A New Functional Suppressor tRNA/ Aminoacyl-tRNA Synthetase Pair for the in Vivo Incorporation of Unnatural Amino Acids into Proteins

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Received February 18, 2000

General methods for selectively incorporating unnatural amino acids into proteins in vivo, directly from the growth media, would greatly expand our ability to manipulate protein structure and function.1 For example, the ability to place fluorophores selectively into proteins in vivo would provide powerful tools for cell biology, or the ability to generate large quantities of proteins with metal binding or keto amino acids might lead to proteins with enhanced physical or catalytic properties. Our approach involves the generation of a suppressor tRNA/ aminoaacyl-tRNA synthetase (tRNA_{CUA}/TyrRS) pair that is orthogonal to Escherichia coli endogenous tRNA/synthetase pairs; that is, the orthogonal tRNA is not a substrate for any endogenous synthetases and the orthogonal synthetase does not recognize any endogenous tRNAs.2,3 The specificity of this synthetase is then altered so that it charges the tRNA_{CUA} only with a desired unnatural amino acid. One such orthogonal pair for use in E. coli was developed from the tRNA_{Glu}/GlnRS pair from Saccharomyces cerevisiae.4 The development of additional orthogonal tRNA/aaRS pairs may allow the simultaneous incorporation of multiple unnatural amino acids into proteins. Moreover, different aminoaacyl synthetases may be better starting points for generating active sites with particular specificities (e.g., specificity for large hydrophobic vs small hydrophilic amino acids). To this end, we have analyzed biochemical data available for tRNA^Tyr/TyrRS pairs from a variety of organisms. This analysis, together with in vivo complementation assays, has afforded a new orthogonal tRNA^Tyr/TyrRS pair as well as insights into the development of additional pairs.

The identity elements of prokaryotic tRNA^Tyr include a long variable arm in contrast to the short arm of eukaryotic tRNA Tyr .4,5 In vitro studies have also shown that tRNA^Tyr of S. cerevisiae6 and Homo sapiens8 cannot be aminoaacylated by bacterial synthetases, nor do their TyrRS aminoacylate tRNAs in orthogonal pairs. To test whether tRNA_{CUA}/TyrRS pairs from these organisms are orthogonal in E. coli, an in vivo complementation assay was used that is based on suppression of an amber stop codon in a nonessential position of the TEM-1 β-lactamase gene encoded in plasmid pBLAM.5 If the newly introduced suppressor tRNA_{CUA} is aminoaacylated by any endogenous E. coli synthetases, cells will grow in the presence of ampicillin. After expressing these tRNA_{CUA} in E. coli strain DH10B transformed with pBLAM, cells survive at very high concentrations of ampicillin, greater than 1200 μg/mL (interpolated from IC_{50} curves in Figure 1) for tRNA_{CUA} derived from S. cerevisiae and 234 μg/mL for that from H. sapiens. When S. cerevisiae tRNA_{CUA} which is an orthogonal tRNA, is tested under the same conditions, the cells survive at only 20 μg/mL ampicillin. For comparison, E. coli strains bearing pBLAM alone survive up to 9.7 μg/mL ampicillin (in the absence of any suppressor tRNA). Since the recognition of tRNA by synthetase depends on relative concentrations in the cell,9,10 the concentration of tRNA_{CUA} was decreased by expressing its gene under the weaker lac promoter instead of the strong lpp promoter. The IC_{50}’s decreased to 383 and 84 μg/mL for S. cerevisiae and H. sapiens, respectively, but these values are still potentially too high to allow the use of these tRNAs in orthogonal pairs.

The change of one single nucleotide in the anticodon (G34 to C34) made the S. cerevisiae and H. sapiens tRNA^Tyr susceptible to acylation by the E. coli synthetases. Most tRNAs have positive and negative elements in the acceptor and anticodon domains to ensure accurate aminoaacylation.11,12 Once the anticodon is changed from GUU to CUA, it is possible that nongencode synthetases that recognize tRNAs with similar anticodons have a stronger affinity for tRNA^Tyr, We hypothesized that the introduction of

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additional negative recognition elements (with respect to E. coli synthetases) could restore orthogonality. To test this notion, C1-G72 of S. cerevisiae tRNA^{Tyr}_{CUA} was mutated to G1-C72. This tRNA^{Tyr}_{CUA}(C1G72C) did show a much lower IC_{50} (12.5 μg/mL ampicillin), indicating that it is a poorer substrate for the E. coli synthetases than S. cerevisiae tRNA^{Gln}_{CUA}. Unfortunately, this tRNA is no longer recognized by its cognate TyrRS from S. cerevisiae, since C1-G72 is a critical S. cerevisiae TyrRS positive determinant. Because it is difficult to rationally design both positive and negative recognition elements into tRNAs, we searched for a tRNA with identity elements outside of the anticodon, in particular, the tRNA^{Tyr}_{CUA}/TyrRS pair from the archaeabacterium Methanococcus jannaschii. This Tyr aminoacyl synthetase is missing most of the nonconserved domain binding to the anticodon loop of its tRNA^{Tyr}, but has the CP1 insertion in the active site to discriminate C1:G72 from G1:C72. Thus, the M. jannaschii TyrRS aminoacylates S. cerevisiae but not E. coli crude tRNA. The properties of M. jannaschii TyrRS suggested it should act as an orthogonal TyrRS in E. coli, and furthermore, that its cognate tRNA^{Tyr} may be a good candidate for an orthogonal tRNA^{CUA}.

To test this notion, M. jannaschii tRNA^{Tyr}_{CUA} was expressed in E. coli in the presence of pBLAM. The transformed cells survived at 55.5 μg/mL ampicillin. Coexpression of M. jannaschii tRNA^{Tyr}_{CUA} and TyrRS allowed cells to survive at 1220 μg/mL ampicillin, indicating TyrRS charges the tRNA^{Tyr} efficiently (compared to the natural suppressor tRNA^{supF} for which the IC_{50} is 1700 μg/mL ampicillin). This increase is not due to the aminoacylation by M. jannaschii TyrRS of any endogenous E. coli tRNAs since expression of M. jannaschii TyrRS alone (pAC + pBLAM-JYRS) afforded an IC_{50} value similar to that of cells without any exogenous tRNA or TyrRS (pAC + pBLAM). On the basis of these assays, the M. jannaschii tRNA^{Tyr}_{CUA}/TyrRS pair appears to be a viable orthogonal pair in E. coli. The tRNA alone may be aminoacylated at a very low level by the E. coli synthetases, but coexpression of the M. jannaschii TyrRS is likely to compete with other E. coli endogenous synthetases and further reduce noncognate aminoacylation.

To further test the in vivo complementation results, tRNA^{Tyr}_{CUA} from different organisms was expressed alone or coexpressed with cognate TyrRS in the presence of the β-galactosidase gene containing an amber codon at position 125 in E. coli strain CA274 (HfrC lac_{am125} trp_lacam). Suppression of the amber codon produces specific activity of β-galactosidase (U/mg). Colony color was measured with the Coomassie Plus protein assay reagent of Pierce. Total protein content was measured by the spectrophotometry also indicated that M. jannaschii tRNA^{Tyr}_{CUA} could suppress the amber codon at a low level due to charging by endogenous E. coli synthetases. Suppression is increased 22-fold with coexpression of the cognate TyrRS, which correlates with the in vivo complementation assay. It should be noted that the β-galactosidase activities were not directly proportional to IC_{50} values, presumably because the suppression of the amber codons in β-lactamase and β-galactosidase occurs in different sequence contexts and the two enzymes tolerate different amino acids to different extents at those positions.

In conclusion, we have developed a new functional orthogonal tRNA^{Tyr}_{CUA}/TyrRS pair for use in E. coli by importing the M. jannaschii tRNA^{Tyr}_{CUA}/TyrRS pair into E. coli. In contrast, neither of the Tyr amber suppressor tRNA derived from human nor yeast tRNAs was orthogonal to the E. coli synthetases, and while a mutant of the yeast amber suppressor is orthogonal, it is no longer recognized by its cognate yeast synthetase. Although the M. jannaschii tRNA^{Tyr}_{CUA} is somewhat “less orthogonal” than the yeast tRNA^{Glu}_{CUA} we previously developed, the M. jannaschii synthetase has higher aminoacylation activity toward its cognate tRNA. We therefore view this pair as a promising candidate for the next stage of this overall strategy, which involves modification of the amino acid specificity of the synthetase.

Acknowledgment. We thank Dr. Brian A. Steer and Professor Paul Schimmel for providing plasmids containing genes of M. jannaschii tRNA^{Tyr} and TyrRS and for helpful comments. We also thank Miro Pastrnak for helpful discussions. Funding was provided by the Department of the Army.

Table 1. Suppression of lac_{am125} in E. coli CA274

<table>
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<th>Transformants a</th>
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See caption of Figure 1 for plasmid definition. pBR-XYS was used to express TyrRS of organism X and pBR was the control plasmid lacking TyrRS. Transformed E. coli cells were plated on LB plates containing 35 μg/mL chloramphenicol, 100 μg/mL ampicillin, 0.5 mM IPTG, and 0.004% (w/v) X-Gal. The plates were incubated at 37 °C overnight. β-galactosidase activity was measured with the high sensitivity β-galactosidase assay protocol of Stratagene. Total protein was measured with the Coomassie Plus protein assay reagent of Pierce. One unit of β-galactosidase converts 1 nmol of chlorophenol red-β-d-galactopyranoside to chlorophenol red and galactose in 1 min at 37 °C.