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Characterization of a phenylacetate–CoA ligase from *Penicillium chrysogenum*

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Enzymatic activation of PAA (phenylacetic acid) to phenylacetyl-CoA is an important step in the biosynthesis of the β -lactam antibiotic penicillin G by the fungus *Penicillium chrysogenum*. CoA esters of PAA and POA (phenoxyacetic acid) act as acyl donors in the exchange of the amino adipyl side chain of isopenicillin N to produce penicillin G or penicillin V. The *phl* gene, encoding a PCL (phenylacetate–CoA ligase), was cloned in *Escherichia coli* as a maltose-binding protein fusion and the biochemical properties of the enzyme were characterized. The recombinant fusion protein converted PAA into phenylacetyl-CoA in an ATP- and magnesium-dependent reaction. PCL could also activate POA, but the catalytic efficiency of the enzyme was rather low with k_{cat}/K_m values of 0.23 ± 0.06 and $7.8 \pm 1.2 \text{ mM}^{-1} \cdot \text{s}^{-1}$ for PAA and POA respectively. Surprisingly, PCL

was very efficient in catalysing the conversion of *trans*-cinnamic acids to the corresponding CoA thioesters [$k_{cat}/K_m = (3.1 \pm 0.4) \times 10^2 \text{ mM}^{-1} \cdot \text{s}^{-1}$ for *trans*-cinnamic acid]. Of all the substrates screened, medium-chain fatty acids, which also occur as the side chains of the natural penicillins F, DF, H and K, were the best substrates for PCL. The high preference for fatty acids could be explained by a homology model of PCL that was constructed on the basis of sequence similarity with the Japanese firefly luciferase. The results suggest that PCL has evolved from a fatty-acid-activating ancestral enzyme that may have been involved in the β -oxidation of fatty acids.

Key words: CoA ligase, fatty acid, luciferase, penicillin G, *Penicillium chrysogenum*, phenylacetate–CoA ligase.

INTRODUCTION

The final step in the biosynthesis of β -lactam antibiotics by the fungus *Penicillium chrysogenum* is the exchange of the L- α -amino adipyl side chain for a more hydrophobic group, e.g. a phenylacetyl group in the case of penicillin G biosynthesis (Figure 1). The exchange is catalysed by the enzyme isopenicillin-N acyltransferase [1]. This enzyme requires an activated form of the side chain for acyltransferase activity. Several thioesters can be used by the acyltransferase as side-chain donors *in vitro* [1,2], but it is generally assumed that, *in vivo*, the side-chain donor is a CoA thioester. Although the biosynthetic pathway for penicillin has been studied intensively, the biochemical properties of the CoA ligase(s) responsible for PAA (phenylacetic acid) activation have remained unclear for a long time. An acetate-CoA ligase isolated from *P. chrysogenum* was shown to be able to activate phenylacetate *in vitro* [3]. However, it is uncertain whether this enzyme has a role in penicillin G biosynthesis *in vivo*. Purification of PCL (phenylacetate–CoA ligase) activity from *P. chrysogenum* cultures has also been described, but few data on the catalytic properties of the enzyme were obtained [4,5]. The properties of the PCL are important because the level of phenylacetyl-CoA is a possible bottleneck in the biosynthesis of penicillin G. The introduction of a PCL from *Pseudomonas putida* was described to have a positive effect on the level of penicillin formation [6] and the disruption of a P450 mono-oxygenase that catalyses PAA 2-hydroxylation, a reaction competing for the available PAA with PCL, resulted in a higher production of penicillin [7].

In 2001, the sequence of a gene (*phl*) that encodes a PCL was published in a patent [8], and it has been shown that a PCL

knockout of *P. chrysogenum* had reduced penicillin production, indicating that the enzyme encoded by this gene is responsible for the synthesis of a large part of the phenylacetyl-CoA used for penicillin G biosynthesis [9]. Overexpression of the *phl* gene resulted in a higher level of penicillin production, again indicating that the CoA ligase step may be limiting in penicillin biosynthesis [9]. The amino acid sequence indicates that PCL is a member of the superfamily of adenylate-forming enzymes [10]. CoA activation by these enzymes proceeds in a two-step reaction (Figure 1). In the first half-reaction, the substrate reacts with ATP to form an acyl-adenylate intermediate with the simultaneous formation of pyrophosphate. In the second half-reaction, the adenylate group is replaced by CoA and AMP is released. The formation of an adenylate intermediate is characteristic for all members of the superfamily [10]. Other family members include the adenylation domains of non-ribosomal peptide synthetases, fatty acid-CoA synthetases, luciferases and coumarate-CoA ligases [10]. Bacterial PCLs involved in the catabolism of PAA in bacteria such as *Escherichia coli* K12 and *Azoarcus evansii* [11,12] and the extensively characterized enzyme from *Pseudomonas putida* U [13] are also members of the superfamily of adenylate-forming enzymes, but the sequence identity between these enzymes and PCL from *Penicillium* is very low (approx. 15%). PCL contains a C-terminal peroxisomal SKI (Ser-Lys-Ile)-targeting signal sequence (PTS1) and probably localizes in the peroxisomal matrix [14]. Isopenicillin-N acyltransferase was also shown to be a peroxisomal enzyme [15], indicating that the last two steps of the penicillin biosynthesis pathway are localized in the peroxisome.

Although PCL is important for the biosynthesis of penicillin G, little information is available about its catalytic properties,

Abbreviations used: LB, Luria–Bertani; MBP, maltose-binding protein; Ni-NTA, Ni²⁺-nitrilotriacetate; PAA, phenylacetic acid; PCL, phenylacetate–CoA ligase; POA, phenoxyacetic acid; PTS, peroxisomal targeting signal sequence.

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Figure 1 The final step in the biosynthesis of penicillin G

The L- α -aminoadipyl side chain of isopenicillin N is exchanged for the phenylacetyl group. The enzyme isopenicillin-N acyltransferase, catalysing the exchange, requires the CoA-activated form of the phenylacetyl side chain. The two-step activation of PAA is catalysed by PCL. The adenylate intermediate is characteristic of all members of the superfamily of adenylate-forming enzymes.

which may be partly due to the instability of the enzyme [5]. We have used an MBP (maltose-binding protein) fusion to obtain a high level of expression in *E. coli* and to increase the stability of PCL. In the present paper, we describe the catalytic properties and identify the substrate-binding residues of this important enzyme of the penicillin G biosynthetic route.

EXPERIMENTAL

Vector construction and mutagenesis of PCL

The *phl* gene, encoding PCL from *P. chrysogenum* (GenBank® accession number AJ001540), was PCR-amplified from a cDNA library [16] using the forward primer 5'-CCGAGGATTTGGGCATATGGTTTTTACCTCC-3', introducing an NdeI site (underlined) in front of the start codon, and the reverse primer 5'-CATACAAAAGATGCATTGGCTCGTCATGAAGAGCCTA-CTAGATCTTGCTACCAGCCTTTCTCC-3', introducing an NsiI site (underlined) downstream of the stop codon. The nucleotides between the stop codon and NsiI site are based on the *P. chrysogenum penDE* termination region. The NdeI/NsiI fragment was isolated and cloned in the *P. chrysogenum* expression plasmid pISEWA-n [17], yielding the plasmid pPCLA. From this, the *phl* DNA was amplified using the forward primer 5'-TTTTCAC-CATTATTTTCTAGAATTCCATATGGTTTTTACCTCC-3' and the same reverse primer as above to insert an EcoRI site (underlined) upstream of the start codon. To create a translational fusion of the PCL with MBP, the resulting 1.7 kb EcoRI/NsiI fragment was used to replace the *cefE* gene behind the *malE* gene in the vector pALJ104 [18], which was partially digested with EcoRI and NsiI, yielding pAJLPA. The fused gene was subsequently transferred to the vector pBADmycHIS/N [19]. To obtain a construct with a C-terminal hexahistidine tag, the stop codon of the *phl* gene was removed by amplifying a part of the gene by PCR using the forward primer 5'-ATCTCAGAATTC-CATATGGTTTTTACCT and the reverse primer 5'-TTTTT-AAGCTTGATCTTGCTACCAGC. The restriction sites EcoRI (forward primer) and HindIII (reverse primer) used for cloning are underlined. The 1.7 kb EcoRI/HindIII was used to make an

in-frame fusion with the hexahistidine tag of the pBAD vector. The QuikChange® site-directed mutagenesis kit (Stratagene) was used to introduce alanine codons in the *phl* gene by replacing the target codon with GCT or GCC. All mutations were confirmed by sequencing at GATC Biotech.

Expression and purification of recombinant PCL

E. coli TOP10 cells (Invitrogen) containing pBAD-derived *phl* constructs were pre-cultured overnight in 5 ml of LB (Luria-Bertani) medium. The pre-culture was used to inoculate 1 litre of LB medium containing 0.001% L-arabinose. Cells were harvested after 72 h of growth at 17°C, and resuspended in TANG buffer [50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.002% sodium azide and 10% (w/v) glycerol]. Subsequently, cells were sonicated, and cell debris was removed by ultracentrifugation at 45 000 rev./min for 30 min on a Beckman 70-Ti rotor. The hexahistidine-tagged enzyme was purified from cell-free extract using nickel-affinity chromatography. The enzyme was bound to 3 ml of Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose resin (Qiagen) and eluted using 3 ml of TANG buffer containing 500 mM imidazole. The imidazole was removed using an Econo-Pac 10 DG chromatography column (Bio-Rad Laboratories). Enzyme concentrations were determined spectrophotometrically at 280 nm using a calculated molar absorption coefficient of 137 370 M⁻¹ · cm⁻¹.

To obtain MBP-free protein, the purified MBP-PCL-His₆ was treated with Factor Xa (New England Biolabs). For this, CaCl₂ was added to a final concentration of 2 mM to 4 mg of purified enzyme in 1 ml of TANG buffer. Subsequently, 10 µg of the protease was added, and the sample was incubated for 4 days at 4°C, after which the fusion protein was completely cleaved. The cleaved PCL was purified from the mixture using the C-terminal hexahistidine tag as described above. Cleavage and purity of the resulting MBP-free enzyme were analysed by SDS/PAGE (12% gels). The concentration of the cleaved protein was determined spectrophotometrically at 280 nm using a calculated molar absorption coefficient of 65 695 M⁻¹ · cm⁻¹.

CoA ligase activity assay

PCL activity in cell-free extracts was determined using the hydroxamic acid assay [4]. Assay mixtures contained 50 mM Tris/HCl (pH 8.0), 10 mM PAA, 10 mM ATP, 1 mM CoA and 10 mM MgCl₂. One unit is defined as the catalytic activity resulting in the formation of 1 µmol of phenylacetyl hydroxamate per min.

Ligase activity with *trans*-cinnamic acid derivatives was determined by monitoring the conversion of the respective cinnamic acid to the corresponding CoA thioesters. The increase in absorbance during conversion was monitored using a PerkinElmer spectrophotometer at wavelengths of 309 nm for cinnamoyl-CoA, 340 nm for coumaroyl-CoA, 346 nm for feruloyl-CoA, 346 nm for caffeoyl-CoA acid and 352 nm for sinapoyl-CoA [20]. Molar absorption coefficients for other cinnamoyl-CoA derivatives were estimated by measuring the final absorbance after complete conversion of 25 and 50 µM substrate. Standard reaction mixtures contained 50 mM Tris/HCl (pH 8.5), 200 mM NaCl, 3 mM ATP, 1.5 mM CoA, 5 mM MgCl₂, 10 µM–20 mM substrate and 10 nM purified recombinant enzyme in a total volume of 1 ml. Reactions were carried out at 30°C.

Ligase activity for non-chromogenic substrates was determined by following the substrate-dependent formation of AMP by reverse-phase HPLC under the same reaction conditions as described above. Samples were diluted 50-fold in eluent to quench enzymatic conversion and, subsequently, AMP was measured

using an Alltech Alltima HP C₁₈ column in connection with Jasco PU-980 pumps and a Jasco MD-910 detector set at 259 nm. The nucleotides AMP and ATP were eluted isocratically using a solution containing 100 mM potassium phosphate, 20 mM ethanoic (acetic) acid, 5 mM tetrabutylammonium bisulfate and 10% acetonitrile, which was adjusted to pH 4.0. Typical retention times were 2 min for AMP and 8 min for ATP. Substrate-independent ATP hydrolysis by PCL was not observed ($k_{\text{obs}} < 0.01 \text{ s}^{-1}$).

Determination of kinetic parameters

Apparent kinetic constants K_m and k_{cat} for PCL substrates were determined by varying the concentration of one substrate at fixed concentration of the other substrates. For determining the apparent K_m values with carboxylic acid substrates, fixed concentrations of 3 mM ATP, 1.5 mM CoA and 5 mM Mg²⁺ were used. The apparent kinetic parameters for *trans*-cinnamic acid derivatives were determined by fitting the initial rates of cinnamoyl-CoA formation with the Michaelis–Menten equation. Apparent K_m and k_{cat} values for non-chromogenic substrates were determined by fitting the initial rate of AMP formation. For substrates with low apparent K_m values (fatty acids) the apparent K_m values were determined by fitting the level of inhibition on the initial rate of conversion of the chromogenic substrate *trans*-cinnamic acid [21]. When two substrates are converted by a single enzyme at the same active site, the substrates behave as mutual competitive inhibitors, and the K_m value of the second (invisible) substrate (K_{m,S_2}) is equal to the inhibition constant on the conversion rate of the visible substrate (v_{S_1}) (eqn 1):

$$v_{S_1} = - \frac{E \cdot k_{\text{cat},S_1} [S_1]}{K_{m,S_1} (1 + [S_2]/K_{m,S_2}) + [S_1]} \quad (1)$$

Apparent k_{cat} values for these invisible substrates were determined by following the production of AMP using reverse-phase HPLC at concentrations of at least ten times that of the apparent K_m value. For the determination of the apparent kinetic parameters of the co-substrates ATP and CoA, fixed concentrations of 1.5 mM cinnamic acid or 60 mM PAA were used. When ATP was varied, 1.5 mM of CoA was used. When CoA was varied, the ATP concentration was 3 mM. All determinations of kinetic parameters were performed at least in duplicate.

Sequence comparison and modelling

The program BLAST was used to search for homologous proteins of PCL in the NCBI and PDB databases. Multiple sequence alignments were made using the program ClustalW, and results were visualized using the program Boxshade. The CPHmodels 2.0 Server (<http://www.cbs.dtu.dk/services/CPHmodels>) was used to build a structural model of PCL using the crystal structure of the luciferase from firefly species *Luciola cruciata* as template (PDB code 2D1S). Figures were prepared using PyMOL software (<http://www.pymol.org>).

RESULTS

Expression and purification of PCL

CoA activation of PAA is an essential step in penicillin G biosynthesis. However, the biochemical properties of the enzyme catalysing this reaction have never been extensively characterized. We have expressed PCL from *P. chrysogenum* in *E. coli* in order to obtain large amounts of recombinant enzyme for characterization.

The expression of active PCL in *E. coli* initially proved to be difficult. An extensive investigation of the expression conditions was performed by varying the *E. coli* host strain, culture volume and aeration, growth temperature, arabinose concentrations and time of induction, but PCL was not detected. SDS/PAGE analysis of whole-cell lysates did not show the presence of the expected protein band at 62.6 kDa, and CoA ligase activity was never detected in cell extracts, indicating that neither active nor inactive enzyme was produced. However, a high level of expression of the CoA ligase was observed for a construct in which the CoA ligase was fused to the C-terminus of MBP. SDS/PAGE analysis showed that up to 20% of all protein in the cell-free extract of *E. coli* TOP10 was MBP–PCL, and the hydroxamic acid assay showed that the cell-free extract contained up to 20 m-units of PCL activity per mg of protein.

Purification of MBP–PCL using amylose-affinity chromatography was unsuccessful because the fusion protein did not bind to the amylose resin. Further characterization of PCL was therefore performed using a hexahistidine-tagged MBP-fusion enzyme. PCR was used to create a construct encoding a fusion protein that contained the N-terminal MBP that appeared to be essential for expression of the enzyme as well as a C-terminal hexahistidine tag for purification. The recombinant protein was purified to homogeneity using Ni-NTA–agarose, yielding a single protein band upon SDS/PAGE. From a culture of 500 ml, typically 20 mg of recombinant enzyme was obtained. The presence of 200 mM NaCl was essential for the stability of the enzyme and storage or conversions in buffer lacking NaCl resulted in rapid loss of activity. The presence of glycerol protected the enzyme when stored at -20°C . Purified protein could be stored in TANG buffer at -20°C for several months without significant loss of activity.

Substrate specificity of PCL

Purified hexahistidine-tagged MBP–PCL was used to investigate the substrate specificity of PCL. The kinetic parameters for the physiological substrate PAA and for POA (phenoxyacetic acid) were determined using the AMP formation assay (Table 1). The enzyme had an apparent k_{cat} of 1.4 s^{-1} and an apparent K_m of 6.1 mM for PAA under the conditions used. POA appeared to be a much better substrate for the enzyme than PAA having both a 10-fold higher apparent k_{cat} value and a 3-fold lower apparent K_m . PCL also catalysed the CoA activation of several substituted PAAs. Hydrophobic *meta*- or *para*-substituted PAAs were substrates for PCL. The larger 3-phenylpropionic acid was also a reasonable substrate for PCL, whereas the shorter benzoic acid was not converted. The amino acids phenylalanine, tyrosine, alanine and glycine were not substrates for PCL.

We also tested CoA ligase activity with *trans*-cinnamic acids, since these are the substrates for the homologous coumarate–CoA ligases from plants. The formation of cinnamoyl-CoAs can easily be monitored because of the strong absorbance of the product at around 309 nm. Both *trans*-cinnamic acid and *trans*-4-coumaric acid were converted very efficiently by PCL, providing a convenient spectrophotometric assay for the enzyme. The catalytic efficiency for cinnamic acid was more than 1000-fold higher than that for PAA. The difference is caused by the lower apparent k_{cat} and a relatively high apparent K_m value for the physiological substrate. The more substituted compounds ferulic acid, caffeic acid and sinapic acid, which are substrates for most 4-coumarate–CoA ligases [22], were very poor substrates for PCL. Surprisingly, the very similar substrates 3-methoxy-, 3-ethoxy- and 3,4-methylenedioxy-cinnamic acid were very good substrates for PCL. To investigate whether the relatively low activity of

Table 1 Steady-state apparent kinetic parameters of PCL

*Data for these compounds were determined at pH 7.5. †No detectable activity at 4 mM substrate ($< 0.01 \text{ s}^{-1}$). ‡No detectable activity at 20 mM substrate ($< 0.01 \text{ s}^{-1}$).

Substrate	Structure	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Aromatic acids				
Phenylacetic acid		1.4 ± 0.3	6.1 ± 0.3	$(2.3 \pm 0.6) \times 10^{-1}$
Phenoxyacetic acid		15.6 ± 1.6	2.0 ± 0.2	7.8 ± 1.2
3-Phenylpropionic acid		22.7 ± 1.9	2.1 ± 0.4	10.8 ± 2.3
<i>trans</i> -Cinnamic acid		43.5 ± 2.7	0.14 ± 0.01	$(3.1 \pm 0.4) \times 10^2$
<i>trans</i> -4-Coumaric acid		3.4 ± 0.5	0.47 ± 0.08	7.2 ± 1.7
Caffeic acid*		> 0.02	> 1.0	$(2 \pm 1) \times 10^{-2}$
Ferulic acid*		> 0.07	> 0.5	$(7 \pm 1) \times 10^{-2}$
Sinapic acid*		NA†	NA†	NA†
3-Methoxycinnamic acid		11.6 ± 0.3	0.84 ± 0.09	14 ± 2
3-Ethoxycinnamic acid		12.1 ± 0.7	0.82 ± 0.02	15 ± 2
3,4-Methylenedioxycinnamic acid		11.2 ± 1.9	0.54 ± 0.04	21 ± 5
Aliphatic acids				
Ethanoic acid		NA‡	NA‡	NA‡
Propionic acid		> 1	> 80	$(1.3 \pm 0.1) \times 10^{-2}$
Butyric acid		9.7 ± 0.4	24.4 ± 3.9	$(4.0 \pm 0.7) \times 10^{-1}$
Valeric acid		18.5 ± 0.9	0.7 ± 0.2	$(2.6 \pm 0.9) \times 10^2$
Caproic acid		15.8 ± 0.5	0.24 ± 0.02	66 ± 7

Table 1 (Contd.)

Substrate	Structure	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Enanthic acid		12.3 ± 3.9	0.05 ± 0.03	$(2.5 \pm 1.8) \times 10^2$
Caprylic acid		11.2 ± 0.7	0.06 ± 0.01	$(1.9 \pm 0.4) \times 10^2$
Capric acid		8.2 ± 1.7	$(0.2 \pm 0.1) \times 10^{-2}$	$(4.1 \pm 2.3) \times 10^3$
Lauric acid		8.5 ± 1.8	$(0.4 \pm 0.2) \times 10^{-3}$	$(2.1 \pm 1.2) \times 10^4$
Myristic acid		2.3 ± 0.1	< 0.0005	$> 4.6 \times 10^3$

Table 2 Apparent kinetic parameters and σ_p^- values for *para*-substituted *trans*-cinnamic acids σ_p^- values are from Hansch et al. [23].

Substrate	Parameter			
	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	σ_p^-
4-Hydroxycinnamic acid	3.4 ± 0.5	0.47 ± 0.08	7.2 ± 1.7	-0.37
4-Methoxycinnamic acid	23.9 ± 3.2	0.18 ± 0.01	$(1.3 \pm 0.3) \times 10^2$	-0.26
4-Methylcinnamic acid	21.0 ± 0.1	0.32 ± 0.01	66 ± 3	-0.17
4-Isopropylcinnamic acid	9.6 ± 0.3	0.07 ± 0.02	$(1.4 \pm 0.5) \times 10^2$	-0.16
4-Fluorocinnamic acid	35.5 ± 1.4	0.084 ± 0.017	$(4.2 \pm 0.9) \times 10^2$	-0.03
Cinnamic acid	43.5 ± 2.7	0.14 ± 0.01	$(3.1 \pm 0.4) \times 10^2$	0.00
4-Chlorocinnamic acid	29.1 ± 0.6	0.16 ± 0.05	$(1.8 \pm 0.6) \times 10^2$	0.19
4-Trifluoromethylcinnamic acid	11.3 ± 1.2	0.19 ± 0.07	60 ± 3	0.65
4-Nitrocinnamic acid	2.8 ± 0.1	0.51 ± 0.01	5.5 ± 0.3	1.27

para-hydroxy-substituted compounds was caused by the strong electron-donating substituent of the substrate, we tested a set of *para*-substituted cinnamic acids with both electron-withdrawing and -donating groups (Table 2). The apparent K_{m} values for these substituted *trans*-cinnamic acids were very similar to that for cinnamic acid and these substrates probably bind in a similar manner in the enzyme active site. The apparent k_{cat} values varied between 2 and 50 s^{-1} when the substituent on the cinnamic acid phenyl ring was changed. A Hammett plot of $\log(k_{\text{cat,X}}/k_{\text{cat,H}})$ against σ_p^- [23] shows a correlation between the observed apparent k_{cat} and the substituent for both electron-donating and electron-withdrawing groups (Figure 2). For substrates with electron-donating groups, the slope is 2.5 ($r = 0.69$) and for substrates with electron-withdrawing groups, the slope is -0.94 ($r = 1.00$). The Hammett plot is biphasic, indicating that both electron-donating and electron-withdrawing groups slow down the reaction. The observed correlation between the substituent and the apparent k_{cat} suggests the formation or disappearance of a negative charge in the rate-limiting step. The biphasic relationship suggests a change in the rate-determining step for substrates with electron-withdrawing substituents compared with substrates with electron-donating substituents [24].

The initial rate of cinnamic acid conversion was used to determine biochemical properties such as optimum pH and temperature and the kinetic parameters for the co-substrates ATP

Figure 2 A Hammett plot of $\log(k_{\text{cat,X}}/k_{\text{cat,H}})$ against σ_p^- for the CoA ligase reaction with *para*-substituted cinnamic acids is biphasic

For substrates with electron-donating groups, the slope is 2.5 ($r = 0.69$) and for substrates with electron-withdrawing groups, the slope is -0.94 ($r = 1.00$).

and CoA and the cofactor magnesium. The pH optimum of the enzyme was 8.5, and the optimum temperature was between 35 and 40°C . The optimal Mg^{2+} concentration was determined by varying the concentration of MgCl_2 at fixed concentrations of ATP (3 mM), CoA (1.5 mM) and cinnamic acid (1.5 mM) and was found to be 5 mM. At higher concentrations, inhibition was observed. When cinnamic acid was used as the substrate to determine the kinetic parameters of the co-substrates, the apparent $K_{\text{m}}^{\text{ATP}}$ was 0.48 ± 0.06 (apparent k_{cat} was $42.9 \pm 1.0 \text{ s}^{-1}$) and the apparent $K_{\text{m}}^{\text{CoA}}$ was $0.94 \pm 0.10 \text{ mM}$ (apparent k_{cat} was $60.5 \pm 3.2 \text{ s}^{-1}$). When PAA was present as the carboxylic acid substrate and the AMP formation assay was used to determine the kinetic parameters, the apparent $K_{\text{m}}^{\text{ATP}}$ was $0.65 \pm 0.03 \text{ mM}$ (apparent k_{cat} was $1.7 \pm 0.2 \text{ s}^{-1}$), and the apparent $K_{\text{m}}^{\text{CoA}}$ was $0.12 \pm 0.01 \text{ mM}$ (apparent k_{cat} $1.5 \pm 0.2 \text{ s}^{-1}$).

Of the carboxylic acid substrates for which we screened, the highest activities were found for fatty acids (Table 1). With the exception of ethanoic acid, all short- and medium-chain fatty acids tested were converted by PCL. With increasing chain length, the apparent K_{m} value decreased from more than 80 mM for

Table 3 CoA ligase activity of PCL with side-chain precursors of natural penicillins

Results are apparent kinetic parameters.

Product	Side-chain precursor	Structure	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Penicillin G	Phenylacetic acid		1.4 ± 0.3	6.1 ± 0.3	$(2.3 \pm 0.6) \times 10^{-1}$
Penicillin V	Phenoxyacetic acid		15.6 ± 1.6	2.0 ± 0.2	7.8 ± 1.2
Penicillin F	<i>trans</i> -3-Hexenoic acid		14.7 ± 1.2	0.12 ± 0.01	$(1.2 \pm 0.2) \times 10^2$
Penicillin DF	Caproic acid		15.8 ± 0.5	0.24 ± 0.02	66 ± 7
Penicillin H	Enanthic acid		12.3 ± 3.9	0.05 ± 0.03	$(2.5 \pm 1.8) \times 10^2$
Penicillin K	Caprylic acid		11.2 ± 0.7	0.06 ± 0.01	$(1.9 \pm 0.4) \times 10^2$
Penicillin X	Hydroxyphenylacetic acid		0.08 ± 0.02	5.7 ± 0.4	$(1.4 \pm 0.4) \times 10^{-2}$

propionic acid (C_3) to less than $1 \mu\text{M}$ for myristic acid (C_{14}). The apparent K_{m} values for fatty acids with low K_{m} values (C_{10} and larger) were determined by fitting the level of inhibition on the initial rate of conversion of the chromogenic substrate *trans*-cinnamic acid as described in the Experimental section. The apparent k_{cat} values were in the same range for all fatty acids (Table 1), but a small decrease with increasing chain length was observed. The apparent k_{cat} value for the long-chain fatty acid myristic acid was approx. 10-fold lower than for the short-chain fatty acid valeric acid (C_5). The cause of the observed decrease with increasing chain length is not immediately apparent, but may be related to poor positioning of the larger substrate for nucleophilic attack on ATP, slow product release, or both.

During penicillin G-producing fermentations of *P. chrysogenum*, the formation of β -lactam side products has been reported [25]. The main side products that are detected are the penicillins K, F and DF, and these penicillins have caprylic acid, *trans*-3-hexenoic acid and caproic acid as side chains respectively. PCL is able to activate all the side chains of these naturally occurring lactam side products (Table 3) and thus may be responsible for their formation during penicillin G production.

To determine whether the MBP-fusion protein behaves similarly to the native PCL protein, we prepared PCL without the N-terminal MBP tag. For this, purified hexahistidine-tagged MBP-PCL was treated with Factor Xa protease. This yielded the two separate proteins, MBP and the hexahistidine-tagged PCL. The PCL protein was purified from this mixture using the C-terminal hexahistidine tag. The cleaved PCL-His₆ had the same steady-state kinetic parameters for cinnamic acid as well as for PAA and POA as MBP-PCL-His₆ (PAA: K_{m} $5.2 \pm 0.9 \text{ mM}$ and k_{cat} $1.9 \pm 0.2 \text{ s}^{-1}$; POA: K_{m} $2.1 \pm 0.1 \text{ mM}$ and k_{cat} $12 \pm 2.1 \text{ s}^{-1}$; and cinnamic acid: K_{m} $0.19 \pm 0.04 \text{ mM}$ and k_{cat} $41.2 \pm 1.4 \text{ s}^{-1}$). The cleaved enzyme was less stable than the MBP-fusion enzyme and precipitation was observed during concentrating or freeze/thawing of the enzyme. Factor Xa cleavage of an MBP-PCL fusion protein that contained the hexahistidine tag to the N-terminus of the MBP instead of the C-terminus of PCL resulted in an even less stable PCL. The fusion protein His₆-MBP-PCL expressed normally in *E. coli* and could be purified using the hexahistidine tag and showed a similar activity with *trans*-cinnamic acid, indicating that functional PCL was

produced. However, Factor Xa cleavage of His₆-MBP-PCL to obtain untagged PCL resulted in almost immediate precipitation of the protein after the His₆-MBP part was separated from PCL. Both the N-terminal MBP and the C-terminal hexahistidine tag appear to stabilize PCL.

Homology modelling

The BLAST program was used to search the NCBI database with the amino acid sequence of PCL. This yielded strong hits with the extensively characterized 4-coumarate-CoA ligases from *Arabidopsis thaliana*, involved in the biosynthesis of plant phenylpropanoids, and a very similar bacterial cinnamate-CoA ligase from *Streptomyces coelicolor* [22,26,27]. These enzymes are also able to catalyse the activation of *trans*-cinnamic acid derivatives to the corresponding CoA esters.

Many structures of members of the superfamily of adenylate-forming enzymes were found in the PDB, including two acetate-CoA ligases [28,29], a chlorobenzoate-CoA ligase [30] and a fatty-acid-CoA ligase [31]. These enzymes have less than 25 % sequence identity with PCL. Surprisingly, the most related sequences to PCL are from two luciferases. The first luciferase structure that was solved is that of the North American firefly *Photinus pyralis* [32]. The structure was solved in the absence of substrate and the identification of the active site, and the residues important for substrate binding remained unclear. More recently, the structure of the Japanese firefly (*Luciola cruciata*) luciferase was solved [33]. This structure was determined in complex with an adenylate intermediate analogue at a resolution of $1.3 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$), and was used as the template for homology modelling of PCL which was possible because the sequence identity was 29 %. Figure 3 shows a sequence alignment of PCL with the homologous CoA ligases and luciferases around the substrate-binding pocket.

The model shows that PCL is composed of a large N-terminal domain and a small C-terminal domain similar to the other members of the adenylate-forming superfamily. In the model, 12 residues form the substrate-binding pocket: His²⁶⁵, Ile²⁶⁶, Tyr²⁶⁷, Val²⁷⁰, Phe³⁰⁷, Phe³³⁵, Gly³³⁷, Ala³³⁸, Gly³⁶¹, Thr³⁶⁹, Val³⁷⁰ and Lys⁵⁵⁷ (Figure 4A). Lys⁵⁵⁷ is located in the C-terminal

		--Motif 1--
PCL	216	SELPGKGMISHRNVIANTLQIKAFQNYRDGGGTPASTEVALGLRQSHI VALWIGHA
LcrLuc	204	TGLPGKGVQLTHEINIVTRFSDHARDP IY----GNOVS P-GTAVLTVVRFHNGFMEHTLG-
PpyLuc	202	TGLPGKGVLPHTACVRFSDHARDP IF----GNQII P-DTALISVVRFHNGFMEHTLG-
ScCCL	183	TGTPKGVMLTHRQIATNLAQLP-----SMPSAPGDRVLAVLFPHHYGLALMNA
At4CL2	207	TGLPGKGVMLTHKGLVT SVAQVQDGEN-----PNLYFNRRDVLICVWFMEHTYALSSIMLC
At4CL1	214	TGLPGKGVMLTHKGLVT SVAQVQDGEN-----PNLYFHSDDVILCVLFMEHTYALSSIMLC
At4CL3	217	TGLPGKGVMLTHKSLIT SVAQVQDGDN-----PNLYLKSNDVILCVLFMEHTYALSSVLLN
PCL	276	GAYRGDQTVLVPKFKLSYENAIQYKISALFLVFPPIIHMLGTQDVCSSKYDLSVSTSL
LcrLuc	257	YLICGFRVVMILTKFDEETPKTKLDYKCTSVLVTFLFAILN-KSELLNKYDLSNLVEIP
PpyLuc	255	YLICGFRVVMILYRFEELFPRSLQDYKIQSACLVPTLFSFFA-KSTLIDKYDLSNLHEIP
ScCCL	234	PLRLGATVVLPFRDLQEPFAAIAQNHRTSLVYAPPIVLALA-KHPLVADYDLSLRVYIP
At4CL2	262	SLRIGATILIMPKEFTLLLEQIQCKVTVMVVPPIVLAIA-KSPETEKYDLSVRVMP
At4CL1	269	GLRVGAAILIMPKEFTINLLLEQIQCKVTVMVVPPIVLAIA-KSSETEKYDLSIRVMP
At4CL3	272	SLRS GATVLMHKEEIGALLDQIRHRTVIALEVEPLVLAIA-KNPTVNSYDLSVRFVMP
		--Motif 2--
PCL	336	TEAAPLGMETADFLKLYPNILIRCSGLTETGTWVSTH----PHDIWLGSSEALLPG
LcrLuc	316	SGAPLSKEVGEAVARFNLPGVRCGSLTETTSALITP-----EGDDKPGASGVVLP
PpyLuc	314	SGAPLSKEVGEAVAKRFHLPGIRCSGLTETTSALITP-----EGDDKPGAVGVVLP
ScCCL	293	SAAPLGDARLAAACQRLGLPFGVCGAMGTELSPGTHVVP--LADADAPGTGVRGIAG
At4CL2	321	SGAPLGELEDAISAKFPNKLQCGHNTGACPVYLAISLGFAPKFPVKS GACSTVVRN
At4CL1	328	SGAPLGELEDAVNAKFPNKLQCGHNTGACPVYLAISLGFAPKFPVKS GACSTVVRN
At4CL3	331	SEAPLGELEODSLRRRLPOAILGCGHNTGACPVYLAISLGFAPKFPVKS GACSTVVRN

Figure 3 Sequence alignment of PCL, firefly luciferases and coumarate-CoA ligases

The following sequences were used (EMBL accession numbers are given in parentheses): LcrLuc, luciferase from *Luciola cruciata* (BAE0731); PpyLuc, luciferase from *Photinus pyralis* (BAF48390); ScCCL, cinnamate-CoA ligase from *Streptomyces coelicolor* A3(2) (CAB95894); At4CL, 4-coumarate-CoA ligase from *Arabidopsis thaliana*, At4CL1 (AAD47191); At4CL2 (AAD47193); At4CL3 (AAD47195). The region that contains the substrate-binding residues in LcrLuc is located around conserved motifs 1 and 2 involved in ATP and magnesium binding respectively. Conserved residues are shaded grey, and the residues that form the substrate-binding pocket in LcrLuc and the putative substrate-binding residues in 4-coumarate CoA ligases [26] as well as the corresponding residues in PCL are highlighted in black.

domain, and this invariant residue is involved in formation of the adenylate intermediate [34,35]. The other residues are located at the interface between the two domains and form the wall of a long tunnel for substrate binding. Close to the carboxylate-binding site, the tunnel is rather narrow because of the backbone of Gly³³⁷, Ala³³⁸ and Gly³⁶¹ and the side chains of Ile²⁶⁶ and Thr³⁶⁹. When PAA is modelled in the substrate-binding channel, there seems to be a steric conflict between the phenyl ring of PAA and the wall of the substrate-binding pocket formed by the backbone of Gly³³⁷ and Ala³³⁸. This may explain the high apparent K_m value observed for PAA. The additional oxygen atom in POA and carbon atom in *trans*-cinnamic acid may cause the phenyl ring of these substrates to become located in the tunnel; these substrates have low apparent K_m values. In the model, the fatty acids are also able to rotate their aliphatic chain into the tunnel and do not have a steric conflict with the wall of the pocket (Figure 4B).

Mutagenesis of substrate-binding pocket residues

The role of the side chains of His²⁶⁵, Ile²⁶⁶, Tyr²⁶⁷, Val²⁷⁰, Phe³⁰⁷, Phe³³⁵, Thr³⁶⁹ and Val³⁷⁰ was investigated by mutating these residues to alanine. All alanine mutations were made in the hexahistidine-tagged MBP–PCL enzyme, and mutant enzymes were expressed and purified as described for wild-type MBP–PCL. The kinetic parameters of the mutants for PAA and POA were determined. The effect of the mutations on the activity with the caproic acid and with *trans*-cinnamic and *p*-coumaric acid was also investigated (Table 4).

The affinity of wild-type PCL for PAA and POA was relatively low, with apparent K_m values in the millimolar range. None of the alanine mutations had a significant effect on the affinity for PAA, since the apparent K_m of all the mutants was unaffected. However, the apparent k_{cat} for PAA was strongly influenced by the mutations, indicating that the residues are important for CoA ligase activity. The relatively high apparent K_m of wild-type PCL for PAA, and the small effect that the mutations have on the affinity for PAA, indicate that the substrate-binding pocket of

PCL is not selectively optimized for PAA. The observed effect on apparent k_{cat} might be caused by changes in orientation of the carboxy group for reaction with ATP in the first half-reaction. Interestingly, the T369A mutant had a 3.7-fold increased apparent k_{cat} for PAA. In the model, Thr³⁶⁹ is located at the top of the tunnel close to the carboxylate-binding site. Removal of the side chain of Thr³⁶⁹ may create some more space at the neck of the substrate-binding tunnel, allowing the substrate to bind more favourably for catalysis. The increased apparent k_{cat} value of mutant T369A was also observed for the substrates caproic acid, cinnamic acid and coumaric acid, but not for POA.

Activity with POA was affected in a similar way by the mutations as activity for PAA. The apparent k_{cat} was affected strongly by all the mutations, but the effect on the apparent K_m was relatively small. The mutants Y267A and F307A and F335A did show a moderate (3–5-fold) increase in apparent K_m for POA. In the model, the phenyl ring of the substrate POA possibly interacts with the phenyl rings of Tyr²⁶⁷ and with Phe³⁰⁷ and Phe³³⁵ located further away in the substrate-binding tunnel. Mutation of one of these residues into alanine removes this interaction causing a lower affinity for the substrate. This effect on apparent K_m was also observed for the other aromatic substrates cinnamic acid and coumaric acid, but not for the aliphatic caproic acid, suggesting that these residues are important for the binding of aromatic substrates. This effect on affinity was not observed for PAA. The model suggests that the phenyl ring of the smaller substrate PAA is unable to rotate towards the substrate-binding pocket, making it impossible for PAA to interact with these aromatic residues (Figure 4B).

The V270A and V370A mutants had relatively small effects on CoA ligase activity for all five substrates. The apparent K_m values for both mutants were similar to those of wild-type PCL. The effect on the apparent k_{cat} was also relatively small, with the exception that the V270A mutant showed a 10-fold lower apparent k_{cat} for POA and the V370A mutant showed a 10-fold lower apparent k_{cat} for PAA. These valine residues may help to form a hydrophobic substrate-binding pocket and are probably not very important in determining the substrate specificity.

DISCUSSION

CoA activation of PAA is an essential step in the biosynthesis of penicillin G by *P. chrysogenum*. Previous work has indicated that multiple CoA ligases may play a role in the formation of phenylacetyl-CoA, but a large part is formed by PCL [9]. Purification of PCL activity from *P. chrysogenum* cultures has been reported, but biochemical data on the enzyme responsible for this activity are scarce, which may be due to the reported instability of the enzyme [5]. In the present paper, we report the overexpression of PCL as an MBP fusion in *E. coli* to produce a stable fusion enzyme, and the characterization of this key enzyme for penicillin G biosynthesis.

MBP from *E. coli* is often used as an expression tag to increase the recombinant expression of proteins [36]. In the case of PCL, the fusion not only increased the expression level of the protein, but also improved the stability of the protein. The MBP part of the MBP–PCL–His₆ protein could be removed by digestion with Factor Xa protease, and the cleaved PCL–His₆ could be purified and showed CoA ligase activity, but was less stable.

Recombinant MBP–PCL–His₆ was used to investigate the substrate specificity of the CoA ligase. For cinnamic acids, the spectrophotometric assay was used to follow CoA ligase activity. The molar absorption coefficients of the aromatic enoyl-CoA products are relatively high (approx. 20 mM⁻¹ · cm⁻¹), providing

Table 4 Apparent kinetic parameters of wild-type and mutant PCL with substrates PAA, POA, caproic acid, *trans*-cinnamic acid and *p*-coumaric acid

PCL	PAA		POA		Caproic acid		Cinnamic acid		Coumaric acid	
	<i>k</i> _{cat} (s ^{−1})	<i>K</i> _m (mM)	<i>k</i> _{cat} (s ^{−1})	<i>K</i> _m (mM)	<i>k</i> _{cat} (s ^{−1})	<i>K</i> _m (mM)	<i>k</i> _{cat} (s ^{−1})	<i>K</i> _m (mM)	<i>k</i> _{cat} (s ^{−1})	<i>K</i> _m (mM)
Wild-type	1.4 ± 0.3	6.1 ± 0.3	15.6 ± 1.6	2.0 ± 0.2	15.8 ± 0.5	0.24 ± 0.02	43.5 ± 2.7	0.14 ± 0.01	3.4 ± 0.5	0.47 ± 0.08
H265A	0.009 ± 0.001	8.5 ± 0.8	0.41 ± 0.09	4.8 ± 0.7	4.4 ± 0.3	1.6 ± 0.2	1.2 ± 0.2	4.0 ± 0.2	0.08 ± 0.03	2.8 ± 0.6
I266A	0.49 ± 0.05	5.5 ± 1.2	1.1 ± 0.1	3.3 ± 0.4	41.8 ± 1.6	0.30 ± 0.01	10.4 ± 1.1	0.8 ± 0.3	0.6 ± 0.1	1.8 ± 0.3
Y267A	0.025 ± 0.002	6.3 ± 1.1	0.13 ± 0.01	10.7 ± 1.5	0.13 ± 0.02	0.13 ± 0.01	0.4 ± 0.2	3.0 ± 1.0	0.011 ± 0.001	0.57 ± 0.03
V270A	0.68 ± 0.04	3.9 ± 0.3	1.5 ± 0.1	2.4 ± 0.2	10.0 ± 1.5	0.13 ± 0.01	11.5 ± 2.8	0.54 ± 0.04	0.30 ± 0.01	0.41 ± 0.03
F307A	0.017 ± 0.002	6.8 ± 0.3	0.53 ± 0.08	5.3 ± 0.7	5.3 ± 0.7	0.24 ± 0.08	4.5 ± 0.3	1.0 ± 0.3	0.11 ± 0.02	1.1 ± 0.1
F335A	0.04 ± 0.01	8.0 ± 0.5	0.91 ± 0.09	10.3 ± 0.9	11.3 ± 0.2	0.8 ± 0.2	12.1 ± 1.4	2.5 ± 0.4	0.8 ± 0.2	0.17 ± 0.02
T369A	5.2 ± 0.6	4.7 ± 0.3	2.2 ± 0.2	1.3 ± 0.2	47.6 ± 1.3	0.11 ± 0.01	56 ± 11	0.5 ± 0.2	3.0 ± 0.2	0.47 ± 0.01
V370A	0.14 ± 0.01	7.6 ± 1.0	4.2 ± 0.3	4.0 ± 0.5	13.9 ± 1.2	0.15 ± 0.04	24.6 ± 2.9	0.25 ± 0.03	1.3 ± 0.1	0.14 ± 0.02

Figure 4 Active-site residues in the structure of LcrLucif (luciferase from *Luciola cruciata*) and the homology model of PCL

(A) The structure of LcrLucif was determined in the presence of an adenylate-intermediate analogue shown in green. The phenylacetyl-adenylate intermediate was modelled into the active site of PCL. (B) Modelling of adenylate intermediates in the PCL active site. Ile²⁶⁶ causes the substrate-binding pocket to be very narrow close to the carboxylate-binding site. This indicates steric hindrance between the phenyl ring and the pocket wall formed by Gly³³⁷ and Ala³³⁸ when PAA is bound (green). The additional oxygen atom in POA (purple) and carbon atom in *trans*-cinnamic acid (yellow) allows the phenyl ring to rotate into the binding pocket. The aliphatic chains of fatty acids are also able to rotate into the pocket (light blue).

a practical and sensitive activity assay for PCL. Formation of as little as 1 μM product is easily detected. To measure CoA ligase activity with other carboxylic acids, the AMP formation assay was preferred over the hydroxamate assay. The release of the co-product AMP is independent of the carboxylic acid substrate used and is also sensitive because of the high UV absorbance of nucleotides at 259 nm. In the hydroxamate assay, the molar absorption coefficient of the iron–hydroxamate complex formed depends on the acid used and is relatively low (e.g. $\epsilon_{\text{max}} = 0.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for phenylacetyl hydroxamate), making the assay not very sensitive. For some substrates, such as the amino acid phenylglycine, the molar absorption coefficient was even 10-fold lower, making it almost impossible to detect CoA ligase activity with such a substrate. To validate the use of AMP detection as a CoA ligase activity assay, we monitored the conversion of *trans*-cinnamic acid both with the spectrophotometric method as well as with AMP formation on HPLC, which yielded similar results.

Comparison of the biochemical properties indicates that PCL, encoded by *phl*, differs from the CoA ligase that was purified previously from *P. chrysogenum* by Kogekar and Deshpande [5]. We found apparent *K*_m values of 6.1 and 2.0 mM for PAA and

POA respectively, whereas Kogekar and Deshpande [5] reported an apparent *K*_m of 2.9 μM for both substrates. Furthermore, unlike PCL, the latter enzyme was unable to use phenylpropionic acid or fatty acids as substrates. The previously constructed *P. chrysogenum phl* knockout was shown to be still able to produce some penicillin G [9] and consequently there must be at least one more enzyme with PCL activity, which may be the enzyme purified by Kogekar and Deshpande [5]. Recently, the cloning of a peroxisomal CoA ligase from *P. chrysogenum* was reported, but the biochemical properties were not investigated in detail [37]. The recombinant enzyme was reported to have PCL activity, and it was suggested that this enzyme may be involved in penicillin biosyntheses, but no quantitative activity data were given. A further candidate for phenylacetate activation that could be considered in *P. chrysogenum* is the previously purified acetate-CoA ligase [3]. This protein was described to activate PAA and may be responsible for the remaining PCL activity that is used for penicillin G biosynthesis in a *phl*-knockout strain [9]. However, this acetate-CoA ligase does not contain a PTS1 or PTS2 sequence [38] and probably localizes in the cytosol, and it is unclear whether PAA activated in the cytosol can be used for the acyltransferase reaction which occurs in the peroxisome [15].

The substrate range of PCL is rather broad. The enzyme showed CoA ligase activity with PAA and with POA, but the activity with these substrates was low compared with that with *trans*-cinnamic acids and fatty acids. Nonetheless, we maintain the name PCL because of its role in penicillin G biosynthesis. The broad substrate range combined with relatively low activity for PAA makes PCL very different from PCLs involved in PAA catabolism from bacteria such as *A. evansii* [12] and *Pseudomonas putida* [13]. Indeed, sequence comparison shows that PCL from *P. chrysogenum* is more closely related to plant coumarate–CoA ligases and firefly luciferases than to the bacterial PCLs. The bacterial CoA ligases are specifically expressed in the presence of PAA, and the substrate profile shows that these enzymes are highly specific for PAA. Despite the strong preference for PAA, the *Pseudomonas putida* CoA ligase was reported to accept a broad range of acid substrates in reactions coupled with isopenicillin–N acyltransferase from *P. chrysogenum*. In these *in vitro* experiments, the CoA ligase was found to activate a wide range of side chains leading to the formation of many both natural and non-natural types of penicillin [39].

The highest activities of PCL were observed for medium-chain fatty acids. Fatty acid activation by CoA ligases is also the first step in β -oxidation of fatty acids, which occurs in the peroxisomal matrix. PCL contains a C-terminal PTS1 sequence (SKI) for targeting and probably localizes in the peroxisomal matrix. Maximal CoA ligase activity was found at a pH of 8.5, indicating that PCL is optimized for the peroxisome, which has a slightly alkaline pH [40]. The β -oxidation of fatty acids has been investigated extensively in yeasts, and it is believed that there are two routes for transport of fatty-acid–CoAs to the peroxisomal matrix. Fatty acids are either activated in the cytosol by a cytosolic ligase and then transported to the peroxisome as CoA thioesters, or the fatty acids are coupled to CoA during transport of the fatty acids across the peroxisomal membrane by a membrane-associated ligase [41]. CoA activation of fatty acids inside the peroxisome by a matrix protein has not been reported to play a role in β -oxidation in yeasts. It is unclear whether fatty acid activation by PCL plays a physiological role in β -oxidation in *Penicillium*, but the formation of β -lactam side products containing fatty acid side chains during fermentations is an indication that fatty acid activation occurs under penicillin-producing conditions.

The formation of β -lactam side products containing these fatty acid side chains has been reported in penicillin G- and V-producing cultures of *P. chrysogenum* [25]. The main β -lactam side products that are formed are the natural penicillin K, F and DF, which contain caprylic acid, *trans*-3-hexenoic acid and caproic acid as side chains respectively. The original strain from which current production strains are derived, *Penicillium chrysogenum* NRRL 1951, and early derivatives of this original isolate (e.g. strain Q176) were shown to produce a mixture of these penicillins when no (additional) PAA was supplied in the feed [42]. It was shown that in a defined medium without any PAA, penicillin G is not produced, and the addition of medium- or long-chain fatty acids results in higher levels of penicillin K, F and DF [25,43]. The formation of these side products occurs because isopenicillin–N acyltransferase is able to accept many CoA-activated fatty acids as a donor in the acyltransferase reaction with isopenicillin N [1,39,43]. The results of the present study show that PCL also has a broad substrate range and is able to activate the side chains of all of the natural β -lactam side products that are observed.

The selectivity observed could be explained by homology modelling. The model shows that PCL has a large narrow active site which is formed by hydrophobic residues. The large hydrophobic active site explains the broad substrate range observed for hydrophobic acids. The fact that the apparent K_m

value for the substrates PAA and POA is much higher than for fatty acid substrates indicates that PCL does not have an optimized binding site for PAA or POA. The alanine mutations do not cause a large increase in the apparent K_m , also indicating that the substrate-binding pocket is not optimized for these substrates. Modelling of acid substrates in the active site shows that α -unsubstituted and mono- β -substituted carboxylic acids are the best substrates for PCL. The narrow neck of the substrate-binding pocket prevents the binding of α -substituted acids such as the amino acids and also the binding of the mono- α -substituted PAA is not optimal. The longer acids POA, *trans*-cinnamic acid and fatty acids do not have substituents on the α -carbon and the β -substituent and can rotate into the substrate-binding tunnel. Therefore these substrates have relatively low apparent K_m values and high turnover numbers.

In summary, the homology model and the substrate specificity suggest that PCL is not optimized for the activation of PAA and POA, but for medium-chain fatty acids, and PCL may have evolved from an enzyme that activated fatty acids, probably involved in the β -oxidation of fatty acids in the peroxisome. The broad substrate range of PCL for relatively large hydrophobic acids may have allowed the enzyme to become involved in the activation of penicillin side chains. During the evolution of β -lactam biosynthesis and perhaps even during the subsequent strain improvement towards higher penicillin G-producing strains, this and other peroxisomal CoA ligases may have been recruited for penicillin G biosynthesis. Interestingly, at the genetic level, there is also evidence for the different evolutionary origin of PCL. The *phl* gene is not part of gene clusters that contain the other genes of penicillin biosynthesis, *pcbAB*, *pcbC* and *penDE* [44]. The recruitment of a peroxisomal fatty-acid–CoA ligase has also been proposed for the evolution of firefly luciferases [45]. The CoA ligase activity observed in some luciferases indicates the close relationship between these two enzyme activities.

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