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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 (ΔΔTY1), 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 of choice, it is important to note that this does not necessarily require a dimodular construct or an intermediate acyl transfer step.

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 from 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)-D-valine, by δ-(L-α-aminoadipyl)-L-cysteinyl-D-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, ΔΔTY1, comprising residue His18 to Ser534 of TY1 plus a C-terminal tail of 10 residues, was expressed as a His_6-tagged fusion in *Escherichia coli* and shown to be active in the L-phenylalanine-dependent ATP–PP_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.

2. Materials and methods

2.1. Expression and protein purification

For the construction of the His_6-tagged truncated tycA fragment, plasmid pD1 [5] was restricted with *Sph*I and *Kpn*I, and ligated into the pQE31 His_6 tag fusion vector from Qiagen (Hilden, Germany). The His_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 ΔΔTY1 (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 $MgCl_2$. 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_6-tagged fusion protein (His_6ΔΔTY1) expressed in *E. coli*. The fragment retained catalytic activity and substrate specificity in the L-phenylalanine-dependent ATP–[^{32}P]PP_i exchange reaction, as previously described [6]. The reaction mixture, containing ΔΔTY1 in the presence of saturating concentration of L-Phe and $MgATP^{2-}$, was incubated at 30°C. Aliquots were withdrawn at intervals and subjected to HPLC analysis, with reference to a standard mixture presented in Fig. 1. The chromatogram of the reaction mixture (Fig. 2) showed the appearance of a distinct major product (R_t 20.783) exhibiting a chromatographic mobility corresponding to the standard for L-Phe-L-Phe (R_t 20.742). Only minor hydrolysis under generation of AMP could be detected, indicating that the aminoacyl adenylate does not exhibit increased reactivity toward water. Analysis of adenylate stability has confirmed an extremely high stability of the L-phenyl-

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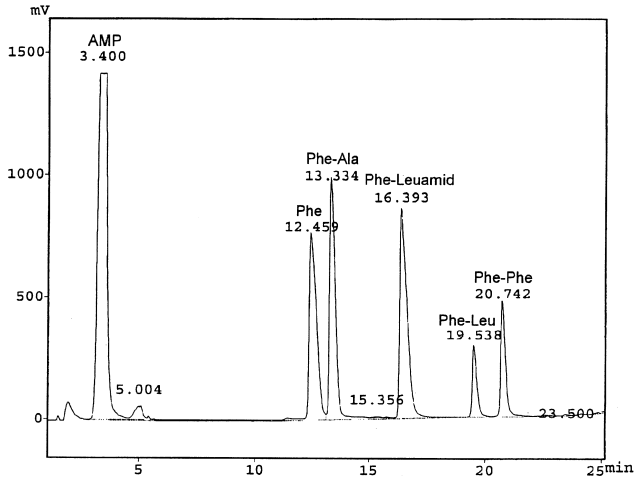

Fig. 1. HPLC profile of the standard mixture containing AMP, L-Phe and various L-phenylalanyl dipeptides. The sample was applied onto a C18 column and separated by isocratic elution with 0.08% TFA for 2 min, followed by a gradient of acetonitrile (0–30%) in 0.08% TFA for 15 min at a flow rate of 1 ml/min and detection wavelength of 210 nm.

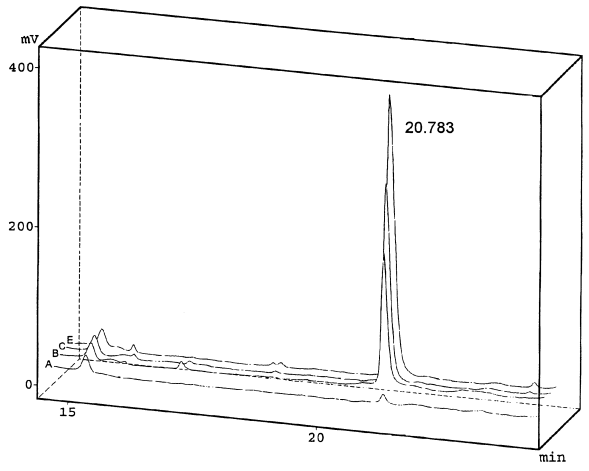

Fig. 2. Time-dependent HPLC profile of L-Phe-L-Phe generation by ΔΔTY1 in the presence of L-Phe and $MgATP^{2-}$. At intervals aliquots of the reaction solution were applied onto a C18 column and analysed by isocratic elution with 0.08% TFA for 2 min, followed by a gradient of acetonitrile (0–30%) in 0.08% TFA for 15 min at a flow rate of 1 ml/min and detection wavelength of 210 nm.

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 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-$PheNH_2$ or L-$LeuNH_2$, 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-$LeuNH_2$ increased the turnover rate of dipeptide formation from k 0.0167 to 0.0186 and 0.0190 min^{-1}, respectively. However, incorporation of L-$PheNH_2$

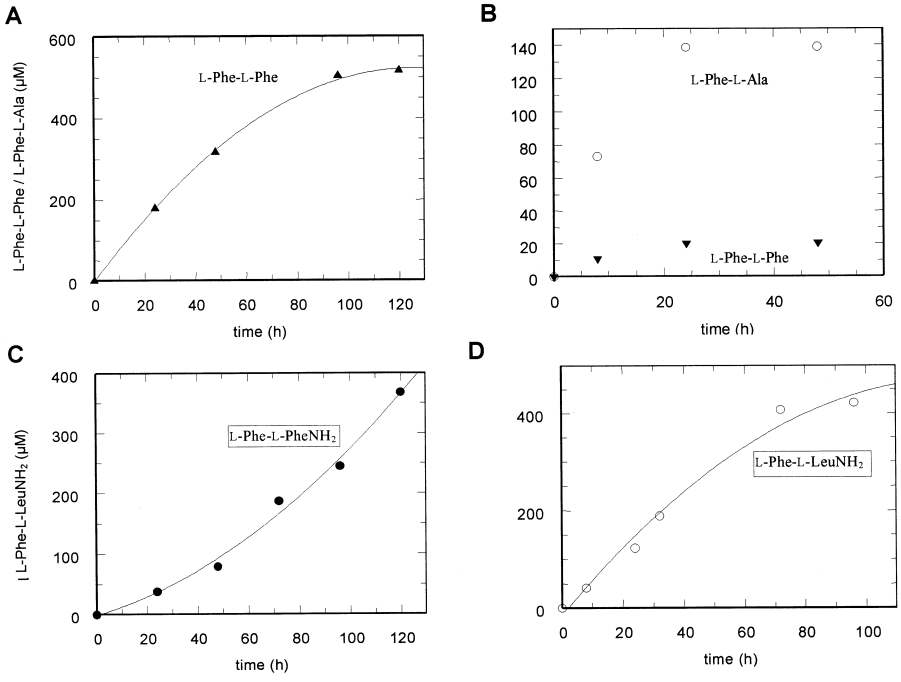

Fig. 3. Time course of synthesis of dipeptide analogues by ΔΔTY1 in the presence of L-Phe, $MgATP^{2-}$ (A) and in the presence of L-Phe, $MgATP^{2-}$, and a non-cognate nucleophile amino acid analogue: (B) L-Phe-L-Ala and L-Phe-L-Phe, (C) L-Phe-L-$PheNH_2$, and (D) L-Phe-L-$LeuNH_2$. The reaction was followed by HPLC analysis relative to the standard mixture.

Table 1
Turnover of dipeptide generation by the isolated adenylation domain of TY1 (ΔΔTY1) in the presence of L-Phe, $MgATP^{2-}$ and various nucleophile substrate analogues

Dipeptide	k (min^{-1})
L-Phe-L-Phe	0.0167
L-Phe-L-Ala	0.0186
L-Phe-L-$LeuNH_2$	0.0190
L-Phe-L-$PheNH_2$	0.0090

into the peptide product proceeds at a slower rate of k 0.009 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 stereoselectively 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 adenylates 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 adenylate-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 0.009–0.019 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 *O*-methylserinyl-D,L-valine. By O^{18}-labelling of the valine carboxyl group dipeptide formation has been shown to proceed at least partially from L-cysteinyl-adenylate or *O*-methyl-L-serinyl adenylate intermediates [3]. The rates of this process have been estimated to about 1–2% of tripeptide biosynthesis, which by itself represents a sequence of 10 enzymatic reactions with an overall turnover number of approximately 10 min^{-1}. An enhancement of the rate of dipeptide formation had been achieved by replacing the initiating amino acid L-α-aminoadipate by L-glutamate, which does form an adenylate, but does not enter the thiotemplate elongation cycle. The formation of glutamyl adenylate is thought to induce a conformational change enhancing this side reaction.

In this cycle NRPS catalyse the formation of peptide bonds from thioester-bound amino(imino) acyl intermediates. These intermediates reside on 4′-phosphopantetheine cofactors covalently attached to carrier protein domains, which are integrated in the modular NRPS structure. Two carrier domains, one adjacent and one non-adjacent, have to interact with a specific condensation domain, where peptide bond formation occurs [9]. The resulting peptidyl intermediate then rests at the non-adjacent carrier domain as a thioester. This process reflects the ribosomal elongation cycle, with the carrier domains representing aminoacyl-tRNAs and the condensation domain the peptidyl transferase [10]. Both aminoacyl thioesters and aminoacyl-tRNAs stabilise the primary activation products, the amino(imino) acyl adenylates, which are formed by 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 adenylate binding site have been shown to be modified in situ by aminoacylation, 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 $Arg\text{-}tRNA^{Arg}$ is catalysed in the presence of cysteine with the formation of Arg-Cys with a k_{cat} of 18 min^{-1}, and a K_M of 150 mM. For free $Arg\text{-}tRNA^{Arg}$ 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 intramolecular cyclisations in lysyl-tRNA synthetase: misacylated ornithine is released by cyclisation with a k_{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 produce 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 intermediate.

References

[1] von Döhren, H., Keller, U., Vater, J. and Zocher, R. (1997) Chem. Rev. 97, 2675–2705.
[2] Marahiel, M.A., Stachelhaus, T. and Mootz, H.D. (1997) Chem. Rev. 97, 2651–2673.
[3] Shiau, C.-Y., Byford, M.F., Aplin, R.T., Baldwin, J.E. and Schofield, C.J. (1997) Biochemistry 36, 8798–8806.
[4] Shiau, C.-Y., Baldwin, J.E., Byford, M.F. and Schofield, C.J. (1995) FEBS Lett. 373, 303–306.
[5] Dieckmann, R., Lee, Y.O., van Liempt, H., von Döhren, H. and Kleinkauf, H. (1995) FEBS Lett. 357, 212–216.
[6] Dieckmann, R., Pavela-Vrancic, M., von Döhren, H. and Kleinkauf, H. (1999) J. Mol. Biol. 288, 129–140.
[7] Conti, E., Stachelhaus, T., Marahiel, M.A. and Brick, P. (1997) EMBO J. 16, 4174–4183.
[8] Pavela-Vrancic, M., Dieckmann, R. and von Döhren, H. (1999) Biochem. J. 342, 715–719.
[9] Linne, U. and Marahiel, M.A. (2000) Biochemistry 39, 10439–10447.

[10] von Döhren, H., Dieckmann, R. and Pavela-Vrancic, M. (1999) Chem. Biol. 6, R273–R279.
[11] Gillet, S., Hountondji, C., Schmitter, J.M. and Blanquet, S. (1997) Protein Sci. 6, 2426–2435.
[12] Jakubowski, H. (1995) Nucleic Acids Res. 23, 4608–4615.
[13] Jakubowski, H. (1997) Biochemistry 36, 11077–11085.
[14] Jakubowski, H. (1999) Biochemistry 38, 8088–8093.
[15] Schneider, A., Stachelhaus, T. and Marahiel, M.A. (1998) Mol. Gen. Genet. 257, 308–318.
[16] Doekel, S. and Marahiel, M.A. (2000) Chem. Biol. 7, 373–384.
[17] Schauwecker, F., Pfennig, F., Grammel, N. and Keller, U. (2000) Chem. Biol. 7, 287–297.