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Peptidyl-prolyl-tRNA at the ribosomal P-site reacts poorly with puromycin

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Abstract

Despite remarkable recent progress in our chemical and structural understanding of the mechanisms of peptide bond formation by the ribosome, only very limited information is available about whether amino acid side chains affect the rate of peptide bond formation. Here, we generated a series of peptidyl-tRNAs that end with different tRNA-attached amino acids in the P-site of the *Escherichia coli* ribosome and compared their reactivity with puromycin, a rapidly A-site-accessing analog of aminoacyl-tRNAs. Among the 20 amino acids examined, proline was found to receive exceptionally slow peptidyl transfer to puromycin. These results raise a possibility that the peptidyl transferase activity of the ribosome may have some specificity with regard to the P-site amino acids.

Keywords: Ribosome; Peptidyl transferase; Puromycin; Amino acid specificity; Translation

In the elongation cycle of translation, the ester carbonyl group of nascent peptidyl-tRNA in the P-site of the ribosome receives a nucleophilic attack by the α-amino group of the A-site-located aminoacyl-tRNA, resulting in the formation of an additional peptide bond between the last amino acid of the peptidyl-tRNA and the A-site aminoacyl-tRNA. This reaction is catalyzed by the peptidyl transferase center (PTC) of the ribosome, in which specific residues of 23S rRNA (in the case of prokaryotic ribosomes) play pivotal roles [1–3]. Different mechanisms have been proposed for the peptidyl transfer catalysis by the PTC, including the general acid–base catalysis, positioning of the substrate moieties, and electrostatic stabilization of the reaction intermediate [2]. Recent studies indicate that ribosome employs the strategies of lowering the entropy of activation rather than the general acid–base catalysis to facilitate peptide bond formation [3–5].

It is believed that the entry of an aminoacyl-tRNA to the A-site rather than the transpeptidation reaction itself is the rate-limiting factor in translation elongation [5]. Therefore, the rate of peptide bond formation can only be measured by using a small molecule analog of aminoacyl-tRNA, including puromycin [6], which enters the A-site very rapidly [4,7] and binds to the 23S rRNA PTC region including a small loop that contains G2553, a residue that specifically interacts with C75 of aminoacyl-tRNA [8,9].

Although different species of aminoacyl-tRNAs are known to enter the A-site at a uniform rate, which is determined by combinations of tRNAs and amino acids [10,11], there is no information whether amino acid side chains have any effect on the intrinsic rate of peptide bond formation. In our previous studies of in vitro translation of SecM using truncated messenger RNAs and the translation system (PURE SYSTEM) composed of all purified components, we observed that peptidyl-tRNA having proline at the P-site only slowly reacted with puromycin [12]. This phenomenon, observed independently of the elongation-arresting property of SecM [13,14], prompted us to examine systematically and using a SecM-unrelated polypeptide sequence whether P-site amino acids affect rates of transpeptidation to puromycin. Results obtained indicate that

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proline is indeed unique in that it reacts very slowly with puromycin.

Materials and methods

Coupled transcription–translation in vitro. For in vitro translation of $OmpA_{1-154}$ and its derivatives, we used PURE SYSTEM [15], purchased from Post Genome Institute (Tokyo Japan). The "classic standard" reaction mixture contained 32 purified components including T7 RNA polymerase, *Escherichia coli* ribosomes, all the initiation, elongation and termination factors as well as purified aminoacyl-tRNA synthetases, in addition to tRNAs, nucleotides, amino acids and other ingredients necessary for protein biosynthesis. We also used a specially prepared reaction mixture that lacked proline. Reactions were directed by DNA templates (5 μg/ml) described below in the presence or absence of ^{35}S-methionine (12.3 MBq/ml with specific radioactivity of 43.5 TBq/mmol obtained from American Radiolabeled Chemicals) at 37 °C according to the manufacturer's instruction.

DNA templates for coupled transcription–translation. Templates were prepared in two steps as instructed by PURE SYSTEM protocols. The second PCR was to attach the T7 promoter and an SD sequence carried on the "universal" upstream primer provided by the supplier for in vitro transcription and translation. In the first PCR, a segment of *ompA* corresponding to the first to the 154th codon, was amplified from plasmid pHM525 (carrying *ompA(Cys290Gly)-his$_6$-myc*) [16] using the upstream primer, 5′-*AAGGAGATATACCA*ATGAAAAAGACAGCTATCGCG-3′ (the initiation codon of *ompA* is underlined and residues complementary to the universal primer are italicized) and the downstream primer, 5′-AGGAGTGATCGCGTACTCAACACCGCC-3′ (the triplet that is complementary to the 154th codon, CCT for proline, of *ompA* is underlined). We also used down stream primers having different triplets that were complementary to codons for other 19 amino acids at the above position. The codons chosen were (the numbers in parentheses indicate the usage frequency per thousand in *E. coli* W3110 listed at http://www.kazusa.or.jp/codon/): Pro, CCU (7.0); Ala, GCU (15.2); Arg, CGU (21.0); Asn, AAC (21.6); Asp, GAC (19.1): Cys, UGU (5.1); Gln, CAG (29.0); Glu, GAA (39.7); Gly, GGU (24.7); His, CAC (9.8); Ile, AUC (25.2); Leu, CUG (53.1); Lys, AAA (33.6); Met, AUG (27.8); Phe, UUC (16.5); Ser, UCU (8.4); Thr, ACC (23.5); Tyr, UAC (12.2); Trp, UGG (15.2); Val, GUU (18.2). The PCR products of ~500 bp were purified by 1% agarose gel electrophoresis and used as templates for the second PCRs, which used the universal primer (5′-GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCA-3′) and the respective same downstream primers as used in the first PCRs. Note that *Pfu* Turbo DNA polymerase (Stratagene) was used for the second PCR to avoid an A/T addition to the 3′ ends of the products. The template DNAs were finally purified by MicroSpin S-400 HR column (GE Helthcare).

Puromycin release assay. PURE SYSTEM reactions were carried out for 30 min (experiments for Figs. 2 and 3 and Table 1) or 60 min (experiment for Fig. 1) either in the presence (experiments for Figs. 2 and 3 and Table 1) or in the absence (experiment for Fig. 1) of ^{35}S-methionine. Puromycin (Sigma) was added at specified concentrations (results were unchanged between 2 μg/ml and 1 mg/ml) and incubation continued for additional 0.5 or 3 min. Samples were mixed immediately with an equal volume of RNA*secure* (Ambion)-treated 125 mM Tris–HCl (pH 6.8) containing 4% SDS, 20% glycerol, and a trace of bromophenol blue, and electrophoresed through a 12% NuPAGE Bis-Tris gel (Invitrogen) with MES SDS running buffer at a constant voltage of 200 V. The peptidyl-tRNA ester bond is preserved during separation with this neutral pH SDS–PAGE system [12], allowing $OmpA_{1-154}$-tRNA to migrate at a position of ~36 kDa. $OmpA_{1-154}$-puromycin migrated at a position of ~13 kDa along with a background radioactive material, which was observed even without added template (data not shown) or in the absence of an amino acid (see Fig. 3, lanes 1–3). Radioactive products were visualized and quantified using a phosphor imager, BAS1800 (Fuji Film). $OmpA_{1-154}$-tRNA having $tRNA^{Pro}$ and its variants having either $tRNA^{Ala}$ or $tRNA^{Gly}$ were also detected by northern hybridization exactly as described previously [12]. To allow incorporation of a proline analog, we used a PURE SYSTEM custom product that did not contain proline. It was supplemented either with L-proline (0.1 mM), L-azetidne-2-carboxylic acid (azetidine, obtained from Sigma; 2 mM), L-thiazolidine-4-carboxylic acid (thiaproline, from BACKEM; 2 mM) or 3, 4-dehydro-L-proline (dehydroproline, from Sigma; 2 mM).

Table 1
Reactivities of puromycin with different amino acids

Last amino acid of peptidyl-tRNA	Peptidyl-tRNA remaining (%)[a]			
	0.5 min		3 min	
	+Puromycin	−Puromycin	+Puromycin	−Puromycin
Pro§	92	99	72	95
Ala§	33	103	21	100
Arg‡	45	122	31	126
Asn	41	97	23	91
Asp‡	47	99	36	94
Cys	61	99	37	90
Gln‡	44	97	27	92
Glu	50	102	32	99
Gly§	43	100	28	97
His	54	106	38	101
Ile	43	100	27	111
Leu	44	101	23	93
Lys§	47	103	28	98
Met	33	100	20	91
Phe‡	50	100	29	99
Ser‡	35	107	16	103
Thr‡	46	102	30	95
Tyr‡	52	99	25	95
Trp	53	102	28	101
Val	44	102	35	99

[a] Reactions were carried out as described in the legend to Fig. 2 and the values were calculated as described in the legend to Fig. 3. Shown are values from a single experiment, whereas amino acids with § were examined in two additional experiments and those with ‡ were examined in one additional experiment with similar results.

Results

Puromycin-sensitivities of proOmpA$_{1-154}$ nascent chain and its Pro154Ala and Pro154Gly variants as detected by tRNA hybridization

We previously examined puromycin-sensitivities of the translation products directed by truncated *secM* templates by NuPAGE separation followed by northern hybridization using probes specific for $tRNA^{Pro}$, $tRNA^{Ala}$, and $tRNA^{Gly}$ [12]. $SecM_{1-153}$ having proline as the last amino acid that was attached to $tRNA^{Pro}$ was partially puromycin-resistant whereas products ending with alanine or glycine were released rapidly by puromycin, except for $SecM_{1-166}$ that contained glycine-ending but puromycin-resistant component because of the "arrest sequence" unique to SecM [12,14]. To allow a rigorous comparison of these amino acids with respect to their reactivity with puromycin, we constructed truncated templates with an upstream sequence (codons 1–153 of *ompA*) that was entirely unrelated to SecM and ending either with proline (the native 154th codon of *ompA*), glycine or alanine. They