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Probing the Synthetic Capabilities of a Center of Biochemical Catalysis

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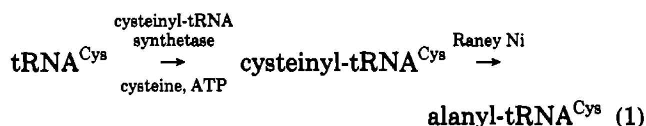
Ribosomally mediated peptide bond formation is the predominant source of peptides and proteins in nature and has been studied intensively at a biochemical level.[1] In overall terms, peptide bonds are formed at the peptidyltransferase site; in prokaryotes, this catalytic center is part of the 50S ribosomal subunit.[2,3] Many details are lacking, however, about the precise chemical mechanism of peptide bond formation.

The formation of a new peptide bond occurs when the peptidyl moiety of a transfer RNA (tRNA) in the ribosomal P (peptidyl/donor) site becomes attached to the aminoacyl moiety of a tRNA in the (adjacent) ribosomal A (aminoacyl/acceptor) site (Scheme I). The formation of amide bonds between individual amino acids is consistent with the reactive nature of the intermediate aminoacyl (peptidyl)-tRNAs involved; it seems likely that these bonds are formed by nucleophilic attack of N^{α} of the aminoacyl moiety of the A-site tRNA on the activated carboxylate ester of the peptidyl-tRNA (Scheme I), although a peptidyl–ribosome intermediate has also been suggested.[4] The sequence and fidelity of amino acid placement within the nascent polypeptide is dependent on codon–anticodon interaction, which orients the appropriate aminoacyl-tRNA in response to each messenger RNA (mRNA) codon as the latter approaches the peptidyltransferase center through a series of translocation events.[3]

As predicted independently by Crick[5] and Hoagland[6] (the adaptor hypothesis) the fidelity of protein synthesis also depends on the accuracy with which each tRNA is activated with its cognate amino acid. While editing mechanisms minimize misacylations during tRNA activation,[7] apparently none of these functions after release of the formed aminoacyl-tRNAs by the activating enzymes. This was demonstrated directly by Chapeville et al.,[8] who prepared a cysteine-specific tRNA activated with alanine (alanyl-$tRNA^{Cys}$) by Raney Ni-mediated desulfurization of cysteinyl-$tRNA^{Cys}$ (eq 1). The use of this misacylated tRNA in an in vitro protein biosynthesizing system programmed with globin mRNA afforded an analog of globin in which the β-chain contained alanine in place of cysteine. The validity of this concept was also demonstrated more quantitatively using misacylated *E. coli* $tRNA^{Lys}$.[9]



$$tRNA^{Cys} \xrightarrow[\text{cysteine, ATP}]{\text{cysteinyl-tRNA synthetase}} \text{cysteinyl-}tRNA^{Cys} \xrightarrow{\text{Raney Ni}} \text{alanyl-}tRNA^{Cys} \quad (1)$$

The experiment of Chapeville et al.[8] used a misacylated tRNA containing one of the 20 amino acids that normally participates in ribosomally-mediated protein biosynthesis. More recently, my laboratory has described a technique ("chemical aminoacylation") that permits the preparation of tRNAs activated with an enormous variety of amino acids and amino acid analogs.[10] This Account describes the preparation of these peptidyl- and aminoacyl-tRNA analogs and their

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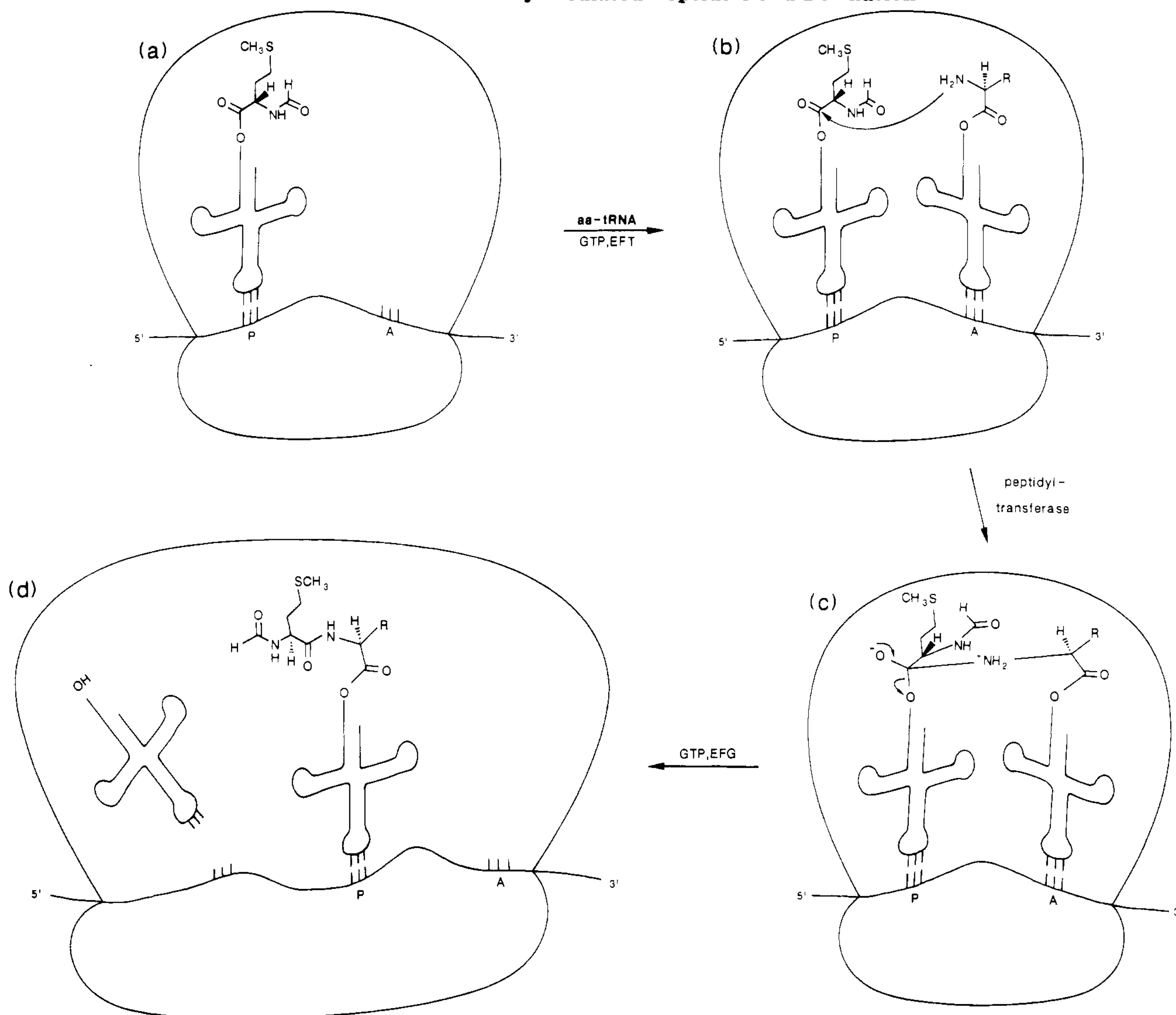
Scheme I. Ribosomally Mediated Peptide Bond Formation[a]



[a] The bond-forming event shown involved N-formylated methionyl-tRNA, which initiates protein synthesis in prokaryotes.

behavior in the peptidyltransferase reaction. It focuses on the chemistry that peptidyltransferase is *capable* of doing, rather than that which normally obtains, as the former can provide key insights into the nature of the peptidyltransferase center and its catalytic capabilities. Also described are novel chemical products of the peptidyltransferase reaction that can provide access to structurally unique polypeptide-derived catalysts not readily accessible by other means.

Preparation of Misacylated tRNAs

To date, three methods have been described for the preparation of tRNAs activated with noncognate amino acids. One of these involves the aminoacyl-tRNA synthetase-catalyzed misacylation of noncognate tRNAs in the presence, e.g., of organic solvents or unusual amounts of Mg^{2+} and ATP.[7c,d] Although generally employed for the substitution of one naturally occurring amino acid by another, aminoacyl-tRNAs containing amino acid analogs have also been prepared.[11]

A second method involves aminoacyl-tRNA synthetase-catalyzed activation of a tRNA with its cognate amino acid, followed by chemical transformation of the aminoacyl moiety. In addition to alanyl-tRNA^{Cys},[8] N^{ϵ}-acetyllysyl-tRNA^{Lys} [12] (1) and phenyllactyl-tRNA^{Phe} [13]

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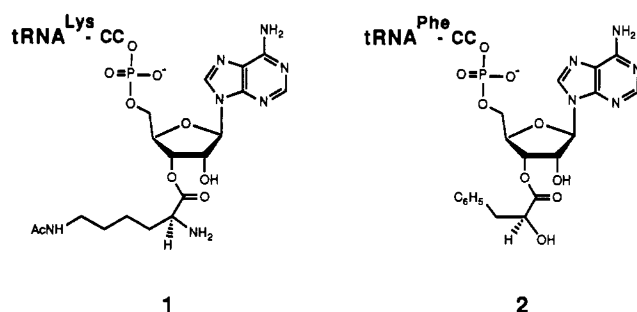

Figure 1. Misacylated tRNAs prepared by chemical alteration of enzymatically activated tRNAs.

(**2**) have been prepared in this fashion (Figure 1). All three of these misacylated tRNAs have been reported to participate in the peptidyltransferase reaction, affording polypeptides other than those which would normally have obtained. The participation of phenyllactyl-$tRNA^{Phe}$ in the peptidyltransferase reaction is of particular interest, as the products are polyesters and depsipeptides rather than polypeptides.

A radically different strategy is outlined in Scheme II. In this case, the bond formed to produce the misacylated tRNA is a phosphate ester between two nucleotides (b, Scheme II), rather than a carboxylate ester between the amino acid and tRNA (a, Scheme II). The "activating enzyme" is T4 RNA ligase, which effects the ligation of an aminoacylated dinucleotide structurally identical with the 3′-end of an aminoacyl-tRNA (the "donor" oligonucleotide) with a tRNA from which the last two nucleotides have been removed (the "acceptor" oligonucleotide). T4 RNA ligase is known to have broad substrate specificity with regard to the donor[14] and utilizes numerous (amino)acylated pCpA derivatives as substrates (vide infra).[10] As a consequence, this method is much more versatile than the two discussed above.

Removal of the 3′-terminal nucleotides from intact tRNAs can be accomplished chemically[15] or enzymatically;[1e,10d] the aminoacylated pCpA derivatives are prepared by chemical synthesis.[10] Because free aminoacyl-tRNAs are readily hydrolyzed to the corresponding amino acid and tRNA, the ligation reaction must be done in a fashion that preserves the activated ester. Where the intended products are N-acylated aminoacyl-tRNAs (i.e., peptidyl-tRNA analogs), the corresponding N-acylated aminoacyl pCpA derivatives are sufficiently stable hydrolytically to be used directly. Where the desired products are free aminoacyl-tRNAs, stabilization of the aminoacyl pCpAs can be achieved by suitable protection of N^{α} of the aminoacyl moiety; following ligation of the N-protected aminoacyl pCpA and tRNA-C_{OH}, deblocking affords the requisite aminoacyl-tRNA. The *o*-nitrophenylsulfenyl protecting group (NPS, removable with sodium thiosulfate) was introduced for this purpose in 1978[10a] and has also been employed by the Schultz laboratory; these workers have also utilized a nitroveratryl group.[16] Recently, we have prepared *N*-pyroglutamylaminoacyl-tRNAs[10d] (removable enzymatically with pyroglutamate aminopeptidase) (Scheme III). The broad substrate specificity of this enzyme and lack of contaminating esterase activities in the commercial preparation make this a convenient method for the preparation of free aminoacyl-tRNAs. Numerous peptidyl- and (protected) aminoacyl-tRNAs can be prepared in this fashion.[10]

Because the aminoacyl protecting group must be hydrolytically stable but subsequently sensitive to some reagent that affects neither the tRNA nor its activated ester, relatively few chemical strategies have proven successful. An interesting alternative developed in the Brunner laboratory[17] involves the use of free aminoacyl pCpA in a ligation reaction of limited duration.

In Vitro Synthesis of Peptides and Proteins

Although the ribosomally mediated synthesis of proteins is a highly efficient process in intact cells, all cell-free preparations described to date elaborate products with considerably less facility. The absence of any dramatic increase in efficiency for an in vitro system reconstituted from the purified individual components[18] is consistent with the view that diminished efficiency results from the loss of organization imposed on the process in an intact cell.

The in vitro synthesis of polypeptides in response to RNA homopolymers and polynucleotides is presumed to result in the formation of oligo- and polypeptides of varying length since the normal start and stop signals are absent. Such mixtures preclude definition of the ability of individual misacylated tRNAs to function in the peptidyltransferase reaction; however, by the use of a ribosome preparation lacking elongation factor G (EF-G), translocation is precluded and dipeptides of uniform composition can be isolated for quantification of yields[10d,19] and structural analysis.[19b,20] Dipeptide formation is carried out as a stepwise process, initiated by the binding of a (modified) peptidyl-tRNA to the ribosomal P-site, and is roughly stoichimetric with the number of ribosomes initially containing bound peptidyl-tRNAs.

As noted, the synthesis of intact, functional proteins in a cell-free system is problematical, at best. Zubay and his co-workers devised a coupled transcription–translation system; this has been used by a number of laboratories and has been reported, e.g., to afford 10–12 molecules of fully functional tryptophan synthase α-subunit for each DNA plasmid employed as a template.[21] An important technical advance was reported by Spirin et al.[22] who hypothesized that because protein synthesis occurs as a high molecular weight aggregate of proteins and nucleic acids, it should be possible to create a "continuous flow reactor" by replenishing reactants such as ATP, GTP, and amino acids and removing products including synthesized

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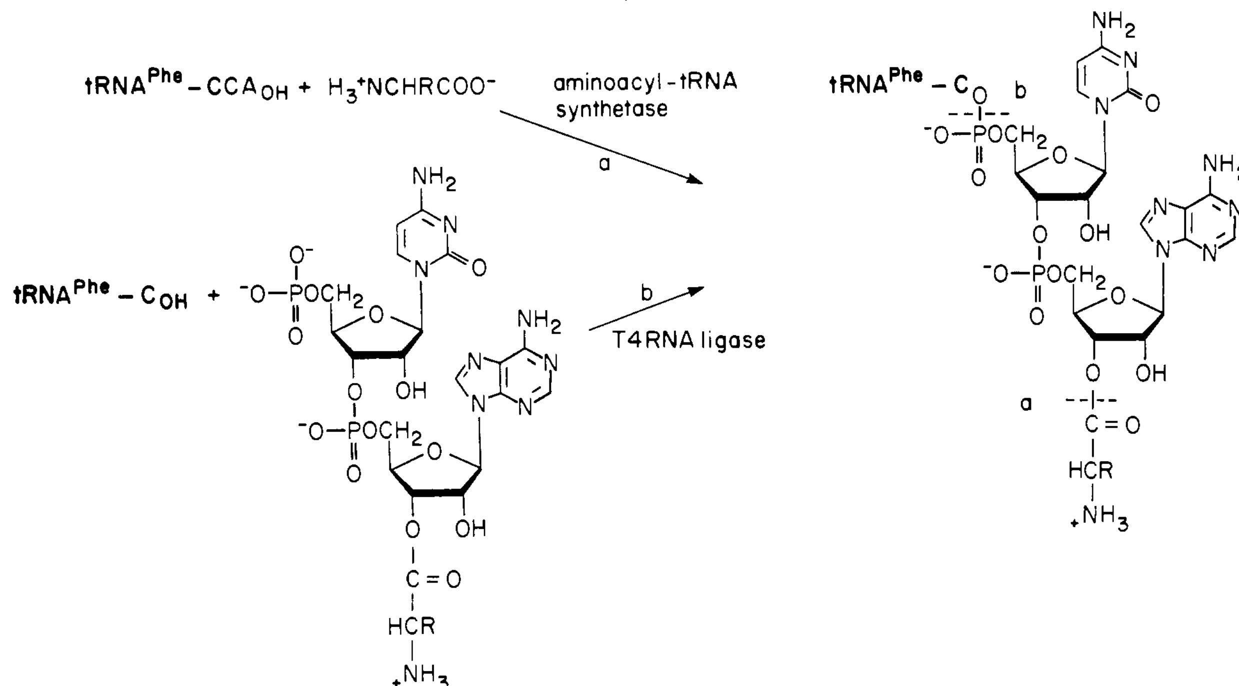
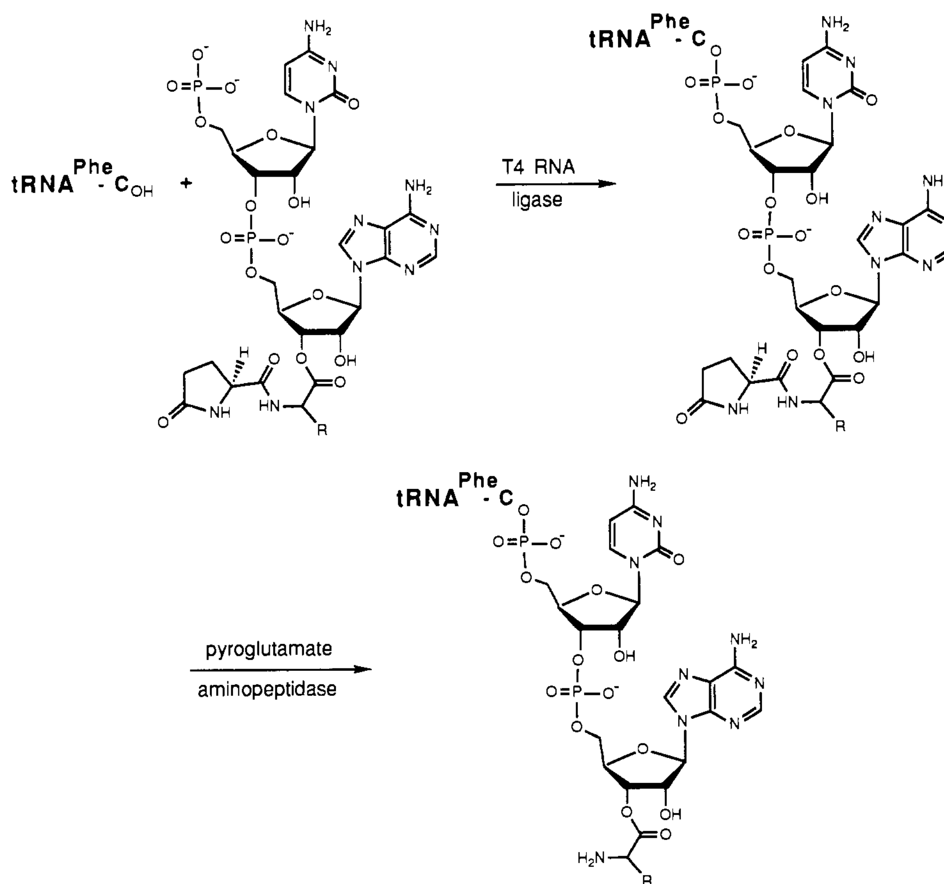
Scheme II. Comparison of Bonds Established during Preparation of Aminoacyl-tRNAs by Enzymatic (a) and "Chemical" (b) Activation



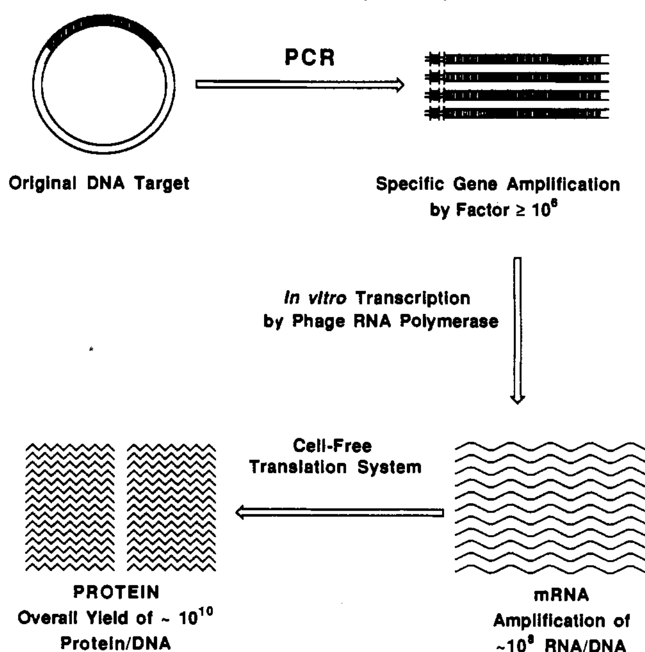
Scheme III. Preparation and Enzymatic Deblocking of *N*-Pyroglutamylaminoacyl-tRNA^Phe^s



polypeptides through a semipermeable membrane. Although the absolute amounts of product were not given, up to 300 copies of calcitonin polypeptide were obtained for each mRNA employed. Recently, Resto et al. have defined an experimental system (gene amplification with transcription/translation; GATT) in which the polymerase chain reaction is coupled to transcription and translation. The latter two processes have also been optimized, such that enormous numbers of protein molecules can be produced for each DNA plasmid employed initially (Scheme IV).[23] In the example optimized by Resto et al., 10^9–10^{11} copies of

(23) Resto, E.; Iida, A.; Van Cleve, M. D.; Hecht, S. M. *Nucleic Acids Res.*, in press.

Scheme IV. Large Scale Production of Proteins in Vitro by Gene Amplification with Transcription/Translation (GATT)



fully functional *E. coli* dihydrofolate reductase were produced for each DNA molecule used! Although the generality of this finding is presently unknown, it has the potential for solving the problem of protein elaboration in vitro on a preparative scale.

Synthesis of Dipeptides

The ribosomal synthesis of a dipeptide involves amide bond formation (Scheme I). In parallel with the chemical synthesis of peptides, the "donor" amino acid is N-protected and has a carboxylate moiety that is activated toward nucleophilic attack; in the present case, there is a good leaving group in the form of a transfer RNA. In a prokaryotic protein biosynthesizing system, the facility of dipeptide formation can be measured by monitoring the efficiency of transfer of *N*-formylmethionine from *N*-formylmethionyl-$tRNA_f^{Met}$ to the aminoacyl moiety of the tRNA occupying the second codon triplet of the mRNA (cf. Scheme I). More conveniently, *N*-acetylphenylalanyl-$tRNA^{Phe}$ can be used as the initiator tRNA in response to poly(uridylic) acid. Although this mRNA contains neither start nor stop signals, it is degenerate and recognizes exclusively $tRNA^{Phe}$, such that the dipeptide produced in systems lacking ribosomal elongation factor G is always *N*-acetylphenylalanylphenylalanine, regardless of the site on the message used to initiate protein synthesis. Clearly, by replacing the P-site tRNA (*N*-acetylphenylalanyl-$tRNA^{Phe}$) or A-site tRNA (phenylalanyl-$tRNA^{Phe}$) with an analog, the effect of the structural alteration on the facility of peptide bond formation can be determined. Aminoacylated oligonucleotides identical to the 3′-termini of tRNA's have also been used to study the mechanism of peptidyltransferase.[1g,1h]

Several structurally altered derivatives (**4**–**9**) of *N*-acetylphenylalanyl-$tRNA^{Phe}$ (**3**) (Figure 2) have been prepared and assayed as donors using high salt wash *E. coli* ribosomes.[19] As is clear from Table I, tRNAs containing D-amino acids (*N*-acetyl-D-phenylalanyl-

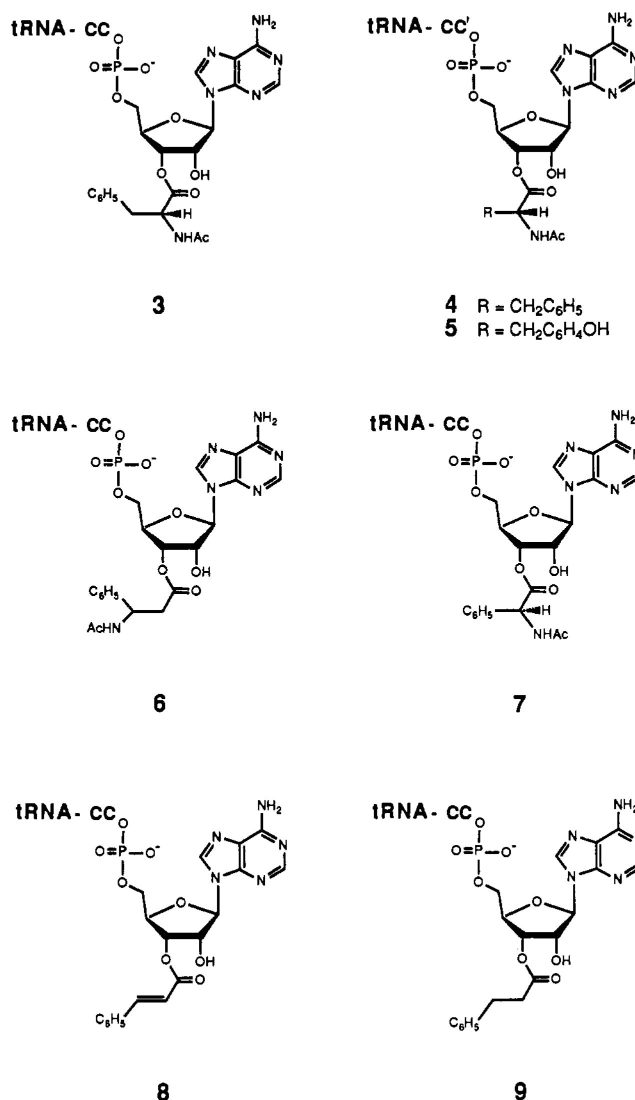


Figure 2. Structures of peptidyl-$tRNA^{Phe}$ analogs studied. The 2′(3′)-*O*-acyl moieties are shown arbitrarily on the 3′-position of ribose.

$tRNA^{Phe}$ (**4**) and *N*-acetyl-D-tyrosyl-$tRNA^{Phe}$ (**5**)) functioned poorly as donors in the peptidyltransferase reaction. In contrast, most of the other tRNAs studied functioned well, including those activated with *trans*-cinnamic acid (**8**) and 3-phenylpropionic acid (**9**). Thus the inability of tRNAs **4** and **5** to participate as donors must reflect some negative steric interaction between the D-amino acids and peptidyltransferase center since the results with **8** and **9** indicate that no amino group is required for competence as a donor. Also of interest was the finding that *N*-acetyl-D,L-phenylglycyl-$tRNA^{Phe}$ (**7**) produced dipeptide to the extent of 93%, in comparison with *N*-acetyl-L-phenylalanyl-$tRNA^{Phe}$. Since D-amino acids are not efficient donors (vide supra), the high efficiency of this racemic analog likely reflects the selective ribosomal binding of the L-isomer of *N*-acetylphenylglycyl-$tRNA^{Phe}$.

Interestingly, the lack of participation of *N*-acetyl-D-aminoacyl-tRNAs as donors was found to obtain only when the amino group was connected to C^α of the amino acid. *N*-Acetyl-D,L-β-phenylalanyl-$tRNA^{Phe}$ (**6**) formed dipeptide to a slightly greater extent (110–130%) than *N*-acetyl-L-phenylalanyl-$tRNA^{Phe}$ and at a faster rate. Participation of the D-isomer was confirmed by the preparation of single isomers of *N*-acetyl-β-phenyl-

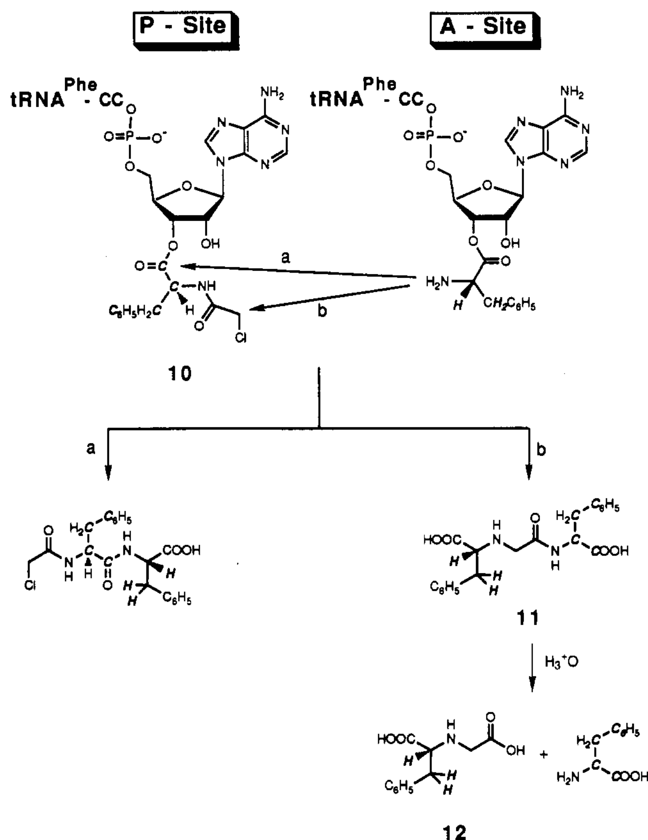
alanyl-tRNA^{Phe}, each of which functioned equally well as a donor. Although the greater yield of dipeptide obtained from tRNAs 6 as compared with tRNA 3 was confusing at first, it must reflect the fact that only 70–90% of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**3**) initially bound to the ribosomal P-site was actually utilized in dipeptide formation upon admixture of L-phenylalanyl-tRNA.[19b] Presumably, a larger fraction of the species bound initially to the ribosomal P-site was utilized for dipeptide formation when *N*-acetyl-β-phenylalanyl-tRNA^{Phe} (**6**) was employed as the donor. This observation has important implications, as it indicates both that the position of the *N*-acetyl amino group can have a facilitating effect on peptide bond formation and also that "normal" amino acids are not necessarily the optimal species for participation.

Also studied was the ability of representative misacylated aminoacyl-tRNAs to function as acceptors in the peptidyltransferase reaction. As indicated (Table II), neither D-phenylalanyl-tRNA^{Phe} nor D-tyrosyl-tRNA^{Phe} functioned as an acceptor, a characteristic that paralleled their marginal activities as donors. Once again, two tRNAs misacylated with L-amino acids (L-phenylglycyl-tRNA^{Phe} and L-*O*-methyltyrosyl-tRNA^{Phe}) acted as acceptors, producing dipeptides in 70% yield. When the same assay was carried out using D,L-β-phenylalanyl-tRNA^{Phe}, the yield of dipeptide was only 8%, i.e. very different than obtained using the same (N-acetylated) tRNA as a donor. In spite of the low yield, the results are important mechanistically as they demonstrate that an amino acid bearing the amino group at a position other than C^{α} can act as a nucleophile in the peptidyltransferase reaction.

The P-site tRNA *N*-(chloroacetyl)phenylalanyl-tRNA^{Phe} (**10**) is of special interest (Scheme V).[20] It has two electrophilic sites either of which could, in principle, react with the nucleophilic N^{α} amino group of the A-site tRNA. In fact, admixture of L-phenylalanyl-tRNA^{Phe} to tRNA **10** + 70S *E. coli* ribosomes + poly(U) afforded two products separable by DEAE-cellulose chromatography. One product, formed in 20–25% yield, was shown to be *N*-chloroacetylphenylalanylphenylalanine by HPLC analysis in comparison with an authentic synthetic sample. The other, formed in comparable yield, was also analyzed by reverse-phase HPLC and found to coelute with an authentic sample of dipeptide analog **11**. Further support for the assigned structure was obtained by treatment of the ribosomally-derived material (putative **11**) with concentrated HCl (100 °C, 6 h), a procedure shown to convert authentic **11** to *N*-(carboxymethyl)phenylalanine (**12**) and phenylalanine. HPLC analysis revealed that one product coeluted with authentic *N*-(carboxymethyl)phenylalanine and another with phenylalanine. Repetition of the experiment using [^{14}C]phenylalanine for preparation of the P-site tRNA (**10**) and an A-site tRNA activated with [^{3}H]phenylalanine resulted in the formation of doubly-labeled **11**; hydrolysis afforded ^{3}H-labeled *N*-(carboxymethyl)phenylalanine (**12**). This experiment thus verified the "transfer" of the two-carbon substituent from [^{14}C]phenylalanine to [^{3}H]phenylalanine, consistent with the intermediacy of dipeptide analog **11**.

In addition to representing the first example of the ribosomally mediated formation of a peptide analog of altered connectivity, the formation of **11** has important mechanistic implications. Specifically, the formation of comparable amounts of products derived from nucleophilic attack at each electrophilic center in **12** implies that there is considerably more flexibility at the peptidyltransferase center than has generally been assumed. In contrast to relatively rigid models that posit the binding of tRNAs to the A- and P-sites such that the reactive groups in each are held in close proximity, the foregoing results imply either greater flexibility in the way that peptidyltransferase promotes the reaction between the ribosome-bound aminoacyl-tRNAs or else the possibility that peptide bond formation occurs with facility between two bound species based on intrinsic chemical reactivity, but *not* due to the intervention of the peptidyltransferase center in a mechanistic sense.

Scheme V. Formation of Dipeptide Analogs from *N*-(Chloroacetyl)phenylalanyl-tRNA^{Phe}



Synthesis of Peptides and Proteins

The incorporation of a (modified) amino acid into a single site within a polypeptide or protein requires that the mRNA employed contain a unique codon at that site. This can be accomplished in a few ways, the most obvious of which involves the use of a protein that naturally lacks some amino acid and, hence, whose mRNA lacks any codon for that amino acid. A good example would be the α-subunit of tryptophan synthase, a polypeptide chain that contains no tryptophan, perhaps because it participates in the biosynthesis of tryptophan. While proteins that lack some amino acid might be predicted to be relatively rare, inspection of a list of amino acid compositions for proteins of known sequence indicates that this property is actually not

unusual.[24] For example, human myoglobin lacks cysteine, horse cytochrome C contains no serine, and *Clostridium pasteurianum* rubredoxin lacks alanine, arginine, glutamine, histidine, and serine.

An alternative approach makes use of nonsense codons, which appear spontaneously within the coding region of proteins in nature and can now be incorporated into virtually any position in a mRNA via recombinant DNA technology. These (termination) codons, having the sequences 5'UAG, 5'UAA, and 5'UGA, normally occur at the end of the coding region of mRNAs and effect termination of polypeptide synthesis because there are no corresponding tRNAs.[25] In cells containing terminator codons within what is normally a polypeptide coding region, there can be a compensatory DNA mutation that leads to the production of a mutant tRNA that recognizes a mRNA nonsense codon after activation with a normal amino acid. This process is termed suppression;[26] the mutated tRNA is a suppressor tRNA. Many modified suppressor tRNAs have now been prepared[27] and these can be adapted for the types of studies of interest here. For mRNAs derived from genetically engineered DNAs, readthrough of the authentic terminator codons can be precluded by using one of the three codons having a different nucleotide sequence than that of the nonsense codon.

The Chamberlin laboratory has recently described the elaboration a 16-residue polypeptide from a synthetic mRNA that contains a 5'UAG codon at position 9.[28] Translation of the mRNA with a suppressor tRNA activated with [^{125}I]iodotyrosine gave readthrough corresponding to a suppression efficiency of 30% (i.e., the efficiency of formation of full-length transcript); in the absence of the suppressor tRNA, readthrough at position 9 was only 3–4%. In addition to providing a direct measure of the efficiency of suppression, the identities of the products (i.e., polypeptides having 8 or 16 amino acids) were confirmed by HPLC comparison of the translation products with authentic synthetic standards.

This system has also been used to compare the relative efficiencies of suppression when the same suppressor tRNA was activated with each of several structurally altered amino acids.[29] As shown (Table III), tRNAs activated with iodotyrosine or glycine functioned well in suppression, but a D-phenylalanyl-tRNA did not, in agreement with findings from the Hecht laboratory.[19] Also in good agreement were the values obtained for L-phenylglycine (51% suppression vs 93% donor and 70% acceptor activities, cf. Tables I and II) and for L-*O*-methyltyrosine (54% suppression vs 70% acceptor activity). The suppression value obtained for 3-aminopropionic acid (9%) was in good agreement with those obtained for β-phenylalanine (110–130% donor and 8% acceptor activities). Also of particular interest was the data for phenyllactic acid (46% suppression) and glycylglycine (0% suppression), which were in good agreement with data obtained previously for hydroxy acids[13] and glycylglycine.[30] The modified amino acids *N*-methylphenylalanine and L-2-cyclohexylalanine were incorporated into the polypeptide with reasonable efficiency, but 5-aminovaleric acid and L-2-amino-3,3-dimethylbutyric acid were not.

To date, less work has been reported dealing with amino acid substitutions of modified amino acids into intact proteins. The earliest report is that of Payne et al.[21] who elaborated functional tryptophan synthase α-subunit in a cell-free system. By the use of phenylalanyl-$tRNA^{Val}$, they replaced all (17) of the valine residues in the subunit; the derived protein had the expected size, but was completely dysfunctional. Baldini et al.[17] employed an *E. coli* $tRNA^{Phe}$ that was

Table I. Peptidyltransferase-Mediated Dipeptide Synthesis Using Analogs of *N*-Acetyl-L-phenylalanyl-$tRNA^{Phe}$

peptidyl-$tRNA^{Phe}$ analog	isolated yield of dipeptide: pmol/A_{260} unit of ribosomes	isolated yield of dipeptide: % relative to peptidyl-tRNA 3
N-acetyl-L-phenylalanyl-$tRNA^{Phe}$ (3)[a]	1.5	100
N-acetyl-D-phenylalanyl-$tRNA^{Phe}$ (4)[b]	0.1	7
N-acetyl-D-tyrosyl-$tRNA^{Phe}$ (5)[b]	0.2	13
N-acetyl-D,L-β-phenylalanyl-$tRNA^{Phe}$ (6)[b]	1.7–2.0	110–130
N-acetyl-D,L-β-phenylglycyl-$tRNA^{Phe}$ (7)[b]	1.4	93
trans-cinnamyl-$tRNA^{Phe}$ (8)[b]	0.8	53
3-phenylpropionyl-$tRNA^{Phe}$ (9)[b]	1.2	80

[a] Prepared by activation of $tRNA^{Phe}$-CC3'dA with phenylalanine in the presence of *E. coli* phenylalanyl-tRNA synthetase, followed by chemical acetylation with *N*-acetoxysuccinimide. [b] Prepared by "chemical aminoacylation".[10]

Table II. Peptidyltransferase-Mediated Dipeptide Synthesis Using Analogs of Aminoacyl-$tRNA^{Phe}$s

misacylated tRNA[a]	isolated yield of dipeptide, μM[b]
L-phenylalanyl-$tRNA^{Phe}$	100
D-phenylalanyl-$tRNA^{Phe}$	0
D-tyrosyl-$tRNA^{Phe}$	0
L-*O*-methyltyrosyl-$tRNA^{Phe}$	70
L-phenylglycyl-$tRNA^{Phe}$	70
D,L-β-phenylalanyl-$tRNA^{Phe}$	8

[a] Produced in situ from the corresponding *N*-pyroglutamyl derivative via the agency of pyroglutamate aminopeptidase. [b] Yield relative to the amount of dipeptide obtained with authentic *E. coli* phenylalanyl-$tRNA^{Phe}$ under comparable conditions.

Table III. Suppression Efficiencies of Several Misacylated $tRNA^{Gly}_{CUA}$-dCAs[29]

misacylated tRNA	suppression efficiency, %
L-(3-iodo)tyrosyl-$tRNA^{Gly}_{CUA}$-dCA	65
glycyl-$tRNA^{Gly}_{CUA}$-dCA	79
D-phenylalanyl-$tRNA^{Gly}_{CUA}$-dCA	0
N-methyl-L-phenylalanyl-$tRNA^{Gly}_{CUA}$-dCA	72
L-phenyllactyl-$tRNA^{Gly}_{CUA}$-dCA	46
3-aminopropionyl-$tRNA^{Gly}_{CUA}$-dCA	9
glycylglycyl-$tRNA^{Gly}_{CUA}$-dCA	0
L-phenylglycyl-$tRNA^{Gly}_{CUA}$-dCA	51
L-*O*-methyltyrosyl-$tRNA^{Gly}_{CUA}$-dCA	54
2-amino-4-phosphonobutyryl-$tRNA^{Gly}_{CUA}$-dCA	61

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mischarged with the photoactivatable phenylalanine analog L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine. This misacylated tRNA was shown to stimulate protein synthesis in a rabbit reticulocyte system. More recently, Noren et al.[16a] replaced the conserved phenylalanine at position-66 in *E. coli* β-lactamase with each of several synthetic amino acids. The position of the inserted amino acid was localized to a single proteolytic digest fragment by peptide mapping. The catalytic properties of three of the mutants were also studied. For example, a mutant β-lactamase containing *p*-nitrophenylalanine at position-66 had a K_m very similar to that of the wild-type enzyme (57 vs 55 μM), but a somewhat smaller k_{cat} value (880 vs 370 s^{-1}). Another study from the same laboratory deals with the incorporation of several nonnatural amino acids into T4 lysozyme.[31] Of particular interest in the latter study was the successful incorporation of α-aminobutyric acid, which had been reported earlier[1g] not to be a peptidyltransferase substrate.

Future Prospects

With the development of methods for the preparation of misacylated tRNAs and of mRNAs that can be translated in vitro, it will be possible to determine the capabilities that the ribosome has for mediating numerous chemical transformations. Misacylated tRNAs can also be used as probes of the chemical and physical properties of the peptidyltransferase center, thus allowing the dissection of this center of biochemical catalysis at a structural and functional level. At the level of technological applications, the ability to insert amino acids of diverse structures into predetermined sites of peptides and proteins provides a powerful experimental tool that can greatly extend the present horizons afforded by site-directed mutagenesis.

At a technical level, there have been two problems that have tended to limit experimental progress and the potential utility of the technology. One of these, concerning the amount of protein accessible, would seem to be resolved with our development of the GATT procedure. The other involves the difficulty in preparation of the misacylated tRNAs. While this limitation is now less serious, due to substantial improvements in the experimental procedures, it is still reasonably arduous. It would be of great advantage to be able to effect tRNA misacylations *enzymatically*, e.g. with an aminoacyl-tRNA synthetase having diminished recognition properties for the amino acid moiety that can be attached to the cognate tRNA. Nonetheless, even without the development of such an enzymatic method, the tools for achieving the key goals in this area would seem to be in hand.

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