Editing of non-cognate aminoacyl adenylates by peptide synthetases

Maja PAVELA-VRANCIC*, Ralf DIECKMANN†, Hans von DÖHREN† and Horst KLEINKAUF†

*Faculty of Natural Sciences, Mathematics and Education, Department of Chemistry, University of Split, N. Tesle 12, 21000 Split, Croatia, and †Max-Volmer-Institute of Biophysical Chemistry and Biochemistry, Technical University Berlin, Franklinstrasse 29, D-10587 Berlin, Germany

Non-ribosomally formed peptides display both highly conserved and variable amino acid positions, the variations leading to a wide range of peptide families. Activation of the amino acid substrate proceeds in analogy to the ribosomal biosynthetic mechanism generating aminoacyl adenylate and acyl intermediates. To approach the mechanism of fidelity of amino acid selection, the stability of the aminoacyl adenylates was studied by employing a continuous coupled spectrophotometric assay. The apo-form of tyrocidine synthetase 1 (apo-TY1) was used, generating an l-phenylalanyl-adenylate intermediate stabilized by the interaction of two structural subdomains of the adenylation domain. Adenylates of substrate analogues have shown variable and reduced degrees of stability, thus leading to an enhanced generation of pyrophosphate due to hydrolysis and continuous adenylate formation. Discrimination of the non-adenylate formation, or missing interdomain interactions.

To investigate the proposed editing mechanism, direct measurements of aminoacyl-adenylate stability were required. So far, kinetic studies of the aminoacyl-adenylation reaction have relied primarily upon the use of the amino acid-dependent formation of [32P]ATP via the ATP-[32P]PP exchange reaction. Here, we have applied a continuous spectrophotometric technique based on coupling of the apo-form of tyrocidine synthetase 1 (apo-TY1)-dependent generation of PP1 to a two-enzyme detection system involving inorganic pyrophosphatase (IPP) and purine nucleoside phosphorylase (PNP). This was to assay the peptide synthetases in the first step of the amino acid-activation reaction in the forward direction [12–17]. Pyrophosphatase cleaves PP1 into two molecules of phosphate, which in turn is used in the PNP-catalysed phosphorylization of the chromogenic 2-amino-6-mercapto-7-methylpurine ribonucleoside (AMMPR) to yield ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine, monitored as an absorption increase at 360 nm.

TY1 was chosen as a representative model system, catalysing the activation of a single amino acid, l-phenylalanine. It is the initial component of a multifunctional enzyme system composed of three complementary enzymes constituting the non-ribosomal biosynthetic system for the production of the cyclodecapeptide antibiotic tyrocidine in Bacillus brevis (A.T.C.C. 8185). No product formation occurs, but enzyme-bound intermediates are generated. A comparative analysis of the recombinant protein, expressed in Escherichia coli, and wild-type TY1 has shown that the over-expressed protein is devoid of the 4-phosphopantetheine cofactor. This produces an enzyme (apo-TY1) that can catalyse the first step of the activation reaction, whereby the overall process ceases with a tightly bound adenylate in the active site.

INTRODUCTION

A wide variety of bioactive linear, cyclic and branched cyclic peptide products are synthesized via the non-ribosomal protein thiopeptide synthesis mechanism catalysed by multifunctional peptide synthetases [1,2]. The ribosomal system is restricted to the 20 proteinogenic amino acids, whereas more than 300 derivatives, including hydroxy, N-methylated and d-amino acids, are known to be incorporated into these secondary metabolites. The carboxyl substrate is initially activated utilizing the energy of ATP hydrolysis to form an acyladenylate–enzyme complex with generation of a stoichiometric amount of pyrophosphate (PP1). The adenylate is subsequently cleaved by the action of the thiol moiety of an enzyme-bound cofactor, 4'-phosphopantetheine, to yield an active thioester and AMP. Peptide-bond formation proceeds by directed transfer to the next thioacyl intermediate for condensation.

Peptide synthetases often do not display unique substrate specificity, as observed with aminoacyl-tRNA synthetases [3], allowing the synthesis of a variety of isofoms of the respective peptide product. Nevertheless, they may exhibit various degrees of selection, providing highly conserved positions in the peptide structure [4–9]. The fidelity of product formation in the ribosomal system depends on the high specificity of aminoacyl-tRNA synthetases maintained by an enzyme-catalysed proof-reading mechanism based on hydrolysis of the non-cognate aminoacyl intermediate [10]. The energy cost of l-(d-α-aminoacipilyl)-l-cysteinyl-d-valine (ACV) synthesis at limiting amino acid concentration has revealed that more than 20 molecules of ATP are consumed per tripeptide formed by ACV synthetase [11]. This elevated energy input has been attributed to loss of mis-activated intermediates by hydrolysis in an editing process governed by conformational changes induced by adenylate formation, or missing interdomain interactions.

Key words: non-ribosomal, proof reading.

Abbreviations used: apo-TY1, apo-form of tyrocidine synthetase 1; IPP, inorganic pyrophosphatase; PNP, purine nucleoside phosphorylase; AMMPR, 2-amino-6-mercaptop-7-methylpurine ribonucleoside; E, enzyme; PP1, pyrophosphate.

1 To whom correspondence should be addressed (e-mail Doehren@chem.tu-berlin.de).
A significant difference in substrate recognition and binding affinity could not be established between the compared proteins, suggesting that the overall active site of the structure has not been affected [18,19]. The acyladenylate may, however, react in a reverse reaction with MgPP\(^{+}\), or be hydrolysed by H\(_2\)O. The presence of excess pyrophosphatase largely eliminates the reverse reaction, making it a suitable system for the analysis of aminoacyl-adenylate stability in view of the putative editing mechanism based on corrective hydrolysis at the aminoacyl-adenylate level. A series of amino acid analogues, generating non-productive aminoacyl-adenylate complexes with apo-TY1, were used as probes of protein function and dynamics. The distinction of the non-cognate aminoacyl adenylate was assayed as an excessive hydrolysis of ATP to AMP and PP\(_i\), distraction of the non-cognate aminoacyl adenylate was assayed were used as probes of protein function and dynamics. The synthesis of the AMMPR reagent was performed according to [18].

**EXPERIMENTAL**

**Materials**

ATP, AMMPR, calf spleen PNP, yeast IPP and various amino acid analogues were purchased from Sigma.

**Enzyme preparation**

The *E. coli* strain XL1-Blue was used as the host for the plasmids containing the *tycA* (apo-TY1) and mutant genes. The recombinant proteins were isolated and purified according to [18].

**Calculation of the protein extinction coefficient**

The molar extinction coefficient of the denatured protein in 6 M guanidinium chloride was calculated from the number of tryptophan, tyrosine and cysteine residues per molecule, using the molar extinction coefficient of the appropriate model compounds: N-acetyl-L-tryptophanamide (5690 M\(^{-1}\) cm\(^{-1}\)), Gly-L-Tyr-Gly (1280 M\(^{-1}\) cm\(^{-1}\)) and cysteine (120 M\(^{-1}\) cm\(^{-1}\)) at 280 nm [21]. For apo-TY1 a value of \(\varepsilon 136 580\) M\(^{-1}\) cm\(^{-1}\) was estimated.

**Synthesis of AMMPR**

The synthesis of the AMMPR reagent was performed according to [12]. A 1-mM stock solution was prepared by dissolving 6.3 mg of AMMPR in 20 ml of distilled water. Aliquots were stored at \(-20^\circ\)C, and thawed just before use.

**Standard curves**

The construction of standard curves and determination of the extinction coefficient for the PNP-dependent phosphorolysis of AMMPR in the presence of PP\(_i\) and IPP, or inorganic phosphate alone, were necessary to establish the correct response of the assay system. Spectrophotometric determinations were conducted at 30 °C in a 10-mm path length quartz cuvette in a Perkin-Elmer Lambda-2 dual-beam UV/visible light spectrophotometer. Increasing amounts of 50 mM Na\(_2\)P\(_2\)O\(_5\) or KH\(_2\)PO\(_4\) were added to a solution containing 0.2 mM AMMPR, 0.2 units of yeast IPP, 0.2 units of calf spleen PNP and 25 mM Tris/HCl, pH 7.5, in a total volume of 1 ml. Following a 30-min incubation, the absorbance at 360 nm was measured against a control sample without Na\(_2\)P\(_2\)O\(_5\) or KH\(_2\)PO\(_4\). The response of the assay to both PP\(_i\) and P\(_i\) concentration proved to be linear in a range of concentrations from 2.5 to 50 \(\mu\)M PP\(_i\), or P\(_i\). The calculated extinction coefficients for the change in absorbance resulting from phosphorolysis of AMMPR were determined to be, on addition of PP\(_i\), or P\(_i\), 18200 and 9300 M\(^{-1}\)·cm\(^{-1}\), respectively.

The values compare well with those obtained in [16].

**Spectrophotometric determinations**

The assay (1 ml) contained 25 mM Tris/HCl, pH 7.5, 5 mM MgCl\(_2\), 1 mM ATP, the respective amino acid (1 mM), 25 \(\mu\)g/ml apo-TY1, PNP (final concentration 0.2 units/ml), IPP (final concentration 0.2 units/ml) and 0.2 mM AMMPR. Upon addition of the final component, absorbance changes at 360 nm were measured against a reference sample lacking the amino acid substrate. The amount of contaminating P\(_i\) in each reagent was tested by measuring the absorbance of the reaction with and without the reagent subjected to analysis. To prevent contaminating P\(_i\) or PP\(_i\), activity, the assay was allowed to prereact with PNP, IPP and AMMPR for about 10 min before the reaction was initiated.

**RESULTS**

In order to examine the accuracy of amino acid discrimination by peptide synthetases, a profile of different analogues of phenylalanine was assayed in the aminoacyl-adenylate reaction catalysed by apo-TY1. As established from ATP-[\(^{32}\)P]PP, isotope-exchange measurements, apo-TY1 catalyses the activation of both l- and d-Phe [18,19]. Although l-Phe is the preferred substrate, the d-phenylalanyl adenylate is generated at 74 \(\%\) of the rate of the l-isomer. Coupling of the PP\(_i\) production to the phosphate assay enables direct monitoring of the amino acid-activation reaction by recording the absorbance increase at 360 nm, utilizing the spectral shift that accompanies the phosphorylation of AMMPR. The pyrophosphatase reaction measures the total consumption of ATP as the amino acid is first activated to form an enzyme-bound aminoacyl adenylate and is then destroyed in an editing reaction, generating amino acid and AMP. Since the reaction rate is a measure of aminoacyl-adenylate stability, quantification of liberated PP\(_i\) enabled an estimation of the rate constant for aminoacyl-adenylate–enzyme breakdown. Apparent rate constants of \(k = 1.93 \times 10^{-4}\) and 0.18 \(\times 10^{-4}\) s\(^{-1}\) for the decomposition of the d-Phe-AMP–E and the l-Phe-AMP–E complexes (where E is enzyme), respectively, were calculated from the linear portions of the time-dependence curves (Table 1).

**Table 1 The rate of PP\(_i\) production catalysed by apo-TY1 in the presence of ATP and different amino acid analogues**

The reaction was monitored at saturating substrate concentration by recording the phosphorylation of AMMPR in a coupled spectrophotometric assay, as described in the Experimental section.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>(k \times 10^2) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phenylalanine</td>
<td>0.18</td>
</tr>
<tr>
<td>D-Phenylalanine</td>
<td>1.93</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.34</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxy-L-phenylalanine</td>
<td>0.28</td>
</tr>
<tr>
<td>3,4-Dimethoxy-L-phenylalanine</td>
<td>0.42</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>1.20</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1.79</td>
</tr>
<tr>
<td>D-Fluoro-L-phenylalanine</td>
<td>0.32</td>
</tr>
<tr>
<td>D-Chloro-L-phenylalanine</td>
<td>1.27</td>
</tr>
<tr>
<td>D-Amino-L-phenylalanine</td>
<td>4.02</td>
</tr>
<tr>
<td>D-Chloro-D-(2-Thienyl)-L-alanine</td>
<td>4.28</td>
</tr>
<tr>
<td>D-(2-Thienyl)-L-alanine</td>
<td>0.25</td>
</tr>
<tr>
<td>D-Benzoyl-L-serine</td>
<td>0.22</td>
</tr>
<tr>
<td>L-Allo-Threo-D-phenylserine</td>
<td>1.48</td>
</tr>
<tr>
<td>L-(2-Thienyl)-D-serine</td>
<td>2.07</td>
</tr>
</tbody>
</table>
the stability of the respective adenylate–enzyme complex. On the other hand, p-chloro-l-phenylalanyl and p-amino-l-phenylalanyl adenylates with threo-configuration in general were further substantiated by p-chloro-d,l,l-phenylalanyl adenylate, comprising a mixture of both the d- and the l-isomers. Contrary to p-chloro-l-phenylalanyl adenylate, the d-isomer was not capable of forming a long-lived aminoacyl-adenylate intermediate, as manifested by an increased susceptibility to degradation. Decreased and variable degrees of stability exhibited by d,l-threo-β-phenylserine and β-(2-thienyl)-l,ß-serine could be attributed to the steric configuration of the amino acid preparation. Aminoacyl adenylates of alanine and serine analogues, β-(2-thienyl)-l-alanine and O-benzoyl-l-serine, exhibiting variable degrees of catalytic efficiency in the isotope-exchange reaction, were apparently very stable against degradation.

This phenomenon was further analysed for the aminoacyl-adenylate reaction catalysed by engineered mutants of apo-TY1 (Table 2). Here, it was investigated how several specific mutations, created by site-directed mutagenesis, affect the stability of the aminoacyl adenylate. Measurements were performed with mutant proteins, apo-[Arg186]TY1, apo-[Thr186]TY1 and apo-[Thr416]TY1, demonstrating variable degrees of adenylate-forming efficiency, as previously established by the radioisotope assay [24,25]. The apo-[Arg186]TY1 mutant acquired an increased editing activity of the aminoacyl intermediate. In contrast, binding of the adenylate by the apo-[Thr186]TY1 and apo-[Thr416]TY1 mutants lowered its susceptibility to hydrolysis by one order of magnitude.

## DISCUSSION

The adenylate-forming domains of peptide synthetases represent the non-ribosomal code for amino acid selection and their incorporation into the peptide product. The first level of discrimination occurs during substrate recognition through rejection of the non-cognate amino acid by the active site providing a characteristic substrate profile for each adenylate-forming domain. The amino acid-activating domains of the tyrocidine-biosynthesis system can either accurately distinguish between two structurally related substrates or exhibit a relaxed but characteristic substrate profile for each adenylate-forming domain. The amino acid-activating domains of the tyrocidine-biosynthesis system can either accurately distinguish between two structurally related substrates or exhibit a relaxed but characteristic substrate profile for each adenylate-forming domain. The amino acid-activating domains of the tyrocidine-biosynthesis system can either accurately distinguish between two structurally related substrates or exhibit a relaxed but characteristic substrate profile for each adenylate-forming domain. The amino acid-activating domains of the tyrocidine-biosynthesis system can either accurately distinguish between two structurally related substrates or exhibit a relaxed but characteristic substrate profile for each adenylate-forming domain.

![Figure 1](image)

The rate of adenylate formation by apo-TY1 as a function of pH

The absorbance change was measured at 360 nm following a 15-min incubation at 30 °C in 25 mM Tris/HCl of variable pH containing 2–25 µg/ml apo-TY1, 1 mM ATP, 1 mM d-Phe, 5 mM MgCl₂, 0.2 mM AMPR, 0.2 units of IPP and 0.2 units of PNP.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>k × 10² (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-TY1</td>
<td>1.93</td>
</tr>
<tr>
<td>Apo-[Arg186]TY1</td>
<td>3.15</td>
</tr>
<tr>
<td>Apo-[Thr186]TY1</td>
<td>0.63</td>
</tr>
<tr>
<td>Apo-[Thr416]TY1</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 2: The stability of the d-phenylalanyl adenylate in complex with apo-TY1 or mutant proteins

The reaction rate was monitored by measuring time-dependent PPi production using the coupled spectrophotometric assay as described in the Experimental section.
activates l-Gln, and, although only slightly, l-Val and l-Leu, whereas TyrA7 activates both l-Trp and l-Tyr and to a lesser extent l-Leu and l-Phe. Varying degrees of selection between l- and d-forms of amino acids can be found. Most activation sites in peptide synthetases show strict stereospecificity and no adenylate formation is found with the alternative isomer. Some enzymes such as TY1 accept both phenylalanine isomers; however, only the l-form is epimerized and channelled to initiate peptide synthesis [5], supported by an extremely stable l-phenylalanyl adenylate. On the contrary, the d-phenylalanyl adenylate displays increased susceptibility to hydrolysis. The l-Leu-activating site of gramicidin S-synthetase 2 has been shown to accept d-Leu, but it is not able to support continued elongation [6].

Since amino acid activation by peptide synthetases is a two-step process consisting of aminoacyl-adenylate formation followed by aminoacylation of the enzyme-bound cofactor, editing can occur by rejection of either the mis-formed aminoacyl adenylate or the mis-acylated thioester. Conformational changes induced by adenylate formation or interdomain interactions may be essential for protection of the reaction-intermediate from decomposition by hydrolysis or its escape from the enzyme during the catalytic process. Kinetic analysis of firefly luciferase points to substantial conformational changes upon substrate binding, resulting in increased hydrophobicity of the binding pocket [28]. Superposition of the crystal structure of the phenylalanine-activating subunit of gramicidin synthetase 1 (PheA), in ternary complex with l-Phe and AMP [29], with that of non-ligated homologous firefly luciferase [28] reveals considerable motion during adenylate formation and product release. However, not much is known about how modules interact and how this interaction may affect the stability of the transition state. Both Lys-186 and Arg-416 from TY1 are located in regions of exceptionally high mobility, and appear to be implicated in the conformational changes that occur when the l-Phe-AMP-E complex is formed [24,25,28–30]. Mutation of the highly conserved residues destabilizes the transition state for the formation of the aminoacyl adenylate, as evidenced by a reduced kinetic rate of adenylate formation monitored by the ATP-[32P]PP exchange reaction [24,25]. The consequences of the mutation are also reflected in an altered level of aminoacyl-adenylate stability. Apo-[Arg186]TY1 demonstrates an enhanced breakdown of the adenylate complex, presumably due to less-productive contacts.

A comparative analysis of a series of amino acid analogues has shown that apo-TY1 exhibits less-specific initial substrate recognition; however, an efficient editing of the mis-activated amino acid occurs with preference for the d-configuration. A rapid loss of the non-cognate intermediates relative to the l-phenylalanyl adenylate may explain an apparent corrective mechanism that could maintain the accuracy of aminoacylation. Only very dissimilar non-cognate complexes should dissociate rapidly to compete significantly with transfer [31–33]. The breakdown of the aminoacyl adenylate may take place by essentially two routes: first, dissociation of the complex into solution to give free aminoacyl adenylates with subsequent hydrolysis, and secondly, hydrolysis of the aminoacyl adenylate when bound to the enzyme. In solution at pH 7.8 and 25 °C, the first-order rate constants for the hydrolysis of isoleucyl, valyl and tyrosyl adenylate are 0.29 × 10⁻², 0.37 × 10⁻² and 0.76 × 10⁻² s⁻¹, respectively [34]. The relative contributions of the individual corrective processes to the overall editing could not be estimated. If the dissociation rate constant is only slightly faster than the pre-transfer hydrolysis rate constant a large proportion of the hydrolysis is expected to occur on the enzyme.

Limited selectivity in pre-transfer hydrolysis of the non-cognate adenylate, as substantiated by a comparable stability of certain aminoacyl adenylates, suggests that discrimination between l-Phe and the mis-activated amino acid should be strongly reduced. A pre-transfer proof-reading mechanism, however, could be related to the requirement of peptide synthetases to undergo the aminoacylation reaction. Although similar hydrolysis rates characterize the phenylalanyl and tyrosyl adenylates in complex with phenylalanyl-tRNA synthetase in the absence of tRNA₆₅₆, binding of tRNA₆₅₆ leads to a preferential increase of the pre-transfer hydrolysis of the mis-activated tyrosyl adenylate [32,35]. A model has been proposed involving an unstable, strained, tetrahedral intermediate which in the cognate system gives rise preferentially to aminoacylation; this is contrary to the non-cognate system, where the strained intermediate decomposes with formation of free amino acid and AMP. It has been suggested that thioacetylation of peptide synthetases must be faster than hydrolysis, or that it regulates the conformational transition, leading to a faster transfer [18]. The affinity for the substrate amino acid is much higher at the thio template than in the adenylation centres; consequently the activation equilibria are quantitatively shifted towards thioester formation [36,37]. The significance of a putative editing mechanism involving the thiolation site remains to be investigated further using the respective holo-enzymes.

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