Dipeptide synthesis by an isolated adenylate-forming domain of non-ribosomal peptide synthetases (NRPS)

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Abstract A deletion mutant of tyrocidine synthetase 1 (ΔTY1), comprising the adenylation domain of TY1 as an independent functional adenylate-forming unit, was used to investigate the ability of the adenylation domain in non-ribosomal peptide synthetases to catalyse peptide bond formation from the aminoacyl adenylate intermediate. The results demonstrate that only one substrate amino acid needs to be activated as an aminoacyl adenylate. In view of the potential exploitation of peptide synthetases for enzymatic synthesis of dipeptides, it is important to note that this does not necessarily require a dimodular construct or an intermediate acyl transfer step. 

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Key words: Peptide synthetase; Tyrocidine synthetase 1; Adenylation domain; Dipeptide generation

1. Introduction

Non-ribosomal peptide synthetases (NRPS) activate their amino acid substrates as amino- or iminoacyl adenylates, whereas peptide bond formation is catalysed by thioesters produced by relocation of the acyl moiety to the thiol of a 4'-phosphopantetheine cofactor on an adjacent peptidyl carrier protein, PCP [1,2]. Peptide bond formation occurs in an amino–carboxy-terminal elongation reaction supported by the condensation domain. This ‘thiotemplate mechanism’ has recently been questioned by Shiau et al. [3,4] having shown the synthesis of dipeptides, L-O-(methylserinyl)-L-valine and L-O-(methylserinyl)-L-cysteinyl-L-valine synthetase (ACVS). Employing \(^{18}O\)-labelled amino acids, the maintenance of label in the carboxyl group indicates direct aminolysis of the mixed anhydride intermediate.

To rule out the participation or assistance of carrier or condensation domains in such peptide-forming side reactions we have investigated peptide bond formation by an isolated adenylate-forming domain, the phenylalanyl-activating domain of tyrocidine synthetase 1 (TY1). A deletion mutant encoding the adenylation domain of TY1, ΔTY1, comprising the pQE31 His\textsubscript{6} tag fusion vector from Qiagen (Hilden, Germany). The His\textsubscript{6}-tagged truncated adenylation domain of TY1 was expressed in E. coli M15[pREP4] and purified on Ni-NTA resin (Qiagen). Elution was performed with a gradient of 0-0.5 M imidazole in 50 mM Na-phosphate, pH 6, containing 300 mM NaCl, and 10% glycerol.

2. Materials and methods

2.1. Expression and protein purification

For the construction of the His\textsubscript{6}-tagged truncated tycA fragment, plasmid pDH1 [5] was restricted with SphI and KpnI and ligated into the pQE31 His\textsubscript{6} tag fusion vector from Qiagen (Hilden, Germany). The His\textsubscript{6}-tagged truncated adenylation domain of TY1 was expressed in E. coli M15[pREP4] and purified on Ni-NTA resin (Qiagen). Elution was performed with a gradient of 0-0.5 M imidazole in 50 mM Na-phosphate, pH 6, containing 300 mM NaCl, and 10% glycerol.

2.2. Dipeptide synthesis

Dipeptide synthesis was monitored in a reaction solution containing ΔTY1 (5 μM) in 50 mM Tris-HCl, pH 7.5, in the presence of 8 mM L-Phe and 100 mM of nucleophile non-cognate substrate when indicated in the text, 20 mM ATP, and 20 mM MgCl\textsubscript{2}. The reaction mixture was incubated over several days at 30°C. At intervals aliquots of the reaction solution were heated at 95°C for 5 min, and the precipitated protein separated by centrifugation. The supernatant was applied onto a C18 column and separated by HPLC using isocratic elution with 0.08% trifluoroacetic acid (TFA) for 2 min, followed by a gradient of acetonitrile (0-30%) in 0.08% TFA for 15 min, at a flow rate of 1 ml/min and detection wavelength of 210 nm. The peptides were identified by comparison of their retention times to that of a standard mixture.

3. Results

An N-terminally truncated fragment similar in size to PheA [7], and comprising the adenylation domain of TY1 (His18 to Ser534 plus a C-terminal tail of 10 residues), was constructed as a His\textsubscript{6}-tagged fusion protein (His\textsubscript{6}ΔTY1) expressed in E. coli. The fragment retained catalytic activity and substrate specificity in the L-phenylalanine-dependent ATP-[\(^{32}\)P]PP\textsubscript{i} exchange reaction [5,6]. The enzyme catalyses the first step of the L-Phe activation reaction, generating an enzyme-bound adenylate in the active site.

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alanyl intermediate [8]. The time course of dipeptide generation (Fig. 3) shows a linear rate dependence of dipeptide synthesis over a period of 100 h. Quantification proceeded by calculation of the peak area relative to that of a standard peptide, achieving a maximum concentration of 520 μM L-Phe-L-Phe at a turnover of $k = 0.0174 \text{ min}^{-1}$ (Table 1).

When the reaction mixture was supplemented with a non-cognate amino acid or an amino acid amide, such as L-Ala or L-PheNH₂ or L-LeuNH₂, the L-phenylalanyl adenylate was cleaved under generation of the corresponding mixed dipeptide (Fig. 3). The nature of the newly synthesised product was established by comparison with commercial standards of the respective dipeptide. In the presence of the non-cognate nucleophile in excess, the L-Phe-L-Phe generation was fully suppressed leading to preferential synthesis of the respective dipeptide analogue (Table 1). Substitution of the nucleophile L-Phe with L-Ala or L-LeuNH₂ increased the turnover rate of dipeptide formation from $k = 0.0167$ to $0.0186$ and $0.0190 \text{ min}^{-1}$, respectively. However, incorporation of L-PheNH₂
into the peptide product proceeds at a slower rate of $k 0.009\text{ min}^{-1}$.

4. Discussion

We have shown that an isolated adenylate-forming domain may act as a synthetase scaffold introducing an aminoacyl adenylate to aminolytic substitution. Adenylation domains may thus serve to stereospecifically catalyse carboxyl activation of amino acids, to be followed by non-enzymatic aminolysis in the synthesis of amides, small peptides and related substitution products. In these templates the adenylylating are stabilised by interactions of the two subdomains of the adenylation domain, and are subject to hydrolytic cleavage depending on the stability of the respective intermediate complex. We have recently shown that enzyme-bound aminoacyl adenylates of structural analogues of L-Phe have different stabilities reflected by their hydrolytic cleavage rates [8]. Likewise, adenylates bound to domains altered by point mutations are also affected in stability. These structural parameters may be exploited in optimisation of adenylation-forming domains mediating peptide bond formation, and hence the peptide synthetase may be rationally designed to efficiently synthesise small peptides of choice. Such design is obviously required, considering the rates of condensation reactions observed in the range of 0.009–0.018 min$^{-1}$.

The tripeptide-forming ACVS has been shown to catalyse the formation of small but detectable amounts of dipeptides, such as L-cysteinyl-d-valine and the formation of small but detectable amounts of dipeptides, By O$_{18}$-labelling of the valine carboxyl group dipeptide formation has been shown to proceed at least partially from represents a sequence of 10 enzymatic reactions with an overall to about 1–2% of tripeptide biosynthesis, which by itself rep-

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>$k \text{ (min}^{-1})$</th>
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<tr>
<td>L-Phe-L-Phe</td>
<td>0.0167</td>
</tr>
<tr>
<td>L-Phe-L-Ala</td>
<td>0.0186</td>
</tr>
<tr>
<td>L-Phe-L-LeuNH$_2$</td>
<td>0.0190</td>
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<td>L-Phe-L-PheNH$_2$</td>
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The turnover of dipeptide generation by the isolated adenylation do-

| Table 1: Turnover of dipeptide generation by the isolated adenylation do-

aminoacyl-tRNA synthetases (ARS) or adenylate domains of

NRPS. Adenylates formed in both ribosomal and non-ribosomal systems as mixed anhydrides are highly reactive with amino acceptors or free thiols.

Even adjacent enzyme amino groups might act as acceptors. In methionyl-tRNA synthetase, lysine residues adjacent to the adenylation binding site have been shown to be modified in situ by aminolysis, leading to alterations of enzymatic properties [11].

The formation of dipeptides by ARS has been analysed in the case of the arginyl-tRNA synthetase [12]. The deacylation of Arg-tRNA$^{\text{Arb}}$ is catalysed in the presence of cysteine with the formation of Arg-Cys with a $k_{\text{cat}}$ of 18 min$^{-1}$, and a $K_M$ of 150 mM. For free Arg-tRNA$^{\text{Arb}}$ a deacylation rate of 0.04 min$^{-1}$ was observed, which in the presence of the synthetase even decreased to 0.003 min$^{-1}$. This reaction is unique to certain thiol-containing compounds, and is thought to involve the thioester intermediate S-(Arg)-Cys, rapidly rearranging to the stable dipeptide. Similar reactions are catalysed by other class II ARS [13]. However, direct peptide bond formation from adenylates by ARS is not known, except for intramolecu-

lar cyclisations in lysyl-tRNA synthetase: misacylated ornithine is released by cyclisation with a $k_{\text{cat}}$ of 9 min$^{-1}$, compared to the sterically unfavourable reaction of lysine with 0.039 min$^{-1}$ [14].

NRPS have been engineered by domain exchanges to pro-

duce novel di- and tripeptides [9,15–17]. Such constructs exhibit significant decreases in overall reaction rates, which are currently not understood in detail. Determined rates range between 0.04 and 2.2 min$^{-1}$ [9,16]. It is of particular interest if peptides are generated with the stereospecific thiotemplate condensation, or, as has been demonstrated in the case of ACVS, by presumably non-enzymatic aminolysis of adenylates. Our data obtained on the reactivity of enzyme-bound aminoacyl adenylates indicate that such reactions proceed at rates of about 0.01–0.02 min$^{-1}$. Peptide formation observed at similar rates in NRPS systems is thus likely to proceed non-enzymatically from the mixed anhydride intermedi-

References