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Relationship between activating and editing functions of the adenylation domain of apo-tyrocidin synthetase 1 (apo-TY1)

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Abstract

Tyrocidine synthetase 1 (TY1), the initial monomodular constituent of the tyrocidine biosynthetic system, exhibits relaxed substrate specificity, however an efficient editing of the mis-activated amino acid provides for fidelity of product formation. We chose to analyse the consequence of single amino acid substitutions, in the amino acid activation site of apo-TY1, on the editing functions of the enzyme. Discrimination between L-Phe and D-Phe by apo-TY1 depends primarily on the editing reaction. Distraction of unnatural amino acid substrates, such as L-PheSer, implies that editing is not designated to select a specific mis-activated amino acid, but instead to discriminate all mis-activated amino acid analogues. It was shown that active site residues which interact with the adenylate are essential for both activation and editing. Substitution of Lys186 with arginine substantially reduces the editing capacity of the protein. Loss of amino acid discrimination ability by the apo-K186T and apo-R416T mutant proteins suggests a role of active site residues in maintaining the structural determinants for substrate selection. Inadequate conformational changes, induced by non-cognate amino acid substrates, promote ATP breakdown yielding P_i and ADP. Replacement of residue Lys186 or Arg416 enhances ATP hydrolysis implying a role in binding or adjusting of the triphosphate chain for adenylate formation and pyrophosphate cleavage.

Keywords: Non-ribosomal peptide synthetases (NRPS); Adenylation domain; Aminoacyl adenylate stability; Editing

1. Introduction

Amino acids can be incorporated into peptides by two peptide-forming systems, the ribosomal system where amino acids are activated by the aminoacyl-tRNA synthetases (aaRSs) as aminoacyl-tRNAs and peptide bond formation is directed by the ribosome, and the non-ribosomal system where amino acids are activated on multifunctional enzyme complexes (non-ribosomal peptide synthetases (NRPS)), also directing peptide bond formation [1,2]. NRPS are organised on modular basis, each module comprising a set of structural and functional domains supporting a single elongation step. The reaction mechanism involves two steps: (i) ATP-linked substrate activation in form of an enzyme-stabilised acyladenylate under release of pyrophosphate (PP_i); (ii) subsequent transfer of the acyl moiety to the sulfhydryl group of a covalently bound 4′-phosphopantetheine cofactor to form an activated thioacyl intermediate and AMP. Activated amino acids are linked into the polypeptide chain by sequential condensation of thioester intermediates, with subsequent release of the peptide product either by cyclisation or hydrolysis.

Contrary to the aaRSs, displaying unique substrate specificity, NRPS adenylation domains may exhibit various degrees of substrate selection, generating positions of variable composition, as well as highly conserved constituents in the peptide structure [1,3]. Amino acid discrimination by the NRPS during substrate binding and activation provides a characteristic substrate profile for each adenylate-forming domain [4].

Abbreviations: aaRS, aminoacyl-tRNA synthetase; AMMP, 2-amino-6-mercapto-7-methylpurine; AMMPR, 2-amino-6-mercapto-7-methylpurine ribonucleoside; Apo-TY1, TY1 devoid of the 4′-phosphopantetheine cofactor; GS1, gramicidin S synthetase 1; IPP, inorganic pyrophosphatase; L-PheSer, L-phenylserine; NRPS, non-ribosomal peptide synthetases; P_i, inorganic phosphate; PheA, L-Phe activating domain of GS1; PNP, purine nucleoside phosphorylase; TY1, tyrocidine synthetase 1.

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Although various natural and synthetic analogues of the cognate amino acid substrate are activated in the adenylate formation reaction, not all acyl intermediates are incorporated into the peptide product. Excessive consumption of ATP indicates that amino acid analogues are hydrolytically removed, but the molecular bases of editing and its relation to the synthetic activity of NRPS are unknown.

The cyclodecapeptide antibiotic tyrocidine, cyclo[DPhe-LPro-LPhe-DPhe-LAsn-LGln-LTyr-LVal-LOrn-LLeu], from *Bacillus brevis* is produced as a mixture of four cyclic decapeptides containing tryptophan in place of phenylalanine in position 3, 4 or 7 [3]. Although the adenylation domain of tyrocidine synthetase 1 (TY1) displays relaxed amino acid specificity, activating a set of amino acid analogues in the first step of the aminoacylation reaction, position 1 is restricted to L-Phe, which is subsequently epimerised into D-Phe [4]. To investigate the mechanism of substrate selection and discrimination, direct measurements of adenylate stability were required. TY1 is a representative example of a monomodular peptide synthetase, containing all functions associated with adenylate formation and aminoacylation. To approach the stability of the aminoacyl adenylate intermediate, the reaction was monitored using the apo-form of TY1. Apo-TY1, generated by expression of the *tycA* gene in *E. coli*, lacks the 4′-phosphopantetheine cofactor [4]. No peptide product generation occurs, however the enzyme catalyses the first step of the amino acid activation reaction, whereby the overall process ceases with an enzyme-bound adenylate in the active site. The fate of the intermediate is determined by several competing reactions. In the reverse reaction with $MgPP_i$, amino acid and ATP are formed. Alternatively, it can be hydrolysed to give free amino acid and AMP. The presence of excess inorganic pyrophosphatase (IPP) largely eliminates the reverse reaction, making the rate of PP_i liberation a suitable measure of aminoacyl adenylate stability.

Fidelity of substrate selection may be 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. In peptide synthetases, the adenylation domain is composed of a large N-terminal and a small C-terminal subdomain, connected by a flexible linker sequence. Superposition of the crystal structure of the L-Phe activating domain (PheA) of gramicidin S synthetase 1 (GS1) in complex with L-Phe and AMP, and the unliganded homologous firefly luciferase has highlighted two regions of poorly defined electron density, implying high flexibility and mobility of the loop structures [5]. In the course of the catalytic process, a series of rearrangements are expected to occur in order to ensure the proper stereochemistry of the catalytic groups, prevent decomposition by hydrolysis, and provide a kinetic barrier for the escape of the reaction intermediates. Residues Lys186 and Arg416 from TY1, located in flexible loop regions, appear to be implicated in conformational changes that occur when the aminoacyl adenylate/enzyme complex is formed [6]. Here we have analysed how replacement changes at Lys186 and Arg416 in the active site of apo-TY1 affect the editing functions of the enzyme. The distraction of the non-cognate aminoacyl adenylate was assayed as excessive hydrolysis of ATP to AMP and PP_i. The generation of PP_i was coupled to a two enzyme detection system involving hydrolysis by IPP, and purine nucleoside phosphorylase (PNP)-dependent phosphorolysis of 2-amino-6-mercapto-7-methylpurine ribonucleoside (AMMPR) [7–9].

2. Materials and methods

2.1. Enzyme preparation

The *E. coli* strain XL1-Blue was used as the host for plasmids containing the *tycA* (apo-TY1) gene and mutants thereof (K186TR, K186T, and R416T). The recombinant proteins were isolated and purified to homogeneity according to [4]. Protein content and purity have been monitored using SDS-PAGE. Protein concentration was determined according to [10].

2.2. Synthesis of AMMPR

The synthesis of the AMMPR reagent was performed according to [7]. 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 °C, and thawed just before use.

2.3. Standard curves

The standard curve for PNP-dependent phosphorolysis of AMMPR, in the presence of P_i or PP_i, was constructed from spectrophotometric determination of the absorbance change at 360 nm in a reaction solution containing 25 mM Tris–HCl, pH 7.5, 0.2 mM AMMPR, 0.2 units ml^{-1} PNP, 0.2 units ml^{-1} inorganic pyrophophatase (IPP) and increasing amounts of KH_2PO_4 or $Na_4P_2O_7$, respectively. Measurements were conducted, following a 30-min incubation, against a control sample without KH_2PO_4 or $Na_4P_2O_7$. The response of the assay to the P_i and PP_i concentration proved to be linear in a range of concentrations up to 60 μM. The calculated extinction coefficient for the change in absorbance, resulting from phosphorolysis of AMMPR, was determined to be 9300 and 17800 M^{-1} cm^{-1} for P_i and PP_i, respectively.

2.4. In vitro editing assay

The rate constant for the overall editing reaction was determined by following the apo-TY1-dependent consumption of ATP to AMP and PP_i, as the non-cognate amino acid is activated in the synthetic reaction and destroyed in the editing reaction. The reaction mixture contained 200 nM enzyme in 25 mM Tris–HCl, pH 7.5, 1 mM ATP, 10 mM magnesium acetate, 1 mM amino acid, 0.2 mM AMMPR, 0.2 units ml^{-1} PNP. The assay solution was allowed to pre-react with