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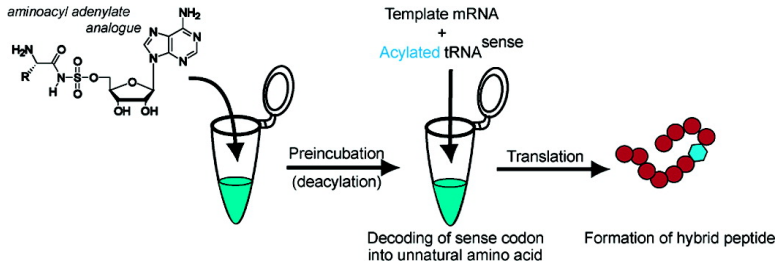
J. Am. Chem. Soc., **2005**, 127 (22), 7998-7999• DOI: 10.1021/ja0502977 • Publication Date (Web): 13 May 2005



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Published on Web 05/13/2005

A Small-Molecule-Based Approach to Sense Codon-Templated Natural-Unnatural Hybrid Peptides. Selective Silencing and Reassignment of the Sense Codon by Orthogonal Reacylation Stalling at the Single-Codon Level

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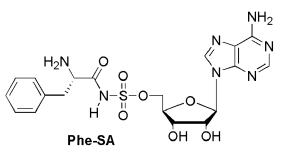
The ribosome is a highly sophisticated RNA machine that directs the mRNA-templated process of amino acid polymerization, which ultimately produces size-, shape-, and sequence-defined polymers (i.e., proteins). The previous demonstration of the "adapter hypothesis"[1] and intensive work to incorporate unnatural substrates with chemically misacylated tRNAs[2] have led to the current challenge of using in vivo[3] or in vitro sense codon-decoding[4−7] translation systems to synthesize sequence-programmed peptidomimetics and unnatural biopolymers. However, because of the unavoidable competition with endogenous tRNA, effective in vitro sense codon suppression has been limited to rare or minor codons.[5,7c] More abundant codons could also be suppressed with misacylated tRNAs to give fully unnatural peptidomimetics but only in endogenous RS/tRNA-free[6] or tRNA-depleted[7] translation systems. Although these elaborate systems allow sense codon-based decoding, the reconstitution and modification procedures require specialized techniques, thus severely restricting their broad applications. We designed a different and simple route, an in situ "orthogonal reacylation stalling" method, to achieve sense codon reassignment. This new route is based on the successful demonstration that the translation pauses codon-selectively in a cell-free system prepared from specific tRNA synthetase (RS)-defective *E. coli* strains.[8] Here, we demonstrate that unnatural substrates [e.g., hydrophobic naphthylalanine (Nap) in this report] can be decoded at the sense codon when the reacylation of the residue-specific tRNA is stalled by inhibiting the responsible aminoacyl tRNA synthetase (aa-RS) activity.[9]

To achieve specific aa-RS deactivation under homogeneous conditions, we focused on 5′-*O*-[*N*-(aminoacyl)sulfamoyl] adenosine (aa-SA), a well-known analogue of aminoacyl adenylate.[10] During the past decade, Ala, Arg, Asp, Cys, Gln, Gly, Lys, Pro, Ser, and Thr analogues of aa-SA have been reported as effective binders or inhibitors of the corresponding prokaryotic or eukaryotic aa-RS. However, at least two prerequisites remain to achieve selective reacylation stalling. One is the efficiency of the inhibition at the single-codon level, and the other is the orthogonality of the aa-SA-aa-RS interactions at practical drug concentrations.[11]



Phe-SA

As our first target, we selected the UUU/UUC codon for phenylalanine (Phe), one of the most abundant codons. We prepared a template mRNA of *E. coli* dihydrofolate reductase (DHFR[6]), containing six Phe codons (three UUU and three UUC codons), with a T7 tag at the *N* terminal. We also prepared a modified mRNA template for DHFR[0], in which all six Phe codons were mutated (see Supporting Information for details). This DHFR[0] contained 19 amino acids but no Phe and thus served as a control to investigate the practical orthogonality. These two templates were translated in a reconstituted prokaryotic cell-free system in the absence or presence of 5 μM of Phe-SA (Figure 1). In the absence of Phe-SA, both mRNAs afforded the full-length DHFR, as shown in lane 2 (DHFR[6]) and lane 5 (DHFR[0]). The presence of Phe-SA strongly suppressed DHFR[6] to <20% (lane 8) but had little effect on the efficient synthesis of DHFR[0] (≥90%) (lane 11). This indicates the practical orthogonality of the Phe-SA-Phe-RS interaction.[11] The small but by no means negligible amount of DHFR[6] in the Phe-SA-inhibited run (lane 8) might have been derived from the single-turnover reaction of the precharged $tRNA^{Phe}$. If so, the chemically unstable α-amino acid functionality in it could be broken simply upon preincubation. HPLC analysis showed that the phenylalanyl-pdCpA[12] underwent deacylation at 37 °C with a rate constant of 2.8×10^{-2} min^{-1} (half-life, 25 min) and at 50 °C with a rate constant of 7.3×10^{-2} min^{-1} (half-life, 9.5 min) (Figure S1). On the basis of this information, the mixture was preincubated at 37 °C for 30 or 60 min under reacylation-stalling (Phe-SA+) conditions before starting the translation. This treatment almost completely suppressed the yields of DHFR[6] (lanes 9 and 10) without significantly decreasing those of DHFR[0] (e.g., the yields in lanes 12 and 13 were ≥90% of the yields in lanes 6 and 7).[13,14]

To analyze the precharged $tRNA^{Phe}$ at the single-codon level, we prepared DHFR[1] mRNA with a single Phe codon (UUC) at position 137. Whereas DHFR[1] was translated with ∼100% yield in the presence of Phe-SA, preincubation with Phe-SA at 37 °C for 60 min suppressed the yield of DHFR[1] to 32% (±10%), and preincubation at 50 °C for 30 min suppressed the yield to 30% (±4%) (Figure S2), relative to the yields in the absence of Phe-SA. Thus, the level of surviving precharged phenylalanyl-$tRNA^{Phe}$ or misreading tRNA if any is insufficient to embed a single Phe codon. When translated in the presence of yeast $tRNA^{Phe}_{GAA}$ misacylated with naphthylalanine (Nap-$tRNA^{Phe}_{GAA}$) in a pre-incubated (50 °C for 30 min with Phe-SA) reaction mixture, full-length DHFR[1] was recovered in ∼100% yield (Figure S3). The high recovery yield of DHFR[1] might remove the concern that deacylated endogenous $tRNA^{Phe}$ serves as an inhibitor or competitor of the exogenous Nap-$tRNA^{Phe}_{GAA}$.

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10.1021/ja0502977 CCC: $30.25

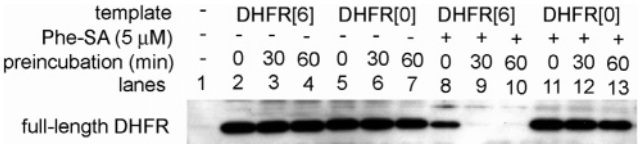

Figure 1. Western-blot analysis of the production of DHFR[6] (lanes 2−4 and 8−10) and DHFR[0] (lanes 5−7 and 11−13) at 37 °C for 60 min in the presence (+) or absence (−) of Phe-SA (5 μM) after preincubation at 37 °C for 0, 30, or 60 min.

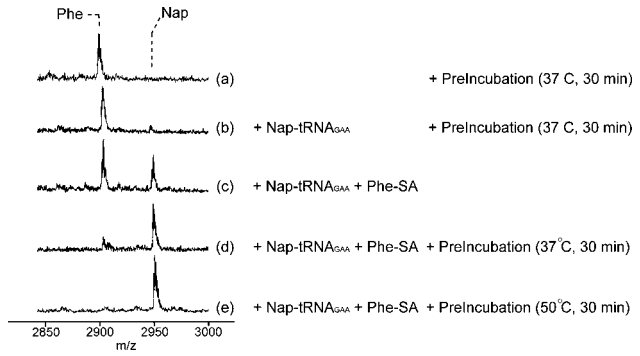

Figure 2. MALDI-TOF mass spectra for the oligopeptides produced at 37 °C for 60 min under various Nap-tRNA$^{Phe}_{GAA}$ (±), Phe-SA (±), and preincubation (±) conditions.

To provide a deeper insight into the Phe-to-Nap competition, we applied this simple sense-codon silencing and reassignment method to the templated synthesis of natural-unnatural hybrid peptides with unnatural Nap reassigned to the Phe codon. To test the selective reassignment of Phe to Nap, we prepared mRNA for a 24-mer oligopeptide H_2N-fMKETAAAK**F**ERQHMDSDYKDDDDK-CO_2H (m/z = 2902.26) with a single Phe (UUC codon at position 9) together with other 12 amino acids. The *N*-terminal 16-mer and the *C*-terminal 8-mer (underlined) regions are identical to the S-tag and FLAG-tag sequences, respectively. The FLAG-tag sequence was used to isolate the translated peptide products. Reassignment of the Phe codon to Nap was confirmed by MALDI-TOF-MS analysis (Figure 2). Incorporating Nap in place of Phe should produce the resulting peptide with a mass of m/z = 2952.28 and a mass shift of (Nap-Phe) +50.02 Da. The peptide product obtained under normal translation conditions showed only one signal at a mass of 2901.76, in a yield of 260 ± 52 ng of purified peptide from a 50 μL translation mixture, as determined by the S-tag assay (Figure 2a). Addition of Nap-tRNA$^{Phe}_{GAA}$ resulted in the appearance of a second, very weak signal at 2951.44 (Figure 2b). In contrast, catalytic reacylation of Phe was inhibited by the addition of Phe-SA, so that the second or higher signal corresponding to the Nap-containing hybrid peptide at 2951.59 was easily detectable (Figure 2c). Preincubation with Phe-SA at 37 °C for 30 min (Figure 2d) or at 50 °C for 30 min (Figure 2e) yielded the mutated Nap peptide as the sole major product together with a just-detectable amount of the nonmutated Phe peptide. These results illustrate that the UUC-Phe codon was rendered vacant upon treatment or preincubation with the inhibitor Phe-SA and was reassigned to the Nap contained in the suppressor tRNA. As far as the mass intensities were concerned, the Phe-to-Nap reassignment under the present conditions was efficient, although still not perfect, and selective or orthogonal.

In summary, our data show that the sense codon for Phe can be silenced and reassigned to Nap simply with a Phe derivative of sulfamoyladenosine (SA), which suppresses the phenylalanyl-tRNA synthetase activity. In principle, our orthogonal reacylation stalling method can be applied to any other sense codons and may provide a novel route to multiply mutated proteins and oligopeptides having two or more unnatural components. The inhibitors may be of the same SA type or a new design based on SA, considering the editing or double-sieving properties of the enzymes.[15] We confirmed that the Ile codon (AUC), in the presence of Ile-SA, can also be reassigned to Nap using yeast tRNA$^{Phe}_{GAU}$. Improved RS deactivation and further consumption of endogenous precharged tRNA would widen the scope of adaptable foreign substrates to include less competitive ones such as polar amino acids. Sense codon reassignment can be achieved using elaborate reconstituted systems[16] such as RS-free[6,8,17] and endogenous tRNA-free[6,7] systems. In principle, the present small-molecule-based approach would work in nonreconstituted normal cell-free systems. The in-cell production of unnatural proteins using nonauxotrophic cells is an intriguing application.[3] Further work is now under way along these lines.

Acknowledgment. We thank Dr. M. Endo of Osaka University and Dr. Y. Shimizu of the Post Genome Institute for helpful discussions and the kind gift of the plasmids used in this research.

Supporting Information Available: General preparation of the aminoacyl tRNA, construction of expression templates, translation conditions, mass analysis conditions, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0502977