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Aminoacyl-coenzyme A synthesis catalyzed by a CoA ligase from *Penicillium chrysogenum*

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ABSTRACT

Coenzyme A ligases play an important role in metabolism by catalyzing the activation of carboxylic acids. In this study we describe the synthesis of aminoacyl-coenzyme As (CoAs) catalyzed by a CoA ligase from *Penicillium chrysogenum*. The enzyme accepted medium-chain length fatty acids as the best substrates, but the proteinogenic amino acids L-phenylalanine and L-tyrosine, as well as the non-proteinogenic amino acids D-phenylalanine, D-tyrosine and (*R*)- and (*S*)-β-phenylalanine were also accepted. Of these amino acids, the highest activity was found for (*R*)-β-phenylalanine, forming (*R*)-β-phenylalanyl-CoA. Homology modeling suggested that alanine 312 is part of the active site cavity, and mutagenesis (A312G) yielded a variant that has an enhanced catalytic efficiency with β-phenylalanines and D-α-phenylalanine.

1. Introduction

Coenzyme A (CoA) ligases (or CoA synthetases) play an important role in nature by catalyzing the activation of carboxylic acids which allows for their further metabolism. For example, CoA ligases activate fatty acids to initiate β-oxidation [1] and are important in the biosynthesis of natural products such as lignin [2] and penicillins [3] (Fig. 1). The mammalian fatty-acyl-CoA ligases also function in the detoxification of diverse xenobiotic compounds such as pesticides and drugs [4]. CoA ligases are members of the superfamily of adenylate-forming enzymes and activation proceeds in a two-step reaction (Fig. 2a). In the first half reaction, the substrate reacts with adenosine 5′-triphosphate (ATP) to form an acyl-adenylate intermediate with the simultaneous release of pyrophosphate. In the second half reaction, the adenylate group is replaced by CoA and adenosine 5′-monophosphate (AMP) is released.

The family of CoA ligases accepts a wide range of substrates. For example, the group of fatty-acyl-CoA synthetases activates fatty acids such as the short-chain acetate to the long-chain acid linoleic acid (C20). Despite this broad substrate range, amino acids have not been described to be accepted as substrates.

Activated amino acids play an important role in metabolic processes such as protein and peptide synthesis, but the activation is then not catalyzed by CoA ligases. In protein biosynthesis amino acid activation is achieved by aminoacyl tRNA-synthetases, whereas in the biosynthesis of small peptides, such as the antibiotic gramicidin S, amino acid activation is catalyzed by the adenylation domains of non-ribosomal peptide synthetases (NRPSs) [5]. The aminoacyl tRNA synthetases and the adenylation domains of the NRPSs are also members of the superfamily of adenylate-forming enzymes. All these enzymes share formation of an adenylate intermediate after the first reaction step (Fig. 2b). In the CoA ligases this intermediate reacts with CoA to form the CoA-thioester while in the tRNA synthetases and in the non-ribosomal peptide synthetases the activated (α-amino)acyl group is transferred to the 3′ hydroxyl group of the tRNA molecule or the phosphopantheteine group of a peptidyl carrier domain, respectively.

A low level of aminoacyl-CoA ligase activity has previously been described as a promiscuous activity of aminoacyl tRNA-synthetases [6] and of the adenylation domains of some NRPSs [7], suggesting there might also be CoA ligases that are able to catalyze CoA activation of amino acids. Aminoacyl-CoA ligase activity was also suggested to be important for the epimerization of the side chain of isopenicillin N in *Penicillium chrysogenum* [8], but the enzyme responsible for CoA activation of isopenicillin N was not characterized.

Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CoA, coenzyme A; HPLC, high-performance liquid chromatography; NRPS, non-ribosomal peptide synthetase; PPi, inorganic pyrophosphate

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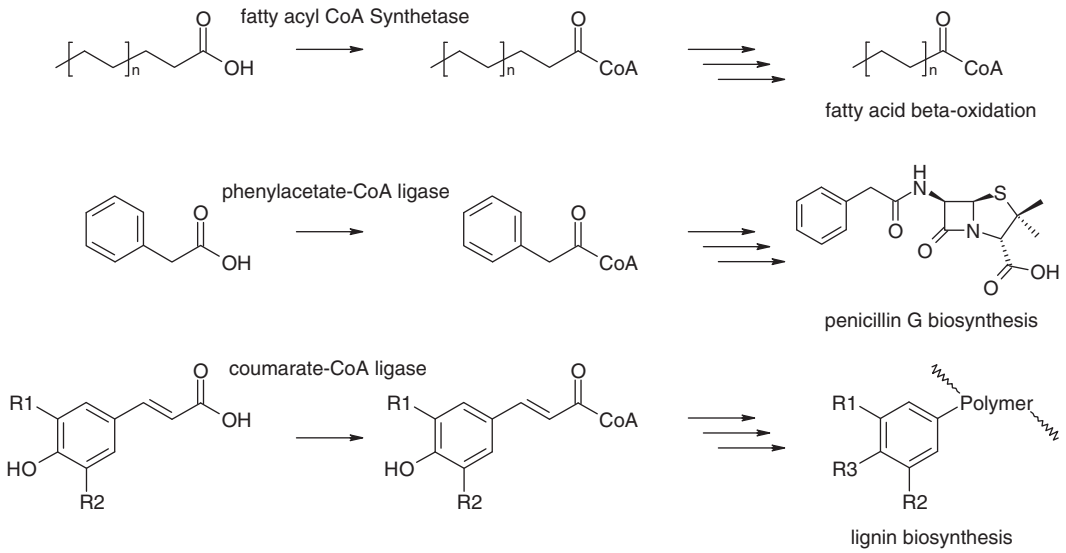

Fig. 1. Examples of CoA ligases in metabolic pathways. Fatty-acyl-CoA synthetases catalyze the first step in the β-oxidation of fatty acids. The activation of the penicillin G side chain phenylacetic acid is catalyzed by phenylacetate-CoA ligase in *Penicillium chrysogenum*. CoA activation of coumarate derivatives in the biosynthesis of the complex phenolic polymer lignin, in cells of higher plants.

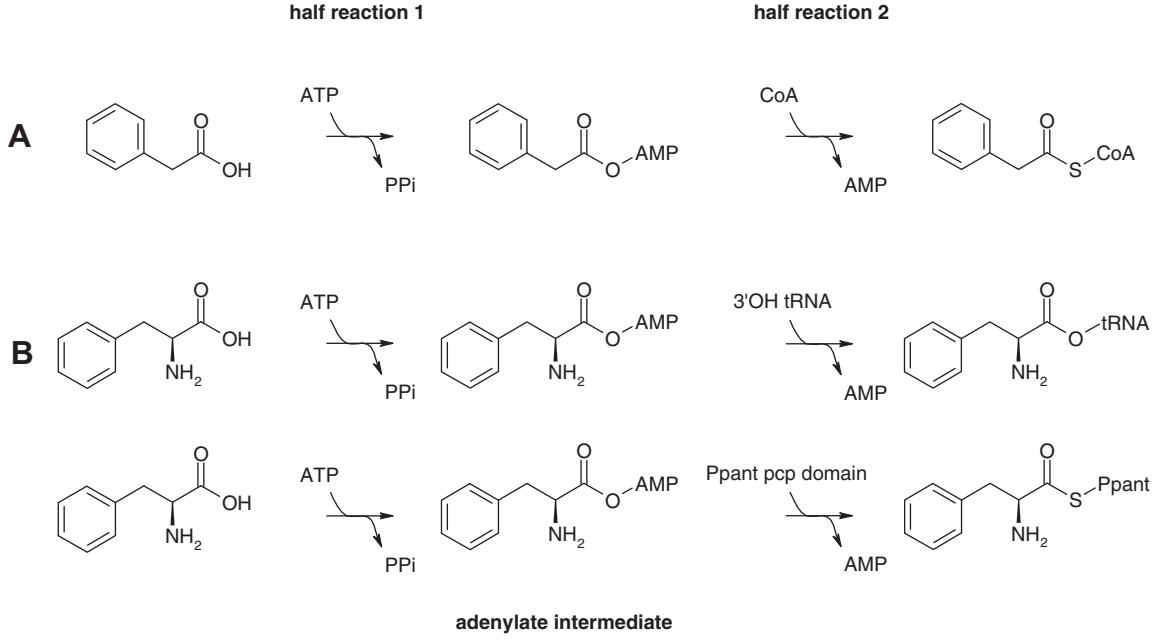

Fig. 2. Reactions catalyzed by members of the family of adenylate-forming enzymes. (A) phenylacetic acid activation catalyzed by a CoA ligase. (B) Ribosomal and non-ribosomal activation of L-phenylalanine.

The genome of *P. chrysogenum* contains a large number of genes encoding CoA ligases [9], several of which have been characterized. A cytosolic acetate-CoA synthetase was shown to have a broad substrate range and was also able to catalyze the activation of side chains that can be coupled to the β-lactam nucleus in vitro [10]. We have recently characterized a phenylacetyl-CoA ligase (PCL, Pc22g14900) and an acyl-CoA ligase (ACLA, Pc22g20270) and although these enzymes are involved in the side-chain activation of penicillins and cephalosporins, respectively, they both are acyl-CoA ligases with broad substrate specificities that show the highest catalytic activities towards medium and long-chain fatty acids. In the present work we describe the cloning and overexpression of a homologous and uncharacterized acyl-CoA ligase from *P. chrysogenum* that has the ability to catalyze the synthesis of aminoacyl-CoAs.

2. Materials and methods

2.1. Materials

We used the CoA ligase, encoded by gene Pc21g20650, from *P. chrysogenum* Wisconsin 54-1255 [9]. The gene was amplified from a cDNA library [11] and subsequently cloned downstream of the *malE* gene and in frame with a hexahistidine tag of vector pBAD-MBP [12]. The resulting construct, encoding an MBP fusion protein, was introduced in *Escherichia coli* using the pBAD vector (Invitrogen) and expressed as described previously [13]. The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce mutation A312G using primers Pc21g20650A312Gfw (5′-CAAGA TCATGTCTGCCGGCGCACCCCTTACTATT G-3′, mutated codon is underlined) and Pc21g20650A312Grv (5′-CAATAGTAAGGGGTGC

GCCGGCAGACATGATCT TG-3′, mutated triplet is underlined). Production and purification of wild-type and mutant CoA ligase were also done essentially as described earlier [13]. The amino acids (*S*)- and (*R*)-β-phenylalanine were obtained from Peptech Corporation. All other chemicals were from Sigma–Aldrich.

2.2. CoA ligase activity assay

CoA ligase activity was determined by following the substrate-dependent formation of AMP by reversed-phase high-performance liquid chromatography (HPLC) under essentially the same conditions as described previously [13]. Standard reaction mixtures contained 50 mM Tris–HCl, 200 mM NaCl, 3 mM ATP, 1.5 mM CoA, 5 mM $MgCl_2$, 50 μM to 100 mM of substrate, and 10 nM–2 μM of purified recombinant enzyme in a total volume of 200 μl. Reactions were carried out at 30 °C and at pH 8.5. Substrate-independent ATP hydrolysis by the CoA ligase was not observed ($k_{obs} < 0.01\ s^{-1}$).

Apparent kinetic constants K_m and k_{cat} for CoA ligase substrates were determined by varying the concentration of one substrate at fixed concentrations 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. Kinetic parameters for cosubstrates ATP and CoA were determined using (*R*)-β-phenylalanine, fixed at 35 mM as the substrate.

2.3. LC–MS/MS identification of aminoacyl-CoAs

The formation of aminoacyl-CoAs was confirmed by electrospray mass spectrometry using a modified version of a described protocol [14] in an LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific). Separation of reaction components was performed by LC using acidic eluent on a C18, 3.5-μm column. Aminoacyl-CoAs were eluted using a linear gradient (eluent A, 10:90 acetonitrile/water containing 0.1% TFA and eluent B, 100% acetonitrile containing 0.1% TFA). Positive ion mode mass spectra were collected over a scan range of *m*/*z* 200–1000.

2.4. Homology modeling of aminoacyl-CoA ligase and docking

For homology model building and docking both online servers (http://www.cbs.dtu.dk/services/CPHmodels/ and http://swissmodel.expasy.org/) and YASARA software (www.yasara.org) were used [15–17]. CPHmodels3.0 selected 3A9V as a template while Swiss model selected 2D1R. YASARA made models based on PDB structures of luciferase (2D1T, 2D1R, 2D1S, 3IEP, 1BA3 [18–20]), long-chain-fatty-acid CoA ligase (3G7S), and coumarate-CoA ligase (3A9U, 3A9V, 3NI2 [21]) as templates. Docking was carried out with the YASARA model using AutoDock4 [22] (350 separate runs). Figures were prepared using Pymol software (www.pymol.org).

3. Results and discussion

3.1. Formation of aminoacyl-coenzyme A

The low levels of aminoacyl-CoA ligase activity observed in the structurally related NRPSs and in tRNA synthetases suggest the existence of CoA ligases that possess aminoacyl-CoA ligase activity. In this study, the CoA ligase from *P. chrysogenum* encoded by the gene Pc21g20650 was tested for the ability to catalyze the synthesis of various aminoacyl-CoAs. The protein shares sequence similarity to *P. chrysogenum* CoA ligases (PCL, ACLA) involved in antibiotic synthesis that were previously characterized [3,13].

Analysis of CoA-dependent AMP production was used to determine which (amino) acids were accepted as substrates. We tested the proteinogenic amino acids and the amino acids D-Ala, D-Val, D-Leu, D-Ser, D-Thr, D-Met, D-Tyr, D-Phe, (*R*)- and (*S*)-norleucine, (*R*)- and (*S*)-phenylglycine, (*R*)- and (*S*)-hydroxyphenylglycine and (*R*)- and (*S*)-β-phenylalanine. Both L-phenylalanine and L-tyrosine were accepted, and activity was also observed with D-phenylalanine, D-tyrosine and (*R*)- and (*S*)-β-phenylalanine. The enzyme also had activity with the medium-chain fatty acids hexanoic, heptanoic, and octanoic acid, which are the preferred substrates of PCL and ACLA.

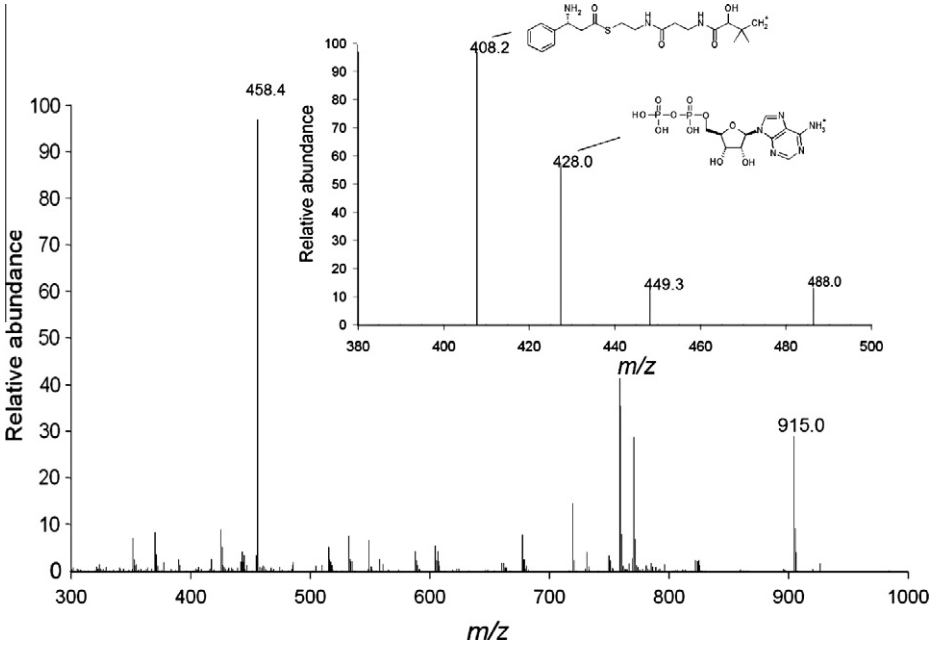

Fig. 3. MS and MS/MS fragmentation of protonated (*R*)-β-phenylalanyl-CoA. The MS shows the parent ion in the single (*m*/*z* of 915) and double (*m*/*z* of 458) protonated form. The MS/MS fragmentation (parent ion 915 *m*/*z*, collision energy 35 eV) shows the fragmented (*R*)-β-phenylalanylpantetheine molecule after a neutral loss of 507 (*m*/*z* of 408) in the positive MS/MS mode. Similar results were obtained for the other isomers L-α-, D-α and (*S*)-β-phenylalanyl-CoA.

Table 1
Steady-state kinetic parameters of (amino)acyl-CoA ligase activity of acyl-CoA ligase (Pc21g20650) from *P. chrysogenum*.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
L-α-Phenylalanine	0.37 ± 0.13	44.7 ± 2.8	$(8.2 \pm 2.9) \times 10^{-3}$
L-α-Tyrosine	0.007 ± 0.004	3.5 ± 1.2	$(2.0 \pm 1.4) \times 10^{-3}$
D-α-Phenylalanine	1.4 ± 0.2	83.0 ± 6.4	$(1.7 \pm 0.3) \times 10^{-2}$
D-α-Tyrosine	0.06 ± 0.03	1.6 ± 0.8	$(3.8 \pm 2.7) \times 10^{-2}$
(*S*)-β-Phenylalanine	0.25 ± 0.02	33.9 ± 0.1	$(7.4 \pm 0.7) \times 10^{-3}$
(*R*)-β-Phenylalanine	3.0 ± 0.2	3.5 ± 0.4	0.86 ± 0.12
ATP	3.3 ± 0.4	0.78 ± 0.01	
CoA	3.9 ± 1.1	2.0 ± 0.4	
Hexanoic acid	17.4 ± 2.22	1.63 ± 0.21	$(1.1 \pm 0.2) \times 10^{1}$
Heptanoic acid	10.8 ± 0.05	0.94 ± 0.06	$(1.2 \pm 0.1) \times 10^{1}$
Octanoic acid	7.3 ± 0.2	0.15 ± 0.01	$(4.9 \pm 0.4) \times 10^{1}$

Table 2
Comparison of steady state kinetic parameters of phenylalanine activation catalyzed by the CoA ligase, tRNA-synthetase and by the adenylation domain of non-ribosomal peptide synthetases.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	Reference
Acyl-CoA ligase				
L-α-Phenylalanine	0.37	44.7	0.0082	This work
D-α-Phenylalanine	1.4	83.0	0.017	This work
(*R*)-β-Phenylalanine	3.0	3.5	0.86	This work
Phenylalanyl tRNA synthetase				
L-α-Phenylalanine[a]	240	0.0042	57,000	[26]
L-α-Phenylalanine[b]	1.72	0.0103	177	[25]
A-domain PheATE NRPS Gramicidin S				
L-α-Phenylalanine[c]	0.001	0.03	0.033	[24]
D-α-Phenylalanine[c]	0.001	0.02	0.05	[24]

[a] Phenylalanine-tRNA synthetase from *Saccharomyces cerevisiae*.
[b] Phenylalanine-tRNA synthetase from *Escherichia coli*.
[c] Kinetic parameters of the adenylation reaction. The low turnover number is due to the slow release of phenylalanyl adenylate product.

HPLC–MS/MS analysis was used to detect the formation of the aminoacyl-CoA adducts from phenylalanines. The observed cleavage of ATP was coupled to the formation of phenylalanyl-CoA. For both enantiomers of α- and β-phenylalanine, the MS data showed the presence of the parent ion in the single (m/z of 915) and double (m/z of 458) protonated form (Fig. 3). The MS/MS fragmentation of protonated phenylalanyl-CoA showed the diffracted phenylalanyl pantetheine molecule with a neutral loss of 507 (m/z of 408) in the positive MS/MS mode.

The kinetic parameters of aminoacyl-CoA formation were determined by fitting the initial rate of AMP formation with the Michaelis–Menten equation (Table 1). The CoA ligase showed a preference for the *R*-enantiomers of the substrates. As shown in Table 1, the turnover number for (*R*)-D-phenylalanine, (*R*)-D-tyrosine and (*R*)-β-phenylalanine was approximately 10-fold higher than with the corresponding (*S*)-enantiomers. For β-phenylalanine the affinity was also 10-fold higher for the (*R*)-enantiomer, resulting in the relatively high catalytic efficiency observed for (*R*)-β-phenylalanine.

The measured apparent k_{cat} values for the acylation of the amino acids are at least 10-fold lower than for the fatty acids hexanoic, heptanoic and octanoic acid (Table 1) and also the apparent K_m values are higher for the amino acids. The kinetic parameters for the fatty acids are very similar to what was found for PCL and ACLA [3,13] suggesting that the CoA ligase is also a fatty acyl-CoA ligase. The proteinogenic amino acids phenylalanine, tyrosine, alanine and glycine were also tested as substrates for PCL and ACLA but no activity was found [3, unpublished data] suggesting that the ability to accept amino acids as a substrate is not a general feature of the CoA ligases from *P. chrysogenum*.

The apparent turnover number measured with CoA ligase Pc21g20650 was several orders of magnitude higher than what was reported for the aminoacylation of CoA catalyzed by the tRNA-synthetases [6,23]. However, in this case the aminoacyl-CoA formation is a promiscuous activity with CoA replacing the natural nucleophile of the second half reaction (Fig. 2), which may explain the low turnover rates. Accordingly, the natural phenylalanine activation reactions catalyzed by tRNA synthetases have much higher observed turnover rates (Table 2). On the other hand, compared to adenylation (A) domains of non-ribosomal peptide synthetases (NRPS), the rates observed with the CoA ligase described here appear higher [7,24], but it should be noted that the low turnover number observed with the adenylation domain of the gramicidin S synthetase initiation module (PheATE) is caused by the slow release of the phenyladenylate. However, the affinity of the Pc21g20650 CoA ligase for phenylalanine is very low compared to that measured with the tRNA synthetases and the A domains of the NRPS, which all have K_m values in the micromolar range [24–26]. Summarized, the current CoA ligase is an unspecific acyl-CoA ligase accepting phenylalanine as a substrate, whereas the tRNA synthetases and the NRPSs are very specific for their amino acid substrate and tolerate CoA as an alternative nucleophile in the second half reaction.

3.2. Improved conversion of amino acids by mutagenesis of the putative binding site

Phenylalanine is a rather good substrate for the CoA ligase, but the specificity for this substrate is low compared to activation of fatty acids or compared to activation of (natural) amino acids catalyzed by tRNA synthetases and NRPSs. This is caused by the relatively low affinity for the amino acid (Table 2). To obtain further insight in the causes of the selectivity of Pc21g20650 CoA ligase with amino acids and carboxylic acids, we used homology modeling and site-directed mutagenesis.

Homology modeling was used to detect residues that may influence substrate selectivity. Three crystal structures of proteins that have considerable sequence similarity to CoA ligase were used: luciferase, long-chain fatty acid CoA ligase and coumarate-CoA ligase. A comparison between the homology models prepared by CPHmodels3.0, SWISS-MODEL and YASARA (see Section 2) yielded model structures with similar backbone folds (backbone RMSD 3.8–12.1 Å), with minor differences near the active site. The backbone RMSD within a range of 6 Å of the bound intermediates in Fig. 4B is only 1.5–3.0 Å. Besides these relatively mild differences in backbone positions there are more pronounced differences in the predicted position of the side chains, as shown in Fig. 4A for the predicted substrate binding site residues A312, Y242 and H240. A YASARA predicted structure based on coumarate-CoA ligase (3A9V, sequence identity 33%) was selected for docking since it had the highest score for structural quality. Automated docking of the (*R*)-β-Phe-AMP intermediate resulted in a predicted binding mode where the AMP tail is almost at the same position as that of a co-crystallized intermediate in coumarate-CoA ligase (3NI2, yellow in Fig. 4B). The (*R*)-β-Phe group of the docked (*R*)-β-Phe-AMP is at a slightly different position as compared to the phenolic group in 3NI2. However, both in the crystallographic coumarate intermediate and the docked the (*R*)-β-Phe intermediate, the C_α and C_β of the activated substrate would be near the position of A312 in the aligned structures. This residue is on a loop that protrudes into the binding pocket of the intermediate. Thus, even though the homology modeling did not produce atomistic detail, it suggested that A312 is near the C_α or C_β atom of the bound activated substrate.

To test the putative role of A312 in substrate binding and to check the possibility of improving the activation of amino acids through mutagenesis, we constructed mutant A312G, which was expected to have a larger pocket. The mutation had clear effects

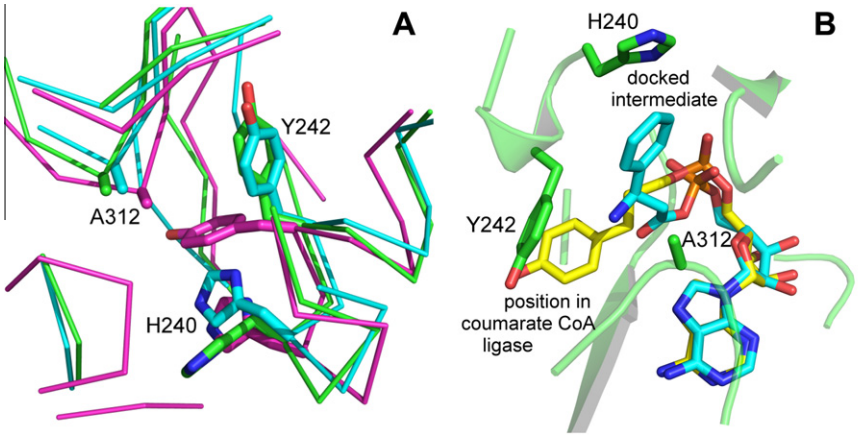

Fig. 4. Homology modeling and docking of substrate. (A) Superposition of three separately prepared homology models of *P. chrysogenum* CoA ligase. Protein backbones within 6 Å of the displayed binding pocket residues are shown as C_α atom traces. Light-blue, model produced by the CPH models server; purple, model produced by the Swiss-Model server; green, top-ranked model produced by YASARA software. (B) Comparison of the top-ranked docked intermediate (light blue in green protein) with the position of the intermediate observed in the crystal structure of coumarate-CoA ligase (yellow).

Table 3
Steady-state kinetic parameters of mutant CoA ligase A312G.

Substrate	k_{cat}		K_m		k_{cat}/K_m	
	(s^{-1})	*r*	(mM)	*r*	(mM s^{-1})	*r*
L-α-Phenylalanine	0.14 ± 0.02	0.4	38.7 ± 3.2	0.9	$(3.6 \pm 0.6) \times 10^{-3}$	0.4
L-α-Tyrosine	0.035 ± 0.01	5	5.9 ± 0.4	1.7	$(6 \pm 2) \times 10^{-3}$	3
D-α-Phenylalanine	3.2 ± 0.2	2	28.7 ± 2.9	0.3	$(1.1 \pm 0.2) \times 10^{-1}$	6.5
D-α-Tyrosine	0.43 ± 0.12	7	2.7 ± 0.7	1.7	$(1.6 \pm 0.7) \times 10^{-1}$	4.2
(*S*)-β-Phenylalanine	1.7 ± 0.3	7	24.2 ± 1.8	0.7	$(7.0 \pm 1.4) \times 10^{-2}$	9.5
(*R*)-β-Phenylalanine	0.23 ± 0.03	0.08	0.068 ± 0.008	0.02	3.4 ± 0.6	4.0

r is the ratio of kinetic parameter of the mutant divided by the kinetic parameter for that substrate of the WT enzyme.

on the apparent k_{cat} and K_m with most substrates (Table 3), including an increased k_{cat} with L- and D-tyrosine and with (*S*)-β-phenylalanine. Most K_m values were less affected, but a strong decrease was observed with (*R*)-β-Phe, resulting in a net fourfold increase of the selectivity constant with this substrate. The best substrate for the mutant enzyme still is (*R*)-β-phenylalanine, but the largest increase in activity was observed for (*S*)-β-phenylalanine (Table 3). The fact that the A312G mutation enhances the conversion of various amino acids indicates that A312 is indeed located near the binding site of the activated substrate, although subtle differences cannot be explained in the absence of a crystallographic structure of the enzyme.

3.3. Role of activated amino acids in natural product biosynthesis

To our knowledge this is the first paper describing a CoA ligase that has considerable activity with aromatic proteinogenic amino acids, as well as with β-phenylalanines. This raises the possibility that CoA ligases are involved in some routes leading to the synthesis of natural products that contain amino acid groups, or that CoA ligases may be used in engineered routes towards natural products. For example, the activation of (*R*)-β-phenylalanine results in the formation of (*R*)-β-phenylalanyl-CoA, which is an important intermediate in taxol biosynthesis in *Taxus brevifolia* [27]. Taxol is an important anti-cancer medicine. In one of the last steps of taxol biosynthesis an acyltransferase couples the (*R*)-β-phenylalanyl side chain to the taxol nucleus. The acyltransferase requires a CoA-activated side-chain precursor [27], but the enzyme responsible for the formation has not been identified. Although the catalytic efficiency of the wild-type CoA ligase described here may be insufficient for production of taxol in engineered systems, the observation that the A312G mutant has already fourfold improved catalytic efficiency bodes well for further improvement by protein engineering.

Nature appears to have evolved two major strategies to activate carboxylic acids to thioesters. Most carboxylic acids are activated by CoA ligases, but this reaction is uncommon for amino acids. CoA activation of α-amino acids would result in the formation of α-aminoacyl thioesters which are less stable than normal acyl-thioesters. This lower stability may be the reason that α-aminoacyl-CoAs are not used as intermediates in secondary product biosynthesis. For protein synthesis, amino acids are activated as esters with tRNA. In the NRPSs the thioester-activated amino acids remain covalently linked to the enzyme, preventing hydrolysis of the energy rich intermediates. In contrast, β-aminoacyl-CoAs are found as intermediates in β-alanine metabolism in *Clostridium propionicum* [28] and in taxol biosynthesis [27], indicating that at least some aminoacyl-CoAs are stable enough to occur in metabolic routes. Although these β-aminoacyl thioesters are more stable than their α-amino acid counterparts, the life-time of free α-aminoacyl-CoAs has been shown to be in the order of several minutes [29], sufficient to allow a role of α-aminoacyl-CoAs a role in biosynthetic routes.

In summary, in this paper we show that α-aminoacyl-CoAs can be formed in a reaction catalyzed by a CoA ligase from *P. chrysogenum*. This CoA ligase catalyzes the CoA activation (*R*)-β-phenylalanine.

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