

# Expression of an active adenylate-forming domain of peptide synthetases corresponding to acyl-CoA-synthetases

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**Abstract** Peptide synthetases and acyl-CoA-synthetases form acyl adenylates which are transferred to CoA or enzyme-bound pantetheine. To verify the existence of an adenylate domain in peptide synthetases, a 60.8 kDa fragment of tyrocidine 1-synthetase was constructed by a 1629 bp deletion, expressed in *Escherichia coli*, and characterized. The truncated multienzyme activated phenylalanine and substrate analogues with comparable kinetics as the over-expressed synthetase, as judged by ATP-[<sup>32</sup>P]PP<sub>i</sub> exchange reaction. Thus the N-terminal domain resembling an acyl-CoA-synthetase is an autonomous structural element. This N-terminal domain is followed by a cofactor binding domain, resembling acyl carrier proteins involved in polyketide formation.

**Key words:** Tyrocidine synthetase; Multienzyme; Peptide synthetase; Acyl-CoA-synthetase; Pantetheine; CoA

## 1. Introduction

A superfamily of carboxyl-group activating enzymes contains peptide synthetases, acyl-CoA-synthetases, insect luciferases, and certain amino acid-adding enzymes [1–3]. Common to these adenylate-forming enzymes are a group of motifs identified by Bairoch, who described these as AMP-binding motifs [4]. In the meantime, sequences of at least 25 peptide synthetases, comprising altogether 58 respective domains, and 38 other proteins have been obtained [1,3] and additional motifs described (A–I; Fig. 1) [1,5–8]. This superfamily of enzymes and multienzymes has been subdivided according to structural similarities into four classes [2]. Class 1 is dominated by eukaryotic acyl-CoA-synthetases, class 2 consists mainly of peptide synthetases, class 3 are acetyl-CoA-synthetases, and class 4 luciferases and other acyl-CoA-synthetases of prokaryotic and plant origin. Only the second class of these AMP-binding proteins (SWISS-PROT) extends in size considerably, and, contrary to the rest, does not transfer the respective activated acyl-compounds to CoA [1]. Instead, an intra-enzymatic transfer to a covalently attached 4'-phospho-pantetheine group occurs [10]. To these thioester intermediates likewise activated intermediates are transferred, either as CoA derivatives or as enzyme-attached esters, and undergo a directed condensation reaction [10,11].

For these additional catalytic properties a cofactor-attachment motif has been identified (J), and other motifs (K–Q) have been suggested to function in condensation or substrate modification reactions [8] (Fig. 1). Peptide synthetases are con-

structed of modules, each contributing one amino- or hydroxy acid to the peptide structure. Each module contains an adenylate-forming domain, a cofactor binding site, and a condensation/modification domain. The adenylate forming domains display significant similarity to acyl-CoA-synthetases and related proteins. We have predicted, by alignment of sequences of these three classes and those of acyl carrier proteins also binding 4'-phosphopantetheine, the boundaries of adenylate and cofactor domains. The linker region is not rich in Ala, Pro and Glu residues, as in fatty acid synthases and 2-oxo-acid dehydrogenases containing similar cofactor domains. Still, amino acids frequently found in linker regions are dominating. Upon deletion of the cofactor and following functional domains of tyrocidine synthetase 1, a stable N-terminal fragment was obtained which catalysed adenylate formation with similar kinetic properties as the apo-form of the peptide synthetase [8].

## 2. Materials and methods

### 2.1. Materials

DNA restriction endonucleases were purchased from Gibco BRL and Appligene. T4 DNA ligase and Klenow fragment were products of Boehringer-Mannheim. [<sup>32</sup>P]PP<sub>i</sub> was from DuPont, <sup>14</sup>C-labeled L-phenylalanine from Amersham/Buchler, ATP and dithioerythritol (DTE) were purchased from Sigma.

### 2.2. Bacterial strains and growth conditions

The *Escherichia coli* strain used for plasmid propagation and enzyme expression was XL1-blue [12]. Transformants of this strain carrying recombinant plasmids were cultured in Luria-Bertani medium supplemented with 50 µg/ml ampicillin.

### 2.3. Isolation of plasmid DNA

The plasmid pGC12 contains a 3.5 kb *HincII* fragment (containing the TY1 coding region) from pBT2 [13] and carries the ampicillin resistance gene and a portion of the *lac* operon, including the *lac* promoter, operator and part of the *Z* gene. Plasmid DNA was isolated by the alkali method of Birnboim and Doly [14].

### 2.4. DNA manipulations

Digestions, ligation, filling in of nucleotides, and other DNA manipulations were performed as described by Sambrook et al. [15].

### 2.5. Transformation and selection

Competent *E. coli* XL1-blue cells were transformed using the procedure of Mandel and Higa [16] or Dagert and Ehrlich [17]. Transformants were selected on plates containing 50 µg/ml ampicillin.

### 2.6. Enzyme purification

All operations were carried out at 0–4°C, except the final purification step on TMAE which was performed at room temperature. The harvested cells were resuspended in a threefold volume of 100 mM Tris-HCl (pH 7.5), containing 10 mM dithioerythritol (DTE), 1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, 2 mM MgSO<sub>4</sub>, 2 µg/ml DNase and 300 µg/ml lysozyme. After being stirred for 1 h, the suspension was sonified. The cell debris was sedimented by centrifugation at 16,000 rpm for 25 min and discarded. Polyethylenimine (PEI) was added to the

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supernatant to give a final concentration of 0.1%; the mixture was stirred for 30 min and centrifuged at 18,000 rpm for 10 min.  $(\text{NH}_4)_2\text{SO}_4$  was added as a saturated solution to give a final concentration of 60%, and the mixture was stirred for 1 h. The precipitate was dissolved in a minimal volume of buffer A (50 mM Tris, pH 7.5, 1 mM DTE, 0.1 mM EDTA, 10% glycerol) containing 150 mM NaCl, and was eluted from a Sephacryl S-300 HR column (Pharmacia) with buffer A. Fractions, which showed activity in the ATP- $[\text{P}^{32}]\text{PP}_i$  exchange assay, were pooled, dialysed against buffer A and applied to an anion-exchange DEAE-Sepharose CL-6B column (Pharmacia). The column was eluted with a linear gradient of 0–400 mM NaCl in buffer A. Active fractions were pooled, adjusted to 1 M NaCl in buffer B (50 mM Tris, pH 7.5, 1 mM DTE, 0.1 mM EDTA), and applied to a phenyl-Sepharose CL-4B column (Pharmacia). Elution was performed with a linear gradient from 1 M to 0 M NaCl in buffer B. After dialysis against buffer B, the enzyme was applied to Fractogel TSK TMAE-650 in a Superformance 150-10 column (Merck). Elution was carried out using a linear gradient from 0 to 500 mM NaCl in buffer B.

### 2.7. SDS-PAGE and determination of N-terminal sequence

SDS-PAGE was performed in 10% polyacrylamide gels according to the method of Laemmli [18]. After electrophoresis, gels were stained with Coomassie brilliant blue R-250. Alternatively proteins were transferred to Immobilon polyvinylidene difluoride membranes. N-Terminal sequencing was done with an Applied Biosystems protein sequencer (model 473A).

### 2.8. Western blot

Western blot analysis was performed according to Towbin et al. [19].

### 2.9. Enzyme assays

ATP- $[\text{P}^{32}]\text{PP}_i$  exchange was measured essentially as described by Pavela-Vrancic [20]. The reaction mixture contained, in a final volume of 70  $\mu\text{l}$ , 50 mM Tris-HCl, pH 7.4, 0.14 mM ATP, 0.7 mM  $\text{Mg}^{2+}$ , 0.07 mM EDTA, 1 mM DTE, 0.07 mM  $\text{PP}_i$ , 0.1  $\mu\text{Ci}$   $[\text{P}^{32}]\text{PP}_i$  and 1.4 mM phenylalanine. The reaction was started by addition of enzyme and allowed to proceed for 15 min at 37°C. For kinetic measurements ATP- $[\text{P}^{32}]\text{PP}_i$  exchange was performed at varying substrate concentrations, 0.01–0.1 mM D-Phe and 0.01–0.4 mM ATP.

### 2.10. Determination of protein content

The protein concentration was measured by the Bradford method [21] with bovine serum albumin as standard.

### 2.11. Isoelectric focusing

The pI of the enzyme was determined on Servalyt Precotes (pH range 3–10) and Servalyt PreNets (pH range 4–6) from Serva (Heidelberg, Germany). The purified enzyme preparations were previously desalted by dialysis.

### 2.12. Mass spectrometric analysis

Mass spectrometric analysis was performed with a Bruker Reflex laser desorption mass spectrometer using sinapinic acid as a matrix (Bruker-Franzen Analytic GmbH, Bremen, Germany).

## 3. Results

### 3.1. Construction of deletion and expression

Within the C-terminal region of the *tycA* gene (3261 bp) a 1629 bp-deletion was constructed making use of *Cla*I and *Eco*RV restriction sites (Fig. 2). In the resulting truncated multienzyme, 543 amino acids between residues Ser<sup>534</sup> and Ile<sup>1077</sup> have been deleted, combining the N-terminal 533 amino acids with the C-terminal decapeptide, with a reduction in the calculated molecular mass from 122,590 to 60,762 Da. Expression was conducted in *E. coli* XL1-blue without induction, presumably using the *tycA* promoter. The corresponding gene product was identified using TY1-specific antibodies. After disintegration of cells, 80% of the over-expressed 61 kDa protein remained in the insoluble fraction. The soluble protein was purified

by 60% ammonium sulfate precipitation, gel-filtration on Sephacryl S-300, anion-exchange chromatography on DEAE-CL-6B, hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B, and anion-exchange chromatography on Fractogel TMAE-650. The homogeneity of the protein was demonstrated by SDS-PAGE (Fig. 3) and isoelectric focusing (pI 5.1) (data not shown).

### 3.2. N-Terminal sequencing and mass spectrometric analysis

The N-terminal sequence (MLANQANLIDN) of the deletion mutant was shown to be identical to TY1. A sample of the purified TY1 fragment expressed in *E. coli* was analyzed by laser desorption mass spectrometry. The mass was determined to be 60,975 Da, which is within the experimental error of the calculated value of 60,762 Da (data not shown).

### 3.3. Enzymatic characterization of the TY1 fragment

The enzyme fragment was shown to retain adenylating activity by ATP- $[\text{P}^{32}]\text{PP}_i$  exchange, although the ability to thioesterify phenylalanine was totally lost. The kinetics of phenylalanine activation by the fragment was studied by ATP- $[\text{P}^{32}]\text{PP}_i$  exchange. The turnover number ( $k_{\text{cat}}$  0.4 s<sup>-1</sup>) obtained by ATP- $[\text{P}^{32}]\text{PP}_i$  exchange activity measurements is in good agreement with the value obtained for TY1 expressed in *E. coli* ( $k_{\text{cat}}$  0.57 s<sup>-1</sup>) [8]. Both enzymes show similar kinetic patterns, and the dissociation constant for L-phenylalanine, which was determined from Lineweaver-Burk plots to be  $K_d = 0.1 \pm 0.02$  mM, corresponds to the values obtained for the wild-type enzyme and the expressed protein ( $K_d = 0.04$ ) [8].

### 3.4. Substitution of phenylalanine by other amino acids and substrate analogues in ATP- $[\text{P}^{32}]\text{PP}_i$ exchange

Substrate specificity with regard to amino acid activation was examined by measuring the ATP- $[\text{P}^{32}]\text{PP}_i$  exchange in the presence of a series of amino acids and phenylalanine analogues (Table 1). The highest activity was obtained with the natural substrate L-phenylalanine. The enzyme strongly prefers amino acids carrying aromatic side chains, tryptophan and kynurenine were not accepted. Glycine, alanine, serine, threonine, isoleucine, cysteine, glutamine, asparagine, and proline were not recognized, while valine, leucine, aspartate, glutamate, and lysine showed a slight reaction (<1%). Surprisingly activation



Fig. 1. Organization of adenylate-forming enzymes involved in carboxyl group activations. (A) Acyl-CoA synthetases; (B1) peptide synthetases; (B2) peptide synthetases with epimerizing function. The boxed motifs of peptide synthetases represent the following core sequences [1,8]: A, LTXXELXXXAXLXR; B, AVXXAXXYVXIDXXYPXER; C, YTSGETTGXPKG; D, IIXXYGXT; E, GELXIXGXXVAR; F, RLYRTGDL; G, IEYLGRXDXQVKIRXXRIELGEIE; H, LXXYMVP; I, LTXXGKLXRKAL; J, LGGXSIAXI; K, (B2) XXLXP-IQXWFXXX, (B1) YPSVXXQXRMYL; L, (B1) LIXRHEXL, (B2) LXXXHDXX; M, (B1) DMHHIXDGXXXIX, (B2) XXHHXXV-DXVSXWIL; N, (B1) LSKXGXQDIIXGTPXAGR, (B2) VXXEGHGRE; O, (B1) IXGMFVNTXLALR, (B2) TVGWFTXXXPPXX; P, PXXGXGY; Q, VXFNYLG.

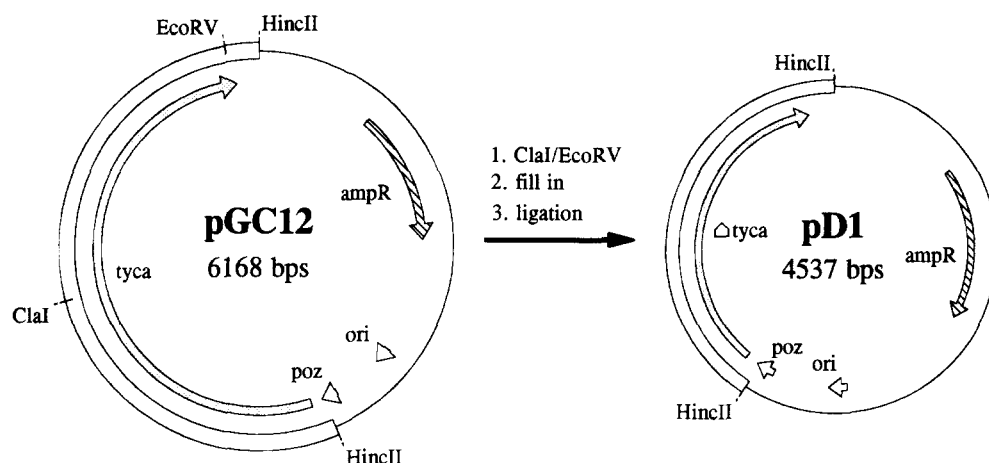


Fig. 2. Construction of pD1, containing 1632 bp of the *tycA* gene (3261 bp).

of methionine was observed, which so far has not been detected in enzymatically formed peptide structures. From these results it can be concluded that amino acid substrate specificity of the synthetase fragment is essentially unchanged when compared to the wild-type enzyme [8].

#### 4. Discussion

Acyl-CoA-synthetases and peptide synthetases share a number of common motifs, termed A–I as described and defined in Fig. 1. In peptide synthetases these motifs are found in the N-terminal parts of the multienzyme or their respective modules, and are followed by a pantetheine attachment motif J [5,8,22,23]. Sequence alignments as shown in Fig. 4 indicate that the region comprising motifs A–I is an N-terminal domain catalyzing the formation of acyl adenylates, which is followed by a cofactor binding domain concluded by certain similarities to acyl carrier proteins likewise binding 4'-phosphopantetheine. Surprisingly the proposed linker region between these domains

did not show the Ala/Pro/Glu-enriched sequences detected in fatty acid synthases [24], polyketide synthases [25] or 2-oxo-dehydrogenase cofactor binding domains [26]. In an analysis of domain linker regions, a preference of certain amino acids has been confirmed [27], including Thr, Ser, Pro, Asp, Gly, while others are rare (Trp, Cys, His, Phe, Met). Such a preference is indeed observed here, together with a very low conservation of residues. This apparently higher mutation rate ('hot spot') is considered an indication of a domain boundary [28].

Additional information on possible structural domains or structural modules was thought to be derived from exon structures [29]. The class of peptide synthetases, however, do not contain introns, despite their considerable gene sizes of up to 45.6 kb in eukaryotic organisms [30]. From exon data of the acyl-CoA-synthetase and luciferase structures [31–33], no clues could be obtained on possible substructures within the adenylate domain. A recent analysis of the exon theory concluded that no correspondence exists between protein structure and exons [34].

More reliable evidence can be obtained from limited proteolysis data, as this method is generally used to prepare stable domains. Tryptic digestion of gramicidin S synthetase 2, a

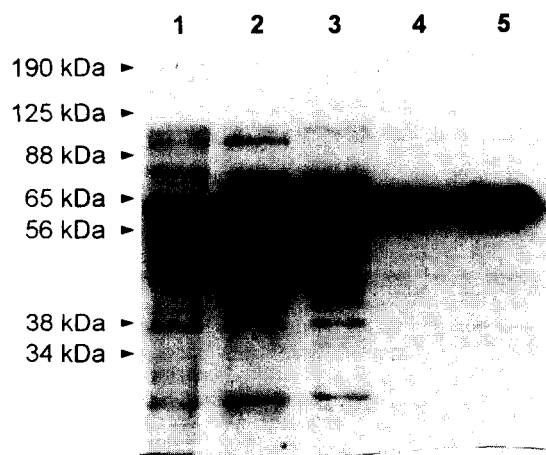


Fig. 3. SDS-PAGE analysis of the truncated tyrocidine synthetase 1 summarizing purification: lane 1, crude extract; lane 2, after purification by gel-filtration on Sephacryl S-300; lane 3, after anion-exchange chromatography on DEAE-Sephacryl; lane 4, after chromatography on phenyl-Sepharose; lane 5, after anion-exchange chromatography on Fractogel TMAE-650 (10% SDS-PAGE).

Table 1  
ATP-[<sup>32</sup>P]PP<sub>i</sub> exchange activity of the truncated tyrocidine synthetase 1 in the presence of various amino acids and phenylalanine analogues

Amino acid	Activity (%)
L-Phenylalanine	100
3-(2'-Thienyl)-L-alanine	95
D-Phenylalanine	86
p-Fluoro-phenyl-L-alanine	77
L-Phenylserine	47
p-Chlorophenyl-L-alanine	45
L-Tyrosine	16
3-(1'-Naphthyl)-L-alanine	9
3,4-Dimethoxy-phenyl-L-alanine	5
o-Benzyl-L-serine	3
4-Hydroxy-3-methoxy-phenyl-L-alanine	1
L-Kynurenine	0
L-Methionine	5

Activity was calculated relative to the values obtained with L-phenylalanine.

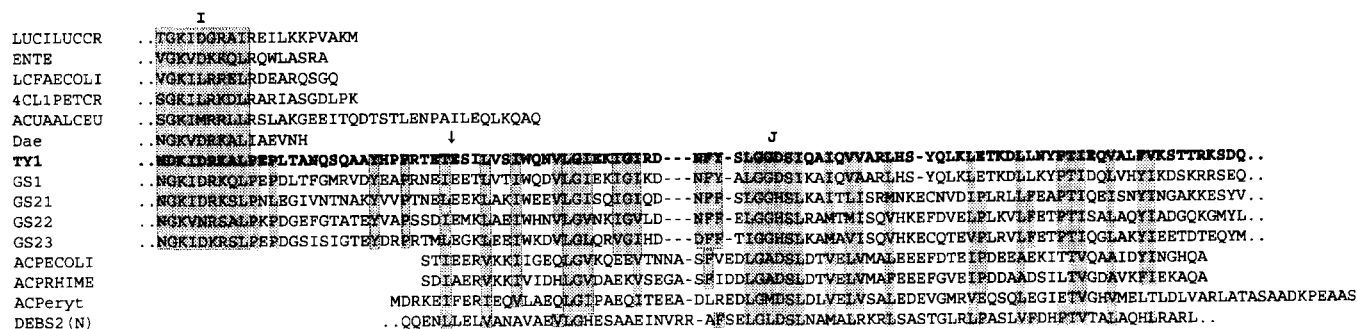


Fig. 4. Alignment of peptide synthetase sequences (TY1, GSX), with acyl-CoA synthetases, luciferase and carboxylic acid- and amino acid-activating enzyme, respectively, and acyl carrier proteins type 1 and type 2. The cleavage site (↓) is located between the I and J Box. LUCILUCCR, luciferin 4-monooxygenase from *Luciola cruciata*; ENTE, enterobactin synthetase component E (dihydroxybenzoic acid-activating enzyme) from *E. coli*; LCFAECOLI, long-chain fatty acid-CoA ligase from *E. coli*; 4CL1PETCR, 4-coumarate-CoA ligase 1 from *Petroselinum crispum* (parsley); ACUAALCEU, acetyl-CoA synthetase from *Alcaligenes eutrophus*; Dae, D-alanine-activating enzyme from *Lactobacillus casei*; TY1, tyrocidine synthetase 1 from *Bacillus brevis*; GS1, gramicidine synthetase 1 from *Bacillus brevis*; GS2, gramicidine synthetase 2 from *Bacillus brevis*; ACPECOLI, acyl carrier protein (ACP) from *E. coli*; ACPRHIME, ACP from *Rhizobium meliloti*; ACPERYT, ACP from *Saccharopolyspora erythraea*; DEBS2 (N), ACP region of 6-deoxyerythronolide-B synthase 2 from *Saccharopolyspora erythraea*.

512 kDa multienzyme integrating 4 modules, led to amino acid-activating domains of 110–115 kDa [35–37]. The Pro-activating domain has been analysed in detail, subcloned, and can be concluded to be truncated before the J-motif [38]. The  $\delta$ -(L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine synthetase, containing three modules, has been digested by subtilisin, and some fragments have been identified by N-terminal sequencing [5]. Cleavage sites are found between the I and J, and the J and K motifs.

To construct a truncated peptide synthetase module, the *tycA* gene was used, containing a *Cla*I site permitting cleavage between the I and J box, and an *Eco*RV site leaving the C-terminal decapeptide. TY1 or tyrocidine synthetase 1 is involved in the initiation of biosynthesis of the cyclodecapeptide tyrocidine. It activates phenylalanine as aminoacyl adenylate, which is used to aminoacylate an enzyme thiol group, presumably 4'-phosphopantetheine. This thioester intermediate is epimerized before its transfer to a prolyl-thioester bound to tyrocidine synthetase 2. Removal of the J box is thus expected to abolish both thioester formation and epimerization, which has been confirmed (results not shown). Ser-to-Ala point mutations in TY1 and surfactin synthetases within the J box motif have also demonstrated loss of aminoacylation ability [39,40].

Attempts to over-express peptide synthetases or modules for detailed characterization have been carried out with gramicidin S synthetase 1 [38], tyrocidine synthetase 1 [8], the serine-activating enzyme of the enterobactin pathway [41], and the aminoacyl activating module of ACV synthetase [42]. In none of the cases have enzyme activities comparable to the wild-type enzymes been reported concerning aminoacylation, which has been attributed to incomplete post-translational processing. At least in the first two cases, aminoacyl adenylate formation seemed to be conserved; however, a more detailed analysis of the over-expressed tyrocidine synthetase 1 revealed a significantly decreased rate in ATP-[ $^{32}$ P]PP<sub>i</sub> exchange [8]. This decreased rate, which is attributed to the apo-enzyme, has been confirmed for the fragment described here. Thus the adenylate domain retained an essentially unchanged structure compared to the apo-enzyme, which again showed unchanged binding constants when compared to the wild-type isolate, probably representing the holo form.

This result suggests that, in enzymatic peptide biosynthesis, amino acid activation proceeds in complete analogy to acyl activation, preceding transfer to coenzyme A. Presumably for reasons of stability, free aminoacyl CoA esters are not observed in the cellular context. Aminoacyl adenylates either react directly with respective acceptors (amino acid-adding enzymes) or aminoacylate enzyme-attached 4'-phosphopantetheine. To what extent acyl-CoA-synthetase-corresponding fragments of peptide synthetases have maintained binding and transfer properties of and to CoA remains to be studied. Fragments of this size may have unchanged substrate binding properties in general, and may thus be suited for module identification and studies of specificity alterations. Over-expression studies have been unsuccessful in several cases employing complete modules or larger multienzyme fragments ([42,43], H. van Liempt, personal communication).

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