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SUBSTRATE SPECIFICITY OF THE SHORT CHAIN FATTY ACYL-COENZYME A SYNTHETASE OF *PINUS RADIATA*

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Key Word Index—*Pinus radiata*; Pinaceae; fatty acyl-CoA synthetase; acetate; propionate; butyrate; chlorinated carboxylic acids; halogenated carboxylic acids; fluoroacetate; dicarboxylic acids; acetate-related compounds; propionate-related compounds.

Abstract—The short chain fatty acid-CoA synthetase of the female gametophytic tissue of *Pinus radiata* exhibited highest affinity for propionate and butyrate ($K_{m(app.)}$ = 0.073 mM for both substrates) when examined by ^{32}PPi–ATP exchange. Relative to the activity at 1 mM propionate, partially purified enzyme supported exchange with acetate (100%), acrylate (114%), crotonate (107%) and thioglycollate (70%), but with varying degrees of affinity as reflected by their different $K_{m(app.)}$ values (2.56, 1.41, 0.45 and 1.26 mM, respectively). All other unhalogenated C_2, C_3 and C_4 carboxylic acids examined did not support PPi–ATP exchange. Partially purified enzyme supported low rates of exchange in the presence of 2-chloroacetate, 2-bromoacetate, 3-chloropropionate, 3-bromopropionate, 2-chlorobutyrate and 2-bromobutyrate; the apparent K_m values were 2.74, 1.03, 0.15, 0.17, 0.85 and 0.23 mM, respectively. In the presence of propionate and one of the unhalogenated or halogenated substrates, the PPi–ATP exchange was consistent with competition for a common substrate binding site. All other halogenated derivatives of acetate and propionate did not support PPi–ATP exchange. The enzyme displayed an absolute requirement for Mg^{2+} (optimum concentration 1.5 mM).

INTRODUCTION

Acetyl-CoA is an essential metabolite involved in many biochemical pathways such as the biosynthesis of fatty acids, branched chain amino acids, mevalonic acid and isoprenoid lipids. Thus, the enzyme responsible for the synthesis of acetyl-CoA plays a key role in plant metabolism. Various acyl-CoA synthetases have been reported in plants, the most active of which is acetyl-CoA synthetase. Located in the stroma of chloroplasts, it catalyses the activation of acetate to acetyl-CoA, which is required for the long chain fatty acid biosynthetic pathway located in the outer membrane of the chloroplast envelope [1–6].

Germinating fatty seeds have a very active β-oxidation pathway for the mobilization of long chain fatty acids to acetyl-CoA, which is in turn metabolized to succinate via the glyoxylate cycle. This process is initiated by long chain fatty acyl-CoA synthetases. However, the female gametophytic tissue of the lipid-rich seeds of *Pinus radiata* contains a short chain fatty acyl-CoA synthetase (SCFA-CoA synthetase, EC 6.2.1.1) [7, 8]. The enzyme is most active towards acetate, propionate and butyrate although, as determined by fatty acid-dependent PPi–ATP exchange, the affinity for propionate and butyrate is *ca* 25- to 35-fold greater than for acetate. The enzyme also supports low rates of PPi–ATP exchange in the presence of 2-chloroacetate, but not 2,2-dichloroacetate. The activity towards 2-chloroacetate is of interest because various halogenated derivatives of acetate and propionate are phytotoxic, some of which are utilized as herbicides (e.g. 2-chloroacetate, 2,2-dichloropropionate [dalapon] and 2,2,3,3-tetrafluoropropionate [tetrapion]). In this paper we describe a study of the substrate specificity of the SCFA-CoA synthetase of *P. radiata* towards short chain carboxylic acids and various halogenated derivatives of acetate, propionate and butyrate. The physiological role of the enzyme in germinating *Pinus* seed is also discussed.

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RESULTS AND DISCUSSION

Enzyme specificity towards unhalogenated carboxylic acids

The propionate-dependent PPi–ATP exchange of crude extracts was commonly only 5–40% greater than the endogenous exchange. However, simple fractionation with $(NH_4)_2SO_4$ decreased the endogenous activity to 0.5–2% of the rate of exchange with propionate. Partially purified extract supported PPi–ATP exchange activity with acetate, propionate, butyrate, valerate, acrylate,

Table 1. Kinetic parameters of the carboxylic acid substrates of SCFA-CoA synthetase. Activity was determined by carboxylic acid-dependent PPi–ATP exchange with partially purified enzyme

Substrate	pK_a	Apparent K_m (mM)	Apparent $V_{max.}$ relative to propionate* (%)	Concn required to attain $V_{max.}$ (mM)
Acetate	4.76	2.56	100	10–15
2-Bromoacetate	2.90	1.03	13	7.5
2-Chloroacetate	2.81	2.74	8	10–15
2-Fluoroacetate	2.60	2.75	5	20
Propionate	4.88	0.073	100	0.8
3-Bromopropionate		0.17	10	5
3-Chloropropionate	4.10	0.15	9	1–5
Butyrate	4.82	0.073	65	0.8
2-Methylpropionate	4.86	0.25	19	3
2-Bromobutyrate		0.23	4	5
2-Chlorobutyrate		0.85	4	10
Valerate	4.84	0.25	10	5
Acrylate	4.25	1.41	114	7.5
Crotonate	4.70	0.45	107	5
Malonate	2.85, 5.70	0.17	17	2
Thioglycollate		1.26	70	5

*The apparent $V_{max.}$ value for each substrate is expressed as a percentage of the apparent $V_{max.}$ for propionate at 1 mM.

crotonate, thioglycollate and 2-methylpropionate (isobutyrate) as substrates (Table 1). The enzyme preparation supported highest activity with acetate, propionate, acrylate and crotonate; the maximum rate of PPi–ATP exchange was similar for all four substrates. The maximum rate supported by butyrate was somewhat less, but the affinity for propionate and butyrate (both having $K_{m(app.)}$ values of 0.073 mM [Fig. 1, Table 1]) was much greater than for acetate ($K_{m(app.)} = 2.56$ mM). The $K_{m(app.)}$ values for acrylate and crotonate were intermediate ($K_{m(app.)}$ values of 1.41 and 0.45 mM, respectively). Concentrations greater than *ca* 1–2 mM propionate/butyrate and 10–15 mM acetate were inhibitory and all subsequent experiments with these substrates were conducted at non-inhibitory concentrations. All of the halogenated and unhalogenated carboxylic acid substrates were tested by GC for the presence of impurities that could contribute to the PPi–ATP exchange observed for each compound. The average level of impurity as determined by peak area was *ca* 0.5%, with the highest level reaching 2.4%. However, acetate and/or propionate, the most active substrates, were not present as contaminants in the other substrates, suggesting that the impurities did not contribute significantly, if at all, to the activity observed for compounds exhibiting PPi–ATP exchange. The findings for the aliphatic carboxylic acids, acetate, propionate and butyrate, are in general agreement with those reported previously for the more purified enzyme from *P. radiata* [7, 8]. The results for acrylate, crotonate and thioglycollate extend the range of substrates reported for this enzyme.

Importantly, the PPi–ATP exchange catalysed by partially purified extracts in the presence of any two of the substrates, acetate, propionate, butyrate, acrylate and crotonate, never exceeded the sum of the rates for the respective substrates when measured individually (results not shown). Rather, the observed exchange under these conditions was consistent with that predicted for these substrates competing for a common site on a single enzyme in accordance with the kinetic parameters listed in Table 1. Experiments of this kind served to emphasize further the lower affinity of the enzyme for acetate.

The SCFA-CoA synthetase of *Pinus* exhibited low affinity towards a broad but distinct range of substrates and thus can be distinguished from the acetyl-CoA synthetase of leaves [9] and etiolated radish cotyledons [10], which have a very high affinity for acetate, but low affinity towards propionate, and a narrower range of substrates. The acetyl-CoA synthetase from non-photosynthetic tissues such as the enzymes from the glyoxysomal membrane of germinating castor bean [11] and potato tuber [12] also exhibit high specificity towards acetate. The *Pinus* SCFA-CoA synthetase differs from the butyryl-CoA synthetase of the fungus *Paecilomyces varioti*, which exhibits greatest affinity and highest activity towards butyrate, but lower affinity for propionate and valerate [13], and the acetyl-CoA synthetase from *Penicillium chrysogenum*, which has a broad substrate range, utilizing fatty acids with chain lengths from C_2 to C_8 as well as some aromatics such as phenylacetate and 2- and 3-thiopheneacetate [14]. It is also dissimilar to the pimeoyl-CoA synthetase involved in biotin biosynthesis in pea cytosol [15] since the *Pinus* enzyme was active towards malonate, but inactive towards dicarboxylic acids containing four carbon atoms.

The kinetic data suggest that propionate and butyrate are the most likely physiological substrates. However, little is known about mechanisms that might produce

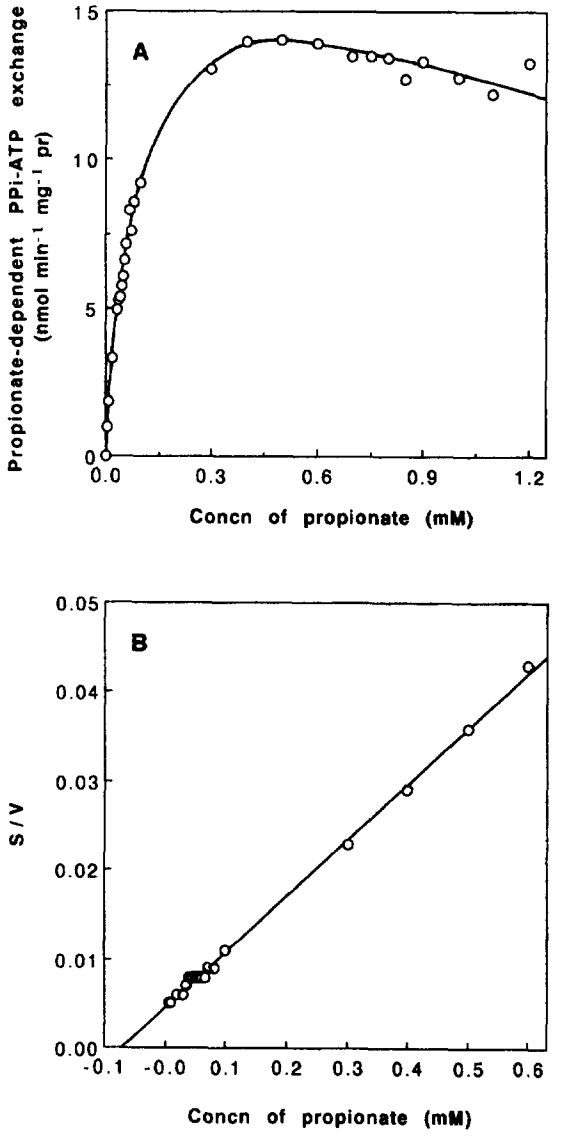

Fig. 1. (A) Primary plot of the effect of propionate concentration on reaction velocity as measured by propionate-dependent PPi–ATP exchange. (B) Hanes plot (S vs. S/V) of the propionate-dependent PPi–ATP exchange shown in Fig. 1(A). The equation for the plot is $y = 4.5531e\text{-}3 + 6.2898e\text{-}2x$ ($R\text{^}2$ value = 0.998). The $K_{m(app.)}$, as derived from the above equation of the X-intercept, is 0.073 mM.

these substrates in free forms in the lipid-rich tissue of the female gametophyte of *Pinus*, especially as the glyoxysomes of oil-rich seeds reportedly contain a very active acetyl-CoA synthetase [11] and since, in theory, the β-oxidation pathway itself results in the production of acetate and, for odd numbered fatty acids, propionate as their CoA thioesters. In spinach leaf, a mitochondrial short chain acyl-CoA hydrolase is responsible for converting the acetyl-CoA produced from pyruvate to free acetate [16], which is transported into the chloroplast where it is reactivated by a highly specific chloroplastic acetyl-CoA synthetase. It is possible that an analogous mechanism, not necessarily involving the same organelles, exists for processing propionyl-CoA in *Pinus*. This would involve hydrolysis of propionyl-CoA in one metabolic compartment of the cell, transport of propionate across a membrane into another metabolic compartment and reformation of the CoA ester through the action of SCFA-CoA synthetase.

Another possible role for the SCFA-CoA synthetase is that it could be involved in metabolizing free propionate and acrylate to acetate via a modified β-oxidation pathway involving malonic semialdehyde as an intermediate [17–22]. This pathway is very active in the alga *Prototheca zopfii* [23, 24]. Another possible function could be that the *Pinus* enzyme supports the synthesis of CoA thioesters, which serve as substrates of short chain fatty acyl-CoA carboxylases in plants, such as propionyl-CoA carboxylase [25, 26] and possibly 3-methylcrotonyl-CoA carboxylase [25–27], although the activity of the *Pinus* enzyme towards 3-methylcrotonate was not examined.

Several other non-halogenated carboxylic acids also supported PPi–ATP exchange. The most active of these was thioglycollate, which exhibited a low $K_{m(app.)}$, second only to propionate and butyrate (Table 1). The enzyme catalysed PPi–ATP exchange with *n*-valerate and malonate at low rates (*ca* 10–20% of the rate with 1 mM propionate), but the following substrates did not support exchange (rates less than 1.5% of the rate with 1 mM propionate): pyruvate, 3-hydroxypyruvate, maleate, malate, succinate, glycine, fumarate and glycollate (refer to Table 2 for concentration ranges tested).

Fatty acid-dependent PPi–ATP exchange was highly dependent on the concentration of $MgCl_2$. In the absence of $MgCl_2$, the rates of exchange supported by 10 mM acetate, 1 mM propionate and 1 mM butyrate were only 2% of those supported by these substrates in the presence of 1.5 mM $MgCl_2$. The optimum concentration, regardless of the fatty acyl substrate, was 1.5 mM (results not shown). Higher concentrations were inhibitory (e.g. 66% inhibition at 5 mM $MgCl_2$ with 1 mM propionate as substrate). This is consistent with the requirement for a divalent cation for the first partial reaction catalysed by acyl-CoA synthetases [28–30].

Halogenated carboxylic acids which act as substrates

Unbranched carboxylic acids containing two or three carbon atoms with a chlorine or bromine substitution on the terminal alkyl carbon atom supported PPi–ATP exchange at rates *ca* 8–13% of the appropriate unhalogenated parent compound (Table 1). C_4 aliphatic carboxylic acids bearing a chlorine or bromine substitution on the terminal carbon atom (C-4) did not support PPi–ATP exchange. However, halogenated butyrates with chlorine or bromine on the C-2 atom supported exchange at 6–7% of the rate with 1 mM butyrate. The affinity of the *Pinus* enzyme towards halogenated fatty acids which supported exchange was closely related to the affinity of the enzyme for the unhalogenated parent compound, i.e. the affinity of the enzyme for the halogenated propionates and butyrates was much

Table 2. Carboxylic acids which supported less than 1.5% of the PPi–ATP exchange activity with 1 mM propionate by partially purified SCFA–CoA synthetase. The carboxylic acids listed in this table are regarded as inactive substrates

Compound	Concentration range tested (mM)
2,2-Dibromoacetate	0.05–5
2,2,2-Tribromoacetate	0.10–7
2,2-Dichloroacetate	0.04–3
2,2,2-Trichloroacetate	0.025–10
2,2-Difluoroacetate	0.25–10
2,2,2-Trifluoroacetate	0.25–12
2-Iodoacetate	0.10–10
2-Bromopropionate	0.05–3
2,3-Dibromopropionate	0.25–10
2-Chloropropionate	0.40–20
2,2-Dichloropropionate	0.25–20
2,3-Dichloropropionate	0.25–20
2,2,3,3-Tetrafluoropropionate	0.05–10
3-Iodopropionate	0.01–10
2,3-Dichloro-2-methylpropionate	0.10–10
4-Bromobutyrate	0.01–10
4-Chlorobutyrate	0.025–30
3,3-Dichloropivalate	0.10–10
Pyruvate	0.10–10
3-Hydroxypyruvate	0.10–10
Maleate	0.10–10
Malate	0.10–10
Succinate	0.05–12
Glycine	0.10–10
Fumarate	0.10–10
Glycollate	0.10–10

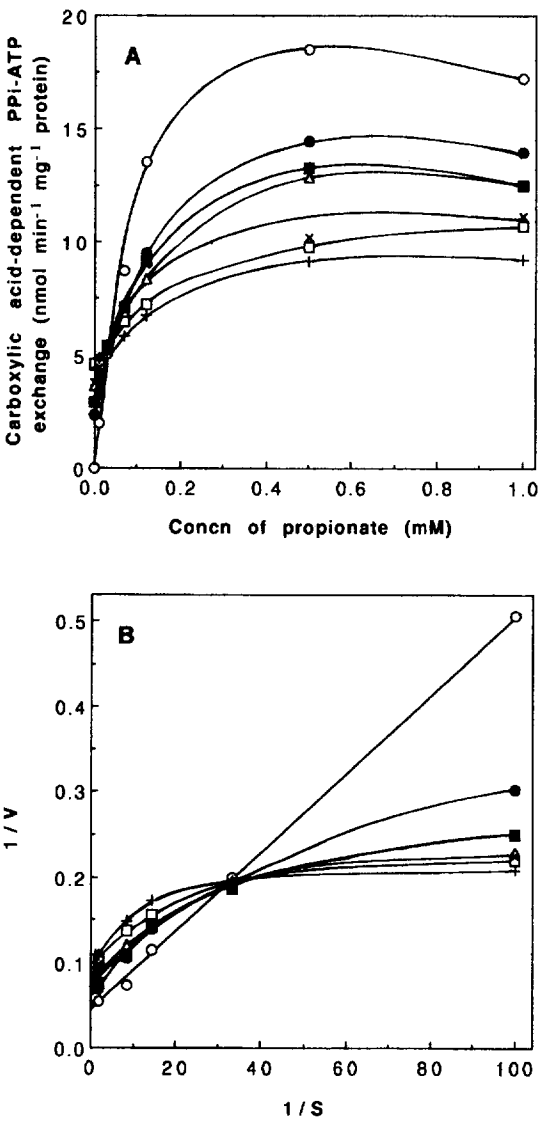

Fig. 2. (A) The effect of 2-chloroacetate and propionate, alone and together, on carboxylic acid-dependent PPi–ATP exchange catalysed by partially purified enzyme from *Pinus radiata*. PPi–ATP exchange with 2-chloroacetate at 0 mM (○), 0.25 mM (●), 0.5 mM (△), 1 mM (□), 3 mM (■), 7.5 mM (×) and 12 mM (+). (B) Double reciprocal plot of the effect of propionate concentration at various concentrations of 2-chloroacetate on PPi–ATP exchange (total velocity, V), for the case when $V_{max.\,(propionate)} > V_{max.\,(2\text{-chloroacetate})}$. The equation with respect to propionate in the absence of 2-chloroacetate is $y = 4.5841e\text{-}2 + 4.5885e\text{-}3x$ ($R\hat{}2 = 0.999$). PPi–ATP exchange with 2-chloroacetate at 0 mM (○), 0.25 mM (●), 0.5 mM (△), 1 mM (□), 3 mM (■), 7.5 mM (×) and 12 mM (+).

greater than for the halogenated acetates. Thus, chain length appears to be the major factor in determining the affinity of the enzyme for the propionate/butyrate and acetate series of compounds. As with the parent carboxylic acids, high concentrations of halogenated carboxylic acids (e.g. 10–15 mM 2-chloroacetate and 1–3 mM 3-chloropropionate) were inhibitory.

The PPi–ATP exchange of incubation mixtures containing pairs of substrates (halogenated and/or unhalogenated) were studied at non-inhibitory concentrations. The activity obtained for such combinations were intermediate between the combined activities for each substrate when supplied alone. Figure 2 shows the PPi–ATP exchange catalysed by partially purified enzyme in the presence of the two substrates, propionate and 2-chloroacetate. As demonstrated in the primary plot (Fig. 2A), the rate of exchange in the presence of the two substrates never exceeds the sum of the two individual rates for each substrate measured alone. The kinetics observed in the double reciprocal plot (Fig. 2B) adhere to the kinetics described by Pocklington and Jeffery [31] for two substrates which compete for a common site on the same enzyme. The results, therefore, are consistent with the proposal that the substrates 2-chloroacetate, 3-chloropropionate, 2-chlorobutyrate and 2-bromobutyrate compete with acetate, propionate and butyrate for a common binding site.

Those compounds which supported less than 1.5% of the activity observed with 1 mM propionate were deemed to be inactive as substrates and are listed in Table 2 along with the concentration range at which they were tested. 2-Fluoroacetate (0.10–20 mM), the active

constituent of the vertebrate pesticide 1080, did not support significant PPi–ATP exchange (e.g. the activity at 20 mM was less than 5% of the activity with 1 mM propionate). The toxic effect of fluoroacetate in animals is thought to involve its incorporation into fluorocitrate, presumably via the intermediate fluoroacetyl-CoA [32], which in turn involves activation of fluoroacetate in place of acetate via an acetyl-CoA synthetase. The fluorocitrate formed in this way is regarded as a lethal synthesis since it is a potent inhibitor of aconitase and thus inhibits the tricarboxylic acid cycle [33–36]. Plants, by contrast, are relatively insensitive to fluoroacetate and indeed some species (e.g. *Acacia georginae* and many species of *Gastrolobium* and *Dichapetalum*) contain high concentrations of fluoroacetate [36, 37]). The negligible activity of the *Pinus* SCFA-CoA synthetase towards 2-fluoroacetate implies that 2-fluoroacetate is unlikely to have any substantial effect on physiological processes involving SCFA-CoA synthetase in *Pinus* female gametophytic tissue.

Treble *et al.* [38] have reported that the aconitases of plants are *ca* 2000-fold less sensitive to fluorocitrate than pig heart aconitase. Thus, if plants, including those which produce fluoroacetate, incorporate this product into fluorocitrate, this is unlikely to result in a lethal synthesis. Nonetheless, it appears that other mechanisms are also involved in minimizing fluoroacetate toxicity in plants. Recently, a fluoroacetyl-CoA hydrolase which hydrolyses fluoroacetyl-CoA, but not acetyl-CoA, has been identified in tissue cultures of the fluoroacetate producer, *Dichapetalum cymosum* [39]. However, if the acetyl-CoA and SCFA-CoA synthetases of other plant species exhibit the same lack of activity towards fluoroacetate as the SCFA-CoA synthetase of *Pinus*, then this would imply that the principal reason for the low toxicity of fluoroacetate in plants is that it is not activated to its CoA derivative. The acetyl-CoA synthetase from potato tuber is also inactive towards fluoroacetate [12]. Whilst the incorporation of ^{14}C-acetate into acetyl-CoA by the acetyl-CoA synthetase from etiolated radish seedlings is inhibited weakly by fluoroacetate (8 and 11% inhibition at 2 and 5 mM fluoroacetate, respectively), activation of fluoroacetate itself was not reported [10].

The activity of SCFA-CoA synthetase towards the halogenated substrates does not appear to be related to the dimensions of the halogen since substitution of a methyl hydrogen atom in acetate with chlorine or bromine results in the formation of an active substrate, but replacement with a smaller fluorine atom does not. Rather, the activity appears to be inversely related to the strength of the acid (Table 1) arising from the strong electronegativity of the halogen substitution. The greater the number of substitutions and the closer these substitutions are to the carboxyl group, the stronger the acid. For example, the activity of the enzyme towards the halogenated acetates as determined by $V_{max.}$ values decreases with the pK_a of the acid. Thus, the enzyme was inactive with 2-fluoroacetate, the strongest of the single substituted halogenated acetic acids. Similarly, all disubstituted halogenated acetic and propionic acids which were inactive as substrates had pK_a values less than 2.8.

Some of the compounds which act as substrates of SCFA-CoA synthetase are well known alkylating reagents and have been used as affinity labels to study the amino acids involved at active sites of various enzymes and to evaluate the kinetic parameters. For example, pig heart mitochondrial aspartate aminotransferase is inactivated by 3-bromopropionate at pH 8 due to carboxyethylation of a lysyl residue at the active site which normally binds to one of the carboxyl groups of the various dicarboxylate substrates [40]. In other studies, ribonuclease M from *Aspergillus saitoi* has been shown to be inactivated at acid pH (pH 4 and 6) by 2-bromoacetate involving carboxymethylation at the N-3 position of the histidyl residue at the active site, and at basic pH (pH 8) by 3-bromopropionate via alkylation of a lysyl residue [41]. Thus, it would appear that lysyl and/or histidyl residues are not involved at the acyl-binding site of the SCFA-CoA synthetase of *Pinus*. α-Bromo acids, such as 2-bromopropionate, had markedly lower reactivites towards *Aspergillus* ribonuclease M than those acids with a terminal bromine, probably due to stearic hindrance by large alkyl side chains or the increase in acid strength brought about by the proximity of the halogen to the carboxyl group [41]. These studies have led to the conclusion that an effective halogenated alkylating agent must have an appropriate chain length and a halogen substituent properly distanced from the carboxyl group. However, it appears that these characteristics aptly describe the criteria for halogenated substrates of SCFA-CoA synthetase of *Pinus*, since 2-bromoacetate, 3-bromopropionate and 2-bromobutyrate act as substrates at pH 8. This, and the PPi–ATP exchange kinetics of incubation mixtures containing one of 2-bromoacetate, 3-bromopropionate and 2-bromobutyrate, together with acetate or propionate, imply that the brominated derivatives bind freely to the acyl-binding site and do not irreversibly inhibit the enzyme.

Proposed model for the acyl binding site

Patel and Walt [42] have reported that yeast acetyl-CoA synthetase supports activity principally with acetate (K_m = 0.51 mM), but also with propionate (K_m = 8.3 mM), acrylate and, at much lower activity, propiolate (2-propynate) and 2-methylacrylate. They proposed a model involving three major binding sites R1, R2 and R3. R1 and R2 accommodate small atoms such as hydrogen and fluorine (but not chlorine). R3 shows less specificity and can accommodate hydrophobic groups such as $-CH_3$, $-CH_2Cl$ and $-CH_2Br$, but not $-CH_2OH$ (as in glycollate) or $-CH_2NH_2$ (as in glycine). The high activity with acrylate was attributed to binding of the terminal $=CH_2$ group between the R1 and R2 sites. However, molecules with larger groups (e.g. $=CHCl$, $-CH_2COOH$ or $=CHCH_3$) cannot be accommodated in this way.

Since the SCFA-CoA synthetase from *Pinus* and the acetyl-CoA synthetase from yeast differ greatly in their relative affinities for propionate and acetate, and the

halogenated carboxylic acids they accept as substrates, the proposed model for the binding sites of the yeast enzyme [42] requires some modification to be applicable to the *Pinus* enzyme. The proposed S1 and S2 sites of the *Pinus* enzyme cannot have the spatial restrictions proposed for the yeast enzyme, since 2-chloroacetate and 2-bromoacetate serve as substrates, but the smaller fluorinated analogue, 2-fluoroacetate, does not. However, with further substitutions, spatial restrictions become evident since 2,2-dichloro- and 2,2-dibromoacetate do not support activity. The size of the halogen appears to be important, since no activity was observed with the smallest halogen (fluorine), and activity was found to increase as the halogen size increased from chlorinated to brominated isologues. Indeed, 2-bromoacetate exhibited the lowest $K_{m(app.)}$ and highest rate of PPi–ATP exchange. However, 2-iodoacetate was inhibitory due to its activity as a –SH group reagent [7]. Derivatives of acetate with substitutions similar in size to fluorine (*ca* 19 FW), such as glycine ($-NH_2$ group, *ca* 16 FW) and glycollate ($-OH$ group, *ca* 17 FW), like 2-fluoroacetate, were inactive, though the polarity of the substituent in these molecules could also be important in determining their inactivity as substrates.

Since derivatives of acetate containing a single bromine or chlorine on the C-2 atom support PPi–ATP exchange (albeit at greatly diminished rates with respect to acetate), but derivatives of propionate do not, this implies some form of interaction between the S1 and S2 sites. Presumably, single bulky bromine and chlorine atoms can only bind at the large S3 site if S1 and S2 are occupied only by small hydrogen atoms (as is the case for the substituted acetates), but bromine and chlorine substitutions at C-2 (as in the C-2 substituted propionates) cannot bind at S1 or S2 due to the binding of the large methyl group to S3. Conversely, addition of a single bromine or chlorine at the terminal C atom (C-3) of propionate permits binding at S3 and therefore does not interfere with binding at S1 or S2 although the catalytic rate is diminished.

The S3 site could exhibit some of the general features proposed for the yeast enzyme, but with some important differences. Most importantly, although the enzyme supports rapid rates of exchange with many substrates, the enzyme, as judged by the $K_{m(app.)}$ and $V_{max.}$ values, most readily accepts propionate and butyrate. We therefore suspect that binding of the preferred substrates propionate and butyrate involves all sites (S1, S2 and S3) which recognize parts of the C_2 and C_3 aliphatic chain attached to the carboxyl group and collectively determine the affinity of the enzyme and the maximum reaction rate. The S3 site appears to bind preferentially the larger acyl chains of propionate and butyrate over the smaller hydrogen atom presented at this site by acetate, as indicated by the much lower $K_{m(app.)}$ values of the C_3 and C_4 substrates. Thus, acetate contains no features that interfere with enzyme catalysis (hence high $V_{max.}$ at high concentrations), but does not possess the larger acyl group at S3, hence the high $K_{m(app.)}$ value. The S3 site is relatively non-specific and accepts a variety of hydrophobic moieties as instanced by the acyl chains of *n*-valerate (C_5, unbranched), 2-methylpropionate (C_4, branched) and thioglycollate (replacement of $-CH_3$ group of propionate with –SH), but not compounds with hydrophilic substituents.

C_3 compounds with a terminal halogen, like the analogous C_2 compounds, behave as substrates, with both chlorine and bromine substitutions supporting similar rates. However, the substituted butyrates do not fit this pattern. Presumably, enlarging the acyl side chain by substituting hydrogen at C-4 with bromine or chlorine (4-bromo- and 4-chloro-butyrate) prohibits attachment to the binding site at S3. However, positioning the halogen in close proximity to the carboxyl group, so that it binds at S1 or S2, and does not interfere with the acyl binding site at S3, results in structures that support enzyme activity (although at rates 10-fold lower than that of unhalogenated butyrate).

Relative to the saturated parent compound, the introduction of a double bond into C_3 and C_4 acids (making them more or less planar so that they do not bind in the same orientation as the saturated carboxylic acids) decreases the affinity since the planar orientation makes it more difficult for the $=CH_2$ and $=CHCH_3$ to attach to the S3 site, but once bound, actually promotes $V_{max.}$, suggesting that the planar configuration diminishes binding to the S1 and S2 sites without affecting the catalytic rate. The C_3 compound, acrylate, had an affinity similar to those of the C_2 series and supported activity at a rate even greater than with acetate. The high activity with the higher homologue, crotonate, is consistent with the $K_{m(app.)}$ values displayed by the C_4 series of compounds. However, relative to butyrate, the presence of the double bond between C-2 and C-3 atoms decreases the binding affinity ($K_{m(app.)}$ value six-fold greater than that for butyrate), but enhances maximum enzyme activity by *ca* 40%, to a value even greater than that of propionate.

The C_3 dicarboxylic acid, malonate, displayed a similar $K_{m(app.)}$ value to the C_3 series of carboxylic acids, and supported rates of exchange similar to 3-chloro- and 3-bromopropionate, suggesting that the additional carboxyl group does not interfere with binding of the side chain to S3 any more than a halogen atom does. However, the addition of another carbon into the chain to form the dicarboxylic acid, succinate, creates a compound with no substrate activity. Fumarate, a C_4 dicarboxylic acid with a double bond between C-2 and C-3, was inactive as both a substrate and an inhibitor, suggesting that it does not bind at all to the active site. Maleate (the *cis*-isomer of fumarate) was also inactive as a substrate, though it was a very weak inhibitor of the enzyme (results not shown).

EXPERIMENTAL

Plant material. Seeds of *P. radiata* were obtained from Australian Seed Company, Hazelbrook, N.S.W.

Crude extracts. These were prepd essentially by method B as per ref. [7].

Preparation of partially purified SCFA-CoA synthetase. Whole seeds were imbibed overnight in the dark at 2°, and were used to prepare Me_2CO powders which were stored in a desiccator at −10°. The powder (3–5 g) was extracted with 70 ml 100 mM Tris–HCl (pH 8), containing 0.1 mM EDTA and 5 mM DTT (medium A) and, after centrifugation, the supernatant was fractionated with solid $(NH_4)_2SO_4$ [7]. The ppt. containing the enzyme [30–40% $(NH_4)_2SO_2$] was redissolved in 10 ml medium A and dialysed overnight (with 2 changes) against medium A. This is referred to as partially purified SCFA-CoA synthetase and was used without further purification.

Assay of SCFA-CoA synthetase activity in crude and partially purified extracts. Enzyme activity was measured by carboxylic acid-dependent [^{32}P]PPi–ATP exchange. Incubations were conducted at 30° for 20 min in a final vol. of 1 ml containing 2 mM $Na_4{}^{32}P_2O_7$ (1 mCi mmol^{-1}), 2 mM K_2Na_2ATP, 1.5 mM $MgCl_2$, 60 mM Tris–HCl buffer (pH 8), carboxylic acid (adjusted to pH 8 with KOH) and either crude extract or partially purified enzyme. Standard assays were conducted with 1 mM propionate or 10 mM acetate as substrate. All solns were used within 2 days of their prepn. For other assays, the concn of the carboxylic acid(s) used is specified for each experiment. [^{32}P]ATP was sepd from [^{32}P]PPi as per ref. [7] and ^{32}PPi–ATP exchange is expressed in nmol min^{-1} mg^{-1} protein. Unless otherwise stated, carboxylic acid-dependent activity is expressed relative to a standard assay with 1 mM propionate as substrate. The average absolute rate for propionate-dependent ^{32}PPi–ATP exchange under standard conditions was *ca* 10 nmol min^{-1} mg^{-1} protein.

Analysis of carboxylic acid substrates. The purity of the carboxylic acid substrates was examined by GC. C_1 to C_4 unhalogenated carboxylic acids were adjusted to 500 mM with milliQ water; all remaining carboxylic acids were adjusted to 100 mM with the exception of valerate and 2-chloroacetate, which were adjusted to 50 mM, and analysed after 0, 1 and 4 days storage at 4°. Samples (1 μl) were analysed in a BPX70 column by FID on an HP 5710A GC linked to a Varian Star workstation using 200° oven temp., 250° injector temp. and 300° detector temp., with He as carrier gas at 1.2 ml min^{-1}.

Determination of protein. Protein in both crude and partially purified extracts was determined with a bicinchoninic acid protein assay kit (Sigma Procedure No. TPRO-562) using BSA as standard except that mixts were incubated at 37° for 30 min and cooled to 4° before determining the *A* at 562 nm.

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