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Expanding the Scope of Protein Synthesis Using Modified Ribosomes

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Abstract

The ribosome produces all of the proteins and many of the peptides present in cells. As a macromolecular complex comprised of both RNAs and proteins, it employs a constituent RNA to catalyze the formation of peptide bonds rapidly and with high fidelity. Thus the ribosome can be argued to represent the key link between the RNA World, in which RNAs were the primary catalysts, and present biological systems in which protein catalysts predominate. In spite of the well known phylogenetic conservation of ribosomal RNAs through evolutionary history, ribosomal RNAs can be altered readily when placed under suitable pressure, e.g. in the presence of antibiotics which bind to functionally critical regions of rRNAs. While the structures of rRNAs have been altered intentionally for decades to enable the study of their role(s) in the mechanism of peptide bond formation, it is remarkable that the purposeful alteration of rRNA structure to enable the elaboration of proteins and peptides containing non-canonical amino acids has occurred only recently. In this Perspective, we summarize the history of rRNA modifications, and demonstrate how the intentional modification of 23S rRNA in regions critical for peptide bond formation now enables the direct ribosomal incorporation of D-amino acids, β -amino acids, dipeptides and dipeptidomimetic analogues of the normal proteinogenic L- α -amino acids. While proteins containing metabolically important functional groups such as carbohydrates and phosphate groups are normally elaborated by the post-translational modification of nascent polypeptides, the use of modified ribosomes to produce such polymers directly is also discussed. Finally, we describe the elaboration of such modified proteins both *in vitro* and in bacterial cells, and suggest how such novel biomaterials may be exploited in future studies.

Graphical Abstract

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The authors declare no competing financial interest.

Introduction

The ribosome is an ancient molecular machine responsible for the cellular production of proteins. It was first described by George E. Palade in 1955;^{1–3} on the basis of this research, he was awarded the Nobel Prize in 1974. The structure and function of ribosomes has been studied extensively in both prokaryotes and eukaryotes, and these investigations have defined a peptidyltransferase center (PTC) located on the large ribosomal subunit (50S in bacteria) as the site of peptide bond formation. While the ribosome is a large macromolecular complex comprised of three RNAs and more than 50 proteins, a key finding in relation to ribosome function is that the catalytic event leading to peptide bond formation is mediated by the 23S ribosomal RNA without the direct involvement of any protein constituent.^{4–6} Thus, befitting its ancient origin, the ribosome is a ribozyme,^{5,7} a participant in the RNA World.^{8,9}

As shown in Figure 1, the formation of a peptide bond on the surface of the ribosome during peptide elongation involves activated transfer RNAs, one bound to the ribosomal P site and bearing a peptide, and the other bound to the ribosomal A site and containing an α -amino acid.^{6,10,11} While the complex that results in peptide bond formation requires both protein factors and energy, formation of the peptide bond itself does not require additional energy. Peptide bond formation can be carried out in solution but the reaction is quite slow.¹² The ribosome greatly accelerates this process; ribosomal peptide bond formation is estimated to be 10^5 – 10^7 -fold more rapid than the uncatalyzed reaction.^{10,13}

In recent years, mechanisms suggested to account for ribosomal peptide bond formation have been informed by X-ray crystal structure data. Initially, it was suggested that the mechanism involved the active participation of 23S ribosomal RNA nucleotide A2451 (*E. coli* nomenclature used throughout) via general acid-base catalysis, as this nucleotide had been found to be in close proximity to the 3'-CCA ends of A-site and P-site tRNA substrates.^{11,12,14} However, improved resolution of the crystallographic studies suggested the need for modification of the putative mechanism,^{15,16} as it became clear that N3 of A2451 is not within hydrogen-bonding distance of the α -NH₂ nucleophile throughout the peptide bond-forming reaction. These findings were further supported by the experimental work of Rodnina and co-workers.^{17,18} When the nucleotide A2451 was changed to uridine (A2451U substitution), making peptide bond formation the rate-limiting process, the rate of reaction was found to be pH-independent.^{11,17} Thus, it appears that the ribosome does not

employ general acid-base catalysis for peptide bond formation, but rather participates actively in positioning the esterified tRNA substrates for reaction.^{11,17} Further evidence was obtained by using phenyllactyl-tRNA as an A-site substrate; again, peptidyl transfer was found to be independent of pH at values between 6 and 9.¹⁸ Comparison of crystal structures of the ribosome alone and in complex with its tRNA substrates provided strong evidence for this view.¹⁹ Specifically, it was found that only after binding both substrates did the PTC undergo the substantial conformational changes that induce interaction of the α -amino group of aminoacyl-tRNA with the 3'-terminal peptidyl group of the bound peptidyl-tRNA; this process is also supported by extensive hydrogen bonding between the surrounding nucleotides in this area.

Thus, the peptidyl transferase center (PTC) is a flexible pocket, organized by several conserved nucleotides, which controls substrate positioning and the catalytic step. Another function of the ribosomal PTC is to participate in maintaining the fidelity of translation, preventing the incorporation of amino acids not specified by the genetic code at the level of mRNA translation.^{20,21} As documented in the literature,^{22–27} the orientation of the 3'-end of the aminoacyl-tRNA in the A-site can be a factor in the facility and accuracy of translation, providing a second level of proofreading in addition to recognition/positioning of the anticodon loop of the aminoacyl-tRNA on the mRNA on the small ribosomal subunit within the ribosomal A-site. This level of discrimination is exemplified by tRNAs activated with D-amino acids, which are not utilized to a significant extent by native ribosomes due to a non-productive binding mode.^{28–30}

However, the ability of the ribosome to incorporate multiple proteinogenic amino acids at the peptidyltransferase center suggests the need for significant flexibility in the PTC to allow α -L-amino acids with a variety of side chains to be incorporated. While native aminoacyl-tRNA synthetases assure a good level of fidelity in the activation of individual tRNA isoacceptors with their cognate amino acids, there are now multiple methods for activating individual tRNAs with non-canonical amino acids. These include chemical methods^{31–40} and the use of sets of orthogonal tRNAs and aminoacyl-tRNA synthetases^{41–50} which recognize canonical amino acids poorly if at all, and thus can be employed to introduce non-canonical amino acids into proteins. This strategy effectively bypasses the main mechanism for excluding non-canonical amino acids from proteins synthesized ribosomally. By misacylating suppressor tRNAs, which insert amino acids at what is normally a stop codon (UAG, UAA or UGA),^{51–53} a four- or five-base codon,^{54,55} some combination of the two,⁵⁶ or by codon reassignment,^{57–59} it has been possible to incorporate a large variety of non-canonical amino acids (usually α -L-amino acid analogues) into numerous proteins, confirming the flexibility of the PTC.

However, proteinogenic or otherwise, not all L- α -amino acids can be incorporated efficiently; the size, conformation and ionization status of L- α -amino acid side chains can result in very low yields of proteins. Examples include analogues of the negatively charged proteinogenic amino acid aspartic acid,⁶⁰ glycosylated amino acids,⁶¹ phosphorylated amino acids such as phosphotyrosine,⁶² and multiple incorporations of L-proline.^{24–26} Nature facilitates L-proline incorporation by the use of the protein factor EF-P,²⁵ and introduces phosphotyrosine post-translationally.^{63–65} However, a logical and more general solution

might involve alteration of the bacterial ribosome itself. Modification of ribosome structure could in principle open new opportunities for the elaboration of modified proteins and peptides *in vitro* and *in vivo*, which might allow the more detailed study of cellular processes such as protein aggregation and protein misfolding. The consequences of protein glycosylation or phosphorylation at atypical positions could also be investigated, and artificial zymogens which can be activated by the use of specific intra-or extracellular signals might also be accessible.

Re-engineering of the bacterial ribosome.

Ribosomal re-engineering has a substantial history, and was first used to probe the contributions of specific regions of rRNAs to ribosome activity; this enabled a better understanding the mechanism of ribosome action.^{66–78} Genetic approaches have included the preparation of plasmids carrying a ribosomal operon under the control of an inducible strong promoter, mutagenesis of the operon in a region of interest, then transformation of the mutant construct into *E. coli* cells and studying the effects of the mutation after induction of synthesis of the corresponding rRNA. This approach provided access to new data relevant to ribosome function, and verified regions responsible for binding different co-factors and critical for peptide bond formation, especially conserved nucleotides essential for function. It was demonstrated that substitution at some positions seriously affected cell growth and even induced cell death.^{20,21,66} Combined with X-ray crystallographic studies, these data facilitated an understanding of the flexible architecture of the PTC, which can result in dramatic changes in the positioning and orientation of several conserved nucleotides and neighboring nucleotides after interaction with substrates.^{17,70,79–81} Thus, A2451U substitution resulted in dramatic changes in the accessibility of A2060, U2506, U2585 and A2572 to chemical probes.^{17,70} Mankin and co-workers⁷⁰ found that U2506, U2584 and U2585 were strongly protected by biotinylated N-acetyltyrosyl-tRNA in footprinting experiments. Additionally, it was found that the presence of an A-site substrate analogue induced the conformational shifts of several nucleotides, including U2506, G2583, U2584 and U 2585.⁸¹ X-ray crystallographic studies have verified that the O4 atom of U2585 was at hydrogen bonding distance from the 2'-OH group of an A-site tRNA analogue in the *H. marismortui* 50S ribosomal subunit.⁸² Thus, networks formed between these nucleotides and surrounding nucleotides could be part of a proofreading mechanism. The importance of these regions for positioning of the amino acid moiety of aminoacyl-tRNA has also been demonstrated in other publications.^{16,83}

Another type of ribosome re-engineering study involves the characterization of antibiotics which bind to the ribosome and thereby prevent bacterial growth.^{84–87} The binding sites of such antibiotics are typically 23S RNA, clustered around the PTC of the ribosome (Figure 2); they preclude the binding of molecules essential for ribosome function, e.g. by hindering the structural rearrangements needed for ribosome activity.⁸⁴

Two major mechanisms have been described for the development of antibiotic resistance, namely nucleotide substitutions and methylation.^{88–94} Modifications of at least eight 23S rRNA nucleotides (G748, A1067, C1920, A2058, G2470, U2479, A2503 and G2535) have been associated with a resistant cell phenotype.^{88,89} Five of these nucleotides are situated in

or near the PTC. Additionally, nucleotide substitutions have been found in antibiotic resistant clinical isolates of bacteria such as *Streptococcus pneumoniae*,^{90,91} *Mycobacterium avium*,^{92,93} *Haemophilus influenzae*⁹³ and *Helicobacter pylori*.⁹⁴ Thus, even in nature bacteria can respond to antibiotic pressure by re-engineering the ribosome to create resistant phenotypes.

A number of research groups have modeled antibiotic resistance using a ribosome re-engineering approach.^{95–97} Thus, binding pockets for macrolide group antibiotics have been identified in 23S rRNA nucleotide region 2058–2063, which is the part of the nascent protein exit tunnel. Nucleotide 2058 is believed to be a key nucleotide for macrolide binding pocket formation.⁹⁸ Adenosine has been found in this position of all studied bacteria, in comparison with the appearance of guanosine at the corresponding position in eukaryotes. Unsurprisingly, resistance to macrolide antibiotics associated with this nucleotide has been observed in clinical isolates and biochemical studies. The resistant phenotype of some clinical isolates^{90–92} has been attributed to A2058C, A2058G, A2059G and A2059C mutations. Böttger and co-workers⁹⁵ introduced an A → G mutation in position 2058 of the chromosomal rRNA of *Mycobacterium smegmatis* and demonstrated that the 2058G mutation in the *rrnB* operon dramatically increased the resistance of the bacterium to the macrolide antibiotics telithromycin and clarithromycin (by ~ 250-fold and more than 1000-fold, respectively).

In parallel with ribosome modifications in natural systems associated with antibiotic resistance, the use of antibiotics for the selection of ribosomes with specific biochemical properties has been adopted as a valuable biochemical tool. In fact, this tool proved to be quite important in the development of a strategy for selecting modified ribosomes capable of incorporating specific, very unusual amino acids into proteins (e.g., non L- α -amino acids and amino acids containing functional groups normally introduced post-translationally; *vide infra*).

The feasibility of using ribosome re-engineering as a means of incorporating unusual amino acids into proteins was first investigated in our laboratory about 15 years ago.^{99–101} As discussed above, the alteration of even one nucleotide in a key position of the PTC can dramatically alter tRNA substrate positioning within the PTC, resulting in a significant loss of activity. Nonetheless, it seemed possible that multiple substitutions, accomplished by random mutagenesis within the PTC might result in the formation of a peptidyltransferase center architecture capable not only of incorporating unusual non-canonical amino acids of interest in a pre-determined protein position, but also supporting good fidelity of protein synthesis at unmodified positions.^{100,101} Several types of new modified ribosomes have now been created: individual modified ribosomes capable of the incorporation of D-amino acids, β -amino acids, dipeptides and phosphorylated amino acids have been described.^{62,99–105} As discussed below, the use of an antibiotic that binds in proximity to the PTC of *E. coli* ribosomes proved important in the derivation of the modified ribosomes.

Ribosomes capable of incorporating D-amino acids into proteins.

D-amino acids are widely distributed in living organisms and their active utilization in many cell types has been demonstrated.^{106–109} However, D-amino acids occurring at specific

positions in peptides or proteins result from post-translational modification, not from incorporation during ribosomal protein translation. Several mechanisms exist to prevent the ribosomal incorporation of D-amino acids. These include slower incorporation by the activating enzymes (aminoacyl-tRNA synthetases),^{109–113} and deacylation of formed D-aminoacyl-tRNAs by specific deacylases.^{108,109,111} The observed cell toxicity of D-tyrosine was shown to be suppressed by D-tyrosyl-tRNA^{Tyr} deacylase, while inactivation of the gene encoding this deacylase increased the toxicity of several D-amino acids, including D-tyrosine, D-aspartic acid and D-tryptophan.^{108,109} The toxicity of D-tyrosine was shown to be due to the conversion of available tRNA^{Tyr} to D-tyrosyl-tRNA^{Tyr}, and could be relieved by overexpression of tRNA^{Tyr}. Thus D-aminoacyl-tRNAs can compete with L-aminoacyl-tRNAs for activation^{109–113} and can be incorporated into protein both *in vitro* and *in vivo*.^{111–115} However, the incorporation of D-amino acids into protein is inefficient, and can impede protein biosynthesis.¹¹⁵ The presence of a D-peptidyl-tRNA analogue in the ribosomal P-site also resulted in diminished peptide bond formation.^{33,116}

In order to study the mechanism by which D-amino acids were excluded from protein incorporation, Yonath and co-workers²³ examined the high-resolution structure of the PTC in its native form and in complex with an A-site tRNA acceptor stem mimic, the latter attached either to D-phenylalanine or to L-phenylalanine. They found that a tRNA acceptor stem mimic containing D-phenylalanine could be bound in A-site in a conformation that would in principle permit peptide bond formation, but that in this conformation there would be a collision between C_β of the D-phenylalanine and O4' of U2506. An alternative binding mode achieved by alignment of the P-site and A-site phenylalanine side chains resulted in positioning of the α-amino group of the A-site tRNA within H-bonding distance of U2585. The putative H-bond was suggested to have the ability to lock the D-amino acid in a conformation which precluded peptide bond formation. In a recent X-ray crystallographic study of the 70S *T. thermophilus* ribosome bound to L- and D-aminoacylated analogues of the 3'-end of an aminocyl-tRNA complemented by modeling studies, Melnikov et al.¹¹³ also noted the effect of U2506 on the orientation of a bound D-aminoacylated tRNA analogue. They suggested that orientation of the α-amino moiety precluded in-line attack on the reactive carbonyl group of the P-site tRNA, and this would preclude efficient peptide bond formation involving the D-amino acid (Figure 3). These studies both strongly support the idea that a change in the architecture of the A-site, allowing D-phenylalanyl-tRNA to bind in a conformation that permitted its reactive amino group to approach the carboxylate ester of the P-site tRNA in an in-line fashion without a significant energetic penalty, might allow D-amino acid incorporation into proteins.

Accordingly, nucleotides 2247–2450 of the 23S rRNA operon were chosen for mutagenesis, since this region is in spatial proximity to nucleotides (including A2451) which orient the α-amino group of the aminoacyl-tRNA for peptide bond formation. Thus, mutation of these nucleotides could potentially release the putative locking mechanism of the ribosome toward D-aminoacyl-tRNAs, affording a pool of ribosomes with enhanced ability to synthesize proteins containing D-amino acids.^{99,100} The mutagenesis was carried out as outlined in Figure 4. Randomized primers in a PCR reaction were used to introduce mutations into the region of interest, and the cells containing mutant ribosomes were grown in the presence of different concentrations of chloramphenicol, which is known to bind in proximity to the

mutagenized region. Clones resistant to chloramphenicol, which also had increased doubling times, were presumed to have altered sequences in the region of interest. These clones were sequenced, and used for the S-30 preparations to determine whether they could mediate the incorporation of D-amino acids. Two mutants, having 2447UUGU2450 and 2447UGGC2450 sequences instead of 2447GAUA2450 in wild type, mediated improved incorporation of D-methionine and D-phenylalanine into proteins (Table 1).

Interestingly, both of these ribosome variants involved the substitutions G2447U and U2449G. It had been shown previously that the invariant nucleotide U2449 was dispensable⁷² but that the G2447U single mutant was inviable.⁷¹ However, double mutants containing G2447U/U2449G demonstrated good cell growth *in vivo* and protein synthesis ability *in vitro*, while enabling enhanced incorporation of D-phenylalanine and D-methionine.^{99,100} Thus, multiple mutations in 23S RNA can change PTC architecture sufficiently to confer new functions, while rescuing a normal function deleted by a single nucleotide change in the PTC.

Ribosomes that incorporate β -amino acids into proteins.

Amino acid side chains play critical roles in the secondary structures of proteins and peptides, as exemplified by the role of amino acids such as alanine and leucine in the stabilization of the α -helix motif.^{117–119} Interestingly, a number of synthetic peptides containing β -amino acids have also been shown to have well defined secondary structures, including helical properties.^{120–123} Contributing to their use in peptidomimetic analogues is their enhanced stability to degradation by proteolytic enzymes.^{124–126} While it is logical to anticipate that proteins containing β -amino acids might also have unique and potentially useful properties, the *in vitro* synthesis of proteins containing β -amino acids using bacterial ribosomes has proven infeasible.¹²⁷ In common with the lack of ribosomal incorporation of D-amino acids into proteins, the introduction of a β -aminoacyl-tRNA_{CUA} into a ribosomal system programmed with a mRNA containing a UAG codon failed to result in significant incorporation of the β -amino acid into the target protein. In an earlier experiment, it had been shown that *E. coli* tRNA^{Phe} activated with β -phenylalanine would form a dipeptide with the P-site tRNA *N*-acetyl-L-phenylalanyl-tRNA^{Phe} in the presence of poly(U) and *E. coli* ribosomes, albeit only in low yield; accordingly, β -phenylalanyl-tRNA must have bound to the ribosomal A-site.³⁴ While the peptidyltransferase center of the ribosome clearly exhibits some flexibility, structural studies of the ribosome have also identified H-bonding networks between nucleotides which are critical for positioning the 3'-end of the aminoacyl-tRNA in the A-site at an optimal distance and orientation with respect to the 3'-end of the bound peptidyl-tRNA.¹²⁸ Apparently, placing the nucleophilic NH₂ group of the A-site tRNA on the β -C atom of the amino acid, rather than the α -C atom, precludes efficient peptide bond formation.

Mutagenesis of the 23S rRNA of *E. coli* ribosomes was again employed to create a clonal library containing numerous modified ribosomes, but the procedure was modified in two ways.¹⁰¹ First, the 23S rRNA region 2057–2063 was randomized to produce clones exhibiting moderate resistance to erythromycin, and eight suitable clones were identified. Each of these eight clones was further randomized in one of three regions, two of which

(2496–2501 and 2502–2507) were ultimately found to afford modified ribosomes able to incorporate β -amino acids. The second change in the mutagenesis procedure involved the screening protocol. Unlike the procedure shown in Figure 4, which involved resistance to chloramphenicol, the new procedure sought to identify clones whose resistance to erythromycin was essentially unchanged, but which exhibited enhanced sensitivity to β^3 -puromycin, a structural analogue of the known antibiotic puromycin (Figure 5). Puromycin is an aminoacylated adenosine analogue which is taken up by bacterial cells and binds to the ribosomal A-site, where it accepts a growing polypeptide chain during the elongation phase of protein synthesis.¹²⁹ The resulting polypeptide is attached to the adenosine moiety by a stable amide bond, which can no longer be transferred. The result is cessation of protein synthesis and cell death. It had also been shown that puromycin analogues containing L- β -amino acids bound weakly to rabbit reticulocyte ribosomes, and inhibited protein synthesis *in vitro*.¹³⁰ It seemed possible that an analogue of puromycin containing a β -aminoacyl moiety might bind more avidly to modified ribosomes whose architecture better accommodated a β -NH₂ group, identifying a subset of modified ribosomes potentially able to incorporate β -amino acids into proteins. This dual selection procedure proved quite successful in identifying clones with modified ribosomes capable of mediating the incorporation of β -amino acids into proteins. Carrying out the selection in a background of moderate erythromycin resistance also provided two other benefits. All of the clones harboring plasmids with modified ribosomes also retained their native ribosomes. As clones of interest were identified, it was possible to force those clones to rely increasingly on the presence of the modified ribosomes by increasing the erythromycin concentration, thus compromising the function of the wild-type ribosomes.

It has been shown that the expression of rRNA is gene dose independent; when the number of rRNA operons in the cell is increased by the presence of plasmid-borne operons, the total rRNA synthesis rate remains constant due to feedback control.^{131–133} Therefore, using high copy plasmids for introducing a modified *rrnB* operon in cells, permits an increase in the percentage of modified ribosomes in cells by up to 70%.^{72,100} If the experiments are carried out in the presence of an antibiotic which blocks the activity of wild-type ribosomes, this allows the study of new functional activities not found for the wild-type ribosomes. Thus, as noted above, when studied in the presence of a sufficient concentration of erythromycin, the observed ribosomal function was due predominantly to the newly introduced modified ribosomes.

Evaluation of several modified β -amino acids (Figure 6) as substrates for incorporation by individual ribosomal clones was carried out by first attaching each amino acid to a suppressor tRNA_{CUA} transcript, as illustrated in Scheme 1 for β -alanine. β -Alanine was incorporated into position 10 of *E. coli* dihydrofolate reductase (DHFR) by two modified ribosomes in suppression yields up to 12%, and into position 6 of *Opisthorcanthus madagascariensis* (scorpion) peptide by two modified ribosomes in yields up to 16% (Figure 7).^{101,102}

Also investigated was the ability of the substitution pattern of the aminoacyl moiety of the puromycin derivative used for ribosome selection to identify ribosomes capable of incorporating one or more of the four isomers of methyl- β -alanine (**1–4**, Figure 6). β^2 -

puromycin and β^3 -puromycin (Figure 5) were used for the selection of clones.¹⁰³ Clone 040329 was selected using β^3 -puromycin. An S-30 preparation obtained using this clone mediated the incorporation of all four isomers of methyl- β -alanine into position 10 of DHFR, but regioisomers **3** and **4** (matching the regiochemistry of β^3 -puromycin) were incorporated with better efficiencies than **1** or **2**, and **4** (also having the same stereochemistry as β^3 -puromycin at position 3) was incorporated more efficiently than **3** (Figure 8). Clone 010335 was selected using β^2 -puromycin. Again, an S-30 preparation from this clone could mediate the incorporation of all four isomers of methyl- β -alanine, but **1** and **2**, having the same regiochemistry as β^2 -puromycin, were incorporated to about the same extent and more efficiently than **3** or **4** (Figure 8).¹⁰³ The structures of the modified 23 S rRNAs from clones 040329 and 010335 were modeled based on a reference structure¹³⁴ and both exhibited rearrangements in the positioning of some key nucleotides within the PTC. Accordingly, the proximate hydrogen bond network in loop regions 2057–2063 and 2496–2507 were compared for wild type and the for two variants employed for the incorporation of the four isomers of methyl- β -alanine (Figure 9).¹⁰³ Both clones had a G2061U replacement methyl- β -alanine (Figure 9).¹⁰³ Both clones had a G2061U replacement that alters the space near A2451; this likely affects the 2451–2061 hydrogen bond length and may be more generally relevant for β -amino acids. Quite likely, the combination of this mutation and modification of region 2502–2507 are involved in the observed regio- and stereoselectivity for the two clones analyzed. Additionally, each of clones was predicted to have a region of the 23S rRNA in which several of the contiguous H-bonds present in wild type were absent, and this may also contribute to relaxing the constraints exhibited by wild type in β -amino acid recognition, plausibly by “opening” the structure to accommodate the larger peptide backbone. A more definitive understanding of the mechanism of β -amino acid incorporation will require more detailed structural studies.

The potential utility of modified β -amino acids as protein constituents was illustrated by modifying a putative α -helical structure within the RRM1 domain of human hnRNP LL,¹⁰³ which has recently been shown to bind to an i-motif DNA in the *BCL2* promoter region.^{136–138} The structure of this domain, and its mode of i-motif DNA interaction was first modeled, affording a structure potentially containing two α -helices (Figure 10). Wild-type RRM1 could be expressed either in *E. coli* or in a cell free protein synthesizing system prepared from native ribosomes. The CD spectrum of the derived protein indicated that the RRM1 domain was not well structured, as evidenced by a negative peak centered at 205 nm indicative of random coil (Figure 10B). The *in vitro* protein synthesizing system prepared from ribosomal clone 040329 was used to introduce 3(*S*)-methyl- β -alanine (**4**) into position 35 of RRM1, normally occupied by alanine. Alanine 35 is situated at the beginning of the smaller α -helix (Figure 10A), and was thought to be important to confer α -helix stability. In fact, the CD spectrum of the modified RRM1 exhibited minima at 208 and 222 nm, typical of α -helical secondary structures. The thermal stabilities of the wild-type and modified proteins were then compared using thermal denaturation of both proteins. As shown in Figure 10C, the modified protein containing β -amino acid **4** at position 35 had a T_m value of 65.9 °C, in comparison with a T_m value of 62.8 °C for wild type.

The abilities of the two RRM1 proteins to bind to DNA was studied using two different assays. The i-motif DNA has a characteristic CD peak at 286 nm;¹³⁶ this peak diminishes in intensity as the i-motif structure is unwound by binding to RRM1. When several concentrations of the wild-type or modified RRM1s were added to a solution containing the i-motif DNA, analysis of the normalized signal intensities at 286 nm indicated that wild-type RRM1 had a K_D of 0.27 μ M, while that of the modified RRM1 was 0.26 μ M. The binding of the two RRM1 proteins to the i-motif DNA was also confirmed using an electrophoretic mobility shift assay (EMSA), which gave essentially the same result for both proteins. Thus, the ribosomal incorporation of a methylated β -alanine amino acid into an α -helical domain (RRM1) of hnRNP LL increased the stability of the protein without significantly altering its ability to bind to its DNA substrate.

Söll, Schepartz and coworkers later studied the biosynthesis of a β -amino acid-containing DHFR in *E. coli*.¹³⁹ They found that the wild-type *E. coli* phenylalanyl-tRNA synthetase was able to activate tRNA^{Phe} with β^3 -phenylalanine, and that *E. coli* elongation factor Tu (EF-Tu) was able to bind the misacylated tRNA. Accordingly, by the use of clone 040329, they demonstrated the incorporation of β^3 -(*p*-bromo)phenylalanine into position 128 of DHFR. In addition they explored the effects of introducing further diversity into clone 040329, and isolated clone P7A7 (having the nucleotide sequence 2502UGACUU2507 rather than the sequence 2502UGGCAG2507 present in 040329). The modified ribosomes containing this sequence exhibited enhanced incorporation of β^3 -(*p*-bromo)phenylalanine into DHFR. While not all non- α -amino acids are likely to be recognized by an endogenous aminoacyl-tRNA synthetase, this report represents an important milestone toward the *in cellulo* expression of proteins containing a variety of non- α -amino acids.

Ribosomes that incorporate dipeptides and dipeptidomimetic motifs into proteins.

While peptides and proteins ably mediate large numbers of essential biochemical functions in nature, there is a long history of structure modification of such species, both to understand their limitations and impart improved function. Activity in this area began with peptides, many of which exhibit hormonal properties, and focused on “limitations” in their functions, notably metabolic stability and lack of preorganization in the “biologically active” conformation. The relatively small size and complexity of peptides, as compared with proteins, permitted the chemical synthesis of analogues resistant to enzymatic degradation, able to be transported across key barriers such as the intestinal and blood-brain barriers, and having conformations optimal for supporting specific functions.^{140–142} These efforts have included the development of peptidomimetic motifs, structural elements that can be used as cassettes to enhance or replace structural elements often present in peptides and proteins.¹⁴³

While such structural modifications of proteins have the potential to realize analogous improvements, the larger size and structural complexity of proteins has resulted in the slower development of strategies to effect selective changes. That said, it is now possible to manipulate protein structures, especially for proteins of modest size. The total chemical synthesis of proteins,¹⁴⁴ augmented by native chemical ligation of synthetic peptide fragments,¹⁴⁵ has played an early role, and numerous extensions to the basic strategy have significantly increased the repertoire of structures accessible.^{146–148} Notably, expressed

protein ligation enables the addition of a synthetic peptide to an expressed protein in a chemoselective fashion.^{149–152} The introduction of a thioamide linkage into α -synuclein represents one interesting example.¹⁵³

In principle, a conceptually straightforward alternative to the foregoing ligation strategy would be the use of modified ribosomes capable of selectively recognizing additional peptidomimetic structural motifs not normally found in proteins. The exploration of this possibility was carried out using the same library of modified ribosomes used initially for the selection of ribosomes able to recognize β -amino acids,¹⁰³ but employing a puromycin analogue containing the dipeptide (*p*-methoxy)phenylalanylglycine (Figure 5). A survey of 419 clones from the library revealed 13 the growth of which were inhibited by at least 50% in the presence of 100 μ g/mL dipeptidylpuromycin. Nine of these were investigated further and are summarized in Table 2; four of the nine had the sequence 2502ACGAAG2507 and two had the sequence 2502CUACAG2507.¹⁰⁴ All of these clones afforded S-30 systems that functioned well in protein biosynthesis *in vitro* and one of them, 010326R6, was used for more detailed protein synthesis experiments.

In initial experiments, the dipeptides glycylphenylalanine and phenylalanylglycine were introduced onto a suppressor tRNA transcript (Scheme 1), and then incorporated into position 10 of DHFR by suppression of a UAG codon in the DHFR mRNA, and the presence of the dipeptides was verified by proteolytic digestion and mass spectrometric analysis of the appropriate peptide fragments. An important extension involved the direct incorporation of the thioamide derivative of phenylalanylglycine (**5**, Figure 11), affording the same type of peptide modification accessible by expressed protein ligation.¹⁵³ In addition to verifying the incorporation of the thiodipeptide into position 10 of DHFR by mass spectrometry, the two Phe-Gly dipeptides were also incorporated into position 16 of a DHFR construct that also had 7-methoxycoumarin at position 49. As shown in Figure 12, excitation of 7-methoxycoumarin resulted in strong fluorescence emission when Phe-Gly was at position 16, but the presence of thioPhe-Gly (**5**) in position 16 resulted in fluorescence quenching, as expected.¹⁵³

Another example of the utility of this strategy involved oxazole **6** (Figure 11), which has been introduced into a few different peptides and proteins. When introduced into position 10 of DHFR, the fluorescence emission of **6** was about an order of magnitude greater than that of the free oxazole.¹⁰⁴ Even when **6** was introduced into the middle of the peptide tetraglycine by chemical synthesis, its fluorescence intensity increased three-fold.¹⁰⁴ A more detailed study of the effect of environment was carried out by incorporating 11 different oxazoles and thiazoles into position 66 of green fluorescent protein, which is within the β -barrel structure.¹⁵⁴ Also carried out was a study of the effect of introducing oxazole **6** into position 39 outside the β -barrel structure. As shown in Table 3, the GFP construct having **6** at position 66 was about four times brighter than wild-type GFP; even when the fluorophore was at position 39, the resulting GFP was about twice as bright as wild type. Thus, the introduction of the oxazole amino acid into a peptide/protein structure resulted in a significant enhancement in fluorescence emission, and the environment of the site of substitution also affects the extent of fluorescence emission enhancement.

Incorporation of an oxazole amino acid into proteins *in cellulo*.

One limitation of the use of non-canonical amino acids lacking an α -amino moiety is that they are typically not recognized by aminoacyl-tRNA synthetases, and thus cannot be activated in cells. While the incorporation of β^3 -(*p*-bromo)phenylalanine into DHFR in *E. coli* was enabled by the finding that *E. coli* phenylalanyl-tRNA synthetase could recognize a phenylalanine having the NH₂ group on C β ,¹³⁹ other β -amino acids were activated poorly.¹³⁹

The availability of *E. coli* ribosomes able to incorporate dipeptides and dipeptidomimetics into proteins suggested that it might be possible to incorporate oxazole amino acids into proteins, and study them in a cellular environment, i.e. *in cellulo*. The pyrrollysyl-tRNA synthetase (PylRS) and its cognate tRNA^{Pyl} from *Desulfitobacterium hafniense* have been used extensively as an orthogonal pair both *in vitro* and *in vivo* for incorporating non-canonical amino acids into proteins,^{46,49} PylRS has also been shown to activate amino acids in which the position of the nucleophilic amine has been altered substantially.¹⁵⁵

An *E. coli* ribosomal clone capable of utilizing oxazole amino acids *in vitro*, was transformed with pTECH-Pyl-OP containing the PylRS-tRNA^{Pyl} orthogonal pair, and additionally with one of three plasmids encoding a single protein for expression. Oxazole **7** (Figure 11) was incorporated into position 66 of GFP and the purified protein was characterized both by analysis of the fluorescence emission spectrum and by mass spectrometry of the intact protein. Also expressed in modified form was the bacterial filamentous protein MreB, which is implicated in a number of bacterial processes including maintenance of the rod-like structure of *E. coli*. Oxazole **7** was incorporated into the protein at position 13, suggested by structural data not to be required for self-polymerization or binding with other proteins.¹⁵⁶ In fact, in common with wild-type MreB, the modified protein was isolated only in low yield as the majority underwent polymerization. MreB was expressed *in cellulo* both as wild-type and modified constructs, and both were purified and shown to have the expected mobility. While high resolution fluorescence microscopy will be required to verify the location of the MreB expressed *in cellulo*, the *E. coli* containing the modified MreB were strongly fluorescent (Figure 13), and the *E. coli* retained its rod-like shape.

One obvious application for the oxazole amino acid would be as a replacement for GFP and its related fluorescent proteins, which are commonly used as genetically encodable labels for other proteins. The resulting fusion proteins enable cellular trafficking and protein-protein interactions to be monitored. While the natural fluorescent proteins and their analogues are very useful tools, they have at least two limitations. One is that GFP is not fluorescent as initially translated; it requires cyclization and aerobic oxidation of three contiguous amino acids to generate the requisite fluorophore. For some fluorescent proteins, this process involves a significant delay,¹⁵⁸ such that early events could not be monitored. In addition, the fluorescent proteins are rather large as simple labels, sometimes larger than the proteins whose behavior is being monitored.¹⁵⁹ In at least one case, the labeling of MreB with a fluorescent protein produced results now regarded as artifactual.¹⁶⁰

The success of these studies demonstrated that it is possible to create new fluorescent proteins with diverse photophysical properties by ribosomal synthesis,^{104,105,154} and to contemplate their use for *in cellulo* studies.¹⁵⁷

Modified ribosomes for protein tyrosine phosphorylation.

Proteins contain numerous modifications introduced subsequent to ribosomal synthesis of the nascent polypeptide chain. Phosphorylation, predominantly of tyrosine, serine and threonine residues, is the main form of reversible covalent modification of proteins, responsible for regulating a broad range of cellular activities including the cell cycle, differentiation, metabolism, and neuronal communication.^{161–163} There are several methods, which are used widely for the detection of phosphorylated proteins.^{164–167} One of these involves the use of phospho-antibodies, which can recognize proteins containing phosphotyrosine.¹⁶⁴ In field of proteomic studies, where the analysis of phosphorylated proteins takes place in complex biological samples such as cell lysates, mass spectrometry techniques are used extensively.^{165,166}

These methods work well in the case of functional studies of phosphorylated proteins. However, the identification of the specific phosphorylated residues formed reversibly *in vivo* can be problematic.^{168,169} Some phosphorylation sites exhibit strong sequence homology, especially for closely related species. However, not all phosphorylation sites are positionally conserved, underscoring the need for strategies to produce proteins having phosphorylated amino acids at predetermined positions. A number of approaches have been developed to facilitate the study of phosphorylated proteins.^{170–173} They include chemical and enzymatic modification of specific amino acid side chains of protein, or incorporation of photocaged amino acids in proteins during *in vitro* and *in vivo* translation. Attempts to incorporate phosphorylated amino acids directly into proteins by ribosomal synthesis have resulted in low yields of the final products *in vitro*⁶² and dephosphorylation by phosphatases *in vivo*.¹⁷⁴

To increase the yield of proteins having phosphotyrosine at pre-determined positions, Chen et al.⁶² employed ribosomal clones randomized in regions 2057–2063 and 2600–2605 of *E. coli* 23S rRNA, and used a puromycin derivative containing phosphotyrosine (phosphopuromycin, Figure 5) to identify clones inhibited by this puromycin. The selection procedure identified six modified ribosome variants that exhibited enhanced sensitivity to this analogue of puromycin (Table 4). There were two notable characteristics of the clones inhibited by phosphopuromycin in the region 2600–2605. Mechanistically, the most important nucleotide in this region is A2602. This nucleotide has been reported to exhibit conformational flexibility and to play a role in PTC rearrangement to accommodate substrate, as well as participate in the catalytic step leading to peptide bond formation.^{23,82,83} Four of the six selected clones had A2602G and two had A2602U substitution in this position (Table 4). It was also found that all of the clones were quite G-rich (50–66%, in comparison with 17% for wild type) in the modified region of the 23S rRNA, especially as regards positions 2600, 2602, 2604 and 2605). Plausibly, the basic G residues may be directly involved in the binding of the acidic phosphate moiety of phosphotyrosyl-tRNACUA. The most efficient incorporation of phosphotyrosine into protein was demonstrated for two clones, 030449 and 040412. Phosphotyrosine was activated on a

suppressor tRNA transcript *in vitro* (cf Scheme 1) and then employed for the suppression of a UAG codon at position 42 of the mRNA encoding I κ B- α , an inhibitor of NF- κ B. The use of S-30 preparations from these two clones resulted in suppression yields of 20 and 24%, respectively, relative to wild-type protein synthesis under the same conditions. Interestingly, these clones had three identical nucleotides in modified region 2057–2063 and the same 2600GUUCGG2605 sequence in the second modified region.⁶²

The expressed I κ B- α protein was analyzed by denaturing polyacrylamide gel electrophoresis in comparison with wild type; phosphorimager visualization was enabled by incorporation of ³⁵S-methionine during protein synthesis. As shown in Figure 14, the wild-type and modified proteins co-migrated, as expected. In addition, immunoblotting analysis of the two expressed proteins indicated that only the phosphorylated I κ B- α protein was bound to a phosphotyrosine-specific antibody.

As anticipated based on literature reports,^{175–181} wild-type I κ B- α inhibited the binding of NF- κ B to a DNA duplex sequence identical with the promoter region of IL-2, the expression of which is regulated by I κ B- α . On the basis of inference from cellular experiments which reported that phosphorylation of I κ B- α at position 42 reversed its ability to bind NF- κ B, it was anticipated that the modified I κ B- α containing phosphotyrosine at position 42 would not prevent the binding of NF- κ B to the IL-2 promoter region DNA sequence. Surprisingly, this proved to be inaccurate; phosphorylated I κ B- α still bound weakly to NF- κ B. Further characterization of the system revealed that phosphorylated I κ B- α actually facilitated DNA exchange by NF- κ B (Figure 15), a property that could contribute to its physiological role as a mediator of transcription of numerous cellular genes.¹⁸²

Opportunities and Challenges

The foregoing studies suggest several different types of opportunities which may be realized by the use of modified ribosomes. Among the most obvious are the incorporation of numerous acyclic, cyclic, and heterocyclic amino acids whose amino and carboxylic acid moieties are attached in spatial arrangements not yet studied. In principle, such species could be incorporated into puromycin derivatives and used to select ribosomes capable of incorporating a broad variety of organic frameworks into protein structures. The challenges associated with the synthesis of such modified proteins *in vitro* certainly include the selection of ribosomes which continue to recognize the canonical α -L-amino acids that will constitute the majority of amino acids present in the modified proteins, while also enabling the structurally novel amino acids to be incorporated. In addition, to be utilized *in cellulo* the modified amino acids will have to be attached to a suitable tRNA, presumably by engineered aminoacyl-tRNA synthetases^{41–50} or ribozymes.^{36–40} Cellular incorporation will presumably also require recognition by cellular factors such as elongation factor Tu.

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A related, but technically more complex challenge would involve the assembly of proteins in which the linkages between “amino acid” residues were altered. This has been achieved using native ribosomes for the synthesis of proteins having some ester linkages,^{186–188} and for other functional groups as well,^{189,190} albeit generally only in low yield. Even for

linkages that probably cannot be realized directly, such as thioamides, the use of dipeptidomimetic cassettes could be useful to obtain the desired products, as exemplified in Figure 11.

While numerous proteins containing non-proteinogenic amino acids have been reported and used to facilitate an understanding of the way in which those proteins function, the ability to incorporate non- α -L-amino acids *in cellulo* should significantly enhance the scope of phenomena that can be studied, especially at a cellular level. Possibilities might be thought to include study of the processes that lead to protein misfolding and aggregation, and the use of artificial zymogens expressed *in cellulo* and activated by extra- or intracellular stimuli to control the temporal behavior of specific proteins. The sites of protein glycosylation and (reversible) phosphorylation appear to be carefully chosen in native systems, and might be better understood through the wherewithal to select alternative sites for such modifications, or simply to block phosphatase-mediated dephosphorylations through the intracellular expression of proteins containing methylenephosphonate functional groups rather than phosphates. Challenges expected to be encountered in implementing strategies of this type include the need to enable protein expression in eukaryotic cells using modified ribosomes. The interpretation of the effects observed in such experiments may be complex, as the intracellular presence of some modified proteins may simply trigger repair or compensatory processes designed to maintain cell integrity. Nonetheless, the potential for dissection of intracellular events in fine chemical detail seems significant and worthy of pursuit.

Finally, it may be noted that ribozymes have now been described which mediate chemical reactions not yet found in natural systems. These include cycloaddition reactions accelerated dramatically by the juxtaposition of two intrinsically reactive species on the surface of an RNA template.^{191–195} Conceptually, this does not differ dramatically from the mechanism by which peptide bonds are formed at the ribosomal peptidyltransferase center. Perhaps the selection of ribosomes capable of mediating chemistry such as C-C bond formation can eventually be realized.

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Figure 1.

Chemical mechanism of peptide bond formation during the elongation phase of ribosomal protein synthesis.

Figure 2.

Ribosomal antibiotic binding pockets, illustrating the structural basis for cross-resistance to antibiotics which bind to the peptidyltransferase center (PTC). (A) Diagram of PTC. Green arrows show the nucleotides involved in antibiotic resistance. Red nucleotides are essential for ribosome function; nucleotides shown in yellow can be replaced by at least two other nucleotides. (B) The overlapping three-dimensional positions at which the antibiotics bind to the PTC. Chloramphenicol (yellow), clindamycin (cyan), retapamulin (orange), dalbavancin (magenta) and linezolid (pink) are shown. Reproduced with permission from ref 83.

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Figure 3. Comparison of the observed structures of analogues of the 3'-ends of L- and D-aminoacyl-tRNA analogues bound in the A-site cleft formed by 23S rRNA. Only the aminoacyl-tRNA analogue having an L-amino acid moiety occupies a position that enables an in-line nucleophilic attack on the carbonyl C-atom of the P-site tRNA. Reproduced from ref 113. Copyright 2018 Oxford University Press.

Figure 4.

Strategy for the construction of modified ribosomes capable of incorporating D-amino acids into proteins. Mutagenesis of plasmid pUCrrnB, containing the wild-type *rrnB* operon was carried out in the region corresponding to nucleotides 2447–2450 using randomized primers in a polymerase chain reaction. The region chosen for mutagenesis is known to be close to the chloramphenicol binding site, so this antibiotic was used for the initial selection of mutants. Reproduced from ref. 100. Copyright 2006 American Chemical Society.

Figure 5. Structures of puromycin and several puromycin derivatives used to select ribosomal clones with specific properties from a library of clones having modified 23S ribosomal RNAs.

Figure 6.
 β -Amino acids incorporated into proteins using *E. coli* ribosomes modified in the 23S rRNA.

Figure 7. Strategy used for β -amino acid incorporation into proteins using modified ribosomes.

Figure 8.

Translation of DHFR from wild-type and modified DHFR (lanes 1–5) mRNAs (UAG codon at position 10) using S-30 systems prepared from clones 010335 and 040329 in the presence of different suppressor tRNAs (lanes 1–5). Lane 1-nonacylated tRNA_{CUA}; lane 2-tRNA_{CUA} acylated with amino acid **1**; lane 3 - tRNA_{CUA} acylated with amino acid **2**; lane 4 - tRNA_{CUA} acylated with amino acid **3**; lane 5 - tRNA_{CUA} acylated with amino acid **4**. Reproduced from ref 103. Copyright 2015 American Chemical Society.

Figure 9.

Partial structure of the peptidyltransferase center of *E. coli* strain K12 70S ribosome at the level of 23S rRNA showing nucleotide regions 2057–2063 (yellow) and 2496–2507 (red) where the mutations were introduced. Top panel, wild-type ribosomes; middle panel, clone 040329; bottom panel, clone 010335. The important nucleotide A2451 is shown colored in gray along with possible hydrogen bonding interactions. The *E. coli* 23S rRNA structure (Protein Data Bank entry 2WWQ) was used as the reference,¹³⁴ and the PTC loop regions were defined as reported.¹⁰ Chimera 1.9 was employed for three-dimensional structure visualization.¹³⁵ Reproduced from ref 103. Copyright 2015 American Chemical Society.

Figure 10.

Structural change in RRM1 caused by the incorporation of 3(*S*)-methyl- β -alanine (**4**). (A) Three-dimensional structure of RRM1, highlighting A35, modeled with reference to the solution structure of N-terminal mouse protein BAB28521 (Protein Data Bank entry 1WEX). (B) the CD spectra of RRM1 produced *in vivo* and *in vitro* showed identical patterns of random coil structure, whereas that of RRM1-**4** showed a stable α -helix. (C) Melting points for RRM1wt and RRM1-**4**. Reproduced from ref 103. Copyright 2015 American Chemical Society.

Figure 11.
Representative dipeptides and dipeptidomimetic analogues incorporated into proteins.

Figure 12.

(Top panel) Model of the DHFR construct containing the 7-methoxycoumarin fluorophore in position 49 and thio-Phe-Gly or Phe-Gly at position 16, based on DHFR structure PDB 1RA1. The distance from the oxygen of Met16 (corresponding to the S atom of ThioPhe-Gly in the figure, yellow color) to the α -carbon of Ser49 is 7.7 Å. (Bottom panel) Fluorescence emission of two samples of DHFR (10 ng/ μ L), each having 7-methoxycoumarin in position 49 and Phe-Gly (blue trace) or thioPhe-Gly (orange trace) in position 16, after excitation at 310 nm. Reproduced from ref. 104. Copyright 2015 American Chemical Society.

Figure 13.

Microscopic study of cells, hosting MreB-wt (prepared in the presence and the absence of oxazole **7**); MreB-13 substituted at position 13 by oxazole **7**, and BFP. The images were obtained using a Nikon Eclipse Ti2 Inverted Microscope System. The excitation wavelength was 357 ± 22 nm, and the emission wavelength was 435 ± 25 nm. The inset shows an enlargement of the image of cells harboring the plasmid encoding modified MreB and grown in the presence of oxazole **7**. Reproduced from ref. 157. Copyright 2019 American Chemical Society.

Figure 14.

Immunoblotting analysis of IκB-α samples obtained by *in vitro* translation from the wild-type gene and from the modified (TAG codon in protein position 42) gene in the presence of phosphotyrosyl-tRNA_{CUA} (pTyr) and purified by Ni-NTA-chromatography. Analysis of samples by 4%–12% SDS-polyacrylamide gradient gel electrophoresis using (A) phosphorimager detection of ³⁵S-methionine and (B) anti-phosphorylated IκB-α (pTyr42) rabbit polyclonal IgG. Reproduced from ref. 62. Copyright 2017 American Chemical Society.

Figure 15.

Time-dependent binding of NF- κ B and DNA. (A) Samples were analyzed by 6% native polyacrylamide gel electrophoresis and quantified using a phosphorimager. Upper panel: binding of NF- κ B and DNA without phosphorylated I κ B- α ; lower panel: binding of NF- κ B and DNA in presence of 20 ng of phosphorylated I κ B- α . Lane 1, incubation for 0 min; lane 2, incubation for 0.5 min; lane 3, incubation for 1 min; lane 4, incubation for 3 min. (B) Relative intensity of NF- κ B–DNA complexes analyzed by the 6% native polyacrylamide gel electrophoresis. The intensity of the NF- κ B–DNA complex in the reaction of NF- κ B and DNA without phosphorylated I κ B- α at 0.5 min was defined as 100%. Reproduced from ref. 62. Copyright 2017 American Chemical Society.

Scheme 1.
Preparation of β -alanyl-tRNA_{CUA}

Table 1.Suppression of a UAG Codon at Position 22 of DHFR mRNA with D-and L-Aminoacyl-tRNACUAs^a

amino acid	suppression (%)	
	wild-type ribosomes	wild-type + mutant ^b ribosomes
L-phenylalanine	58	54
D-phenylalanine	3	12
L-methionine	52	47
D-methionine	5	23
-	0.9	2

^aRelative to the amount of DHFR produced using wild-type mRNA and wild-type ribosomes.^bS-30 preparation from clone having 2447UGGC2450 sequence in 23S rRNA.

Table 2.23S Ribosomal RNA Sequences in the Region 2502–2507 of Clones Inhibited by Dipeptidylpuromycin^a

clones	nucleotide in position					
	2502	2503	2504	2505	2506	2507
010309R3	A	C	G	A	A	G
010326R6	A	C	G	A	A	G
010328R4	A	C	G	A	A	G
010322	A	C	G	A	A	G
010310R1	C	G	C	A	A	U
010310R4	C	C	C	A	C	G
010326R5	C	U	A	U	G	U
010328R2	C	U	A	C	A	G
010326R1	C	U	A	C	A	G

^a All of these clones had the sequence 2057UGCGUGG2063.¹⁰⁰

Table 3.

Estimation of Quantum Yields of Modified Green Fluorescent Proteins in Comparison with GFPwt

protein	$\lambda_{\text{ex}}/\text{abs}$, nm	absorption extinction coefficient, $\text{M}^{-1}\text{cm}^{-1}$	quantum yield, λ_{em}	brightness
GFPwt	395	25,000	0.79 (509 nm)	19,750
GFP66oxazole6	310	90,300	0.91 (378 nm)	82,150
GFP66Gly39oxazole6	310	50,200	0.84 (407 nm)	42,150

Table 4.

Sequences of Nucleotides 2600–2605 of the 23S rRNAs of the Modified Ribosomes Selected Using Phosphopuromycin

clone	nucleotide position					
	2600	2601	2602	2603	2604	2605
030405	G	A	G	U	G	A
030449	G	U	U	C	G	G
030454	G	A	G	A	G	C
040412	G	U	U	C	G	G
040424	U	G	G	U	G	G
040435	G	A	G	A	G	G
wild type	A	C	A	G	U	U