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

Lei Wang, Thomas J. Magliery, David R. Liu,† and Peter G. Schultz*

Department of Chemistry, The Scripps Research Institute 10550 North Torrey Pines Road, La Jolla, California 92037

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/aminoacyl−tRNA synthetase ($tRNA_{CUA}$/aaRS) 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 $tRNA_2^{Gln}$/GlnRS pair from *Saccharomyces cerevisiae*.[3] The development of additional orthogonal tRNA/aaRS pairs may allow the simultaneous incorporation of multiple unnatural amino acids into proteins. Moreover, different aminoacyl 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 $tRNA_{CUA}^{Tyr}$/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] In addition, eukaryotic $tRNA^{Tyr}$ contains a C1:G72 positive recognition element, whereas prokaryotic $tRNA^{Tyr}$ has no such consensus base pair.[5,6] In vitro studies have also shown that $tRNA^{Tyr}$ of *S. cerevisiae*[7] and *Homo sapiens*[8] cannot be aminoacylated by bacterial synthetases, nor do their TyrRS aminoacylate bacterial tRNA. To test whether $tRNA_{CUA}^{Tyr}$/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-lactmase gene encoded in plasmid pBLAM.[3] If the newly introduced suppressor $tRNA_{CUA}$ is aminoacylated by any endogenous *E. coli* synthetases, cells will grow in the presence of ampicillin. After expressing these $tRNA_{CUA}^{Tyr}$ in *E. coli* strain DH10B transformed with pBLAM, cells survive at very high concentrations of ampicillin, greater than 1206 μg/mL (interpolated from IC_{50} curves in Figure 1) for $tRNA_{CUA}^{Tyr}$ derived from *S. cerevisiae* and 234 μg/mL for that from *H. sapiens*. When *S. cerevisiae* $tRNA_{CUA}^{Gln}$, which is an orthogonal tRNA, is tested under the same conditions, the cells survive at only 20 μg/mL ampicillin.[3] 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 $tRNA_{CUA}^{Tyr}$ 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 ampicillin 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.

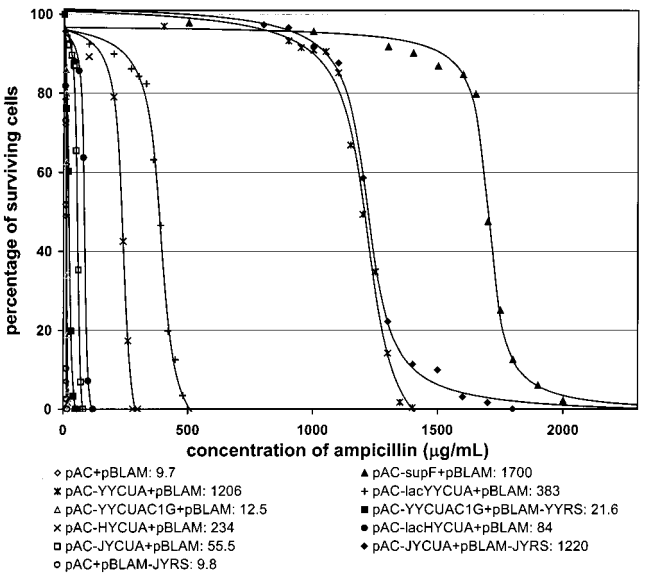

Figure 1. In vivo complementation assays to determine the orthogonality of $tRNA_{CUA}^{Tyr}$'s from various organisms expressed in *E. coli*. pAC-XYCUA was used to express $tRNA_{CUA}^{Tyr}$ of organism X under the control of the *lpp* promoter where X = Y is *S. cerevisiae*, X = H is *H. sapiens* and X = J is *M. jannaschii*. pAC-lacXYCUA is similar to pAC−XYCUA except the *lpp* promoter was changed to *lac*. pAC-supF was used to express *E. coli sup*F tRNA. pAC is a control plasmid without any tRNA gene. pBLAM was used to express the β-lactamase gene with an amber stop codon at Ala-184. pBLAM-XYRS encodes the indicated TyrRS gene. Numbers following legend text are IC_{50} values in units of μg/mL ampicillin.

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 aminoacylation.[11,12] Once the anticodon is changed from GUA to CUA, it is possible that noncognate synthetases that recognize tRNAs with similar anticodons have a stronger affinity for $tRNA_{CUA}^{Tyr}$. We hypothesized that the introduction of

* To whom correspondence should be addressed. Telephone: (858) 784-9300. Fax: (858) 784-9440. E-mail: schultz@scripps.edu.
† Present address: Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138.

(1) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621.
(2) Liu, D. R.; Magliery, T. J.; Pastrnak, M.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10092.
(3) Liu, D. R.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4780.
(4) Himeno, H.; Hasegawa, T.; Ueda, T.; Watanabe, K.; Shimizu, M. *Nucleic Acids Res.* **1990**, *18*, 6815.
(5) Lee, C. P.; RajBhandary, U. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11378.
(6) Quinn, C. L.; Tao, N.; Schimmel, P. *Biochemistry* **1995**, *34*, 12489.
(7) Wakasugi, K.; Quinn, C. L.; Tao, N.; Schimmel, P. *EMBO J.* **1998**, *17*, 297.
(8) Kleeman, T. A.; Wei, D.; Simpson, K. L.; First, E. A. *J. Biol. Chem.* **1997**, *272*, 14420.
(9) Swanson, R.; Hoben, P.; Sumner-Smith, M.; Uemura, H.; Watson, L.; Söll, D. *Science* **1988**, *242*, 1548.
(10) Sherman, J. M.; Rogers, M. J.; Söll, D. *Nucleic Acids Res.* **1992**, *20*, 1547.
(11) Schimmel, P.; Giegé, R.; Moras, D.; Yokoyama, S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8763.
(12) Giegé, R.; Sissler, M.; Florentz, C. *Nucleic Acids Res.* **1998**, *26*, 5017.

10.1021/ja000595y CCC: $19.00
Published on Web 05/04/2000

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 $tRNA^{Tyr}_{CUA}$(C1G G72C) 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.[5] Because it is difficult to rationally design both negative and positive 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 archaebacterium *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.[13] 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 $tRNA^{Tyr}_{CUA}$ efficiently (compared to the natural suppressor tRNA *sup*F 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.[10]

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_{am}).[14] Suppression of the amber codon produces functional β-galactosidase, which turns cells blue in the presence of added X-Gal. As shown in Table 1, transformants with IC_{50} values higher than 84 μg/mL all turned blue. The β-galactosidase activities measured by 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 contrast to the results of Ohno et al.,[15] who reported that expression of *S. cerevisiae* $tRNA^{Tyr}_{CUA}$ alone in CA274 gives white colonies and that *S. cerevisiae* $tRNA^{Tyr}_{CUA}$ is not aminoacylated by *E. coli* synthetases, our result showed that colonies of CA274 with *S. cerevisiae* $tRNA^{Tyr}_{CUA}$ expressed were clearly blue whenever the $tRNA^{Tyr}_{CUA}$ gene is under control of *lpp* or *lac* promoter. Moreover, the β-galactosidase activity is very high and is similar to that afforded by expression of the *sup*F suppressor.

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

transformants[a]	colony color[b]	specific activity of β-galactosidase (U/mg)[c]
pAC + pBR	white	0.0065
pAC-supF + pBR	blue	46
pAC-YYCUA + pBR	blue	33
pAC-lacYYCUA + pBR	blue	28
pAC-YYCUAC1G + pBR	white	0.0075
pAC-YYCUAC1G + pBR-YYRS	white	0.0063
pAC-HYCUA + pBR	blue	4.2
pAC-lacHYCUA + pBR	blue	0.76
pAC-JYCUA + pBR	white	0.11
pAC-JYCUA + pBR-JYRS	blue	2.5
pAC + pBR-JYRS	white	0.0055

[a] See caption of Figure 1 for plasmid definition. pBR-XYRS was used to express TyrRS of organism X and pBR was the control plasmid lacking TyrRS. [b] 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. [c] 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.

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 were 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 $tRNA^{Gln}_{CUA}$ we previously developed,[3] 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.

JA000595Y

(13) Steer, B. A.; Schimmel, P. *J. Biol. Chem.* **1999**, *274*, 35601.
(14) Yamao, F.; Inokuchi, H.; Ozeki, H. *Jpn. J. Genet.* **1988**, *63*, 237.
(15) Ohno, S.; Yokogawa, T.; Fujii, I.; Asahara, H.; Inokuchi, H.; Nishikawa, K. *J. Biochem.* **1998**, *124*, 1065.