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

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Short title: phenylacetate-CoA ligase from *Penicillium*

Abbreviations: PCL, phenylacetate-CoA ligase; PAA, phenylacetic acid; POA,
phenoxyacetic acid; MBP, maltose binding protein; PTS, peroxisomal targeting signal

Keywords: phenylacetate-coenzyme A ligase, CoA ligase, penicillin G, *Penicillium*
chrysogenum

SYNOPSIS

Enzymatic activation of phenylacetic acid (PAA) 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 phenoxyacetic acid (POA) act as acyl donor in the exchange of the aminoadipyl side chain of isopenicillin N to produce penicillin G or penicillin V. The *phl* gene, encoding a 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 to 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 $k_{\text{cat}}/K_{\text{m}}$ values of 0.23 ± 0.06 and $7.8 \pm 1.2 \text{ mM}^{-1} \text{ s}^{-1}$ for PAA and POA, respectively. Surprisingly, PCL was very efficient in catalyzing the conversion of *trans*-cinnamic acids to the corresponding CoA thioesters ($k_{\text{cat}}/K_{\text{m}} = (3.1 \pm 0.4) \cdot 10^2 \text{ mM}^{-1} \text{ s}^{-1}$ 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 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.

INTRODUCTION

The final step in the biosynthesis of β -lactam antibiotics by the fungus *Penicillium chrysogenum* is the exchange of the L- α -aminoadipyl side chain for a more hydrophobic group, *e.g.* a phenylacetyl group in the case of penicillin G

biosynthesis (Fig. 1). The exchange is catalyzed by the enzyme isopenicillin N acyltransferase [1]. This enzyme requires an activated form of the side chain for acyltransferase activity. Several thioesters as 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 activation have remained unclear for a long time. An acetate-CoA ligase isolated from *P. chrysogenum* was shown to be able to CoA activate phenylacetate *in vitro* [3]. However, it is uncertain if this enzyme has a role in penicillin G biosynthesis *in vivo*. Purification of a 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 phenylacetate-CoA ligase are important because the level of phenylacetyl-CoA is a possible bottleneck in the biosynthesis of penicillin G. The introduction of phenylacetate-CoA ligase from *Pseudomonas putida* was described to have a positive effect on the level penicillin formation [6] and the disruption of a P450 monooxygenase catalyzing PAA 2-hydroxylation, a reaction competing for the available PAA with PAA-CoA ligase activity, resulted in a higher production of penicillin [7].

In 2001 the sequence of a gene (*phl*) that encodes a phenylacetate-CoA ligase (PCL) was published in a patent [8], and recently it was 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 in these enzymes proceeds in a two-step reaction (Fig. 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 nonribosomal peptide synthetases, fatty-acid-CoA synthetases, luciferases and coumarate-CoA ligases [10]. Bacterial phenylacetate-CoA ligases involved in the catabolism of PAA in bacteria such as *E. 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 (approximately 15%). PCL contains a C-terminal peroxisomal SKI 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, which may be partly due to the instability of the enzyme [5]. We have used a maltose binding protein (MBP) fusion to obtain a high level of expression in *E. coli* and to increase the stability of PCL. Here 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* (accession number AJ001540), was PCR amplified from a cDNA library [16] using the forward primer 5'-CCGAGGATTTGGGCATATGGTTTTTTTACCTCC-3', introducing a *Nde*I site in front of the start codon, and the reverse primer 5'-CATAAAAAGATGCATTGGCTCGTCATGAAGAGCCTACTAGATCTTGCTACCAGCCTTTCTCC-3', introducing a *Nsi*I site downstream of the stop codon. The nucleotides between the stop codon and *Nsi*I site are based on the *P. chrysogenum penDE* termination region. The *Nde*I-*Nsi*I fragment was isolated and cloned in the *P. chrysogenum* expression plasmid pISEWA-n [17], yielding the plasmid pIPCLA. From this, the *phl* DNA was amplified using the forward primer 5'-TTTTCACCATTATTTTTCTAGAATCCCATATGGTTTTTTTACCTCC-3' and the same reverse primer as above to insert an *Eco*RI restriction site upstream of the start codon. To create a translational fusion of the PCL with maltose binding protein, the resulting 1.7 kb *Eco*RI-*Nsi*I fragment was used to replace the *cefE* gene behind the *malE* gene in the vector pALJ104 [18], which was partially digested with *Eco*RI and *Nsi*I, 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'-ATCTCAGAATCCCATATGGTTTTTTTACCT and the reverse primer 5'-TTTTTTAAGCTTGATCTTGCTACCAGC. The restriction sites *Eco*RI (forward primer) and *Hind*III (reverse primer) used for cloning are underlined. The 1.7 kb *Eco*RI/ *Hind*III was used to make an in-frame fusion with the histidine 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 by GCT or GCC. All mutations were confirmed by sequencing at GATC Biotech (Konstanz, Germany).

Expression and purification of recombinant PCL

E. coli TOP10 cells (Invitrogen) containing pBAD derived *phl* constructs were precultured overnight in 5 ml LB. The preculture was used to inoculate 1 l of LB medium containing 0.001% (w/v) 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% (w/v) NaN₃ and 10% (w/v) glycerol). Subsequently, cells were sonicated and cell debris was removed by ultracentrifugation. The histidine-tagged enzyme was purified from cell-free extract using Ni affinity chromatography. The enzyme was bound to 3 ml of Ni-NTA 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 (Biorad). Enzyme concentrations were determined spectrophotometrically at 280 nm using a calculated extinction coefficient of 137,370 M⁻¹ cm⁻¹.

To obtain MBP-free protein, the purified MBP-PCL-HIS 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 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 histidine tag as described above. Cleavage and purity of the resulting MBP-free enzyme were analyzed by SDS-PAGE. The concentration of

the cleaved protein was determined spectrophotometrically at 280 nm using a calculated extinction coefficient of $65,695 \text{ M}^{-1} \text{ cm}^{-1}$.

CoA ligase activity assay

Phenylacetate-CoA ligase 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 MgCl_2 . One unit is defined as the catalytic activity resulting in the formation of one μmol of phenylacetylhydroxamate per minute.

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 followed in a Perkin Elmer 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]. Extinction 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, 200 mM NaCl, 3 mM ATP, 1.5 mM CoA, 5 mM MgCl_2 , 10 μM to 20 mM of substrate, and 10 nM of purified recombinant enzyme in a total volume of 1 ml. Reactions were carried out at 30°C and at pH 8.5.

Ligase activity for nonchromogenic substrates was determined by following the substrate-dependent formation of AMP by reversed phase HPLC under the same reaction conditions as described above. Samples were diluted 50-fold in eluents 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 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 Mg^{2+} 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 nonchromogenic 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_{mS_2}) is equal to the inhibition constant on the conversion rate of the visible substrate (v_{SI}) (equation 1).

$$v_{SI} = \frac{E \cdot k_{\text{cat}S_1} [S_1]}{K_{mS_1} \cdot (1 + [S_2]/K_{mS_2}) + [S_1]} \quad \text{Eq. 1}$$

Apparent k_{cat} values for these invisible substrates were determined by following the production of AMP using reversed phase HPLC at concentrations of at least ten times

the apparent K_m value. For the determination of the apparent kinetic parameters of the cosubstrates 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 (Protein Data Bank 2D1S). Figures were prepared using Pymol software (www.pymol.org).

RUSULTS

Expression and purification of PCL

CoA activation of PAA is an essential step in penicillin G biosynthesis. However the biochemical properties of the enzyme catalyzing 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 showed that up to 20% of all protein in CFE of *E. coli* TOP 10 was MBP-PCL and the hydroxamic acid assay showed that the CFE contained up to 20 mU phenylacetate-CoA ligase 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. Therefore further characterization of PCL was performed using a histidine-tagged MBP fusion enzyme. PCR was used to create a construct encoding a fusion protein that contained the N-terminal MBP that appeared 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-agarose, yielding a single protein band upon SDS-PAGE. From a culture of 500 ml, typically 20 mg of recombinant enzyme were 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 phenoxyacetic acid (POA) were determined using the AMP formation assay (Table 1). The enzyme had an apparent k_{cat} of $1.4 \pm 0.3 \text{ s}^{-1}$ and an apparent K_m of

6.1 ± 0.3 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 catalyzed the CoA activation of several substituted phenylacetic acids. Hydrophobic *meta*- or *para*-substituted phenylacetic acids were substrates for PCL. The larger 3-phenylpropionic acid was also a reasonable substrate for PCL while 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-CoA can easily be followed 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 a 1,000-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-methylenedioxycinnamic acid were very good substrates for PCL. To investigate whether the relatively low activity of *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 the 2 and 50 s⁻¹ when the substituent on the

cinnamic acid phenyl ring was changed. A Hammett plot of $\log(k_{\text{cat}, x} / k_{\text{cat}, \text{H}})$ vs. σ_p^- [23] shows a correlation between the observed apparent k_{cat} and the substituent for both electron-donating and electron-withdrawing groups (Fig. 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 to 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 cosubstrates ATP 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 cosubstrates, the apparent K_m^{ATP} was 0.48 ± 0.06 (apparent k_{cat} was $42.9 \pm 1.0 \text{ s}^{-1}$) and the apparent K_m^{CoA} was 0.94 ± 0.10 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_m^{ATP} was 0.65 ± 0.03 mM (apparent k_{cat} was $1.7 \pm 0.2 \text{ s}^{-1}$), and the apparent K_m^{CoA} was 0.12 ± 0.01 mM (apparent k_{cat} $1.5 \pm 0.2 \text{ s}^{-1}$).

Of the carboxylic acid substrates that we screened for, the highest activities were found for fatty acids (Table 1). With the exception of acetic acid, all short and medium chain fatty acids tested were converted by PCL. With increasing chain length the apparent K_m value went down from more than 80 mM for propionic acid (C3) to less than 1 μ M for myristic acid (C14). The apparent K_m values for fatty acids with low K_m values (C10 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 Experimental. 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 long chain fatty acid myristic acid was approximately 10-fold lower than for the short chain fatty acid valeric acid (C5). 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 similar to the native PCL protein, we prepared PCL without the N-terminal MBP-tag. For this, purified histidine 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 histidine tag. The cleaved PCL-HIS

had the same steady state kinetic parameters for cinnamic acid as well as for PAA and POA as MBP-PCL-HIS (PAA: K_m 5.2 ± 0.9 mM, k_{cat} 1.9 ± 0.2 s⁻¹; POA: K_m 2.1 ± 0.1 mM, k_{cat} 12 ± 2.1 s⁻¹ and cinnamic acid: K_m 0.19 ± 0.04 mM, k_{cat} 41.2 ± 1.4 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 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 HIS-MBP-PCL expressed normally in *E. coli* and could be purified using the histidine tag and showed a similar activity with *trans*-cinnamic acid, indicating that functional PCL was produced. However, Factor Xa cleavage of HIS-MBP-PCL to obtain untagged PCL resulted in almost immediate precipitation of the protein after the HIS-MBP part was separated from PCL. Both the N-terminal MBP and the C-terminal histidine tag appear to stabilize PCL.

Homology modelling

The program BLAST 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 catalyze 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 database, 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 luciferase (*Luciola cruciata*) was solved [33]. This structure was determined in complex with an adenylate intermediate analogue at a resolution of 1.3 Å, 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 14 residues form the substrate binding pocket: H265, I266, Y267, V270, F307, F335, G337, A338, G361, T369, V370, and K557 (Fig. 4A). Lysine 557 is located in the C-terminal 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 residues G337, A338 and G361 and the side chains of I266 and T369. 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 residues G337 and A338. 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 (Fig. 4B).

Mutagenesis of substrate binding pocket residues

The role of the side chains of residues H265, I266, Y267, V270, F307, F335, T369 and V370 was investigated by mutating these residues to an alanine. All alanine mutations were made in the histidine-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 *para*-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 carboxylate group for reaction with ATP in the first half reaction. Interestingly, mutant T369A had a 3.7-fold increased apparent k_{cat} for PAA. In the model T369 is located at the top of the tunnel close to the carboxylate binding site. Removal of the side chain of residue T369 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. Mutants Y267A and F307A and F335A did show a moderate (3 to 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 Y267 and with residues F307 and F335 located further away in the substrate binding tunnel. Mutation of one of these residues into an 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 suggest 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 (Fig. 4B).

Mutations V270A and V370A 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 mutant V270A showed a 10-fold lower apparent k_{cat} for POA and mutant V370A 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 PAA-CoA ligase 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 this study we report the overexpression of phenylacetate-CoA ligase 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-HIS protein could be removed by digestion with Factor Xa protease, but the cleaved PCL-HIS could be purified and showed CoA ligase activity but was less stable.

Recombinant MBP-PCL-HIS 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 extinction coefficients of the aromatic enoyl-CoA products is relatively high (around $20 \text{ mM}^{-1} \text{ cm}^{-1}$), providing a practical and sensitive activity assay for PCL. Formation of as little as one micromolar of 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 coproduct 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 extinction coefficient of the formed iron-hydroxamate complex depends on the acid used and is relatively low (*e.g.* $\epsilon_{\max} = 0.9 \text{ mM}^{-1} \text{ cm}^{-1}$ for phenylacetyl-hydroxamate), making the assay not very sensitive. For some substrates, such as the amino acid phenylglycine, the extinction coefficient was even ten times 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 followed the conversion of *trans*-cinnamic acid both with the spectrophotometric method as well as with AMP formation on HPLC, which yielded the same results.

Comparison of the biochemical properties indicates that PCL, encoded by *phl*, differs from the CoA ligase that was earlier purified from *P. chrysogenum* by Kogekar *et al.* [5]. We found apparent K_m values of 6.1 and 2.0 mM for PAA and POA, respectively, whereas Kogekar *et al.* 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 phenylacetyl-CoA ligase activity, which may be the enzyme purified by Kogekar *et al.* 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 phenylacetyl-CoA ligase 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 phenylacetic acid and may be responsible for the remaining phenylacetyl-CoA ligase activity that is used for penicillin G biosynthesis in a PCL

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 phenylacetic acid 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 to that with *trans*-cinnamic acids and fatty acids. Nonetheless, we maintain the name phenylacetate-CoA ligase because of its role in penicillin G biosynthesis. The broad substrate range combined with relatively low activity for PAA makes PCL very different from phenylacetate-CoA ligases involved in PAA catabolism from bacteria such as *A. evansii* [12] and *P. 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 phenylacetate-CoA ligases. 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 *P. 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 penicillins [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 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 acyl-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 CoA activation occurs 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 if fatty acid activation by PCL plays a physiological role in β -oxidation in *Penicillium*, but the formation of β -lactam side products containing fatty acyl 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 the side chain. 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 presented here show that PCL also

has a broad substrate range and is able to activate the side chains of all the natural β -lactam side products that are observed.

The observed selectivity 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 observed broad substrate range 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, likely 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 the 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 the penicillin biosynthesis, *penAB*, *penC* and *penD* [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 relation between these two enzyme activities.

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Table 1. Steady state kinetic parameters of PCL.

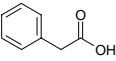
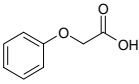
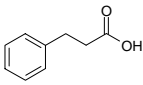
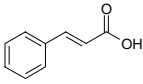
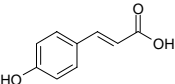
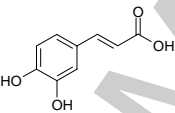
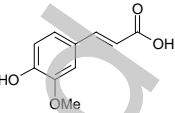
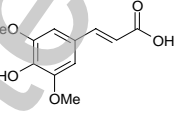
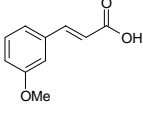
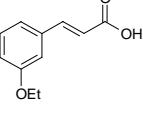
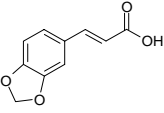










Substrate		k_{cat}	K_{m}	$k_{\text{cat}} / K_{\text{m}}$
<i>aromatic acids</i>		(s^{-1})	(mM)	($\text{mM}^{-1} \text{s}^{-1}$)
phenylacetic acid		1.4 ± 0.3	6.1 ± 0.3	0.23 ± 0.06
phenoxyacetic acid		15.6 ± 1.6	2.0 ± 0.2	7.8 ± 1.2
3-phenylpropionic acid		22.7 ± 1.9	2.1 ± 0.4	10.8 ± 2.3
<i>trans</i> -cinnamic acid		43.5 ± 2.7	0.14 ± 0.01	$(3.1 \pm 0.4) \cdot 10^2$
<i>trans</i> -4-coumaric acid		3.4 ± 0.5	0.47 ± 0.08	7.2 ± 1.7
caffeic acid ^a		>0.02	>1.0	0.02 ± 0.01
ferulic acid ^a		>0.07	>0.5	0.07 ± 0.01
sinapic acid ^a		na ^b	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-methylenedioxy cinnamic acid		11.2 ± 1.9	0.54 ± 0.04	21 ± 5

Table 1. continued

Substrate		k_{cat}	K_{m}	$k_{\text{cat}} / K_{\text{m}}$
<i>aliphatic acids</i>		(s^{-1})	(mM)	($\text{mM}^{-1} \text{s}^{-1}$)
acetic acid		na ^c	na	na
propionic acid		>1	>80	$(1.3 \pm 0.1) \cdot 10^{-2}$
butyric acid		9.7 ± 0.4	24.4 ± 3.9	$(4.0 \pm 0.7) \cdot 10^{-1}$
valeric acid		18.5 ± 0.9	0.7 ± 0.2	$(2.6 \pm 0.9) \cdot 10^2$
caproic acid		15.8 ± 0.5	0.24 ± 0.02	$(6.6 \pm 0.7) \cdot 10^1$
enanthic acid		12.3 ± 3.9	0.05 ± 0.03	$(2.5 \pm 1.8) \cdot 10^2$
caprylic acid		11.2 ± 0.7	0.06 ± 0.01	$(1.9 \pm 0.4) \cdot 10^2$
capric acid		8.2 ± 1.7	$(0.2 \pm 0.1) \cdot 10^{-2}$	$(4.1 \pm 2.3) \cdot 10^3$
lauric acid		8.5 ± 1.8	$(0.4 \pm 0.2) \cdot 10^{-3}$	$(2.1 \pm 1.2) \cdot 10^4$
myristic acid		2.3 ± 0.1	< 0.0005	$>4.6 \cdot 10^3$

^a Data for these compounds were determined at pH 7.5

^b na, no detectable activity at 4 mM substrate (< 0.01 s^{-1})

^c na, no detectable activity at 20 mM substrate (< 0.01 s^{-1})

Table 2. Kinetic parameters and σ_p^- values for *para* substituted *trans*-cinnamic acids

	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} / K_m (mM ⁻¹ s ⁻¹)	σ_p^- ^a
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 ± 0.3) · 10 ²	-0.26
4-methylcinnamic acid	21.0 ± 0.1	0.32 ± 0.01	(6.6 ± 0.3) · 10 ¹	-0.17
4-isopropylcinnamic acid	9.6 ± 0.3	0.07 ± 0.02	(1.4 ± 0.5) · 10 ²	-0.16
4-fluorocinnamic acid	35.5 ± 1.4	0.084 ± 0.017	(4.2 ± 0.9) · 10 ²	-0.03
cinnamic acid	43.5 ± 2.7	0.14 ± 0.01	(3.1 ± 0.4) · 10 ²	0.00
4-chlorocinnamic acid	29.1 ± 0.6	0.16 ± 0.05	(1.8 ± 0.6) · 10 ²	0.19
4-trifluoromethylcinnamic acid	11.3 ± 1.2	0.19 ± 0.07	(6.0 ± 0.3) · 10 ¹	0.65
4-nitrocinnamic acid	2.8 ± 0.1	0.51 ± 0.01	5.5 ± 0.3	1.27

^a Values from Hansch *et al.* (22)

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Table 3. CoA ligase activity of PCL with side chains precursors of natural penicillins.

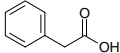
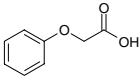
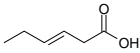
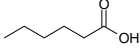
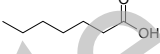
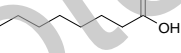
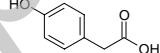
Product	Side-chain precursor	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}} / K_{\text{m}}$ ($\text{mM}^{-1} \text{s}^{-1}$)
penicillin G	phenylacetic acid 	1.4 ± 0.3	6.1 ± 0.3	$(2.3 \pm 0.6) \cdot 10^{-1}$
penicillin V	phenoxyacetic acid 	15.6 ± 1.6	2.0 ± 0.2	7.8 ± 1.2
penicillin F	<i>trans</i> -3-hexenoic acid 	14.7 ± 1.2	0.12 ± 0.01	$(1.2 \pm 0.2) \cdot 10^2$
penicillin DF	caproic acid 	15.8 ± 0.5	0.24 ± 0.02	$(6.6 \pm 0.7) \cdot 10^1$
penicillin H	enanthic acid 	12.3 ± 3.9	0.05 ± 0.03	$(2.5 \pm 1.8) \cdot 10^1$
penicillin K	caprylic acid 	11.2 ± 0.7	0.06 ± 0.01	$(1.9 \pm 0.4) \cdot 10^1$
penicillin X	hydroxyphenylacetic acid 	0.08 ± 0.02	5.7 ± 0.4	$(1.4 \pm 0.4) \cdot 10^{-2}$

Table 4. Kinetic parameters of PCL wild type and mutants with substrates PAA, POA, caproic acid, *trans*-cinnamic acid and *para*-coumaric acid.

	PAA		POA		caproic acid		cinnamic acid		coumaric acid	
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_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	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	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	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 LEGENDS

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, catalyzing the exchange, requires the CoA activated form of the phenylacetyl side chain. The two-step activation of phenylacetic acid is catalyzed by phenylacetate-CoA ligase. The adenylate intermediate is characteristic for all the members of the superfamily of adenylate forming enzymes.

Figure 2. A Hammett plot of $\log(k_{\text{cat, x}}/k_{\text{cat, H}})$ vs. 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 ($r = 1.00$).

Figure 3. Sequence alignment of PCL, firefly luciferases and coumarate-CoA ligases. The following sequences were used (EMBL accession numbers in parentheses): LcrLuc, luciferase form *Luciola cruciata* (BAE80731); PpyLuc, luciferase form *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 LcrLucif 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 LcrLucif and the putative substrate binding residues in 4-coumarate CoA ligases (26) as well as the corresponding residues in PCL are highlighted in black.

Figure 4. Active site residues in the structure of LcrLucif 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. Residue 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).

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Figures

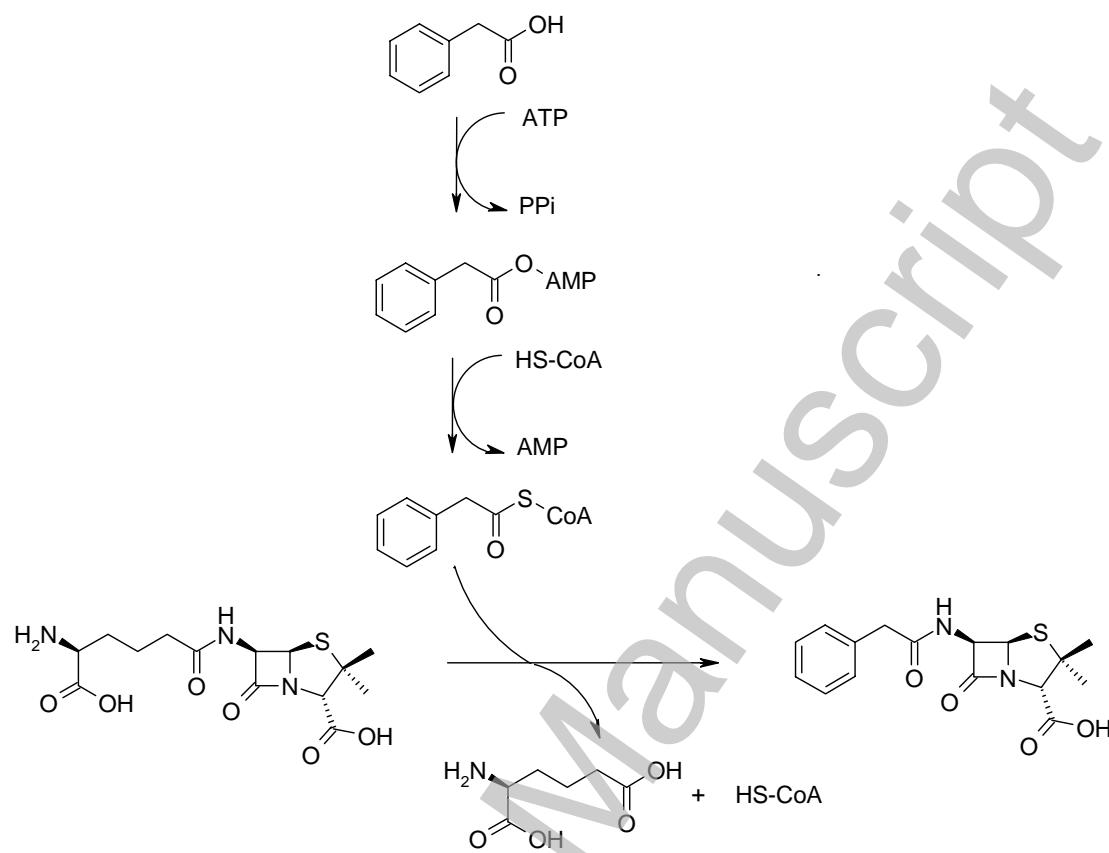


Figure 1

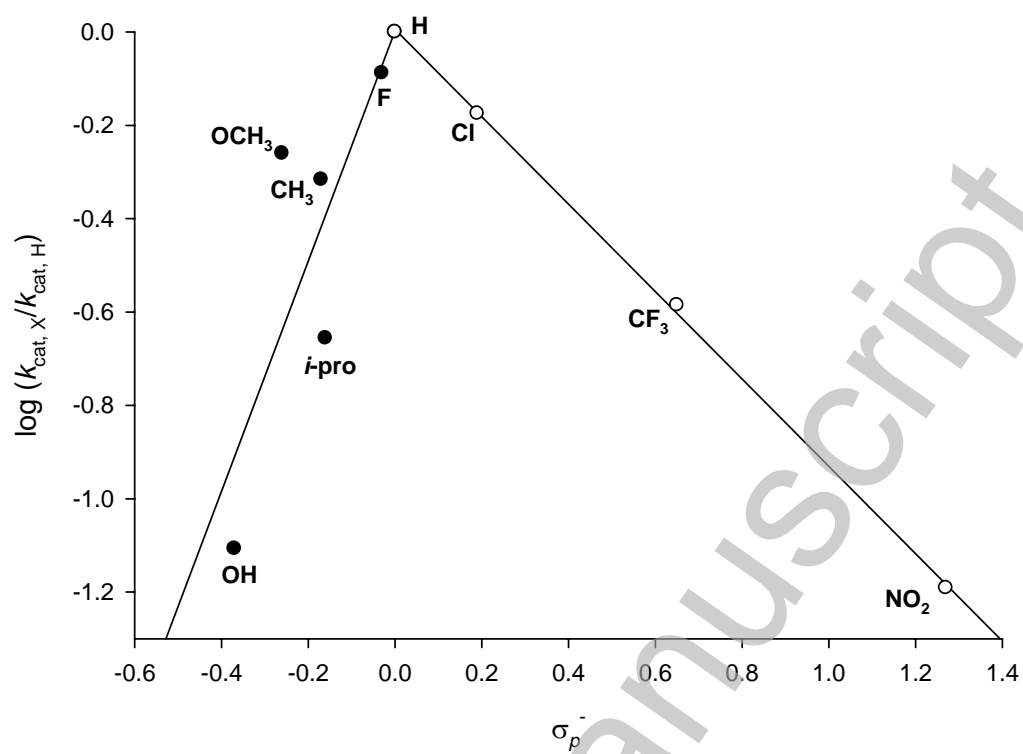


Figure 2

---Motif 1---

PCL	216	SGLPKGVMISHRNVIAN TLQIKAFEQNYRDGGGTPASTEVALG LLEPQSHIYALVWIGHA
LcrLuc	204	TGLPKGVQLTHENIVTRF SHARDPIY-----GNQVSP-GTAVLTVV PFFHHGFGMFTTLG-
PpyLuc	202	TGLPKGV ALPHRTACVRF SHARDPIF-----GNQIIP-DTAILSVV PFFHHGFGMFTTLG-
ScCCL	183	TGTPKGVMLTHRQIATN LAQLEP-----SMPSAPGDRVLAVL PFFHIIYGLTALMNA
At4CL2	207	TGLPKGVMLTHKGLVTSVAQQVDGEN-----PNLYFN RDDVILCVWPMFH IYALNSIMLC
At4CL1	214	TGLPKGVMLTHKGLVTSVAQQVDGEN-----PNLYFH SDDVILCVLPMFH IYALNSIMLC
At4CL3	217	TGLPKGVV LTHKSLITSVAQQVDGDN-----PNLYLKSNDVIL CVLPLFHIYSLNSVLLN

PCL	276	GAYRGDQ TIVLPKFELKSYLNAIQQYKISALFLVPP I I IHMLGTQDVCSKYDLS SVTSLF
LcrLuc	257	YLICGFRVVM LTKFDEETFLKTLQDYKCTSVILVPTLFAILN-KSELLNKYDLSNLVEIA
PpyLuc	255	YLICGFRVVM LMYRFEELFLRSLQDYKIQSALLVPTLFSFFA-KSTLIDKYDLSNLHEIA
ScCCL	234	PLRLGATVVV LPRFDLEQFLAAIQNHRTISLYVAPP IVLALA-KHPLVADYDLS SLRYIV
At4CL2	262	SLRIGATILIMP KFEITLLEQIQRCKVTVAMVVPPIVLAIA-KSPETEKYDLS SVRMVK
At4CL1	269	GLRVGAAI LIMPKFEINLLELIQRCKVTVAPMVPP IVLAIA-KSSETEKYDLS SIRVVK
At4CL3	272	SLRSGATVLLM HKFEIGALLDLIQRHRVTIAALVPP LVIALA-KNPTVNSYDLS SVRFVL

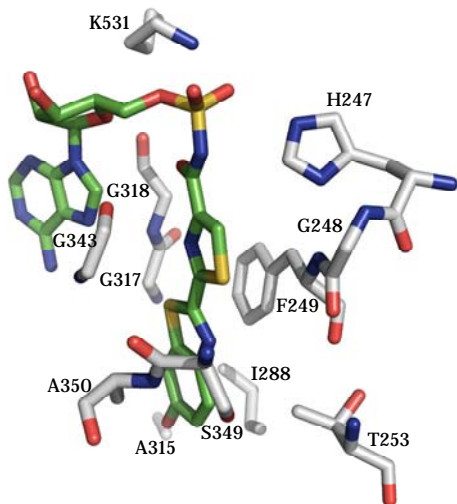
--Motif 2--

PCL	336	TGAAPLGMETAADFLKLYPNILIRQGYGLTETCIVV STH-----PHDIWLGSSGALLPG
LcrLuc	316	SGGAPLSKEVGEAVARRFNLP GVRQGYGLTETTSAIIITP-----EGDDKPGASGKV VPL
PpyLuc	314	SGGAPLSKEVGEAVAKRFHLP GIRQGYGLTETTSAIITP-----EGDDKPGAVGKV VPF
ScCCL	293	SAAAPLDARLAAACSQR LGLPPVQGAYGMTELSPGTHVVP--LDAMADAPP GTVGRLIAG
At4CL2	321	SGAAPLGKELED AISAKFPNAKLGQGYGMTEAGPVLAMSLGFAKEPFPVKSGACGTVVRN
At4CL1	328	SGAAPLGKELED AVNAKFPNAKLGQGYGMTEAGPVLAMSLGFAKEPFPVKSGACGTVVRN
At4CL3	331	SGAAPLGKELQDSLRRRLPQA IILGQGYGMTEAGPVLAMSLGFAKEPIPTKSGSCGTVVRN

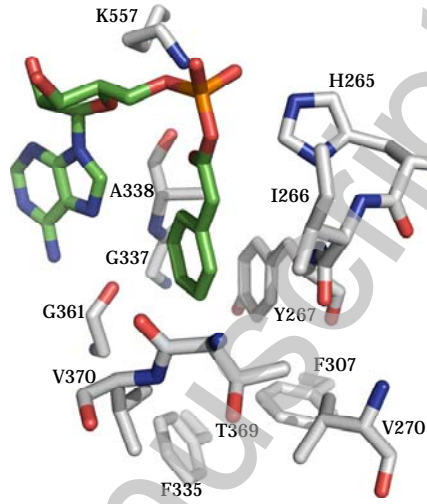
Figure 3

A

LcrLucif



PCL



B

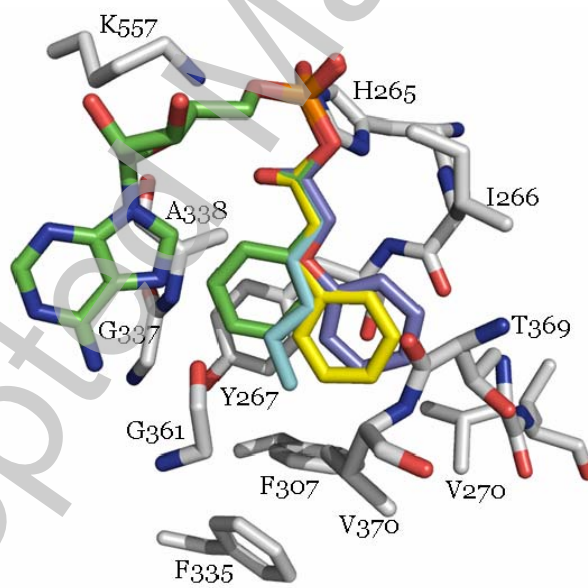


Figure 4

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