was acetyl-AMP or propionyl-AMP.

Methods

Preparation of crude extracts. Method A. Untreated seeds of *P. radiata* D. Don. were obtained from local seed merchants ex Kaingaroa Forest, New Zealand. Seeds were soaked for 1 h in a solution of sodium hypochlorite [3% (w/v) available chlorine] to minimize infection, washed and then placed in the dark for 3 days at 25°C on moist filter pads; the radicle did not rupture the testa in this time. Soaking the seeds on filter pads moistened with penicillin solution (1000 units/ml) did not affect the amount of short-chain fatty acyl-CoA synthetase extracted from the seeds. The testae were removed and the remaining tissue was extracted in a mortar with 5 ml of 100mM-Tris-HCl buffer, pH 8, per g fresh wt. The supernatant solution was recovered by centrifugation (20000g for 30min); the fatty layer overlying the supernatant solution was removed by decanting the supernatant solution through cheesecloth. The supernatant solution was dialysed against 100mM-Tris-HCl buffer, pH 8, and used as a source of fatty acyl-CoA synthetase.

Method B was used to prepare crude extracts to study enzyme activities in relation to germination. Seeds (ex Beechworth, Vic., Australia) were soaked in the dark as described for method A; treatment with sodium hypochlorite was omitted. At specified times,

110 seeds were sampled in duplicate and the testae removed. The remaining tissue was homogenized in 22ml of 100mM-Tris-HCl buffer, pH 8, containing 0.1mM-EDTA and 5mM-dithiothreitol, by using a mortar and pestle and then a Potter-Elvehjem homogenizer. The supernatant solution was recovered as described for method A. A sample of the supernatant solution was used to measure isocitrate lyase activity. The remaining solution was dialysed against 100mM-Tris-HCl buffer, pH 8, containing 0.1mM-EDTA, and used to measure short-chain fatty acyl-CoA synthetase activity. All extractions and subsequent operations were carried out at 2°C.

Purification of short-chain fatty acyl-CoA synthetase. Crude aqueous extracts were an unsatisfactory starting material, since they were invariably contaminated with fat in suspension, and so the following method was used. Seeds were soaked on filter pads for 3 days and homogenized with acetone at -10°C in a Sorvall Omni-Mixer. Coarse material was removed by straining the extract through cheesecloth; remaining material was filtered under vacuum at 2°C. Residual acetone was removed by evaporation at room temperature for 1 h and the powder was stored in a desiccator at -15°C; no loss of activity occurred during 2 weeks. The powder (15g) was extracted with 300ml of 100mM-Tris-HCl, pH 8, containing 0.1mM-EDTA. Particulate material was removed by centrifugation (23000g for 30min); the supernatant is referred to as acetone-dried-powder extract. Solid (NH₄)₂SO₄ was added (0.176g/ml) and precipitated material was discarded. Additional (NH₄)₂SO₄ (0.062g/ml) was added and the precipitate containing the enzyme was recovered by centrifugation and dissolved in 22ml of 20mM-Tris-HCl, pH 8, containing 0.1mM-EDTA (medium 1). Protamine sulphate solution (13.2ml) was added; precipitated material was discarded. The supernatant, referred to as protamine sulphate supernatant, was passed through a Sepharose 4B column (3.5cm×46cm) connected in series with a Sephadex G-50 (medium grade) column (2.7cm×26cm); both columns were pre-equilibrated with medium 1. Active fractions were pooled and are referred to as purified enzyme; dithiothreitol and/or ATP were added [final concentration(s) 5mM] to stabilize the enzyme. Extraction of enzyme and purification was done at 2°C.

Assay of short-chain fatty acyl-CoA synthetase. Enzyme activity was measured by fatty acid-dependent [³²P]PP_i-ATP exchange (method 1). Incubations were conducted at 35°C for 15min in a final volume of 1ml and contained Na₄³²P₂O₇ (0.3-2.0μCi), K₂Na₂ATP, MgCl₂, fatty acid (adjusted to pH 8 with KOH), Tris-HCl, pH 8, other components as specified, and enzyme; concentrations are specified for each experiment. Fatty acid was omitted from control incubations. Reactions were terminated with

2ml of trichloroacetic acid (7.5%, w/v). [^{32}P]ATP was separated from [^{32}P]PP_i and counted for radioactivity as described by Shaw & Anderson (1972). Enzyme activities were calculated by the method of Davie *et al.* (1956) and are expressed as fatty acid-dependent PP_i-ATP exchange in nmol/min (fatty acyl-CoA synthetase units); specific activities are expressed as units/mg of protein or as specified in the text.

Enzyme activity was also measured by fatty acyl-AMP-dependent synthesis of [^{32}P]ATP from [^{32}P]PP_i (method 2). Incubation mixtures contained 6mM- $\text{Na}_4^{32}\text{P}_2\text{O}_7$ (2–4 μCi), 1mM- MgCl_2 , 102mM-Tris-HCl, pH 8, 0.5mM-dithiothreitol, 10 μM -EDTA, fatty acyl-AMP and enzyme in a final volume of 1ml. Fatty acyl-AMP was omitted from control assays. After incubation for 15 min at 35°C, ATP was separated from [^{32}P]PP_i as described for assay method 1 and counted for radioactivity in a gas-flow planchet counter.

The overall reaction catalysed by the enzyme was measured by the formation of [^{14}C]fatty acyl-CoA (method 3). Incubations were conducted at 35°C for 20min in a volume of 0.25ml and contained 4mM- MgCl_2 , 1.84mM-CoA, 2.8mM-dithiothreitol, 2mM- $\text{K}_2\text{Na}_2\text{ATP}$, 48mM-Tris-HCl buffer, pH 8, 40 μM -EDTA, enzyme and [$1\text{-}^{14}\text{C}$]fatty acid. CoA was omitted from control assays. Reaction mixtures containing [^{14}C]acetate and [^{14}C]propionate were terminated with 50 μl of 17.5M-acetic acid and 50 μl of 13.4M-propionic acid respectively. [^{14}C]Fatty acyl-CoA was separated from [^{14}C]fatty acid and determined as described by Huang (1970). Counting efficiency was constant both within and between experiments. Fatty acyl-CoA synthetase activities are expressed as the CoA-dependent synthesis of fatty acyl-CoA in nmol/min per mg of protein.

Assay of isocitrate lyase. Enzyme activity was measured by the method of Dixon & Kornberg (1959). Reactions were conducted at 30°C for 10min in a final volume of 2ml and contained 20mM-trisodium isocitrate, 5mM- MgCl_2 , 3mM-GSH, freshly prepared 17.5mM-phenylhydrazine hydrochloride, 0.75mM-dithiothreitol, 15 μM -EDTA, 240mM-Tris-HCl buffer, pH 7.2, and enzyme. Reactions were terminated by the addition of 2ml of ethanol, and protein was removed by centrifugation. Activity was measured as the E_{324} against a control without isocitrate. Results are expressed as nmol of glyoxylate produced/min per seed or as specified in the text.

Identification of ^{14}C -labelled acetyl- and propionyl-CoA. These were identified as their hydroxamates as described by Huang (1970). Reaction mixtures contained 7.6 μg of purified enzyme protein, 2mM- $\text{K}_2\text{Na}_2\text{ATP}$, 24mM-dithiothreitol, 32 μM -EDTA, 46mM-Tris-HCl, pH 8, 4mM- MgCl_2 , 1.84mM-CoA and 16mM-[$1\text{-}^{14}\text{C}$]acetate or [$1\text{-}^{14}\text{C}$]propionate in a final volume of 0.25ml. Enzyme, MgCl_2 or CoA were omitted from control assays. After incubation for 20min at

35°C, 0.2ml of salt-free 3.25M-hydroxylamine was added and the mixtures were incubated for a further 10min, and then reactions were terminated with 0.1ml of 17.5M-acetic acid or 13.4M-propionic acid; samples (80 μl) were analysed as described by Huang (1970). Acetohydroxamic acid and propionohydroxamic acid were used as markers and were detected as their ferric salts (Stadtman & Barker, 1950).

Chromatography of the ^{32}P -labelled products adsorbed on to charcoal in assay methods 1 and 2. The procedures described by Shaw & Anderson (1971, 1972) were used.

Determination of protein. Protein in crude extracts, $(\text{NH}_4)_2\text{SO}_4$ fractions and fractions treated with protamine sulphate was precipitated with trichloroacetic acid (final concn. 10%, w/v), washed twice with acetone, solubilized with 2M-NaOH at 35°C for 3h and measured by the method of Ellman (1962). Crystalline bovine serum albumin was used as a standard. The protein in solutions subjected to gel filtration was measured by the method of Warburg & Christian (1941).

Determination of fat content of germinating pine seeds. Samples of seeds were killed in boiling acetone and dried to constant weight in a vacuum oven at 85°C. Each sample (approx. 5g dry wt.) was ground in a mortar and the lipid was exhaustively extracted with 180ml of chloroform-methanol (2:1, v/v). The extract was washed twice with 200ml of a solution containing saturated NaCl solution (1 vol.) and 1M-HCl (1 vol.) (Willemot & Stumpf, 1967). The washed lipid extract was dried to constant weight at 70°C under a stream of N_2 .

Results

Properties of the fatty acid-dependent PP_i-ATP exchange reactions catalysed by crude extracts prepared by method A

Propionate-dependent PP_i-ATP exchange was critically dependent on MgCl_2 concentration (Fig. 1); the effect of MgCl_2 concentration on acetate-dependent PP_i-ATP exchange was very similar. Propionate-dependent exchange activity increased with the concentration of MgCl_2 up to 1.5mM, but higher concentrations inhibited activity; a more detailed study of this phenomenon is described by Young & Anderson (1974). Endogenous PP_i-ATP exchange, however, increased with MgCl_2 concentration up to 7mM. Propionate-dependent exchange at 1.5mM- MgCl_2 was 7.5-fold as great as endogenous exchange (Fig. 1), and this concentration was normally used. Propionate-dependent exchange was proportional to crude enzyme concentration up to 1.7mg of protein per mixture, and was insensitive to 10mM-NaF, suggesting that pyrophosphatase activity did not interfere with fatty acid-dependent exchange (Shaw & Anderson, 1971, 1972).

Fig. 1. Effects of $MgCl_2$ concentration on endogenous and propionate-dependent PP_i -ATP exchange catalysed by crude extract of pine seeds

The incubation mixtures (assay method 1) contained $MgCl_2$ as specified, 60mM-Tris-HCl, pH 8, 0.75mM-propionate, 2mM-ATP and 2mM- PP_i . ○, Endogenous PP_i -ATP exchange; ▲, propionate-dependent PP_i -ATP exchange; ●, total PP_i -ATP exchange.

Crude extracts catalysed acetate-, propionate-, *n*-butyrate- and *n*-valerate-dependent PP_i -ATP exchange. The effect of fatty acid concentration on fatty acid-dependent PP_i -ATP exchange did not follow Michaelis-Menten kinetics (Fig. 2). The concentrations required for optimum activity were reproducible features of the particular fatty acids. The optimum concentrations for propionate, *n*-butyrate and *n*-valerate were approx. 1, 1 and 1.5mM respectively, but the optimum concentration for acetate was approx. 10mM. The rate of exchange at the optimum concentration of fatty acid was less for *n*-butyrate and *n*-valerate than for propionate or acetate. The PP_i -ATP exchange catalysed by crude extracts in incubation mixtures containing mixtures of fatty acids indicated that only one enzyme catalysed fatty-acid dependent PP_i -ATP exchange for the four fatty acids, with propionate as the preferred substrate. Crude extracts did not catalyse *n*-hexanoate-, *n*-octanoate-, *n*-decanoate-, dodecanoate- or linoleate-dependent PP_i -ATP exchange. Rather, these fatty acids inhibited endogenous PP_i -ATP exchange; inhibition increased with fatty acid concentration, and fatty acids with the longest chain length were the most effective inhibitors. Inhibition of endogenous exchange was probably due to a non-specific inhibition of endogenous enzyme activities by the

Fig. 2. Effect of fatty acid concentration on fatty acid-dependent PP_i -ATP exchange catalysed by crude extract of pine seeds

The incubation mixtures (assay method 1) contained fatty acid as specified, 60mM-Tris-HCl, pH 8, 1.5mM- $MgCl_2$, 2mM-ATP and 2mM- PP_i . ▲, Propionate; ○, *n*-butyrate; △, *n*-valerate; ●, acetate.

detergent properties of long-chain fatty acid salts (Pande & Mead, 1968; Pande, 1972).

Most (80%) of the acetate- and propionate-dependent PP_i -ATP exchange activity was recovered in the supernatant solution; the fatty layer and the 20000g precipitate, which were normally discarded during the preparation of the supernatant solution, did not catalyse any significant *n*-octanoate-, dodecanoate- or linoleate-dependent PP_i -ATP exchange.

Activity of short-chain fatty acyl-CoA synthetase during seed germination

The activity of the enzyme was examined in relation to fat content and activity of isocitrate lyase, a marker enzyme of glyoxysome activity. Expressed as activity per seed, short-chain fatty acyl-CoA synthetase activity was present in dry ungerminated seeds and remained at approximately the same value for approx. 11 days after imbibition, and then decreased to a negligible value (Fig. 3a). Expressed as activity per mg of extracted protein, activity decreased during germination. The proportion of propionate-/acetate-/*n*-butyrate-dependent PP_i -ATP exchange activities

Fig. 3. Fat content and activities of isocitrate lyase and short-chain fatty acyl-CoA synthetase during germination of pine seeds

Method B crude extracts were prepared at the times specified and assayed as described in the text. Between day 12 and day 16 the residual female gametophyte was discarded; accordingly, at day 17 the seedling only was tested for enzyme activities. Fat content was determined as described in the text. The incubation mixtures (assay method 1) contained 60mM-Tris-HCl, pH 8, 60 μ M-EDTA, 2mM-MgCl₂, 2mM-ATP, 3mM-PP_i, and either 10mM-acetate, 1mM-propionate or 1mM-*n*-butyrate. All results are means of duplicate determinations each on duplicate extractions. (a) ▲, Propionate-dependent PP_i-ATP exchange; ●, acetate-dependent PP_i-ATP exchange; ○, *n*-butyrate-dependent PP_i-ATP exchange. (b) ■, Isocitrate lyase activity; Δ, fat content.

was approximately constant throughout germination. The activity of isocitrate lyase was negligible in dry seeds, increased to a maximum after approx. 11 days and then declined whether activity was expressed per seed (Fig. 3b) or per mg of extracted protein.

At approx. 17 days after imbibition, *P. radiata* seedlings discard the female gametophyte, the principal fat-storage tissue. At this time the activities of both isocitrate lyase and short-chain fatty acyl-CoA synthetase in the seedlings were negligible (Fig. 3), and analysis of the tissues demonstrated that both enzymes were associated with the female gametophyte (Table 1).

Crude extracts of seeds imbibed for 7 and 9 days did not catalyse fatty acid-dependent PP_i-ATP exchange when 0.1mM-linoleate, -palmitate, -dodecanoate or -*n*-octanoate were used as substrates.

Purification of short-chain fatty acyl-CoA synthetase

The fatty acid-dependent PP_i-ATP exchange activity of acetone-dried-powder extracts was proportional to enzyme concentration up to 0.6mg of protein per mixture; the apparent specific activity decreased at higher concentrations of enzyme. Accordingly, the enzyme activity of acetone-dried-powder extracts was monitored at less than 0.6mg of protein per incubation mixture. The enzyme was purified approx. 70-fold with respect to acetone-dried-powder extract (Table 2). Fractionation with (NH₄)₂SO₄ produced the largest increase in specific activity and decreased endogenous PP_i-ATP exchange to a low value. Attempts to purify the enzyme further by chromatography on DEAE-cellulose equilibrated with Tris-HCl buffer, pH 8, containing 2mM-dithiothreitol and 0.1mM-EDTA were unsuccessful, possibly owing to binding of dithiothreitol and EDTA by DEAE-cellulose. Gel filtration of protamine sulphate supernatant solutions prepared and run in the absence of EDTA effected poor resolution of enzyme activity, and the recovery of activity was low compared with the standard procedures, which were conducted in the presence of 0.1mM-EDTA. The acetate- and propionate-dependent PP_i-ATP exchange activities were not separated during purification, and the ratio of these two activities was approximately constant during all stages of purification (Table 1) and during chromatography on DEAE-cellulose.

Purified enzyme did not catalyse PP_i-ATP exchange when K₂SO₄ (40mM) or a mixture of 20 protein L-amino acids (each 1mM) was used in lieu of short-chain fatty acid. This indicates either that ATP sulphurylase and the aminoacyl-tRNA synthetases were absent from purified short-chain fatty acyl-CoA synthetase or that the conditions of assay were unsuitable for detecting activities of these enzymes. Mg²⁺-dependent alkaline pyrophosphatase, which interferes with PP_i-ATP exchange reactions, was absent from purified short-chain fatty acyl-CoA synthetase when assayed by the method of Shaw & Anderson (1972).

Stability of purified fatty acyl-CoA synthetase

Although 0.1mM-EDTA minimized loss of activity during gel filtration, purified enzyme was unstable in the presence of EDTA during storage at both 2°C and -15°C. Addition of 5mM-dithiothreitol retarded the rate of loss of activity during storage at 2°C, and was therefore added to purified enzyme preparations as

Table 1. *Localization of enzyme activities in the tissues of germinating pine seeds*

Extraction of enzymes and assay procedures were as described in Fig. 3 except that 50 seedlings or female gametophytes were homogenized in 20 ml of extracting medium.

Enzyme activity	Tissue			
	Seedling		Female gametophyte	
	Day 9	Day 13	Day 9	Day 13
Isocitrate lyase (nmol of glyoxylate/min per seedling or gametophyte)	1.5	0.8	252	233
$10^2 \times$ Short-chain fatty acyl-CoA synthetase activity (nmol of PP_i /min per seedling or gametophyte)				
Propionate-dependent PP_i -ATP exchange	2.3	6.0	176	139
Acetate-dependent PP_i -ATP exchange	1.8	1.6	144	108
<i>n</i> -Butyrate-dependent PP_i -ATP exchange	2.5	4.0	104	80

Table 2. *Purification of short-chain fatty acyl-CoA synthetase from pine seeds*

Activity was measured by assay method 1. Incubation mixtures contained $20 \mu M$ -EDTA, 1.5 mM - $MgCl_2$, 3 mM -ATP, 3 mM - PP_i and either 10 mM -acetate or 1 mM -propionate. Details are described in the text.

Treatment	Protein(mg)	Fatty acid-dependent PP_i -ATP exchange (nmol/min per mg of protein)		Acetate-dependent exchange
		With acetate	With propionate	Propionate-dependent exchange
Acetone-dried-powder extract	481	1.70	2.30	0.74
$(NH_4)_2SO_4$ fraction	40	44.8	59.4	0.75
Protamine sulphate supernatant	37	46.0	57.8	0.80
Region of peak activity after gel filtration	2.6	127	148	0.86

a routine. The loss of activity of enzyme stored in 5 mM -dithiothreitol and 0.1 mM -EDTA was less at $2^\circ C$ than at $-15^\circ C$. Addition of dithiothreitol to preparations that had lost activity during storage in 0.1 mM -EDTA did not restore activity.

Addition of 5 mM -ATP in lieu of dithiothreitol prolonged full activity of purified fatty acyl-CoA synthetase during storage at $2^\circ C$ for about 8 days; after this time activity declined. $MgCl_2$ (10 mM) and KCl (40 mM) retarded loss of activity, but 5 mM -propionate was ineffective. Storage of the enzyme in 5 mM -ATP plus 5 mM -dithiothreitol was no more effective than storage in ATP alone.

Properties of the fatty acid-dependent PP_i -ATP exchange reaction catalysed by purified enzyme

The rate of propionate-dependent PP_i -ATP exchange in the presence of 0.2 mM -dithiothreitol at $23^\circ C$, $33^\circ C$ and $40^\circ C$ was constant for 60 min. The initial rate at $50^\circ C$ was greater than at $40^\circ C$, but decreased after 10 min. Standard assays were conducted at $35^\circ C$ for 15 min. Pretreatment of the enzyme at $70^\circ C$ for 2 min, with or without ATP, abolished activity. Propionate- and acetate-dependent

PP_i -ATP exchange was proportional to enzyme concentration in assays containing up to $37 \mu g$ of purified protein per incubation mixture.

The ^{32}P -labelled product synthesized from $[^{32}P]$ - PP_i in standard PP_i -exchange assays containing purified enzyme and either propionate (1 mM) or acetate (10 mM) as substrate was confirmed as $[^{32}P]$ ATP by the procedures described by Shaw & Anderson (1971); neither $[^{32}P]$ ADP nor unlabelled AMP was detected. Purified enzyme catalysed propionate- and acetate-dependent PP_i exchange when dATP was used in lieu of ATP; the formation of $[^{32}P]$ dATP was confirmed by the procedures described by Shaw & Anderson (1972). A small amount of radioactive product was adsorbed on to charcoal when ADP was used in lieu of ATP. The enzyme, however, did not catalyse $ADP-PP_i$ exchange, as the radioactive product was identified as $[^{32}P]$ ATP. Trace amounts of ATP were present in the ADP. Purified enzyme did not catalyse propionate- or acetate-dependent PP_i exchange when AMP or GTP was added in lieu of ATP. Similarly the enzyme did not catalyse P_i exchange with ATP, dATP, ADP, AMP or GTP when $[^{32}P]P_i$ was added in lieu of $[^{32}P]PP_i$. The formation of $[^{32}P]$ ATP from

Table 3. Summary of the kinetics of fatty acid-dependent PP_i -ATP exchange

The incubation mixtures for assay method 1 contained fatty acid as specified in the text, 84mM-Tris-HCl, pH 8, 20 μ M-EDTA, 1mM-dithiothreitol, 2mM-MgCl₂, 1mM-ATP and 3mM-PP_i. K_m and V values were calculated from 12 values of [S] for each fatty acid after straight-line fitting by the method of least squares; in each instance the correlation coefficient was greater than 0.995. The observed maximum velocities were measured at the optimum concentration of each fatty acid: 10mM-acetate, 1mM-propionate, 1mM-*n*-butyrate and 1mM-*n*-valerate.

Fatty acid	K_m (mM)	V (nmol/min per mg of protein)	Observed maximum velocity (nmol/min per mg of protein)
Acetate	4.7	268	166
Propionate	0.21	248	207
<i>n</i> -Butyrate	0.33	243	135
<i>n</i> -Valerate	2.1	68	25

[³²P]PP_i was dependent on a complete reaction mixture containing ATP, MgCl₂, fatty acid and enzyme.

With purified enzyme, the effect of MgCl₂ concentration on fatty acid-dependent PP_i-ATP exchange was essentially the same as described for crude extracts obtained by method A (Fig. 1); some fatty acid-dependent PP_i exchange occurred in the absence of MgCl₂, but this activity was attributed to traces of Mg²⁺ in the ATP substrate (12nmol of Mg²⁺/μmol of ATP). In PP_i-ATP exchange assays, Mg²⁺ complexes with ATP and PP_i; at least one complex species, MgP₂O₇²⁻, is probably a reactive substrate for the enzyme (Young & Anderson, 1974). The usual concentration of EDTA in PP_i-ATP exchange assays (20 μM) was low compared with the concentration of PP_i (3mM), so it is unlikely that EDTA would prevent formation of MgP₂O₇²⁻, even though the stability of MgEDTA²⁻ is greater than that of MgP₂O₇²⁻ (O'Sullivan, 1969).

Other bivalent cations supported propionate-dependent PP_i-ATP exchange. The exchange activity of incubation mixtures containing 3mM-ATP, 3mM-PP_i and 1mM-propionate with 2mM-CoCl₂, -CaCl₂ and -ZnCl₂, was 55%, 30% and 15% respectively of the activity with 2mM-MgCl₂. CdI₂, NiSO₄ and MnCl₂ (each 2mM) supported less exchange than did 2mM-ZnCl₂. The optimum concentration of CoCl₂ was approx. 3.5mM. At this concentration propionate-dependent PP_i-dependent PP_i-ATP exchange was 70% of the exchange at the optimum concentration of MgCl₂ (2mM). Dithiothreitol was omitted from this study because of the

different stability constants of bivalent cation-dithiothreitol complexes (Cornell & Crivaro, 1972).

The effect of fatty acid concentration on fatty acid-dependent PP_i-ATP exchange catalysed by purified enzyme was similar to that described for method A crude extracts (Fig. 2). The fatty acid-dependent exchange activity of purified enzyme was studied as a function of low concentrations of fatty acids so that inhibition by fatty acids would be minimal. The concentrations chosen (0–2mM-acetate, 0–0.2mM-propionate, -*n*-butyrate and -*n*-valerate) were less than the optimum concentrations of the fatty acids (Fig. 2). The results were analysed by double-reciprocal plots; extrapolation of the lines of best fit (which were linear) yielded values for K_m and V (Table 3). As judged by K_m values, the affinity of the enzyme was similar for propionate and *n*-butyrate, but the affinity for acetate was lower.

The kinetics of *n*-butyrate/propionate and propionate/acetate competition experiments (Figs. 4 and 5) demonstrate that propionate competes with acetate and *n*-butyrate; competition between acetate and *n*-butyrate was demonstrated in another experiment. The results are consistent with competition of the three fatty acids for the same enzyme, and confirm the lower affinity of the enzyme for acetate (Table 3).

Over a range of concentrations, malonate, citrate, formate, ethanol, propanol, acetaldehyde and dichloroacetate did not significantly stimulate PP_i-ATP exchange when used in lieu of short-chain fatty acid; monochloroacetate and 2-methylpropionate, however, stimulated exchange. The kinetics of 2-methylpropionate-dependent PP_i-ATP exchange were similar to those of *n*-valerate-dependent exchange, but monochloroacetate was less effective as a substrate than *n*-valerate.

The pH optima for propionate- and acetate-dependent PP_i-ATP exchange were 8.1 and 7.5 respectively. In general, propionate-dependent activity was greater than acetate-dependent activity whenever the pH exceeded 8.0; the converse applied when the pH was less than 8.0. The optimum concentrations of acetate and propionate were approx. 10 and 1mM respectively at both pH 7.0 and 8.0.

The enzyme was sensitive to thiol-group reagents. In the absence of dithiothreitol, 1mM-*N*-ethylmaleimide and 2.8 μM-*p*-chloromercuribenzoate inhibited propionate-dependent PP_i-ATP exchange by about 90%. CoA and AMP inhibited fatty acid-dependent PP_i-ATP exchange. With 10mM-acetate or 1mM-propionate, 0.47mM-CoA inhibited exchange by 16–18%. The inhibition by AMP is described by Young & Anderson (1974).

ATP synthesis from fatty acyl-AMP and PP_i

In the presence of MgCl₂ short-chain fatty acyl-CoA synthetase catalysed the synthesis of [³²P]ATP

Fig. 4. Effects of *n*-butyrate and propionate alone and together on fatty acid-dependent PP_i -ATP exchange

The incubation mixtures (assay method 1) contained fatty acid as specified, 84 mM-Tris-HCl, pH 8, 20 μ M-EDTA, 1 mM-dithiothreitol, 2 mM-MgCl₂, 1 mM-ATP and 3 mM- PP_i . ▲, No *n*-butyrate; ○, 0.4 mM-*n*-butyrate; ●, 1 mM-*n*-butyrate.

Fig. 5. Effects of propionate and acetate alone and together on fatty acid-dependent PP_i -ATP exchange

The incubation mixtures (assay method 1) contained fatty acid as specified, 84 mM-Tris-HCl, pH 8, 20 μ M-EDTA, 1 mM-dithiothreitol, 2 mM-MgCl₂, 1 mM-ATP, and 3 mM- PP_i . ●, No propionate; ○, 0.1 mM-propionate; ▲, 1 mM-propionate.

Table 4. Cofactor requirements for the synthesis of ATP

The complete incubation mixtures (assay method 2) contained 0.11 μ mol of acetyl-AMP or propionyl-AMP. The activity of the complete incubation mixture containing propionyl-AMP was equivalent to a propionyl-AMP-dependent synthesis of 176 nmol of ATP/min per mg of protein. Incubation mixtures without enzyme contained an equal volume of medium 1 containing 5 mM-dithiothreitol. Background radioactivity was less than 0.2 c.p.s.

	Radioactivity retained on charcoal (c.p.s.)	
	With acetyl-AMP	With propionyl-AMP
Complete incubation mixture	106	102
Without fatty acyl-AMP	3.7	3.0
Without MgCl ₂	15.2	11.6
Without enzyme	2.4	2.7

from [32 P] PP_i and either propionyl- or acetyl-AMP (Table 4); the formation of [32 P]ATP was demonstrated by the method of Shaw & Anderson (1971). The rate of formation of ATP from propionyl-AMP was constant for 25 min. When AMP, a major contaminant of propionyl-AMP and acetyl-AMP, was used in lieu of fatty acyl-AMP, ATP was not synthesized. Addition of AMP to assays containing propionyl-AMP inhibited ATP synthesis. This indicates that measurements of propionyl-AMP-dependent (and probably acetyl-AMP-dependent) ATP synthesis are underestimated owing to inhibition by contaminating AMP. However, with propionyl-AMP the concentration of contaminating AMP did not cause more than 7% underestimation of activity.

The apparent K_m for propionyl-AMP was 7.5 μ M, about eightfold less than for acetyl-AMP, and V -(propionyl-AMP) was 0.66 of V -(acetyl-AMP). CoA weakly inhibited ATP synthesis: in the presence of 92 μ M- and 18 μ M-propionyl-AMP, 1.25 mM-CoA inhibited ATP formation by 16% and 27% respectively.

Fatty acyl-CoA synthesis

Purified enzyme catalysed the formation of [14 C]-propionyl-CoA and [14 C]acetyl-CoA from [1- 14 C]-propionate and [1- 14 C]acetate respectively. The rate of CoA-dependent fatty acyl-CoA formation was constant for 20 min. The formation of fatty acyl-CoA from fatty acid required ATP, MgCl₂, CoA and enzyme, but dithiothreitol slightly inhibited activity.

The formation of propionyl-CoA and acetyl-CoA was confirmed by the synthesis of their respective hydroxamates.

The apparent K_m was less for propionate than for acetate when measured by the formation of fatty acyl-CoA in the presence of 2mM-ATP or -dATP; the apparent K_m values were 16mM for acetate with ATP, 29mM for acetate with dATP, 7.5mM for propionate with ATP and 12mM for propionate with dATP. dATP was less effective as a source of nucleoside triphosphate than equimolar ATP. Addition of 0.11unit of Mg^{2+} -dependent alkaline pyrophosphatase, purified from spinach by the method of Shaw & Anderson (1972), increased the CoA-dependent synthesis of acetyl-CoA by 46%, suggesting that PP_i is a product of the overall reaction.

Discussion

Several lines of evidence suggest that the short-chain fatty acid-dependent PP_i -ATP exchange is catalysed by a single enzyme. The proportion of propionate-/acetate-/n-butyrate-dependent PP_i -ATP exchange was approximately constant during seed germination (Fig. 3) and the ratio of propionate-/acetate-dependent PP_i -ATP exchange was approximately constant at all stages of purification (Table 2). Competition experiments between propionate and n-butyrate (Fig. 4), acetate and propionate (Fig. 5) and acetate and n-butyrate also demonstrated that acetate, propionate and n-butyrate competed for one enzyme. The affinity of purified enzyme was greater for propionate and n-butyrate than for acetate as judged from the effect of fatty acid concentration on PP_i -ATP exchange at concentrations less than the optimum concentration (Table 3); the competition experiments (Figs. 4 and 5) support this conclusion.

The increased stability of the purified enzyme in the presence of dithiothreitol and EDTA, and the sensitivity of the enzyme to thiol-group reagents, indicates that the enzyme contains a sensitive thiol group(s). ATP retarded loss of enzyme activity on storage, but the effects of ATP and dithiothreitol were not additive; these results suggest that ATP protects a sensitive thiol group(s). Analogous results were reported for the medium-chain fatty acyl-CoA synthetase from pig liver (Jencks & Lipmann, 1957) and the acetyl-CoA synthetase from ox heart mitochondria (Webster & Campagnari, 1962). Stabilization of enzyme activity with ATP has been demonstrated with long-chain fatty acyl-CoA synthetase from rat liver microsomal fraction (Bar-Tana *et al.*, 1972; Pande, 1972).

Berg (1956a) studied the acetyl-CoA synthetase from yeast and postulated that the formation of acetyl-CoA, or more generally fatty acyl-CoA, occurred in two steps:

Partial reaction (i) was demonstrated with the *Pinus* enzyme by assay methods 1 and 2 (Table 4). We have not attempted to demonstrate enzyme-catalysed synthesis of acetyl-AMP or propionyl-AMP with the *Pinus* enzyme. Berg (1956a) established that the time-course of PP_i -ATP exchange for the yeast enzyme was characteristic of exchange at chemical equilibrium, which is consistent with the involvement of enzyme-bound acetyl-AMP (Berg, 1956a; Webster & Campagnari, 1962; Webster, 1963). Experiments described by Young & Anderson (1974) indicate that the PP_i -ATP exchange reaction of the pine seed enzyme occurs at chemical equilibrium. The PP_i -ATP exchange reactions of amino acyl-tRNA synthetases also occur at chemical equilibrium (Santi *et al.*, 1971); in these situations stoichiometric amounts of PP_i and a modified form of the enzyme containing bound aminoacyl-adenylate are produced after a single turnover. The similarity of the PP_i -ATP exchange reactions of the yeast enzyme (Berg, 1956a) and of the aminoacyl-tRNA synthetases (Santi *et al.*, 1971) to the PP_i -ATP exchange reaction of the pine seed enzyme suggests that the fatty acyl adenylates are firmly bound intermediates in this system.

The higher affinity of the pine seed enzyme for propionate than for acetate as judged by PP_i -ATP exchange and fatty acyl-CoA synthesis, and the higher affinity for propionyl-AMP than for acetyl-AMP as judged by ATP synthesis, distinguishes the short-chain fatty acyl-CoA synthetase of *Pinus* seeds from the acetyl-CoA synthetases of spinach (Millard & Bonner, 1954) and potato tuber (Huang & Stumpf, 1970). The spinach enzyme did not catalyse the synthesis of propionyl-CoA, and the affinity of the potato enzyme for propionate was less than for acetate.

Fatty acid-dependent PP_i -ATP exchange was inhibited by fatty acid concentrations greater than 10mM-acetate, 1mM-propionate and 1mM-n-butyrate (Fig. 2), but there was no obvious inhibition of fatty acyl-CoA synthesis by concentrations of fatty acid up to 32mM-acetate and 24mM-propionate. The mechanistic significance of the inhibition of PP_i -ATP exchange by high concentrations of fatty acids is discussed by Young & Anderson (1974).

The pattern of activity of isocitrate lyase during germination of *P. radiata* seeds (Fig. 3b) was similar to that observed during germination of other fatty seeds (Beevers, 1961; Firenzuoli *et al.*, 1968b; Ching, 1970), a finding which suggests that the glyoxylate cycle is operative in germinating seeds of *P. radiata*. During germination the decrease in fat content per seed was correlated with isocitrate lyase activity but not with the activity of short-chain

fatty acyl-CoA synthetase (Fig. 3). Yamada & Stumpf (1965) and Hutton & Stumpf (1969) studied the activities of β -oxidation and component enzymes of β -oxidation in germinating castor-bean endosperm, which constitutes the lipid-storage tissue of these seeds. Castor beans germinate in approximately 1 week. β -Oxidation and component enzymes initially exhibited low specific activities; specific activities dramatically increased to a maximum about 4 days after germination began, and subsequently declined. In contrast, the activity of short-chain fatty acyl-CoA synthetase of pine seeds did not increase during germination irrespective of whether activity was expressed as units per seed (Fig. 3a) or as units per mg of extracted protein. The β -oxidation system of germinating castor bean is involved in the mobilization of lipid during germination (Beevers, 1961; Hutton & Stumpf, 1969) and a similar system is presumed to operate during the germination of fatty coniferous seeds. It is probable that the pine seed short-chain fatty acyl-CoA synthetase plays only an indirect part (if any) in the mobilization of lipid during germination, even though the enzyme is located in female gametophyte tissue (Table 1), the principal fat-storage tissue.

The metabolism of propionate by tissue slices and cell-free extracts of seeds has been reported (Hitchcock & Nichols, 1971). Addition of CoA, ATP and bivalent metal ions was necessary for the metabolism of propionate by mitochondria from peanut cotyledons (Giovannelli & Stumpf, 1957, 1958) and by an extract of wheat germ (Hatch & Stumpf, 1962); these authors implicated propionyl-CoA as an intermediate in propionate metabolism. It is possible that short-chain fatty acyl-CoA synthetase is involved in the first stage of propionate metabolism. Nothing is known about the production of free propionate *in vivo* in germinating fatty seeds, though propionyl-CoA can arise by β -oxidation of odd-numbered fatty acids and monomethyl-substituted fatty acids (Hutton & Stumpf, 1971; Hitchcock & Nichols, 1971). Another possibility is that the enzyme is a remnant from the synthesis of long-chain fatty acids during seed maturation. This hypothesis is consistent with the localization of the enzyme in female gametophyte tissue (Table 1) and its presence in the dry mature seed (Fig. 3).

The fatty acids of *Pinus* spp. seeds are almost entirely C₁₆ to C₁₈ (Firenzuoli *et al.*, 1968a). Accordingly, long-chain fatty acyl-CoA synthetases should be present to initiate β -oxidation on germination. One possible explanation of our failure to demonstrate long-chain fatty acid-dependent PP_i-ATP exchange could be that the reaction mechanism of long-chain fatty acyl-CoA synthetase(s) of pine seeds precludes the measurement of activity by the PP_i-ATP exchange technique.

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