Active site titration of gramicidin S synthetase 2: evidence for misactivation and editing in non-ribosomal peptide biosynthesis

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Received 18 June 1999; received in revised form 13 October 1999

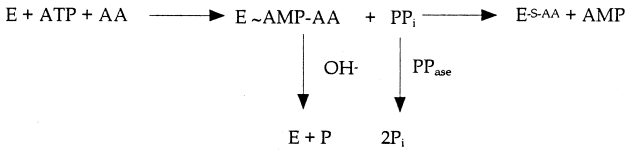
Abstract The catalytic competence of gramicidin S synthetase 2 (GS2) was determined by following the kinetics of PP_i generation using active site titration measurements with [γ-^{32}P]ATP. The initial 'burst' of product formation can be correlated to the generation of the aminoacyl adenylate:enzyme complexes at the four amino acid activation domains and the subsequent aminoacylation of carrier domains, followed by a slow linear turnover of substrate due to breakdown of the intermediate. Simultaneous activation of all four amino acid substrates at a saturating concentration displayed a consumption of 8.3 ATP/GS2. In the presence of single amino acids, a binding stoichiometry higher than the anticipated two ATP per active site was obtained, implying misactivation at non-cognate domains. Breakdown of acyladenylate intermediates reflects a possible corrective mechanism by which the enzyme controls the fidelity of product formation.

Key words: Multienzyme; Peptide synthetase; Gramicidin S synthetase 2; Aminoacyl adenylation; Misactivation; Editing

1. Introduction

A wide variety of bacterial and fungal low molecular weight peptide products are synthesized via the non-ribosomal pathway by multifunctional peptide synthetases [1]. According to the reaction mechanism, one molecule of ATP is consumed for the formation of one peptide bond. To establish the exact molecular mechanism of peptide synthetases, the binding stoichiometries of the ligands must be determined at a high degree of accuracy. The carboxyl substrates are initially activated by the formation of an acyladenylate:enzyme complex with concomitant release of PP_i. The adenylate reacts in a transfer reaction with 4′-phosphopantetheine, a covalently bound enzyme thiol acceptor, to yield an active thioester and AMP (Eq. 1). Upon acylation, a second adenylate should be formed, which cannot react further due to occupancy of the thioester site (Eq. 2). The acyladenylate may, however, react in the reverse reaction with $MgPP_i^{2-}$ or decompose by hydrolysis. The ligand binding stoichiometry was investigated using gramicidin S synthetase 2 (GS2) as a model system [2].

GS2 is a component of the multienzyme complex composed of two complementary multifunctional proteins (GS1 and 2) involved in the biosynthesis of the cyclodecapeptide antibiotic gramicidin, cyclo(D-Phe-L-Pro-L-Val-L-Orn-L-Leu)$_2$, produced by *Bacillus brevis*. GS1 activates L-phenylalanine and catalyzes its epimerization into the D-form. Peptide elongation is initiated by the transfer of the activated aminoacyl group from GS1 to GS2, which is responsible for the sequential activation of the remaining four amino acids, L-Pro, L-Val, L-Orn and L-Leu, and their incorporation into the peptide product. GS2 contains four amino acid binding sites and at least four ATP binding sites. Since the starter amino acid is missing, no products are formed, but enzyme bound intermediates are generated. If each amino acid activating domain of GS2 forms two acyladenylates per active site, one of which is consumed in the thioacylation reaction, a consumption of eight ATP molecules per molecule of enzyme is expected in a simultaneous activation of its cognate amino acid substrates at a saturating concentration.



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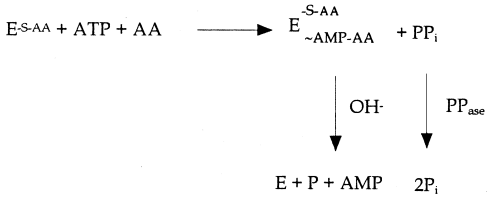

(2)

The catalytic competence of peptide synthetases may be determined either by charging or by reactions depending on aminoacyl adenylation. To assess the stoichiometry of ATP consumption by GS2, we followed the kinetics of PP_i generation using active site titration measurements with [γ-^{32}P]ATP. The method has been successfully applied in determining the number of catalytically competent active sites in aminoacyl-tRNA synthetases based on the stoichiometry of aminoacyl adenylate formation [3]. Such reactions exhibit an initial 'burst' of product generation, corresponding to the formation of the enzyme:aminoacyl adenylate complex, followed by a slower reaction which depends on its rate of decomposition.

2. Materials and methods

2.1. Protein preparation

GS2 was isolated and purified from *B. brevis* ATCC 9999 as de-

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PII: S0014-5793(99)01445-3

scribed by Kittelberger et al. [4]. Protein concentrations were estimated from the measured absorbance at 280 nm using the calculated extinction coefficient.

2.2. Calculation of the protein extinction coefficient

Here, we have applied a method for calculating the molar extinction coefficient at 280 nm from the amino acid composition data using model chromophores for the absorbing residues of the protein [5]. The molar extinction coefficient of the denatured protein in 6 M guanidiniumchloride was calculated from the number of tryptophan (37) and tyrosine (190) residues per molecule using the molar extinction coefficient of the appropriate model compound, *N*-acetyl-L-tryptophanamide (5690 M^{-1} cm^{-1}), and Gly-L-Tyr-Gly (1280 M^{-1} cm^{-1}) in the same solution at 280 nm. A value of $\varepsilon = 453\,730$ mol^{-1} cm^{-1} was obtained for GS2 at 280 nm and 37°C. The molecular mass of GS2 calculated from the sequence data plus four 4′-phosphopantetheine residues (one for each amino acid activating domain) provides a value of 511 372 Da.

2.3. Isotope exchange assay

The radioisotope exchange activity measurements, recording the amount of radioactivity (cpm) incorporated into charcoal-adsorbed ATP, were performed proceeding a 15 min incubation at 37°C in an assay mixture containing in a final volume of 70 μl: 0.2 M cacodyate (pH 5.6–7.4) or 0.2 M glycine buffer (pH 7.6–10.4), GS2 (2 μg/ml), 5 mM $MgCl_2$, 0.5 mM dithioerythritol, 0.025 mM EDTA, 0.1 mM PP_i, 0.1 μCi [^{32}P]PP_i, 1 mM respective amino acid substrate, 1 mM ATP and 1.4 μg of the multienzyme. The reaction is terminated by the addition of an activated 2% charcoal suspension (100 μl) containing 7% perchloric acid and 0.1 M sodium pyrophosphate, the charcoal is separated by filtration through Schleicher and Schuell GF92 filters (2.5 cm diameter), followed by washing with 20 ml deionized water.

2.4. Active site titration

The reaction solution containing [γ-^{32}P]ATP, $MgCl_2$, sodium phosphate, the respective amino acid, DTE, EDTA and yeast inorganic pyrophosphatase at concentrations as indicated in the text was incubated at 37°C. The reaction was initiated by addition of GS2 to the reaction mixture. Aliquots (100 μl) were taken at time intervals and quenched with an activated 2% charcoal suspension (100 μl) containing 7% perchloric acid. Following centrifugation, aliquots (100 μl) of the supernatant were subjected to radioactivity measurements of [^{32}P]P_i.

3. Results

The catalytic competence of GS2 was investigated by measuring the stoichiometric formation of pyrophosphate from ATP on formation of an aminoacyl adenylate. The addition of pyrophosphatase ensures irreversibility of the adenylation step. Under the assumption that both activation reactions in Eqs. 1 and 2 are analogous, a simplified reaction scheme (Eq. 3) may be derived

$$[\gamma\text{-}^{32}\mathrm{P}]\mathrm{ATP} + \mathrm{AA} + \mathrm{E} \xrightarrow{k_1} \mathrm{AA} \sim \mathrm{AMP}\cdot\mathrm{E} \xrightarrow{k_2} \mathrm{E} + \mathrm{AA} + \mathrm{AMP} + [^{32}\mathrm{P}]\mathrm{PP_i} \quad (3)$$

where E equals peptide synthetase, AA is the amino acid, E·AA–AMP the enzyme bound adenylate and k_1 and k_2 are the pseudo-first-order rate constants for the formation and decomposition of the aminoacyl adenylate complex, respectively, at a given amino acid and ATP concentration. The P_i generation-dependence can be expressed with the following equation

$$[\mathrm{P_i}] = n[\mathrm{E}]_o \left(\frac{k_1}{k_1+k_2}\right)\left(\frac{k_1}{k_1+k_2} - \frac{k_1 e^{-(k_1+k_2)t}}{k_1+k_2} + k_2 t\right) \quad (4)$$

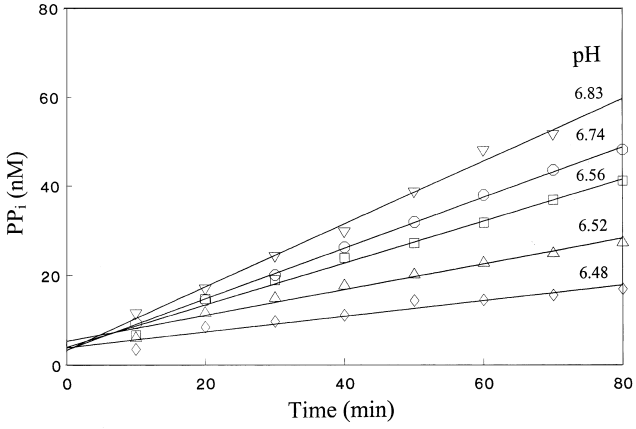

Fig. 1. A time course of PP_i generation in the course of ornithyl-adenylate formation from ornithine (1 mM), [γ-^{32}P]ATP (153 nM) and GS2 (3.3 nM) at 37°C in the presence of inorganic pyrophosphatase (5 U/ml), DTE (10 mM), $MgCl_2$ (10 mM), EDTA (0.25 mM) and 20 mM sodium phosphate buffer at variable pH.

where n equals the number of mol of aminoacyl adenylate per mol enzyme, $[E]_o$ is the total enzyme concentration, t is the reaction time and k_1 and k_2 are the apparent rate constants. In case when $k_1 \gg k_2$ and $t \gg (k_1+k_2)^{-1}$, Eq. 5 is obtained.

$$[\mathrm{P_i}] = n[\mathrm{E_o}] + n[\mathrm{E_o}]k_2 t \quad (5)$$

The ATP depletion rate, expressed as the equimolar amount of [^{32}P]P_i liberated versus time, was examined at various pH conditions using L-Orn (1 mM) as the amino acid substrate (Fig. 1). The concentration of the product initially increases in a rapid exponential phase which is followed by a relatively slow linear turnover of substrate due to decomposition of the aminoacyl adenylate. The rate of decomposition of the enzyme:adenylate complex increases with increasing pH (Fig. 1). The reaction rate increases linearly with the amount of enzyme used (Fig. 2). An apparent rate constant k_2 of 0.1×10^{-3} s^{-1} for the decomposition of the enzyme:adenylate complex

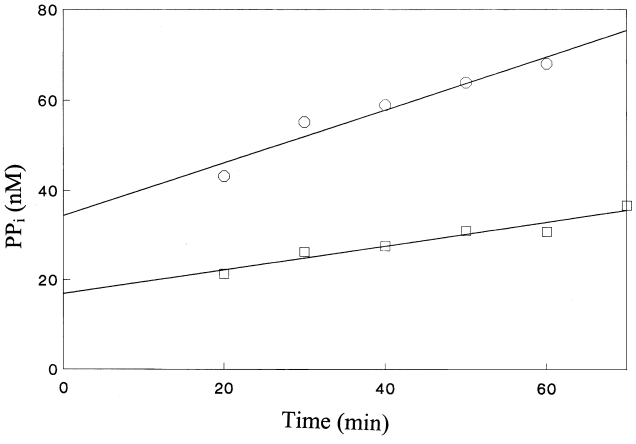

Fig. 2. The time-dependence of P_i generation in the course of ornithyl-adenylate formation at a variable enzyme concentration: (□) 4.6 nM and (○) 9.1 nM GS2. The reaction was conducted in 20 mM sodium phosphate buffer, pH 6.3, containing inorganic pyrophosphatase (5 U/ml), DTE (10 mM), EDTA (0.25 mM), $MgCl_2$ (10 mM), ornithine (1 mM) and [γ-^{32}P]ATP (190 nM) at 37°C.

was calculated from the linear portion of the time-dependence using Eq. 5. An approximation of k_1 to 5×10^{-3} s^{-1} has been obtained from the data shown in Fig. 3 (calculation not shown). The stoichiometry of ATP binding is evaluated by extrapolating the linear portion of the time-dependence back to zero time. A pH profile (pH 5.5–10.5) of the amino acid activation reaction, monitored by the isotope exchange assay, exhibited a bell-shaped dependence with maximum activity at pH 8 for all four amino acids of the GS2 system (Fig. 3). The inflection point (half-maximum activity) corresponds to a pH value of 6.2 ± 0.1. A kinetic analysis of GS2 has previously been performed using the isotope exchange assay [6]. It has been established that the binding affinities of the respective amino acids are of the same order of magnitude, K_d 0.25, 0.40, 0.25 and 0.30 mM for L-Pro, L-Val, L-Orn and L-Leu, respectively, at pH 7.2. Hence, optimization of the reaction conditions was conducted using L-Orn as the amino acid substrate at pH 6.3. Measurement of the catalytic efficiency at varying ornithine concentrations has shown that saturation is obtained at an amino acid concentration of approximately 3 mM (K_m 1 mM). The saturating concentration is 10 times the binding constant (K_d) for L-Orn at pH 6.3. The optimal Mg^{2+} concentration was estimated to be 10 mM $MgCl_2$ (data not shown). The required conditions for a 'burst' assay were satisfied at low concentrations of the nucleotide substrate, so that ATP concentrations in the nM range were used.

At saturating concentrations of single substrates, GS2 forms 2.2 aminoacyl adenylates per molecule of enzyme in the presence of L-Pro, 3.6 with L-Val, 2.7 with L-Orn and 3.7 with L-Leu (Fig. 4). When activity measurements were performed in the presence of varying amino acids at a 3 mM concentration, in addition to 3 mM L-Orn, ratios of 4.9, 7.6 and 6.7 ATP/GS2 for L-Orn+L-Pro, L-Orn+L-Val and L-Orn+L-Leu, respectively, were obtained. Higher binding stoichiometries were found than would be expected from either one or two active sites per enzyme molecule. When all amino acids involved were introduced simultaneously at a saturating substrate concentration, 8.3 aminoacyl adenylates were generated. A time-dependent increase in ATP uptake was observed as the complex decomposes, releasing the enzyme for further aminoacyl adenylate formation. The decomposition rate constants were estimated from the linear portion of the time-dependence using Eq. 5, providing values of 0.138×10^{-3}, 0.07×10^{-3}, 0.09×10^{-3} and 0.07×10^{-3} s^{-1} for the L-prolyl, L-valyl, L-ornityl and L-leucyladenylates, respectively.

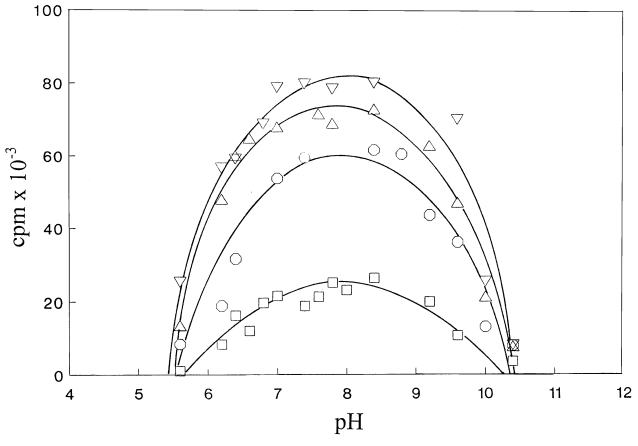

Fig. 3. The pH profile of the adenylate forming activity catalyzed by GS2. The reaction was monitored using the ATP-[^{32}P]PP_i exchange assay as described under Section 2 in the presence of (□) L-Pro, (○) L-Orn, (△) L-Val and (▽) L-Leu as the amino acid substrate.

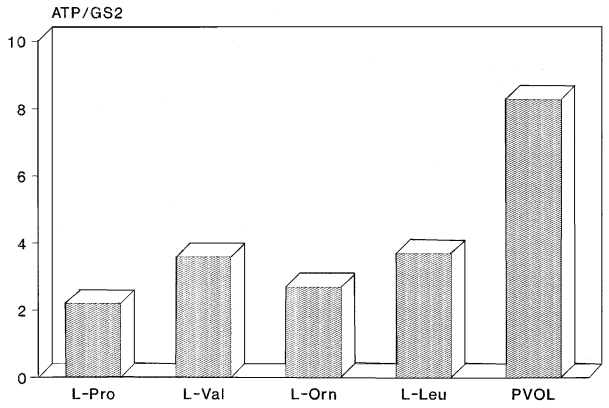

Fig. 4. Stoichiometry of ATP binding to GS2. The stoichiometry of binding was calculated by back extrapolation to $t=0$, as demonstrated in Fig. 2, providing the 'burst' and consequently the n-value. The reaction was conducted in the presence of single amino acids (L-Pro, L-Val, L-Orn or L-Leu) or the full complement (L-Pro, L-Val, L-Orn and L-Leu) at 3 mM each. The reaction was monitored upon incubation at 37°C in 20 mM sodium phosphate buffer, pH 6.3, containing [γ-^{32}P]ATP (350 nM), GS2 (5.5 nM), inorganic pyrophosphatase (5 U/ml), DTE (10 mM), EDTA (0.25 mM) and $MgCl_2$ (10 mM).

4. Discussion

In peptide synthetases, aminoacyl adenylates are cleaved by the action of the enzyme bound thiol, resulting in formation of a thioester bond between the amino acid and the enzyme. It has been assessed in peptide synthetases that at saturating substrate concentrations, one thioester and one adenylate should be formed utilizing two ATP molecules per active site [1,6]. Stoichiometric estimations in these studies, however, relied on inaccurate molecular mass data. We have established from titration data with GS2 at saturating concentrations of substrate a stoichiometry of 8.3 molecules ATP per molecule enzyme, providing evidence in favor of this hypothesis. However, in the presence of a single amino acid substrate, a binding stoichiometry higher than the anticipated two ATP molecules per molecule GS2 was obtained, indicating misactivation at neighboring domains. In vitro synthesis employing gramicidin S synthetase has shown that the complex may generate a peptide analogue with leucine in the place of valine [7]. Activation of L-Leu at the L-Val site in addition to its cognate site substantiates the detected stoichiometry of approximately 3.7 ATP/GS2. The increased stoichiometry of binding obtained with L-Val or L-Orn reflects misactivation at a non-cognate aminoacyl adenylation domain. The burst is followed by a steady-state-dependence resulting from decomposition of the enzyme:aminoacyl adenylate complex. The data do not permit an accurate estimation of the possible decomposition pathway and their contribution to an excess energy consumption. The kinetics of the thioester formation reaction of GS2 were previously investigated [8]. The thioesterification reactions are expected to be faster than hydrolysis and conformational transitions upon adenylate formation may enhance the

transfer rate [9]. Rates have been estimated at 1°C by filter binding assays [10]. Thioester formation with L-Orn and L-Leu proceeded at half-life times of approximately 20 s. The valine activation reaction exhibited a $t_{1/2}$ of 570 s at 1°C with an appreciable temperature-dependence. The apparent affinity for the substrate amino acid is much higher for thioesterification than in the adenylation reactions [10]. However, thioesters are hydrolyzed slowly in neutral solution [11]. Analysis of thioester stability demonstrates that the GS2-L-Pro thioester complex decomposes with a half-life of 62 h at 3°C and pH 7.2.

Breakdown of the adenylate:enzyme complex may take place by hydrolysis of the respective aminoacyl adenylate intermediate. In the ribosomal biosynthesis system, the fidelity of protein synthesis depends on the high specificity of aminoacyl-tRNA synthetases controlled by hydrolytic proof-reading, which may consume several additional molecules of ATP depending on the respective aminoacylation system [12]. Analysis of aminoacyl adenylate stability in the amino acid activation reaction catalyzed by tRNA synthetases has revealed that the rate constant for the hydrolysis of cognate aminoacyl adenylates from the enzyme is in general very low [13–15]. Hence, only non-cognate complexes should decompose rapidly to compete significantly with transfer, reflecting a possible mechanism by which the enzyme aborts the catalytic cycle by releasing the 'incorrect' amino acid into solution. Superposition of the crystal structure of the phenylalanine activating domain of GS1 (PheA), in ternary complex with L-Phe and AMP [16], and that of non-liganded firefly luciferase [17] reveals considerable motion during adenylate formation and product release. Conformational changes induced by adenylate formation or interdomain interaction are expected to protect the reaction intermediates from decomposition by hydrolysis and prevent their dissociation from the enzyme. Decomposition may be in part the result of abortive side reactions, such as the formation of dipeptides from active aminoacyl adenylates. Small but detectable amounts of dipeptides, such as L,D-cisteinyl-valine and L-O-(methylserinyl)-L-valine, are formed by ACV synthetase from interaction of an enzyme bound acyladenylate and an amino group of a free amino acid without the involvement of an activated intermediate thioester moiety [18–21]. The dipeptides were detected in solution as 'shunt' products and not as enzyme bound intermediates. Some members of the adenylate forming superfamily, such as the acyl CoA synthetases [22] and luciferase [23,24], catalyze in the presence of pyrophosphatase and ATP or polyphosphates the formation of a variety of adenosine 5′-polyphosphates (Ap_nA and p_nA) by transferring the adenylyl moiety from the aminoacyl adenylate onto the corresponding acceptor. The results support the occurrence of two binding sites for ATP, one with high affinity for $MgATP^{2-}$ and high specificity to form an E·AA–AMP complex and the other with a low affinity and low specificity, binding ATP, NTP and polyphosphate chains. Comparative studies with nucleotide analogues have postulated the possible existence of a second nucleotide binding site in peptide synthetases [25].

Acknowledgements: The work has been supported by Grants from the BMBF (GW/326G0A61, Structural and functional analysis of multienzyme systems for the production of antibiotics) and by the DFG (Kl 148/31-1, Integrated enzyme systems in peptide synthesis).

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