The *prpE* gene of *Salmonella typhimurium* LT2 encodes propionyl-CoA synthetase

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Biochemical and genetic evidence is presented to demonstrate that the *prpE* gene of *Salmonella typhimurium* encodes propionyl-CoA synthetase, an enzyme required for the catabolism of propionate in this bacterium. While *prpE* mutants used propionate as carbon and energy source, *prpE* mutants that lacked acetyl-CoA synthetase (encoded by *acs*) did not, indicating that *Acs* can compensate for the lack of *PrpE* in *prpE* mutants. Cell-free extracts enriched for *PrpE* catalysed the formation of propionyl-CoA in a propionate-, ATP-, Mg²⁺- and HS-CoA dependent manner. Acetate substituted for propionate in the reaction at 48% the rate of propionate; butyrate was not a substrate for *PrpE*. The propionyl-CoA synthetase activity of *PrpE* was specific for ATP. GTP, ITP, CTP and TTP were not used as substrates by the enzyme. UV-visible spectrophotometry, HPLC and MS data demonstrated that propionyl-CoA was the product of the reaction catalysed by *PrpE*.

Keywords: propionyl-CoA synthetase, short-chain fatty acid catabolism, 2-methylcitric acid cycle enzymes, propionate catabolic genes

INTRODUCTION

Growth of *Salmonella typhimurium* on propionate as a sole carbon and energy source requires enzymes encoded by the *prp* locus (Hammelmann *et al.*, 1996; Horswill & Escalante-Semerena, 1997). Five genes, *prpRBCDE*, make up this locus and four of these genes show DNA and amino acid sequence similarity to proteins with known biochemical activities (Fig. 1). *PrpR* is a member of the family of RpoN (σ^{54}) activators; *PrpB* is homologous to isocitrate lyases; *PrpC* is homologous to citrate synthases; and *PrpE* is homologous to acetyl-CoA synthetases. A similar region in *Escherichia coli* has recently been implicated in the breakdown of propionate by the methylcitric acid cycle and the *PrpC* homologue has been identified as methylcitrate synthase (Gerike *et al.*, 1998; Textor *et al.*, 1997). We have obtained evidence that *S. typhimurium* also catabolizes propionate via this pathway (data to be presented elsewhere).

The activation of propionate to propionyl-CoA is the proposed first step of the methylcitric acid cycle and of all other propionate breakdown pathways in bacteria (Horswill & Escalante-Semerena, 1997; Textor *et al.*, 1997). However, no evidence has been reported identi-

fying a gene encoding propionyl-CoA synthetase (EC 6.2.1.17) activity. This activity has been attributed to the two routes of acetyl-CoA synthesis (Rhie & Dennis, 1995a, b; Van Dyk & LaRossa, 1987), i.e. the acetate kinase (*AckA*) and phosphotransacetylase (*Pta*) pathway, and the acetyl-CoA synthetase (*Acs*) pathway. In a previous paper, we proposed that *PrpE* catalysed the synthesis of propionyl-CoA during propionate breakdown (Horswill & Escalante-Semerena, 1997). However, no evidence to support this hypothesis was presented. The location of *prpE* downstream of the *prpBCD* operon strongly suggested that the putative propionyl-CoA synthetase activity was needed. Failure to isolate *prpE* mutants was attributed to the existence of an enzyme that can substitute for *PrpE* during growth on this carbon source (Horswill & Escalante-Semerena, 1997).

In this paper, we address the function of *PrpE* by demonstrating that: (i) *prpE* is part of the *prpBCD* operon; (ii) acetyl-CoA synthetase compensates for lack of *PrpE*; and (iii) *PrpE* has propionyl-CoA synthetase activity.

METHODS

Culture media and growth conditions. Growth of *S. typhimurium* in rich and minimal media and the concentration of antibiotics were as described previously (Escalante-Semerena & Roth, 1987). The final concentrations of

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

Fig. 1. The *prpBCDE* locus and methylcitric acid cycle of *S. typhimurium*. (a) Graphical representation of the *prp* locus showing homologues and predicted molecular mass of each protein (Horswill & Escalante-Semerena, 1997). (b) Propionate breakdown via the methylcitric acid cycle with the steps catalysed by proteins of the *prp* locus. Evidence for the assignment of PrpB, PrpC and PrpD catabolic steps will be presented elsewhere.

compounds provided in the culture medium were as follows: methionine, 0.5 mM; propionate, 30 mM; acetate, 10 mM. All chemicals were purchased from Sigma unless otherwise stated. Overnight cultures of strains grown in nutrient broth (NB) were subcultured 1:50 (v/v) into 5 ml minimal medium with appropriate supplements. Cultures were incubated in 18 × 150 mm tubes at 37 °C with shaking, and cell growth was monitored at 650 nm with a Spectronic 20D spectrophotometer furnished with a red filter (Milton Roy). A list of strains and plasmids used and their genotypes is provided in Table 1.

Overexpression of *prpE*. Strain JE4184 (pGP1-2 *rpo*⁺ *kan*⁺; pPRP38 *prpE*⁺ *bla*⁺) was used to overexpress *prpE* using the temperature-sensitive T7 system of Tabor (1990). Cell pellets were stored at 4 °C for no longer than 16 h before use.

Genetic crosses. Transductions involving phage P22 *HT105 int201* were performed as described by Chan *et al.* (1972), Schmieger (1971) and Schmieger & Bakhaus (1973). Transductions from *E. coli* into *S. typhimurium* involving phage P1 were performed as reported by O'Brien *et al.* (1992).

Recombinant DNA techniques. Restriction and modification enzymes were purchased from Promega unless otherwise stated and were used according to the manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α/F'. Plasmids were transformed into *E. coli* or *S. typhimurium* by CaCl₂ heat-shock as described by Ausubel *et al.* (1989). Plasmids transferred to *S. typhimurium* were first transformed into recombination-deficient *S. typhimurium* strain JR501 (Tsai *et al.*, 1989). Plasmids from strain JR501

were quick-transformed into other *S. typhimurium* strains as described by Ryu & Hartin (1990).

Plasmid constructions. Plasmid pPRP37: a 3 kb *Hind*III–*Sph*I fragment of pPRP29 containing the *prpE*⁺ gene was ligated into pSU20 *cat*⁺ (Cm^r) (Bartolomé *et al.*, 1991) digested with the same enzymes. Plasmid pPRP38: a 3 kb *Bam*HI–*Hind*III fragment of plasmid pPRP37 *cat*⁺ (Cm^r) was cloned into plasmid pT7-6 *bla*⁺ (Ap^r) (Tabor, 1990) cut with the same enzymes. Plasmid pPRP45: a 2 kb *Nde*I–*Cla*I fragment containing the *prpE*⁺ gene was ligated into pT7-7 *bla*⁺ (Ap^r) (Tabor, 1990) digested with the same enzymes. The *Nde*I site was constructed by PCR mutagenesis at the ATG start codon of *prpE* to allow use of the T7 ribosome-binding site. Plasmid pPRP49: a 350 bp *Hind*III–*Sal*I fragment of plasmid pPRP37 *cat*⁺ (Cm^r) was cloned into plasmid pMAK705 *cat*⁺ (Cm^r) (Hamilton *et al.*, 1989) digested with the same enzymes. Plasmid pPRP50: a 1.3 kb *Pst*I fragment of plasmid pUC4K (Pharmacia) containing the kanamycin (Km) resistance gene was blunt-ended with Klenow fragment of DNA polymerase I and ligated into *Sfi*I-digested plasmid pPRP49. Plasmid pPRP51: an approximately 2.5 kb *Sma*I fragment from plasmid pKT254Ω-*bla*⁺ (Fellay *et al.*, 1987) containing the ampicillin (Ap) resistance gene was ligated into *Sfi*I-digested plasmid pPRP49. Plasmid pPRP54: plasmid pPRP37 *prpE*⁺ *cat*⁺ (Cm^r) was digested with *Hind*III, blunt-ended with Klenow fragment and then digested with *Sph*I. The approximately 3 kb fragment containing the *prpE* gene was cloned into plasmid pBAD30 (*bla*⁺) (Guzman *et al.*, 1995) digested with *Sma*I and *Sph*I.

Table 1. Strains and plasmids used

Strain or plasmid	Genotype	Reference or source*
<i>E. coli</i>		
AJW805	$\Delta lacX74$ <i>thi-1 thr-1</i> (amber) <i>leuB6 metF159</i> (amber) <i>rpsL136</i> $\lambda lacY$ <i>acs::kan-1</i>	A. Wolfe ¹
DH5 α /F'	F' <i>endA1 hsdR17</i> (r_K^+ m_K^+) <i>supE44 thi-1 recA1 gyrA</i> (NaI ^r) <i>relA1</i> $\Delta(lacZYA-argF)$ U169 <i>deoR</i> [$\phi 80d lac\Delta(lacZ)M15$]	New England Biolabs
<i>S. typhimurium</i>†		
JR501	<i>hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719 H1-b H2-en,n,x</i> (Fels2 ⁻) <i>fla-66 nml</i>	Tsai <i>et al.</i> (1989)
MS1933	<i>mutS::Tn10 galE496 metA22 metE55 xyl-404</i> (Fels2 ⁻) <i>H1-b nml H2 enx ilv hsdL6 hsdSA29 rpsL120</i>	S. Maloy via D. Downs ²
TR6583	<i>metE205 ara-9</i>	K. Sanderson via J. Roth ³
Derivatives of TR6583		
JE4182	TR6583/pGP1-2 (T7 <i>rpo</i> ⁺ <i>kan</i> ⁺)	This work
JE4184	TR6583/pGP1-2 (T7 <i>rpo</i> ⁺ <i>kan</i> ⁺) pPRP38 (<i>prpE</i> ⁺ <i>bla</i> ⁺)	This work
JE4271	TR6583/pBAD30 <i>bla</i> ⁺	This work
JE4287	TR6583/pGP1-2 (T7 <i>rpo</i> ⁺ <i>kan</i> ⁺) pT7-6 <i>bla</i> ⁺	This work
JE4288	<i>acs::kan-1</i>	This work
JE4289	<i>prpE212::bla</i> ⁺ <i>acs::kan-1</i>	This work
JE4305	<i>acs::kan-1 nrfA1::Tn10d</i> (Tc)	This work
JE4312	$\Delta 1231$ (<i>acs</i>)	This work
JE4354	JE3056/pPRP12-5.4 [<i>prpBCD</i> ⁺ <i>kan</i> ⁺]	This work
JE4358	JE4336/pPRP12-5.4 [<i>prpBCD</i> ⁺ <i>kan</i> ⁺]	This work
Plasmids		
pPRP29	<i>prpBCDE</i> ⁺ cloned into pBR328 [<i>bla</i> ⁺ <i>cat</i> ⁺ (Ap ^r , Cm ^r)]	Laboratory collection
pPRP12-5.4	<i>prpBCD</i> ⁺ in pSU39; <i>kan</i> ⁺	Laboratory collection

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† All *S. typhimurium* strains are derivatives of LT2.

Construction of *prpE* insertions and deletions. Plasmids pPRP50 and pPRP51 were transformed into *S. typhimurium* TR6583 (*prpE*⁺) and cointegrates were isolated and resolved as described by Hamilton *et al.* (1989). To isolate strains carrying *prpE* insertions from plasmid-containing strains, P22 phage grown on a pool of 10 independently resolved cointegrates was used as donor to transduce strain TR6583 to Ap or Km resistance. P22 crosses were plated on NB with Km for pPRP50 and NB with Ap for pPRP51, and incubated at 44 °C. Km^r Cm^s (for pPRP50) and Ap^r Cm^s (for pPRP51) transductants were saved for further analysis. A strain carrying insertion *nrfA1::Tn10* Δ 16 Δ 17 (hereafter referred to as Tn10d(Tc); Way *et al.*, 1984) was used to isolate an *acs* deletion by the method of Bochner *et al.* (1980) as modified by Maloy & Nunn (1981). A Km^r Tc^s Ace⁻ (JE4312) strain was isolated and saved for further experiments.

Sequencing of *prpE* insertions. The location of the Ap- and Km-resistance cassettes within *prpE* was verified by PCR amplification and sequencing. PCR reactions were prepared using one-tenth volume of boiled template, 50 pmol of each primer,

0.2 mM of each dNTP (Promega) and *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. Reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer) using the following conditions: 30 cycles at 94 °C for 90 s, 50 °C for 30 s, 72 °C for 2 min. The 5' and 3' ends of the *prpE212::bla*⁺ insertion were PCR-amplified independently using the following sets of primers: 5'-CGTGGAGTTTACTGATGGAT-3' (in *prpD*) and 5'-GCATCTTTACTTTTACCAGCG-3' (in the *bla* gene) to generate a 1.5 kb fragment; 5'-ATGGATGAACGAAATAGACAGA-3' (in the *bla* gene) and 5'-GCTCTTCATCGGTCTCTGA-3' (in *prpE*) to generate a 1.4 kb fragment. For *prpE213::kan*⁺ the entire Km^r cassette and flanking DNA was amplified as a 1.8 kb fragment using the *prpD* and *prpE* primers shown above. Amplified DNA was purified using the QIAquick PCR Purification kit (Qiagen). PCR sequencing reactions were prepared using the ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. Reactions were purified in AutoSeq G-50 columns (Pharmacia Biotech), dried in a SpeedVac concen-

trator (Savant Instruments) and sequenced at the Biotechnology Center (University of Wisconsin-Madison, USA).

Sequencing of the *acs::kan-1* insertion. Strain JE4305 [*acs::kan-1 nrfA1::Tn10d(Tc)*] was used to locate the Km resistance cassette in acetyl-CoA synthetase. Primers 5'-TCCATTGCTGTTGACAAAGG-3' for Tn10-L and 5'-ACCCATATAAATCAGCATCC-3' for the Km resistance cassette were used to PCR-amplify the chromosomal DNA between the two insertions. The PCR amplification procedure used to locate *prpE* insertions (described above) was also used to amplify a 2 kb DNA fragment between the *acs* and *nrfA* insertions. Sequence was obtained (as described above) for both ends of the amplified DNA fragment.

Biochemical and spectroscopic techniques

Preparation of dialysed, cell-free extracts. Cell pellets of strain JE4184 grown as described above were resuspended in 40 ml cold, 50 mM potassium phosphate buffer (pH 7.5) and the suspensions were centrifuged at 10500 g for 10 min at 4 °C. The pellet was resuspended in 40 ml phosphate buffer, centrifuged as before and resuspended again in 10 ml phosphate buffer. Cells were kept on ice and broken by sonication (10 min, 50% duty, setting 3) on a model 550 Sonic Dismembrator (Fisher Scientific). Cell debris was removed by centrifugation in 50 ml Nalgene polypropylene copolymer Oakridge tubes (Fisher Scientific) at 31000 g for 1 h at 4 °C. The supernatant was dialysed at 4 °C in Spectra/Por 1 Molecularporous dialysis membrane (Spectrum Medical Industries) against 1 l phosphate buffer (50 mM, pH 7.5, 4 °C). The dialysis buffer was replaced after 2 and 4 h and then allowed to dialyse an additional 16 h. The extracts (6 mg protein ml⁻¹) were maintained at 4 °C for up to 1 week with no detectable loss of activity. Strain JE4287 (pGP1-2 *rpo*⁺ *kan*⁺; pT7-6 *bla*⁺) was used to establish the background acyl-CoA synthetase activity in our *in vitro* assays. Increases in this activity seen in extracts of strain JE4184 were relative to the ones measured for extracts of strain JE4287.

***In vitro* acyl-CoA synthetase assay.** The assay described by Brown *et al.* (1977) was used to monitor the PrpE-dependent synthesis of acyl-CoA compounds. The following were combined in a 1.5 ml reaction volume: HS-CoA, 0.75 µmol; ATP, 1.5 µmol; MgCl₂, 7.5 µmol; hydroxylamine, 450 µmol; crude cell-free extract, 30 µg; phosphate buffer, 75 µmol, pH 7.5. The mixture was preincubated at 37 °C for 10 min and the reaction was started with propionate (7.5 µmol). The reaction mixture was incubated at 37 °C for 30 min and was stopped with 1.5 ml 2.0% FeCl₃/4.0% trichloroacetic acid/2 M HCl reagent. A reaction mixture without HS-CoA was prepared as control for each condition tested. Colour was allowed to develop for at least 10 min but no longer than 45 min. Absorbance at 520 nm was measured using the no HS-CoA controls as blanks. Detection of product was linear for 30 min under these assay conditions. To assess the substrate specificity of PrpE, acetate and butyrate were substituted for propionate at an equal concentration. Similarly, GTP, ITP, CTP and TTP were substituted for ATP at an equimolar concentration. Standard curves were prepared for the assay using propionyl-CoA, acetyl-CoA and butyryl-CoA. A unit of activity (U) was defined as the amount of enzyme required for the synthesis of 1 µmol acyl-CoA min⁻¹.

Chromatography and spectroscopy. For HPLC analysis of the PrpE reaction, we used a modification of the procedure described by Hosokawa *et al.* (1986) to separate propionyl-CoA from reaction mixture substrates. The reaction mixture was resolved using reverse-phase HPLC with a Prodigy 5 ODS-2 column (250 × 4.60 mm; Phenomenex). The column

was developed immediately after injection of the sample with an 80 min convex gradient (Waters curve 3) of acetonitrile/water (10:90) containing 0.2 M ammonium acetate. The flow rate was 1 ml min⁻¹ and column temperature was maintained at 35 °C. Elution was monitored at 260 nm on a Waters model 990 Plus photodiode array detector (Millipore).

For MS of the PrpE product, HPLC fractions containing the PrpE product were combined and solvent was removed in a SpeedVac concentrator. Negative ion electrospray spectra were obtained by resuspending samples in 50% acetonitrile and analysing them with a Perkin Elmer Sciex API 365 triple quadrupole spectrometer equipped with an ion spray source. Authentic propionyl-CoA (Sigma) was subjected to the same manipulations and used as positive control.

Other procedures. Protein concentrations were determined by the method described by Kunitz (1952). Proteins were separated by SDS-PAGE (Laemmli, 1970) using 12% polyacrylamide gels and were visualized with Coomassie blue (Sasse, 1991). Low-range standards (14–97.4 kDa) were used for SDS-PAGE (Bio-Rad). UV-visible spectroscopy was performed in a computer-controlled Lambda 6 UV-vis spectrophotometer (Perkin-Elmer).

RESULTS AND DISCUSSION

Construction and analysis of *prpE* mutants

Chromosomal insertions *prpE212::bla*⁺ and *prpE213::kan* were constructed by gene replacement and the location of both antibiotic markers within *prpE* was verified by sequencing. On propionate medium, *prpE* mutants and the *prpE*⁺ strain grew with very similar doubling times, i.e. 6.6 h for the mutant and 6.1 h for the wild-type. This result suggested that an alternative function compensated for the lack of PrpE. Since bacterial acetyl-CoA synthetases often activate propionate (Maruyama, 1982; Preston *et al.*, 1990; Priefert & Steinbüchel, 1992), we inactivated the *acs* gene which encodes acetyl-CoA synthetase. An *E. coli acs::kan-1* insertion was moved into *S. typhimurium* (Kumari *et al.*, 1995) and the location of the Km resistance cassette was verified by PCR amplification of chromosomal DNA between *acs::kan-1* and *nrfA1::Tn10d(Tc)* in strain JE4305, followed by sequencing of the amplified DNA. The *acs* mutant JE4288 grew in propionate medium with a doubling time of 6.2 h. However, the *acs prpE* double mutant strain JE4289 failed to grow on propionate. Plasmid pPRP54 (P_{BAD-prpE}⁺) allowed strain JE4313 (*metE205 ara-9 Δ1231acs prpE213::kan*⁺) to grow in propionate medium containing arabinose with a doubling time of 8.3 h. This rate of growth was identical to the one measured for the *acs*⁺ *prpE*⁺ strain JE4271. Plasmid pPRP54 also complemented growth of *acs* mutants on low acetate (10 mM) when arabinose was included in the medium, indicating that PrpE can synthesize acetyl-CoA.

On the basis of these data we conclude that acetyl-CoA synthetase can compensate for the lack of propionyl-CoA synthetase activity in *prpE* mutants. PrpE and Acs appear to be the only propionyl-CoA synthetase activities in the cell capable of supporting growth on propionate.

Fig. 2. Overexpression of *prpE*. Lanes: 1 and 2, *prpE* overexpression strain (JE4184); 3 and 4, control strain with no *prpE* insert (JE4287); 1 and 3, dialysed, soluble extract from each strain; 2 and 4, insoluble protein from each strain. Each lane contained approximately 5 µg protein. Numbers on the right indicate the molecular mass of the following proteins (top to bottom): β -phosphorylase, serum albumin, ovalbumin and carbonic anhydrase.

In *S. typhimurium*, the acetate kinase (*ackA*) and phosphotransacetylase (*pta*) pathway of acetyl-CoA synthesis does not appear to be a major contributor (if at all) to the synthesis of propionyl-CoA, since *ackA* mutants of this bacterium are not affected in their ability to grow on propionate (data not shown). These results are consistent with reports that propionate is not a substrate for *AckA* (Fox & Roseman, 1986).

prpE is part of the *prp* operon

To determine if *prpE* was cotranscribed with *prpBCD*, the polarity of a *prpB* insertion on *prpE* was assessed. For this purpose, plasmid pPRP12-5.4 (Horswill & Escalante-Semerena, 1997) was introduced into a tester strain lacking *Acs*. When the growth of strains JE4354 (*prpB121::Tn10d(Tc)*/pPRP12-5.4) and JE4358 (*prpB121::Tn10d(Tc)* Δ 1231 (*acs*)/pPRP12-5.4) was compared, strain JE4358 failed to utilize propionate as carbon and energy source. The polar effect of the *prpB121::Tn10d(Tc)* element on *prpE* lends strong support to the idea that *prpE* is part of the *prp* operon.

Overexpression of *prpE*

Plasmid pPRP38 was used to overexpress *prpE*. Plasmids pPRP38 or pT7-6 (vector-only control) were each transformed into strain JE4182, which carried plasmid pGP1-2 (Tabor, 1990), resulting in strains JE4184 (pPRP38, pGP1-2) and JE4287 (pT7-6, pGP1-2), respectively. These strains were grown under conditions that overexpressed *prpE* and dialysed cell-free extracts of each strain were prepared. Cell-free extracts were fractionated by centrifugation and samples (approx. 5 µg of soluble and insoluble protein) were analysed by SDS-PAGE (Fig. 2). *PrpE* was identified as the 70 kDa band (lane 1) that was absent in the control strain (lanes

Table 2. *PrpE* activity and substrate specificity in dialysed, crude cell-free extracts

Assay conditions	Specific activity [mU (mg protein) ⁻¹]*	Relative activity (%)†
Complete	630	100
– ATP	25	4
– Mg ²⁺	92	15
– Propionate	30	5
– <i>PrpE</i>	23	4
Complete with acetate‡	300	48
Complete with butyrate§	17	3
pT7-6 with propionate	28	4
pT7-6 with acetate	52	8

* A unit of activity (U) is defined as the amount of enzyme that generates 2 µmol product min⁻¹.

† Relative activities were determined by dividing specific activities by that of the *PrpE* reaction with propionate [630 mU (mg protein)⁻¹].

‡ Acetate substituted for propionate.

§ Butyrate substituted for propionate.

|| An equal amount of dialysed cell-free extract of strain JE4287 (vector-only control) was substituted for cell-free extract of the *prpE*⁺-overexpressing strain JE4184.

3 and 4). Under these overexpression conditions, *PrpE* remained mostly soluble, however a small amount was lost as inclusion bodies (lane 2). Overexpression of *prpE* to higher levels using plasmid pPRP45 (*prpE*⁺ in pT7-7) with the T7 ribosome-binding site resulted only in a large increase of insoluble protein (data not shown).

Acyl-CoA synthetase activity

Table 2 presents data which demonstrate that *PrpE* has acyl-CoA synthetase activity. Enzyme activity depended upon the addition of propionate or acetate, HS-CoA, ATP, Mg²⁺ and *PrpE* present in crude cell-free extract. Propionate was the preferred substrate of *PrpE*. The activity of the enzyme with propionate as substrate was assigned the arbitrary value of 100%. *PrpE* synthesized acetyl-CoA at 48% the rate of propionate. Most of this activity was attributed to *PrpE* since the control extract displayed only 8% activity (Table 2); butyrate was not a substrate for *PrpE*. The enzyme displayed high specificity for ATP. GTP, ITP, CTP and TTP failed to substitute for ATP in the reaction. The specificity for ATP contrasts to that of the acetyl-CoA synthetase (ADP-forming) activity of *Pyrococcus furiosus*, which can use GTP and ITP very effectively (Glasemacher *et al.*, 1997). At present, we do not know if *PrpE* is an acyl-CoA (ADP-forming), or acyl-CoA (AMP-forming) synthetase. A careful analysis of the products of the *PrpE* reaction is necessary.

Reaction mixtures lacking Mg²⁺ showed 15% *PrpE* activity, which was attributed to Mg²⁺ present in the

Fig. 3. Negative ion electrospray mass spectra of authentic propionyl-CoA (a), and of the product of the PrpE reaction (b). The inset in (a) shows the structure of propionyl-CoA. In both spectra the negative molecular ion was identified at m/z 822.

crude extracts. Consistent for the need for Mg^{2+} , the propionyl-CoA synthetase activity of PrpE was sensitive to EDTA in the reaction mixture (data not shown).

HPLC purification of PrpE product

The PrpE product was purified by reverse-phase HPLC. The reaction product eluted as unresolved peaks from 39 to 44 min (data not shown), which were collected and analysed by MS. Authentic propionyl-CoA standard dissolved in 50 mM phosphate buffer pH 7.5 had the same elution profile, retention times and UV-visible spectrum when the acetonitrile/ammonium acetate solvent system was used (data not shown). The propionyl-CoA peak was not seen when cell-free extract of strain JE4287 (vector-only control) was substituted for cell-free extract of strain JE4184 (pT7-6 *prpE*⁺) in the reaction mixture (data not shown). If the solvent system was changed to phosphate buffer with methanol (Corkey *et al.*, 1981), both the product of the PrpE reaction and authentic propionyl-CoA eluted as a single peak (data not shown). This result suggested that multiple peaks observed in the acetonitrile/ammonium acetate solvent system were a mixture of different salts of the CoA moiety. The phosphate/methanol solvent system was not used due to difficulties in removing phosphate buffer from the PrpE product.

MS of PrpE product

Negative ion electrospray mass spectra were obtained for authentic propionyl-CoA and for the product of the PrpE-catalysed reaction (Fig. 3a and b). The molecular mass of propionyl-CoA is 823 Da at the neutral state. Fig. 3(a) shows the negative molecular ion ($M-1$) for authentic propionyl-CoA at m/z 822.2. Peaks at m/z 766.2, 860.2 and 898.0 were assigned to CoA-1, the potassium salt of the negative molecular ion ($M-1+K^+$) and the di-potassium salt of the negative molecular ion ($M-1+2K^+$), respectively. Two other prominent peaks (m/z 742.2 and m/z 686.2) were presumed to be fragments of propionyl-CoA, but were not identified. Fig. 3(b) shows that the spectrum obtained for the HPLC-purified product of the PrpE-catalysed reaction displayed the same peaks. This result confirmed that propionyl-CoA was the product of the PrpE reaction.

Implications of the amino acid sequence of PrpE

Moyed & Lipmann (1957) first reported propionyl-CoA synthetase activity in extracts of an unidentified soil bacterium. This paper reports the first identification of a gene encoding propionyl-CoA synthetase. In light of the homology of *prpE* to genes encoding acetyl-CoA synthetases, it is possible that genes annotated in

databases as encoding acetyl-CoA synthetases in other organisms may actually encode propionyl-CoA synthetases. For example, according to BLASTP sequence analysis (Altschul *et al.*, 1997), homologues in *E. coli* (GenBank accession no. U73857), *Lysobacter* (Y07914) and *Pseudomonas putida* (U24215) are 89, 58 and 54% identical to PrpE, respectively. However, Acs of *E. coli* (P27550) is only 37% identical to PrpE and 36–40% identical to the PrpE homologues from *E. coli*, *Lysobacter* and *P. putida*. These differences raise the possibility that these homologues are likely to be propionate-specific acyl-CoA synthetases.

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