Chemistry & Biology, Vol. 11, 575–582, April, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.03.009

Transformation of Aminoacyl tRNAs for the In Vitro Selection of "Drug-like" Molecules

Chuck Merryman and Rachel Green*
Department of Molecular Biology and Genetics
Johns Hopkins School of Medicine
Howard Hughes Medical Institute
702A PCTB
725 North Wolfe Street
Baltimore, Maryland 21205

Summary

Evolutionary approaches are regularly used to isolate single molecules with desired activities from large populations of nucleic acids ($\sim10^{15}$). Several methods have also been developed to generate libraries of mRNA-encoded peptides and proteins for the in vitro selection of functional polypeptides. In principal, such mRNA encoding systems could be used with libraries of nonbiological polymers if the ribosome can be directed to polymerize tRNAs carrying unnatural amino acids. The fundamental problem is that current chemical aminoacylation systems cannot easily produce sufficient amounts of the numerous misacylated tRNAs required to synthesize a complex library of encoded polymers. Here, we show that bulk-aminoacylated tRNA can be transformed into N-monomethylated aminoacyl tRNA and translated. Because poly-N-methyl peptide backbones are refractory to proteases and are membrane permeable, our method provides an uncomplicated means of evolving novel drug candidates.

Introduction

Ribosomes can polymerize well over 100 different amino acid analogs (e.g., [1–5]). As might be predicted from surveying the standard 20 amino acids, a wide variety of side chains are acceptable. Perhaps of greater importance is the fact that unusual backbones can also be synthesized by the ribosome. Currently, the list of potential translation products includes D-polypeptides, polyesters, thioesters, thioamides, phosphinoamides, β-peptides, N-methyl polypeptides, polypeptides with quaternary α-carbons, hydrazino-based polymers, and aminooxy-based polymers [6–13]. Collectively, these data suggest that ribosomes could be used to synthesize libraries with useful physical, chemical, and biological properties by translating randomized pools of mRNA in the presence of tRNAs that bear nonstandard monomers (nonstandard aa-tRNAs). For example, poly-N-methyl peptides (methylated peptides) seem to be both protease resistant and to have the ability to cross cell membranes [14–19], making them an attractive class of compounds for drug discovery.

By itself, the ribosomal synthesis of nonstandard polymers is an interesting but rather involved way of generating libraries that could be made easily by other approaches. However, ribosome synthesis is critical because it allows for the encoding of products with mRNA and the subsequent processing of staggeringly complex libraries ($>10^{13}$) by in vitro selection [20–25]. The unique power of evolutionary methods is that they make it comparatively simple to identify which library members have the desired function. The basic procedure is to use iterative rounds of selection and amplification, where the selective step increases the proportion of functional molecules and amplification increases their number. With each round, the library is exponentially enriched in molecules that satisfy the selective criteria. Thus, an originally diverse population that may contain only a single copy of a desirable molecule quickly evolves into a population dominated by the molecule.

The ribosome-based synthesis of nonstandard polymers was demonstrated over 30 years ago, and, as expected, nonstandard polymers can also be encoded with mRNA [23]. Nevertheless, the significant challenge that remains before evolutionary methods can be employed to select novel molecules is the generation of a diverse and chemically promising starting population. To achieve high diversity with low molecular weight libraries that are suitable for drug discovery (M.W. $\sim$1000; chain length $\sim$10), about 20 different building blocks (i.e., acylated tRNAs) will be required ($10^{13}\sim20^{10}$). The next task, then, is to generate 20 or so nonstandard aa-tRNAs in sufficient quantity for successive rounds of ribosome-based encoding. Because mRNA encoding systems produce about 10^{11} to 10^{12} library members per milliliter, a diverse library of 10^{13} would require a translation reaction as large as 100 ml. At normal in vitro protein synthesis concentrations (1 mg/ml tRNA), a considerable amount of tRNA would be necessary (10–100 mg). To further complicate matters, current chemical misacylation techniques depend on individually isolated tRNAs, separate reactions, multiple steps, and commercially unavailable reagents [1–5]. Overall, this appears to be a cumbersome strategy, even if lower complexity libraries made from small amounts of tRNA are acceptable. And, while it seems likely that improvements in mRNA encoding efficiency as well as optimization of translation reactions will eventually reduce the total amount of tRNA required, as long as one tRNA is consumed for every codon translated (a necessity when enzymes are not capable of recharging expended tRNAs within a translation reaction), library complexity is limited by the amount of nonstandard aa-tRNA that can be made.

An alternative to chemical misacylation is to transform the standard 20 amino acids after they have been attached to their respective tRNAs by aminoacyl tRNA synthetases. In such an approach, readily available and inexpensive bulk tRNA can be used because the synthetases ensure that the correct amino acids are attached to their cognate tRNAs. Subsequent chemical transformation of the whole population of tRNA-bound amino acids then allows for the immediate synthesis of 20

*Correspondence: ragreen@jhmi.edu

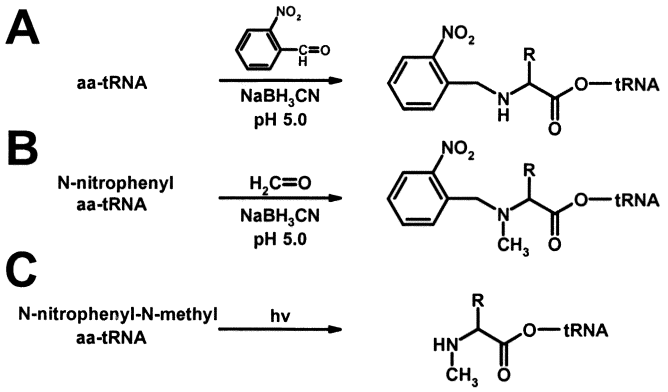

Figure 1. N-Monomethylation Scheme for tRNA-Bound Amino Acids

The structures of the reagents are shown above the arrows, and the aminoacyl tRNA product is on the right. The aminoacyl tRNA substrate (aa-tRNA) and RNA chain of tRNA (tRNA) are abbreviated.

(A) Reductive alkylation with *o*-nitrobenzaldehyde and $NaBH_3CN$ produced the monosubstituted intermediate N-nitrophenyl aminoacyl tRNA. The bulky *o*-nitrobenzaldehyde is incapable of disubstituting the amino acid due to steric hinderance [27].

(B) After a brief incubation, formaldehyde was added directly to the reaction from (A) and produced the disubstituted intermediate N-nitrophenyl-N-methyl aminoacyl tRNA.

(C) N-monomethyl aminoacyl tRNA was produced by photoreversal of the nitrobenzaldehyde adduct with UV light.

altered monomers that are ready for translation. Although conceptually satisfying with respect to the production of nonstandard aa-tRNA in large quantities, this framework is subject to a few demanding constraints: the transformation must ultimately produce polymers with useful properties; chemical manipulations are confined to reactions that proceed in slightly-acidic-aqueous media (the aminoacyl ester bond is base labile and tRNA is insoluble in common organic solvents); side reactions must not inactivate the 25 kDa tRNAs to which the amino acids are attached; and nearly quantitative yields are required so that there is a one-to-one correspondence between the monomer borne by a tRNA and the codon the tRNA recognizes.

Here, we show that reductive alkylation with *o*-nitrobenzaldehyde followed by reductive methylation with formaldehyde and photoreversal of the initial adduct produces tRNAs bearing N-monomethyl amino acids. This simple transformation is quantitative, and most of the N-methyl aminoacyl tRNAs are substrates for the ribosome. Because this approach can be used with bulk aminoacylated tRNA, it is trivial to simultaneously synthesize large amounts of numerous N-methyl aminoacyl tRNAs. By eliminating the substantial synthetic efforts demanded by traditional methods, our approach provides a readily accessible means for producing and searching vast libraries ($\sim 10^{13}$) of "drug-like" methylated peptides via ribosome-based encoding and in vitro selection.

Results

Synthesis of N-Monomethyl Aminoacyl tRNA

The steps used for N-methylation of enzymatically charged tRNA are outlined in Figure 1. In the first step, the amino acids were protected from dimethylation by N-alkylating the tRNA-bound amino acids with *o*-nitrobenzaldehyde and sodium cyanoborohydride ([26]; Figure 1A). Although the α-amine of the amino acid has the potential to undergo a second alkylation, with nitrobenzaldehyde this secondary substitution is sterically prevented [27]. Upon addition of the less hindered formaldehyde, a second reductive derivatization (this time methylation) proceeds smoothly ([26]; Figure 1B). In a final step, the disubstituted intermediate (N-nitrophenyl-N-methyl aminoacyl tRNA) is exposed to UV light to remove the protective nitrobenzaldehyde (Figure 1C) and produce the desired tRNA with an N-monomethyl amino acid.

To optimize the chemistry and allow for product analysis, the above scheme was initially applied to puromycin, a minimal analog of aminoacyl tRNA that is chemically stable and commercially available on the gram scale (Figure 2A). As shown by TLC (Figure 2B), reductive alkylation with nitrobenzaldehyde decreased the mobility of puromycin (Figure 2B, compare lanes 1 and 2), and subsequent addition of formaldehyde to the reaction resulted in conversion to the disubstituted intermediate

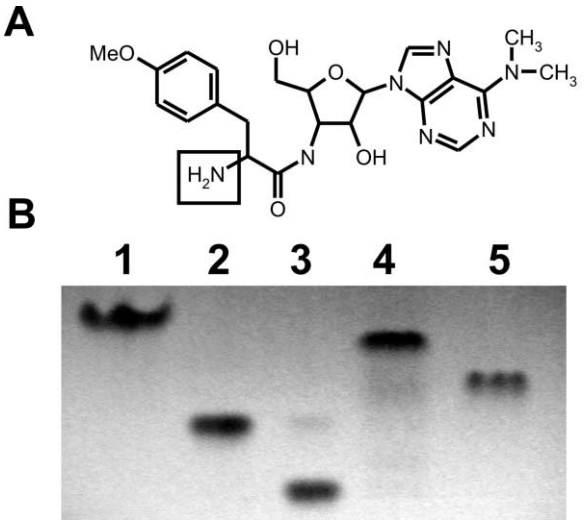

Figure 2. N-Monomethylation of Puromycin

(A) Structure of puromycin. The reactive primary amine of the amino acid moiety (methoxytyrosine) is boxed.

(B) Synthesis of N-monomethyl puromycin. The products of the reaction were separated by TLC, visualized by UV shadowing, and photographed. Lane 1, puromycin; lane 2, N-nitrophenyl puromycin; lane 3, N-nitrophenyl-N-methyl puromycin; lane 4, N-methyl puromycin; lane 5, N,N-dimethyl puromycin.

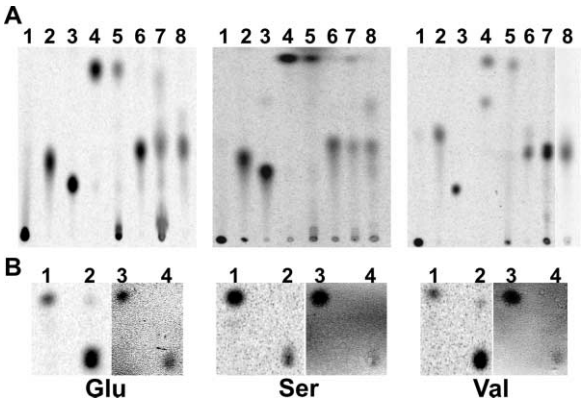

Figure 3. N-Monomethylation of tRNA-Bound Amino Acids

(A) Synthesis of N-monomethyl- Glu, Ser, and Val. The products and intermediates of the reaction were separated by TLC and visualized by phosphorimager analysis. In lanes 2–8, the aminoacyl ester bond was hydrolyzed to free the modified amino acid from its immobile tRNA. Lane 8 employed bulk tRNA that was charged with a complete amino acid mix and a single radiolabeled amino acid. Lane 1, unhydrolyzed N-methyl [^{14}C]-aminoacyl tRNA; lane 2, [^{14}C]-aminoacyl tRNA; lane 3, N,N-dimethyl [^{14}C]-aminoacyl tRNA; lane 4, N-nitrophenyl-N-methyl [^{14}C]-aminoacyl tRNA; lane 5, N-nitrophenyl-N-[^{14}C]-methyl aminoacyl tRNA; lane 6, N-methyl [^{14}C]-aminoacyl tRNA; lane 7, N-[^{14}C]-methyl aminoacyl tRNA; lane 8, N-methyl [^{14}C]-aminoacyl tRNA from bulk tRNA.

(B) Comigration of N-methyl amino acid standards and transformed amino acids. Radiolabeled N-methyl Glu, Ser, and Val were synthesized while bound to their respective tRNA, the aminoacyl-tRNA ester bond was hydrolyzed, and the samples were mixed with excess unlabeled N-methyl-amino acid standards. These mixtures were then subjected to eTLC. Radiolabeled compounds (lanes 1 and 2) were visualized by phosphorimager analysis, and the unlabeled standards (lanes 3 and 4) were visualized by ninhydrin staining. Lane 1, amino acid; lane 2, N-methyl aminoacyl tRNA; lane 3, unlabeled amino acid standard; lane 4, unlabeled N-methyl amino acid standard.

(N-nitrophenyl-N-methyl puromycin; Figure 2B, lane 3). N-monomethyl puromycin was generated by photoreversal of the disubstituted intermediate (Figure 2B, lane 4) and confirmed by mass spectrometry (data not shown). If the protection step was not utilized, formaldehyde treatment led directly to dimethylation, and this product had a distinct mobility (Figure 2B, lane 5).

Next, to demonstrate the generality of the scheme, we performed this chemical transformation on several different aminoacyl tRNAs (Glu, Ser, and Val). These diverse amino acids were selected to substantiate that the methylation protocol was fundamentally independent of the nature of the side chain. As before, products from each of the individual steps were separated by TLC (Figure 3A), but the ester linkage to the tRNA was hydrolyzed before running the TLC to allow direct observation of the amino acid moiety. As with puromycin, we were able to separate the N-methyl products from the amino acids, disubstituted intermediates, and dimethyl amino acids. Reaction precursors and products were followed using either labeled amino acids or labeled formaldehyde, allowing the analysis to establish that the reaction products contained an amino acid and a formaldehyde adduct. The equivalent (fast) migration of the disubstituted intermediates (N-nitrophenyl-N-methyl amino acids) when either labeled amino acid or labeled formaldehyde was used suggested that the desired double transformation had occurred (Figure 3A, lanes 4 and 5). When only formaldehyde was used for derivatization, the dimethylated products were readily identified by their distinctive mobilities (Figure 3A, lane 3). Finally, as expected for a nitrophenyl-protected amine, UV exposure shifted the mobilities of the disubstituted intermediates, and the migrations of the photoreversed products were distinct from those of the amino acids, intermediates, or dimethylated controls (Figure 3A, lanes 6 and 7). Again, irrespective of whether the amino acid or formaldehyde was labeled, the photoreversed products comigrated, consistent with the predicted formation of N-methyl amino acids. Without hydrolysis, the final products of the reaction remained at the origin, demonstrating that the chemistry did not cleave the ester linkage between the tRNA and N-methyl amino acid (Figure 3A, lane 1).

As a final control, methylation of the tRNA-bound amino acids was verified by comparison to commercially available standards (Figure 3B). Here, the samples were analyzed by thin-layer electrophoresis (eTLC) to increase the resolution between the amino acids and their N-methyl analogs. Phosphorimager quantitation of the precursors and products indicated that greater than 95% of the radiolabeled amino acids were transformed into their N-methyl counterparts (Figure 3B, lanes 1 and 2). Unlabeled standards were cospotted, visualized with ninhydrin, photographed, and aligned by marks made on the thin-layer plates with radiolabeled dyes (Figure 3B, lanes 3 and 4). The N-monomethyl amino acids were faintly stained by a modified ninhydrin protocol that allowed for establishment of their mobility (see Experimental Procedures).

Transformation of Bulk tRNA

An important aspect of our procedure is its potential to simultaneously alter many amino acids after a simple enzymatic aminoacylation step is used to attach them to their respective tRNAs. By following this methodology, the need to purify individual tRNA species and subsequently subject each tRNA to a multistep misacylation protocol can be avoided. As shown in Figure 3A, lane 8, our transformation produced N-methyl amino acids from bulk tRNA as readily as from individually purified tRNAs. For these reactions, bulk *E. coli* tRNA was charged with unlabeled amino acids and the single radiolabeled amino acid of interest. With each of the amino acids directly tested, N-methyl products were efficiently produced, and it seems probable that the remaining 18 N-methyl aminoacyl tRNAs were synthesized concurrently (proline should return to its original form after deprotection).

Activity of N-Monomethyl Aminoacyl tRNA

A key requirement for the synthesis of libraries of nonstandard polymers is that the transformed tRNAs be substrates for the ribosome. This first will depend on the methylation chemistry itself not inactivating the tRNA body for interaction with the protein synthesis machinery. Although the standard nucleotides are not

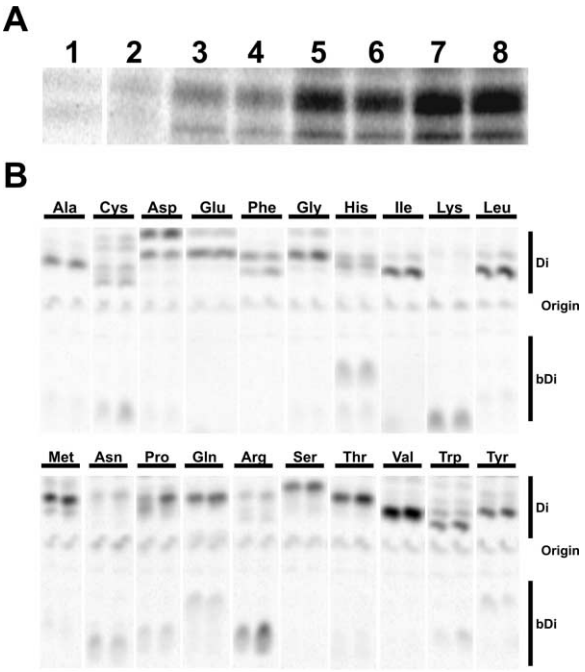

Figure 4. Full Activity of Mock-Treated tRNA

(A) In vitro translation with mock-transformed naked tRNA. As substrates for translation, ribosomes were supplied untreated bulk tRNA (lanes 2, 3, 5, and 7) or naked bulk tRNA that had been treated according to the N-methylation protocol (lanes 4, 6, and 8). Aminoacylation of the tRNAs was performed in the translation mix. Translation products labeled with [^{35}S]-Met were resolved on a 12% NuPAGE gel (Invitrogen) and visualized by phosphorimager analysis. Lane 1, contained no exogenous tRNA and was incubated for 27 min; lane 2, contained no mRNA and was incubated for 27 min; lanes 3 and 4, translation products after 3 min; lanes 5 and 6, products after 9 min; lanes 7 and 8, products after 27 min.

(B) Dipeptide formation with mock-treated tRNA. To test the effect of the N-methylation protocol on the tRNA itself, initiation complexes containing f-[^{35}S]-Met-tRNA^{met} and an appropriate mRNA were supplied bulk tRNA that was aminoacylated following a mock treatment. From left to right, the lanes for each amino acid used normal tRNA or mock-treated tRNA. The dipeptide products were separated by eTLC and visualized by phosphorimager analysis. Dipeptide (Di) and basic dipeptide (bDi) products are indicated on the right-hand side of the figure.

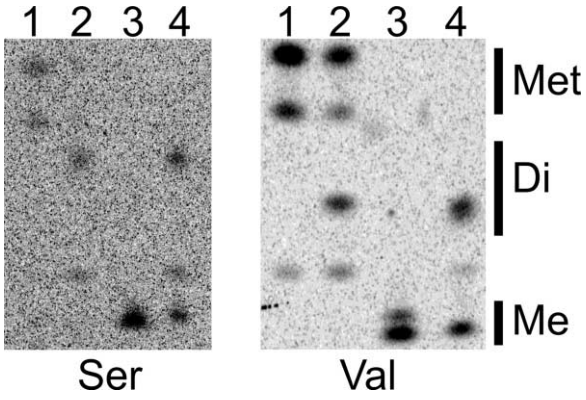

Figure 5. Peptide Acceptor Activity of tRNA Bearing Methylated Ser or Val

Ribosomes loaded with mRNA and fMet-tRNA^{met} were supplied an N-methyl [^{14}C]-aminoacyl tRNA to form dipeptides (lanes 2 and 4). Controls lacked the mRNA, and thus no dipeptide was formed (lanes 1 and 3). In lanes 1 and 2, the initiator tRNA was labeled with [^{35}S]-Met, and before analysis the samples were diluted 100- to 500-fold to equalize band intensity between [^{35}S]- and [^{14}C]-containing samples. To show that N-methyl-[^{14}C]amino acids were incorporated into the dipeptides, lanes 3 and 4 contained unlabeled fMet-tRNA^{met}. The amino acids and dipeptide products were separated by eTLC and visualized by phosphorimager analysis. The fMet (Met) and N-methyl amino acids (Me) as well as the dipeptide products (Di) are indicated at right.

expected to undergo reductive alkylation, the potential for RNA folding to introduce uniquely reactive environments and the presence of primary and secondary amines in the posttranscriptionally modified nucleotides of tRNA suggested that unwanted modifications were likely. Initial experiments with radiolabeled formaldehyde and uncharged tRNA indicated that reductive methylation resulted in about one undesired modification per tRNA. We next performed mock reactions with deacylated tRNA to examine whether such side reactions affected the ability of tRNA to function properly in translation. As shown in Figure 4A, translation of an 88-amino acid-long fragment of β-galactosidase proceeded at the same rate and to the same extent with both treated and untreated tRNA. Furthermore, for each amino acid, mock-treated tRNA was just as competent as untreated tRNA in forming fMet-labeled dipeptides on the ribosome (Figure 4B). Thus, although formaldehyde is incorporated into the body of tRNA (i.e., observe the radiolabeled formaldehyde which remains at the origin with tRNA; Figure 3A, lanes 5 and 7), this limited modification has no detectable consequences for tRNA function. This is presumably because the modifications are distributed randomly and/or occur at positions that are not critical for overall tRNA function.

We next asked whether monomethylated Ser and Val were effectively incorporated by the ribosome. This was examined by testing the ability of the transformed amino acids to form dipeptides encoded by the supplied mRNA. Initiation complexes containing fMet-tRNA^{met} and the appropriate mRNA were formed, and each of the N-methyl aminoacyl tRNAs was added. Production of fMet-N-methyl dipeptides indicated that the transformed tRNAs were recruited by the ribosome and were active in peptide bond formation (Figure 5). This documentation of activity is important for two reasons. First, as above, it confirms that chemical transformation does not meaningfully alter the tRNA itself. Second, the production of dipeptides that contain labeled N-methyl amino acids (Figure 5, lane 4) provides clear evidence that such substrates can be polymerized by the ribosome.

To examine the acceptor activity of each of the N-methyl amino acids, [^{35}S]-Met labeled dipeptides were produced from initiation complexes that were supplied bulk tRNA and an appropriate mRNA. The amount of dipeptide formed when normal tRNA, N-methyl tRNA, or dimethylated tRNA was used as an acceptor substrate is shown in Figure 6. In general, when compared to the amount of dipeptide formed with normal tRNA, the extent of reaction with N-methylated amino acids was lower but still quite respectable (Figure 6, compare lanes

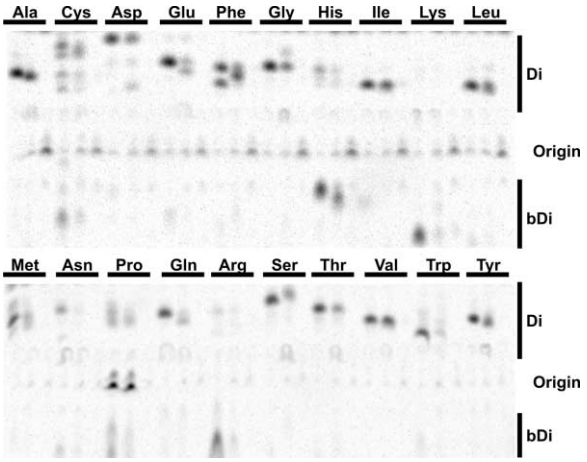

Figure 6. Acceptor Activity of Bulk tRNA Bearing N-Monomethyl Amino Acids

Translation initiation complexes containing f-[^{35}S]-Met-tRNA^{met} and an appropriate mRNA (see Experimental Procedures) were supplied bulk aminoacyl tRNA, and the dipeptides produced were separated by eTLC. From left to right, the lanes for each amino acid used normal tRNA, N-methyl tRNA, or N,N-dimethyl tRNA as the acceptor substrate. The dipeptide (Di) and basic dipeptide (bDi) products are indicated at right.

1 and 2). Several amino acids were efficiently incorporated (>50%; N-methyl- Ala, Phe, His, Ile, Leu and Val), whereas a number of others were incorporated less well but with an activity of at least 30% in comparison to the normal amino acid (N-methyl- Cys, Asp, Gly, Met, Gln, Ser and Thr). Only N-methyl- Glu, Lys, Asn, Arg, and Trp were poor substrates (<15%).

Because product analysis for each of the dipeptide products was not feasible, we used a number of criteria to establish that the primary product species was authentic. First, in a number of cases, the migration of the dipeptide formed with N-methylated tRNA differed from the migration of the dipeptide product formed with untreated aminoacyl tRNA, which suggested that a methyl group was present and that it altered the mobility of the dipeptide (Figure 6; compare lanes 1 and 2 for Ala, His, Gln, Ser, and Tyr). Next, we note that N-methyl aminoacyl tRNAs were almost completely inactive in dipeptide formation at conditions where normal tRNA was fully functional: normal and mock-treated tRNAs were efficient substrates at both low (6 mM) and high (15 mM) magnesium concentration, while the methylated aminoacyl tRNAs were incorporated only at 15 mM magnesium. Because the transformation procedure has no effect on acceptor activity when it is performed before bulk tRNA is aminoacylated (Figure 4B), the magnesium dependence and reduced acceptor activity of the tRNAs when transformation follows aminoacylation must result from derivatization of the amino acids themselves. Finally, we established that alkylation was efficient with each of the amino acids by leaving *o*-nitrobenzaldehyde out of the transformation reaction (so that the tRNA products carried inactive N,N-dimethyl amino acids) and showing that no dipeptide was produced from any of the mRNA templates (Figure 6, lane 3). Thus, it appears that all of the amino acids are efficiently alkylated.

Discussion

Here, we demonstrated that a three-step procedure generated N-monomethylated tRNAs from their aminoacylated tRNA precursors under mildly acidic, aqueous conditions. We then established that most of the N-methylated aminoacyl tRNA substrates had significant acceptor activity on the ribosome in peptidyl transferase assays. As discussed above, this approach is particularly well suited for synthesizing complex libraries of methylated peptides due to the ease with which the nonstandard aa-tRNAs are generated. In conjunction with ribosome-based encoding schemes, this approach opens the prospect that methylated peptides with desirable functions can be identified by in vitro selection from diverse populations ($\sim 10^{13}$).

The ribosome-based production of methylated peptides depends on producing nonstandard aa-tRNAs that reliably incorporate specific N-methyl amino acids in response to specific codons. Our approach uses tRNAs that are charged by highly accurate synthetases, and thus the expected code (N-methyl Ser at Ser codons, etc.) has the potential to be essentially error free. There are, however, mechanisms by which a chemical transformation could disrupt the ability of a tRNA to incorporate the correct monomer into a growing chain. For example, incomplete methylation of an amino acid or its side chain would result in a degenerate tRNA population bearing normal and transformed amino acids. Thus, each mRNA sequence would potentially code for a wide variety of mixed polymers rather than a single methylated peptide. This potential problem is exacerbated by a poorly used N-methyl amino acid, as the unmethylated version, even if present at low concentrations, would be preferentially incorporated by the ribosome. However, the extent of methylation observed (>95%) with three rather different aminoacyl tRNAs suggests that N-methylation is quite robust. Furthermore, as no dipeptides are formed from any of the dimethylated tRNAs (Figure 6, lane 3), complete alkylation appears to be independent of the amino acid, and we therefore expect few problems from untransformed amino acids in the population.

The efficiency of the peptidyl transferase reaction and the apparent homogeneity of the dipeptide product varied across the range of amino acids. In the simplest cases, dipeptide products formed from N-methyl-Ala, His, Gln, Ser and Tyr had slightly different mobilities than the dipeptides formed from the corresponding unmethylated amino acids. These changes in migration are anticipated based on altered chemical properties resulting from the acquisition of a nonpolar methyl group. The fact that a single product was observed for these amino acids argues strongly that the product was the N-methylated version of the desired dipeptide. For a second group of amino acids (Asp, Gly, Ile, Leu, Thr, and Val), the unmethylated and methylated dipeptide products migrated similarly. When assayed directly, radiolabeled N-methyl Val was incorporated into dipeptide (Figure 5) and was fully methylated in bulk tRNA (Figure 3, lane 8). It seems probable that the other dipeptides also contain N-methyl amino acids, but, like N-methyl Val, their incorporation does not alter the mobility of their respective product. This idea is supported by the

fact that following the methylation protocol, all of the N-methyl amino acids were efficiently incorporated only when additional Mg^{+2} was supplied to induce productive interaction with the ribosome. This condition was unnecessary with normal or mock-treated tRNA, and thus the effects must result from alteration of the amino acids themselves.

Multiple dipeptide products were formed from a third group of modified amino acids (Cys, Phe, and Met), indicating that there was some miscoding for these codon:anticodon pairs. In these cases, while the correct N-methylated aminoacyl tRNA appeared to be used, inappropriate, presumably near-cognate tRNAs were selected by the ribosome at some rate. Such miscoding was not wholly unexpected, as the elevated magnesium concentrations that were required to recruit N-methyl aminoacyl tRNAs onto the ribosome are known to decrease fidelity [28]. It remains possible that the codons not tested here might exhibit lower levels of miscoding. These fidelity issues might also be addressed experimentally by further optimization of the reaction conditions. Indeed, when more tRNA was added to these reactions, the overall level of miscoding decreased (data not shown), in agreement with previous results [29]. We anticipate that during library synthesis with nonstandard aa-tRNAs, miscoding issues will resurface as translation irreversibly consumes the preformed tRNAs. It therefore seems possible that library synthesis will require more nonstandard aa-tRNA than the amount used here (1 mg/ml) for the formation of a single peptide bond. Fortunately, unlike recent methods that propose the use of chemically charged tRNA [23, 30, 31], our revamped system can easily supply excess tRNA to limit substrate depletion and the associated problems of miscoding and translation inhibition via the buildup of uncharged tRNA.

A final set of amino acids presents us with the greatest challenge. N-methyl- Glu, Lys, Asn, Arg, and Trp were inefficiently incorporated by our purified translation system, and we have been unable to increase these values with any of the standard approaches. This problem will likely need to be addressed in a different fashion. The simplest approach will be to leave the inefficiently incorporated N-methyl amino acids underivatized in the tRNA substrate mix. While we have focused our attention on evaluating the potential to generate universally methylated peptides, our method provides the basic tools necessary to generate libraries from combinations of normal and N-methyl amino acids. Selective methylation can be envisioned by charging bulk tRNA with a subset of amino acids; after transformation of these residues, the remaining naked tRNAs would be charged with normal amino acids. Because N-methyl amino acids do not appear to be removed by the editing function of crude aminoacyl tRNA synthetases (data not shown), the second round of aminoacylation should not require purified enzymes.

Beyond providing access to libraries of methylated peptides, our approach might be extended to encompass other backbones. This could be accomplished by using different aldehydes or ketones during the reductive alkylation step to add other moieties to the α-amine of the aminoacyl tRNAs. Currently, it is unclear how permissive the ribosome is with respect to substitution at the attacking amine of an amino acid; the experiments reported to date required successful competition of the nonstandard aa-tRNA with release factors, and the translation systems were not customized for optimal tRNA loading [11]. Such issues are likely to cause an underestimate of substrates acceptable to the ribosome itself. While increasing the range of available backbones by changing the alkyl substituent on the α-amine may prove challenging, it could provide a powerful means of steering a library toward useful properties such as extreme protease resistance.

Modification of specific amino acid side chains can easily extend the properties of our libraries by expanding the range of available monomers. For example, derivatizable side chains such as lysine or cysteine could be used to mount diverse groups on normal or suppressor tRNAs for inclusion within libraries of methylated peptides. In previous evolutionary experiments, similar manipulations have produced peptide-drug conjugates whose activity is 100-fold better than the parent compound [32]. With methylated amino acids, the pendant moieties are mounted on a backbone that has the potential to enhance the bioavailability and biostability of the parent compound in addition to improving its affinity or target selectivity. It therefore might prove shrewd to devise selective schemes that are aimed at incorporating properties that are lacking in existing or abandoned compounds.

Significance

The technique described in this communication provides a simple chemical approach for transforming bulk aminoacylated tRNA into a pool of N-methylated aminoacyl tRNA for use in in vitro translation reactions. Our approach is straightforward and economical (both in terms of resources and personnel). These features should allow for the convenient synthesis of diverse libraries ($\sim 10^{13}$) of methylated peptides. Because such molecules are protease resistant and membrane permeable, the method provides a readily accessible means for producing "drug-like" libraries. In conjunction with ribosome-directed encoding schemes, vast libraries could be searched by in vitro selection methods to ultimately identify inhibitors or modulators of essentially any target.

Experimental Procedures

Materials

Ribosomes, S150 enzyme fraction, aminoacyl-tRNAs, in vitro protein synthesis reactions, and mRNAs were made and used as previously described [23]. Individual tRNA species were purchased from Subriden (Rolling Bay, WA) or Sigma (St. Louis, MO). Radiolabeled amino acids, amino acid mixtures, and formaldehyde were obtained from Moravek Biochemicals (Brea, CA), American Radiolabeled Chemicals, (St. Louis, MO) or Sigma. Other materials were purchased from standard sources.

Transformation of Puromycin

N-methyl puromycin was synthesized by incubating 8 mM puromycin, 50 mM *o*-nitrobenzaldehyde, and 20 mM cyanoborohydride in buffer (100 mM NaOAc [pH 5.0], 37°C). After 30 min, 0.2 volumes of 100 mM formaldehyde was added to the reaction and the incubation

continued (ambient; 30 min). The sparingly soluble nitrobenzaldehyde-puromycin derivatives were purified by centrifugation (16,000 × g; 4°C). Precipitates were suspended in ethanol, precipitated from water, and dissolved in ethanol containing 500 mM BME. Nitrobenzaldehyde adducts were removed by spotting the sample in the cap of a microfuge tube under a covered polystyrene Petri dish, the dish was placed on ice, and the sample was exposed to UV for 7 min using a 550 watt Hanovia bulb with water jacket [23]. Assuming complete recovery, 6 nmol was banded on RP-18 F_{254} TLC plates (Merck) and developed in 65% acetonitrile (pH 8.0).

Transformation of Aminoacyl-tRNAs

N-methyl Glu, Ser, and Val were synthesized while attached to their respective tRNAs by incubating 5 μM aminoacyl tRNA or 150 μM bulk tRNA with 30 mM o-nitrobenzaldehyde and 20 mM cyanoborohydride in buffer A (50 mM KOAc [pH 5.0], 5 mM $MgCl_2$) at 37°C for 30 min. After the initial incubation, 0.11 volumes of 100 mM formaldehyde was added and incubation continued (ambient; 30 min). When radiolabeled formaldehyde (Sigma; ~18 mM) was used, the tRNAs were precipitated after the nitrobenzaldehyde reaction and used at 25 μM during the methylation step so that the final formaldehyde concentration could be maintained at 10 mM. The disubstituted tRNAs were purified by ethanol precipitation, suspended in buffer A with 50 mM BME, the nitrobenzaldehyde adduct was removed as before, and the tRNA was precipitated and dissolved in buffer A with 5 mM BME. Aminoacyl-tRNA ester bonds were cleaved in 250 mM KOH (30 min; 37°C). Small aliquots (~10 pmol) were spotted on silica gel G TLC plates and developed in 80%–90% ethanol containing 300 mM NH_4OAc (pH 5.0). For eTLC, the hydrolyzed N-methyl amino acids were mixed with authentic N-methyl amino acids (10 mM; Bachem), spotted on cellulose TLC plates (Merck), and electrophoresed (20 min; 6% formic acid [pH 1.5]). For ninhydrin staining, equal volumes of 0.33% ninhydrin in tert-butanol and glacial acetic-water-pyridine (1:5:5) were mixed and applied to the plate, and the plate was heated to 100°C until developed.

Mock-Treated Bulk tRNA

Naked bulk tRNA at 25 μM was treated according to the N-methylation protocol described above. Aminoacylation and in vitro protein synthesis reactions using treated and untreated tRNA were performed as previously described [23].

Acceptor Activity of Mock-Treated and N-Methyl Aminoacyl tRNA

Dipeptides were formed by incubating various combinations of $fMET\text{-}tRNA^{met}$ and tRNA with 1 μM ribosomes, 20 μg/ml pyruvate kinase, 3 μM each of IF1, IF2, and IF3, 4–20 μM EFTu, 10 mM phosphoenol pyruvate, 2 mM ATP, 500 μM each of GTP, CTP, and UTP, and 2 μM mRNA in low Mg^{2+} buffer (10 mM HEPES [pH 7.5], 6 mM MgOAc, 150 mM NH_4Cl, 0.6 mM spermine, 0.4 mM spermidine, 4 mM BME) or high Mg^{2+} buffer (10 mM Tris [pH 7.5], 15 mM MgOAc, 100 NH_4Cl, 4 mM BME) for 10–30 min at 37°C. The mRNA 5′-GGGU UAA CUU UAG AAG GAG GUA AAA AAA **AUG** (NNN) UUU UUC UUU-3′ was used to direct tRNA binding. The initiator methionine codon (AUG) is shown in bold and is followed by an appropriate codon (NNN) for the respective amino acid (A = GCG, C = UGC, D = GAC, E = GAA, F = UUU, G = GGU, H = CAU, I = AUU, K = AAA, L = CUG, M = AUG, N = AAC, P = CCG, Q = CAG, R = CGG, S = UCU, T = ACC, V = GUC, W = UGG, Y = UAC). When [^{35}S]Met-labeled initiator tRNA was used, its concentration was 0.5–1 μM, and the appropriate N-methyl aminoacyl tRNA was 1 μM (Val), 2 μM (Ser), 25 μM (bulk mock-treated tRNA), or 40 μM (bulk N-methylated tRNA). When unlabeled initiator tRNA was used, its concentration was 1–1.5 μM and the appropriate N-methyl aminoacyl tRNA was 0.5 μM. After incubation, 0.3 volumes of 1M KOH was added to cleave the dipeptides and unreacted amino acids from their respective tRNAs (30 min; 37°C). The samples were spotted on cellulose TLC plates and electrophoresed (30–60 min; 20% acetic acid, 0.5% pyridine [pH 2.75]).

Acknowledgments

We thank Dave Lee and Scott Baskerville for helpful discussions, Phil Cole, Walt Hill, and Jon Lorsch for critical comments on the manuscript, and Wes Barfield and Julie Brunelle for the preparation of numerous reagents. This work was supported by HHMI and the NIH.

Received: January 19, 2004
Revised: February 18, 2004
Accepted: March 1, 2004
Published online: March 18, 2004

References

1. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. (1989). A general method for site-specific incorporation of unnatural amino acids into proteins. Science *244*, 182–188.
2. Bain, J.D., Wacker, D.A., Kuo, E.E., and Chamberlin, A.R. (1991). Site-specific incorporation of nonnatural residues into peptides - effect of residue structure on suppression and translation efficiencies. Tetrahedron *47*, 2389–2400.
3. Mendel, D., Ellman, J., and Schultz, P.G. (1993). Protein-biosynthesis with conformationally restricted amino-acids. J. Am. Chem. Soc. *115*, 4359–4360.
4. Thorson, J.S., Cornish, V.W., Barrett, J.E., Cload, S.T., Yano, T., and Schultz, P.G. (1998).). A biosynthetic approach for the incorporation of unnatural amino acids into proteins. Methods Mol. Biol. *77*, 43–73.
5. Hohsaka, T., Kajihara, D., Ashizuka, Y., Murakami, H., and Sisido, M. (1999).). Efficient incorporation of nonnatural amino acids with large aromatic groups into streptavidin in *in vitro* protein synthesizing systems. J. Am. Chem. Soc. *121*, 34–40.
6. Calendar, R., and Berg, P. (1967). D-Tyrosyl RNA: Formation, hydrolysis and utilization for protein synthesis. J. Mol. Biol. *26*, 39–54.
7. Fahnestock, S., and Rich, A. (1971). Ribosome-catalyzed polyester formation. Science *173*, 340–343.
8. Gooch, J., and Hawtry, A.O. (1975). Synthesis of thiol-containing analogues of puromycin and a study of their interaction with N-acetylphenylalanyl-transfer ribonucleic acid on ribosomes to form thioesters. Biochem. J. *149*, 209–220.
9. Victorova, L.S., Kotusov, V.V., Azhaev, A.V., Krayevsky, A.A., Kukhanova, M.K., and Gottiky, B.P. (1976). Synthesis of thioamide bond catalyzed by E. coli ribosomes. FEBS Lett. *68*, 215–218.
10. Tarussova, N.B., Jacovleva, G.M., Victorova, L.S., Kukhanova, M.K., and Khomutov, R.M. (1981). Synthesis of an unnatural P-N bond catalyzed with Escherichia coli ribosomes. FEBS Lett. *130*, 85–87.
11. Ellman, J.A., Mendel, D., and Schultz, P.G. (1992). Site-specific incorporation of novel backbone structures into proteins. Science *255*, 197–200.
12. Killian, J.A., Van Cleve, M.D., Shayo, Y.F., and Hecht, S.M. (1992). Ribosome-mediated incorporation of hydrazinophenylalanine into modified peptide and protein analogues. J. Am. Chem. Soc. *120*, 3032–3042.
13. Eisenhauer, B.M., and Hecht, S.M. (2002). Site-specific incorporation of (Aminooxy)acetic acid into proteins. Biochemistry *41*, 11472–11478.
14. Pohl, J. (1990). Action of pepsin on peptide substrates containing N-alpha-methyl amino acids, pp. 365–366. In J.E. Rivier and G.R. Marshall, eds., Pept.: Chem., Struct. Biol., Proc. Am. Pept. Symp., 11th.
15. Gordon, D.J., Sciaretta, K.L., and Meredith, S.C. (2001). Inhibition of beta-amyloid(40) firillogenesis and disassembly of beta-amyloid(40) fibrils by short beta-amyloid congeners containing N-methyl amino acids at alternate residues. Biochemistry *40*, 8237–8245.
16. Haviv, F., Fitzpatrick, T.D., Swenson, R.E., Nichols, C.J., Nichols, A.M., Bush, E.N., Diaz, G., Bammert, G., Nguyen, A., Rhutasel, N.S., et al. (2001). Effect of N-methyl substitution of the peptide bonds in luteinizing hormone-releasing hormone agonist. J. Med. Chem. *36*, 363–369.
17. Conradi, R.A., Hilgers, A.R., Ho, N.F.H., and Burton, P.S. (1992). The influence of peptide structure on transport across Caco-2

cells. II. Peptide bond modification which results in improved permeability. Pharm. Res. *9*, 435–439.
18. Chikhale, E.G., Ng, K., Burton, P.S., and Borchardt, R.T. (1994). Hydrogen bonding potential as a determinant of the in vitro and in situ blood-brain barrier permeability of peptides. Pharm. Res. *11*, 412–419.
19. Sheehy, B.A., Ho, N.F.H., Burton, P.S., Day, J.S., Geary, T.G., and Thompson, D.P. (2000). Transport of model peptides across Ascaris suum cuticle. Mol. Biochem. Parasitol. *105*, 39–49.
20. Mattheakis, L.C., Bhatt, R.R., and Dower, W.J. (1994). An in vitro polysome display system for identifying ligands from very large peptide libraries. Proc. Natl. Acad. Sci. USA *91*, 9022–9026.
21. Nemoto, N., Miyamoto-Sato, E., Husimi, Y., and Yanagawa, H. (1997). In vitro virus: bonding of mRNA bearing puromycin at the 3 -terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. FEBS Lett. *414*, 405–408.
22. Roberts, R.W., and Szostak, J.W. (1997). RNA-peptide fusions for the in vitro selection of peptides and proteins. Proc. Natl. Acad. Sci. USA *94*, 12297–12302.
23. Merryman, C., Weinstein, E., Wnuk, S.F., and Bartel, D.P. (2002). A bifunctional tRNA for in vitro selection. Chem. Biol. *9*, 741–746.
24. Baskerville, S., and Bartel, D.P. (2002). A ribozyme that ligates RNA to protein. Proc. Natl. Acad. Sci. USA *99*, 9154–9159.
25. Keefe, A.D., and Szostak, J.W. (2001). Functional proteins from a random-sequence library. Nature *410*, 715–718.
26. Gareau, Y., Zamboni, R., and Wong, A.W. (1993). Total synthesis of N-Methyl LTC4: A novel methodology for the monomethylation of amines. J. Org. Chem. *58*, 1582–1585.
27. Ohfune, Y., Kurokawa, N., Higuchi, N., Saito, M., Hashimoto, M., and Tanaka, T. (1984). An efficient one-step reductive N-monoalkylation of alpha-amino acids. Chem. Lett. (Jpn) *3*, 441–444.
28. Thompson, R.C., Dix, D.B., Gerson, R.B., and Karim, A.M. (1980). Effect of Mg^{2+} concentration, polyamines, streptomycin, and mutations in ribosomal proteins on the accuracy of the two-step selection of aminoacyl-tRNAs in protein biosynthesis. J. Biol. Chem. *256*, 6676–6681.
29. Wagner, E.G., Ehrenberg, M., and Kurland, C.G. (1982). Kinetic suppression of translational errors by (p)ppGpp. Mol. Gen. Genet. *185*, 269–274.
30. Forester, A.C., Tan, Z., Nalam, M.N.L., Lin, H., Qu, H., Cornish, V.W., and Blacklow, S.C. (2003). Programming peptidiomimetic syntheses by translating genetic codes designed de novo. Proc. Natl. Acad. Sci. USA *100*, 6353–6357.
31. Frankel, A., Millward, S.W., and Roberts, R.W. (2003). Chem. Biol. *10*, 1043–1050.
32. Li, S., and Roberts, R.W. (2003). A novel strategy for in vitro selection of peptide-drug conjugates. Chem. Biol. *10*, 233–239.