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Enzymatic aminoacylation of tRNA with unnatural amino acids

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The biochemical flexibility of the cellular translation apparatus offers, in principle, a simple route to the synthesis of drug-like modified peptides and novel biopolymers. However, only ≈75 unnatural building blocks are known to be fully compatible with enzymatic tRNA acylation and subsequent ribosomal synthesis of modified peptides. Although the translation system can reject substrate analogs at several steps along the pathway to peptide synthesis, much of the specificity resides at the level of the aminoacyl-tRNA synthetase (AARS) enzymes that are responsible for charging tRNAs with amino acids. We have developed an AARS assay based on mass spectrometry that can be used to rapidly identify unnatural monomers that can be enzymatically charged onto tRNA. By using this assay, we have found 59 previously unknown AARS substrates. These include numerous side-chain analogs with useful functional properties. Remarkably, many β-amino acids, *N*-methyl amino acids, and α,α-disubstituted amino acids are also AARS substrates. These previously unidentified AARS substrates will be useful in studies of the specificity of subsequent steps in translation and may significantly expand the number of analogs that can be used for the ribosomal synthesis of modified peptides.

aminoacyl-tRNA synthetases | enzyme specificity | MALDI-TOF mass spectrometry | translation

The recent development of translation systems composed entirely of purified components (1, 2) has enabled the ribosomal synthesis of peptides composed primarily of unnatural amino acids (3, 4). The extension of this program to the synthesis of highly modified drug-like peptides and unnatural biopolymers is limited by the number of unnatural building blocks that are compatible with the translation apparatus. Overcoming this limitation would enable the synthesis of extremely large libraries ($>10^{13}$) of novel compounds in a format that is amenable to *in vitro* selection and directed evolution by mRNA-display (5). The chemical aminoacylation of tRNA (6, 7), or the chemical modification of aminoacylated tRNA (8), is often used to allow amino acid analogs to enter the translational pathway. However, the enzymatic aminoacylation of tRNA by aminoacyl-tRNA synthetases (AARS) is much more convenient and practical, especially when considering large numbers of analogs. Effective methods for evolving AARS with relaxed or altered substrate specificities have been developed, and a growing number of interesting amino acid analogs can now be incorporated into peptides through the use of mutant AARS (9–11).

Surprisingly, the substrate specificity of most wild-type AARS enzymes toward unnatural amino acids is poorly understood, and relatively few amino acid analogs have been reported to be clear AARS substrates (12). As part of an ongoing effort to expand the chemical diversity of the monomer building blocks that can be conveniently incorporated into unnatural peptides, we became interested in screening a wide variety of unnatural amino acids for their ability to be enzymatically charged onto tRNA. We soon realized that none of the current AARS assays were appropriate for such a task. For example, the detection of aminoacyl-tRNA (AA-tRNA) by using radiolabeled amino acids (13) is not applicable to screening large numbers of unnatural amino acids. The commonly used ATP-PP_i exchange assay, although very sensitive, does not actually measure the formation of the AA-tRNA product (13). A powerful assay developed by Wolfson and Uhlenbeck (14) allows the observation of AA-tRNA synthesis even with unnatural amino acids through the use of tRNA which is ^{32}P-labeled at the terminal C-*p*-A phosphodiester linkage (4, 15). Because the assay is based on the separation of AMP and esterified AA-AMP by TLC, each amino acid analog must be tested in a separate assay mixture; moreover, this assay cannot generally distinguish between tRNA charged with the desired unnatural amino acid or with contaminating natural amino acid.

Here, we describe a tRNA aminoacylation assay based on MALDI-MS. This assay overcomes several of the problems discussed above and allows rapid qualitative analysis of AA-tRNA synthesis with multiple unnatural amino acid analogs in a single reaction mixture. We have used this assay to screen >190 commercially available unnatural amino acids and have identified >90 unnatural backbone and side-chain analogs that can be enzymatically charged onto tRNA.

Results

We chose to pursue an assay based on MS because of the potential for direct detection of the AA-tRNA product. Moreover, multiple amino acid analogs can be tested together as long as they differ in mass, thus accelerating the screening process. However, an MS approach based on direct detection of the large polyanionic AA-tRNA suffers from both poor sensitivity and the inability to resolve the small mass differences between analogs (16). We decided to address the sensitivity and mass resolution problems by cleaving the AA-tRNAs into mononucleotides (14). Unfortunately, the low molecular weight of AA-AMPs places them in a region of the mass spectrum that is obscured by peaks arising from the matrix material used in MALDI-MS (the most sensitive MS method for nucleotides). To avoid this problem, we derivatized the AA-tRNA on the primary amine of the amino acid to increase the molecular weight of the AA-AMP fragment. We used reductive amination chemistry, which preserves the labile AA-tRNA ester bond (8), to link the amine to an aldehyde containing a triphenylphosphonium group (Fig. 1*A*). The bulky triphenylphosphonium group shifts the AA-AMPs out of the region of the spectra obscured by the matrix, and its fixed positive charge aids in the matrix assisted laser desorption process and thereby improves sensitivity (17). After derivatization, the tRNA is digested with nuclease P1 (14) at pH 5.0, which liberates the derivatized AA-AMP without disrupting the sensitive ester bond. MALDI-MS analysis of the derivatized AA-AMP identifies the amino acids that have been charged (Fig. 1*A*).

Conflict of interest statement: No conflicts declared.

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Abbreviations: AA-tRNA, aminoacyl-tRNA; AARS, aminoacyl-tRNA synthetase(s); *N*-Me, *N*-methyl.

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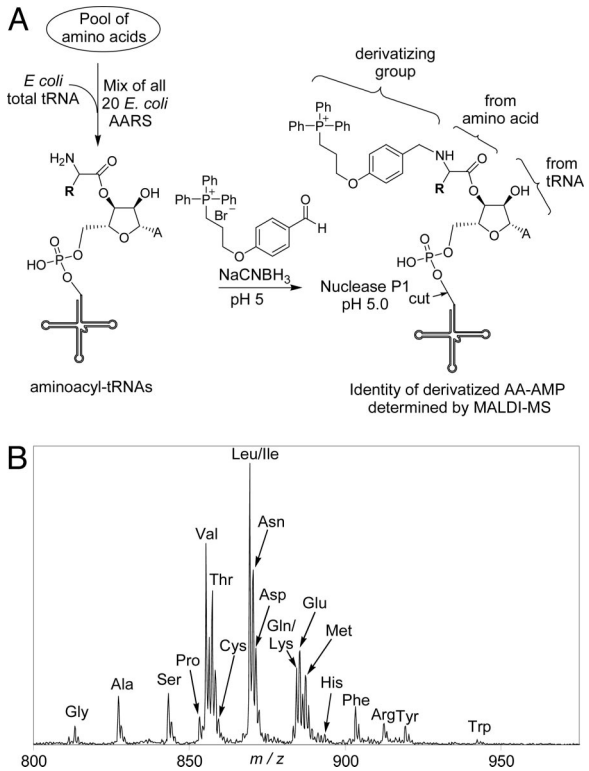

Fig. 1. MALDI-MS detection of tRNA charging with natural amino acids. (*A*) The MALDI-MS AARS assay involves reductive amination of the esterified amino acid with a phosphonium-containing benzaldehyde followed by Nuclease P1 treatment. (*B*) Assay of aminoacylation of bulk tRNA by all 20 natural amino acids and AARS in a single pot reaction.

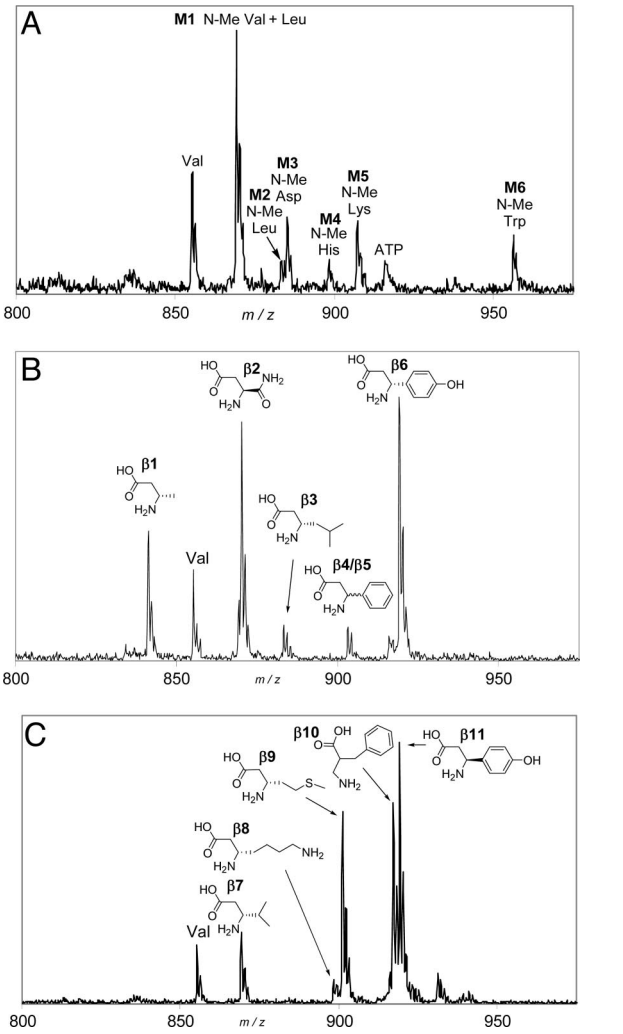

Fig. 2. Assay results with various backbone analog classes. (*A–C*) Assay with *N*-Me amino acids (*A*), selected β-amino acids (*B*), and additional β-amino acids (*C*).

In an initial test, a mixture of the 20 natural amino acids was incubated with the 20 recombinant *Escherichia coli* AARS and bulk *E. coli* tRNA in a single solution (Fig. 1*B*). The derivatized AA-AMP peaks for 18 of the 20 natural amino acids could be readily detected (although Leu/Ile and Gln/Lys have the same masses and cannot be distinguished when assayed together), and small peaks were observed at the expected mass for the remaining two, Cys and His. In separate assays, these two AA-AMPs were readily detected. In an assay lacking any added amino acids (see Fig. 8, which is published as supporting information on the PNAS web site), small peaks at 855, 869, and 916 are observed. The peak at 916 corresponds to derivatized ATP (most likely at the 2-amino group) as evidenced by a decrease in peak intensity upon decreasing the ATP concentration in the assay (results not shown). Mild base treatment removed the peaks at 855 and 869, suggesting that they correspond to aminoacylated AMP species. Because these peaks were still present when the assay was performed with neither amino acids nor AARS, they most likely correspond to aminoacylated Val-tRNA^{Val} or Leu/Ile tRNA$^{Leu/Ile}$ in the commercial tRNA mix, consistent with the greater hydrolytic stability of Ile-tRNA^{Ile} and Val-tRNA^{Val} relative to other AA-tRNAs (18). A peak corresponding to derivatized AMP at mass 756.23 (data not shown) was present in all of the assays and was used to internally calibrate the spectra.

Having established that the assay could detect tRNA acylation with natural amino acids, we then tested a variety of unnatural amino acids. We screened groups of 5–10 analogs with differing masses in each assay using a mixture of all 20 purified AARS. The analogs were sorted so that chemically similar analogs were tested separately to avoid competition between analogs for the same AARS. When an analog was found to be an AARS substrate, the particular AARS involved was determined from subsequent assays with a single AARS.

Because of our interest in preparing peptides with backbone-modified structures, we investigated commercially available *N*-methyl (*N*-Me) amino acids and β-amino acids of various configurations (β_2-amino acids have a single methylene group inserted between the amino group and the α-carbon, whereas β_3-amino acids have a single methylene group inserted between the α-carbon and the carboxylic acid). Remarkably, the *N*-Me and β-amino acids shown in Fig. 2 are AARS substrates. Six *N*–Me amino acids are substrates for AARS (Fig. 2*A*). tRNA aminoacylated with *N*-Me Val (**M1**), *N*-Me Leu (**M2**), *N*-Me Asp (**M3**), *N*-Me His (**M4**), *N*-Me Lys (**M5**), and *N*-Me Trp (**M6**) was detected with the assay, even though the reductive amination step is likely to be more difficult on these secondary amines. The β-amino acid set includes five β_3-amino acids [Ala (***β*1**), Leu (***β*3**), Val (***β*7**), Lys (***β*8**), and Met (***β*9**)], all charged by their cognate synthetases, and one β_2-amino acid [2-aminomethyl-3-phenyl-propionic acid (***β*10**)], a substrate for PheRS. Configurational β_3-isomers of Asn (***β*2**), Phe (***β*4**, ***β*5**), and Tyr (***β*6**, ***β*11**) also appeared to be charged, although because these analogs have the same masses as their natural counterparts it is possible (although unlikely for ***β*4**, ***β*5**, ***β*6**, and ***β*11** because these compounds are not prepared from their natural analogs) that these peaks result from natural amino acid contamination. Interestingly, PheRS and TyrRS activated both the *R* (***β*4**, ***β*6**)

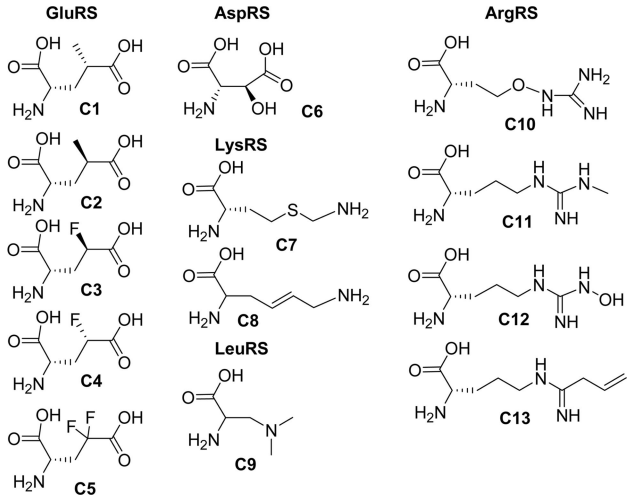

Fig. 3. Charged analogs activated by AARS.

and *S* (***β*5**, ***β*11**) β-amino acid isomers. It should be noted that all of these analogs were charged by the expected synthetases (e.g., LysRS charged ***β*8**, etc.) except for ***β*1** which was charged by ValRS.

We then focused our attention on unnatural side-chain analogs. We examined many analogs with minor chemical alterations, because substitutions with such analogs might be useful in the optimization of binding by selected peptides. We also examined a number of analogs with side chains bearing potentially useful functional groups not found in the set of 20 standard amino acids. Our results are organized by side-chain type. Analogs for which no peak of the appropriate mass could be detected are listed in Figs. 9–15, which are published as supporting information on the PNAS web site.

Side-Chain Analogs with Charged Groups. GluRS and AspRS tolerate a number of substitutions on the γ or β carbons of their natural substrates (Fig. 3). Both 4-methyl Glu isomers, **C1** and **C2**, are substrates in this assay. GluRS will also activate both the mono-4-fluoro Glu isomers **C3** and **C4** as well as the 4,4-difluoro analog **C5**. The only aspartic acid analog found thus far is L-*threo*-β-hydroxy Asp, **C6** (4, 19).

A number of conservatively substituted Arg and Lys analogs are substrates for ArgRS and LysRS. *S*-2-aminoethyl cysteine (**C7**) (20) a well studied analog of lysine, and dehydro Lys **C8** (21) are charged by LysRS. L-canavanine, **C10**, a thoroughly studied Arg analog (22) is an ArgRS substrate, as expected. ArgRS also tolerates methylation (**C11**) or hydroxylation (**C12**) at the terminal guanidine nitrogen or conversion of this guanidine to an amidine by replacement of the nitrogen with a carbon in the case of vinyl-L-NIO, **C13**. The tertiary carbon in leucine can be substituted with a nitrogen, giving a charged leucine analog, aza-leucine, **C9**. LeuRS activation of this analog is consistent with a report describing aza-leucine incorporation *in vivo* (23).

Polar, Uncharged. A number of polar, uncharged amino acid analogs are substrates for AARS (Fig. 4). Three GlnRS substrates (19), L-Glu γ-hydrazide (**P1**) L-albizziine (**P2**), and L-theanine (**P3**), were found. Surprisingly, acivicin (**P4**), a cyclic anticancer agent and γ-glutamyl transpeptidase inhibitor (24), is a substrate for IleRS. β-Hydroxy norvaline (**P5**) was, as expected (4, 22), a substrate for ThrRS. Lastly, we were surprised to find that the methyl esters of Asp (**P6**) and Glu (**P7**) are substrates for MetRS.

Aromatic. Consistent with previous results (12), we found a wide variety of aromatic amino acid analogs to be substrates for AARS

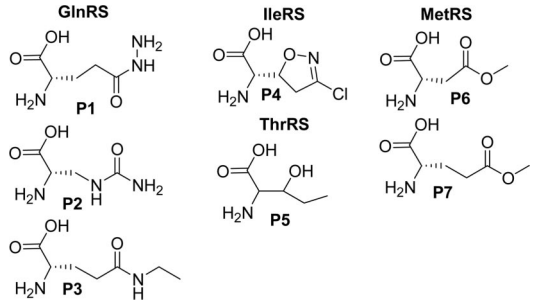

Fig. 4. Polar, uncharged analogs activated by AARS.

(Fig. 5). Two His analogs were found: β-2-thiazolyl-alanine **A1** [this analog is not incorporated into proteins (25), suggesting a block at a subsequent translation step] and triazole analog **A2** (25, 26), which is known to be incorporated into peptides. For TyrRS, 3-fluoro-L-Tyr (**A3**) (22, 27), and 3-nitro-L-Tyr, (**A4**) (28) are both charged onto tRNA [3-nitro Tyr was observed indirectly from its expected decomposition products under MALDI-MS conditions (29)]. PheRS charged the frequently studied analogs 2-fluoro Phe (**A5**) and 2-thienyl Ala (**A6**) (22), as well as β-substituted analogs β-methyl Phe (**A7**) and β-thienyl Ser (**A8**) (28). As expected, use of the PheRS A294G mutant allowed several previously described *p*-substituted analogs (30) (**A9**–**A13**) to be charged onto tRNA. The *p*-azido and *p*-ethynyl Phe derivatives provide useful functionality for posttranslational derivatization (31, 32). *p*-nitro Phe (**A14**) also was attached to tRNA by this synthetase. For TrpRS, the well studied 4-fluoro Trp (**A17**) (12) and the useful fluorescent analogs

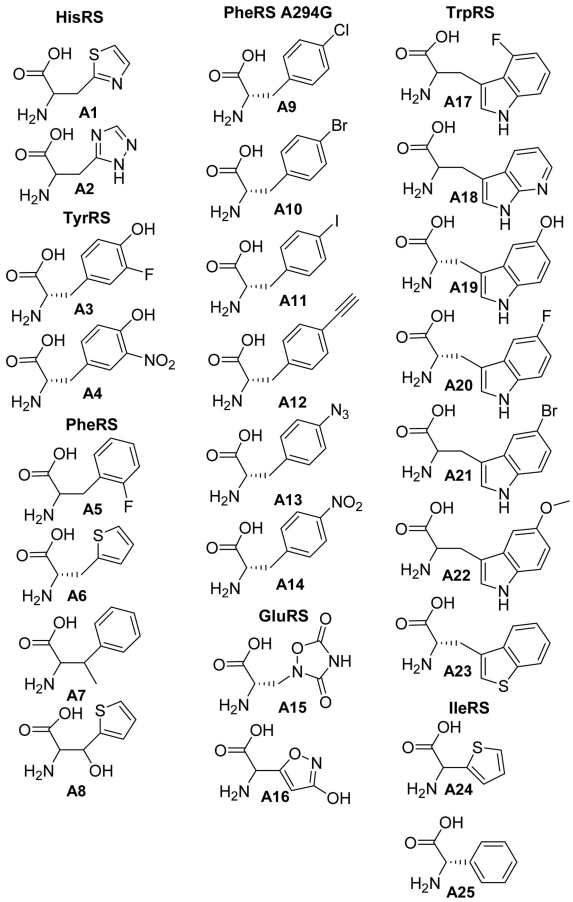

Fig. 5. Aromatic analogs activated by AARS.

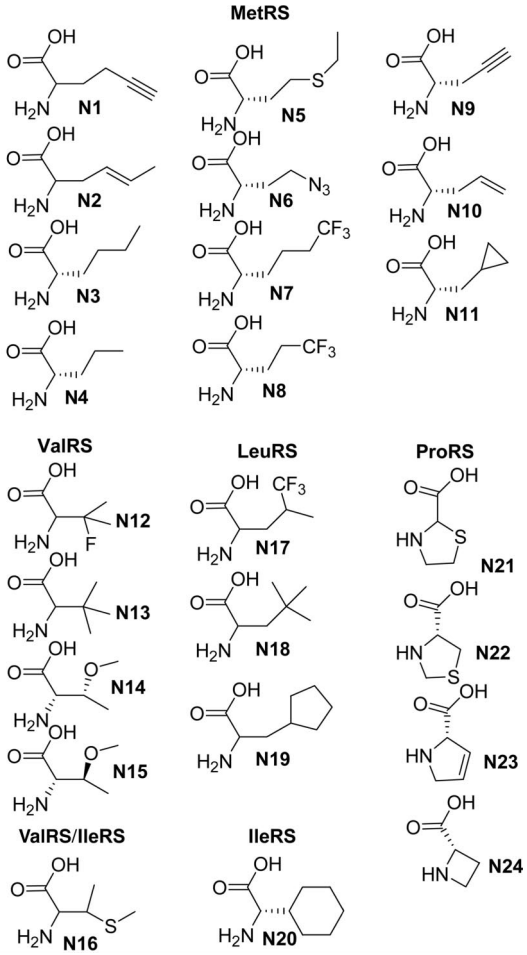

Fig. 6. Nonpolar analogs activated by AARS.

7-aza Trp (**A18**) (22, 33) and 5-hydroxy Trp (**A19**) (34, 35) were confirmed by this assay. The 5-position also can be substituted with fluoro (12), bromo, and methoxy substituents (**A20**–**A22**). TrpRS tolerates a nitrogen-to-sulfur substitution as well; 3-(thianapthen-3-yl)-L-Ala (**A23**) is a substrate. Remarkably, a few aromatic analogs are substrates for GluRS and IleRS. The polar but uncharged glutamate receptor agonists (36, 37) L-quisqualic acid (**A15**) and ibotenic acid (**A16**) resemble Glu in more than just receptor binding, because both are substrates for GluRS. IleRS recognizes both 2-thienyl Gly (**A24**) and L-phenylglycine (**A25**).

Nonpolar. A wide variety of nonpolar amino acid analogs are charged onto tRNA (Fig. 6). The activity of many previously reported MetRS substrates (38–40) was verified by our MALDI-MS assay including 2-amino hex-5-ynoic acid, crotylglycine, norleucine, norvaline, ethionine, and β-azidohomoalanine (**N1**–**N6**). Trifluoro analogs of norleucine and norvaline, **N7** and **N8**, also were substrates. MetRS also activated L-C-propargyl Gly (**N9**) and known substrate L-allyl Gly (**N10**) (41). Compound **N9** was reported to be undetectable as a MetRS substrate (41); our ability to observe it underscores the sensitivity of the MALDI-MS assay. We found β-cyclopropyl Ala, **N11**, to be a substrate for MetRS but not LeuRS [a report that showed ATP/PP_i exchange with LeuRS, but no incorporation into tRNA, suggests that this analog may be rejected by the LeuRS editing activity (42)]. ValRS accepted 3-fluoro-valine (**N12**), L-*t*-butyl-Gly (**N13**), and methyl ether derivatives of both L-Thr (**N14**) (43) and L-*allo*-Thr (**N15**). Interestingly, 4-thia-Ile

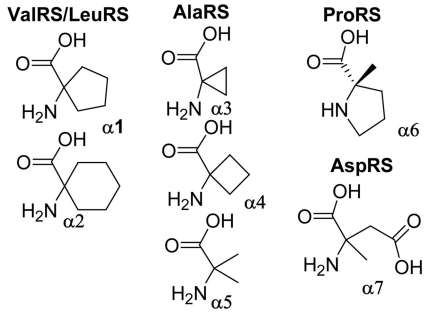

Fig. 7. α,α-disubstituted analogs activated by AARS.

(**N16**) was a substrate for both ValRS and IleRS, but **N14** and **N15** were only ValRS substrates [they may be activated by IleRS but edited (44)]. L-cyclohexyl-Gly **N20** also was an IleRS substrate. Besides the known 5′,5′,5′-trifluoro Leu **N17** (22), LeuRS also charged β-*t*-butyl Ala (**N18**) and β-cyclopentyl Ala (**N19**). Three ProRS substrates were found, thiazolidine-2-carboxylic acid (**N21**) (45), thiazolidine-4-carboxylic acid (**N22**) (46), and 3,4-dehydro Pro (**N23**) (47). One known ProRS substrate, L-azetidine-2-carboxylic acid, **N24** (4, 22), could not be detected by our MS-based aminoacylation assay, perhaps because ring strain prevents formation of the planar Schiff's base complex required for the reductive amination derivatization step [indeed, very few enamines have been formed by ketone or aldehyde condensation with azetidines for this reason (48)].

α,α*-Disubstituted.** Several α,α-disubstituted amino acids are substrates for AARS (Fig. 7). The cyclopentane and cyclohexane containing α,α-disubstituted amino acids ***α*1** and ***α*2** are substrates for both ValRS and LeuRS, and the analogs with cyclopropane and cyclobutane rings (α*3** and ***α*4**) are substrates for AlaRS. The α-methyl analogs of Ala (***α*5**), Pro (***α*6**), and Asp (***α*7**) are substrates for their corresponding AARS.

Quantitation. The nature of the MALDI-MS assay described herein is qualitative. However, when determining a set of analogs suitable for translation, a quantitative measure of their aminoacylation efficiency would be highly desirable. We have approached this problem in two ways. First, we analyzed all of the analogs active in the MS assay with Uhlenbeck's labeled tRNA assay (14). Many (67; 73%) showed >25% charging onto tRNA relative to the natural amino acids (see Fig. 16, which is published as supporting information on the PNAS web site). Considering that AA-tRNAs are catalytically regenerated during translation, we expect that this level of charging will lead to efficient translation. In a second approach, we have adapted our MS assay for quantitative measurements. An isotopically labeled (d_{15}) derivatized-Met-AMP (d_{15}-Met-AMP) [prepared under standard assay conditions by using a deuterated (d_{15}) version of the phosphonium benzaldehyde (Fig. 1*A*) (17)] served as an internal standard in competition assays with several Met analogs. An identical amount of d_{15}-Met-AMP was added to each analog assay; comparison of the ratio of standard Met-AMP with d_{15}-Met-AMP in the presence and absence of the analog allowed measurement of inhibition. Analogs (50 mM) (**N1**–**N3**, **N5**, **N6**, and **P7**) showed >50% inhibition of Met-$tRNA^{Met}$ formation; whereas analogs ***β*9**, **N4**, **N9**, **N10**, **N11**, and **P6** showed little or no inhibition even at 50 mM (see Fig. 17, which is published as supporting information on the PNAS web site). The IC_{50} values of two of the analogs, **N6** and **P7**, then were determined (5.4 and 11 mM, respectively; see Fig. 18, which is published as supporting information on the PNAS web site). The IC_{50} of **N6** (108 times the concentration of Met in the assay) is consistent with the relative

k_{cat}/K_m for **N6** vs. Met of ≈390 (40), suggesting that this assay can be configured for quantitative analysis of analog charging.

Discussion

We have developed a simple and robust procedure, based on chemical derivatization and MS, for monitoring the activity of AARS enzymes. This assay does not require radiolabeled tRNA or substrates and therefore can be applied to screening collections of amino acid analogs. Our procedure also has the distinct advantage of providing confirmation of the identity of the aminoacylated tRNA product, which is particularly important in cases where an analog may be contaminated with the normal amino acid or with other compounds. Although we have used this assay mainly in a qualitative manner to detect novel AARS substrates, we also have shown that the assay can be applied quantitatively by using a deuterium-labeled benzyl phosphonium AA-AMP as an internal standard.

We used our MALDI-MS assay to screen >190 commercially available amino acid analogs and found 92 amino acid analogs that are enzymatically charged onto tRNA. To the best of our knowledge, 59 of these analogs have not been described before. Our survey led to the identification, as AARS substrates, of 68 side-chain analogs, 11 β-analogs, 6 *N*-Me analogs, and 7 α,α-disubstituted analogs. The analogs **C13**, **P1**, **A12**, **A13**, **N1**, **N6**, **N9**, and **N10**, contain alkenes, alkynes, azides, and hydrazides, all of which can be used in chemoselective ligation reactions (40, 49–51). These reactions could be used to further modify and diversify the types of building blocks attached to tRNAs or could be applied to diversify peptides containing these amino acids after translation (40).

Based on the ability of each AARS to discriminate against the other 19 natural amino acids, we were initially surprised that so many analogs were found to be substrates. However, the synthetases have only evolved to discriminate against competing substrates that they encounter in their cellular environment. As a result, some AARS accept remarkably divergent analogs as substrates. The charging of Asp and Glu methyl esters by MetRS is an interesting example, because the ester introduces a polar carbonyl branch into the side chain, whereas all of the other known MetRS substrates are unbranched and hydrophobic. Also surprising were two substrates for IleRS, L-phenylglycine and 2-thienylglycine, which have aromatic rings in the place of the β-branched Ile side chain. The existence of analogs that are so different from the natural amino acid they replace further stresses the importance of screening each analog with a mixture of all 20 AARS. If conventional approaches had been used, these substrate analogs would not have been uncovered, because they would not have been tested with the correct AARS.

The ability of the translation machinery to incorporate these unnatural amino acids into peptides needs further investigation. The translation apparatus will accept L-amino acids with bulkier side chains than any found in this study (52), although recent studies suggest that a certain threshold EF-Tu affinity is required for an AA-tRNA to enter the ribosomal A-site (53). Because this affinity is partially determined by the amino acid (54, 55), some side-chain analogs may be inefficiently translated. Our preliminary results, however, suggest that most, if not all, of the side-chain analogs that we studied are translated with high efficiency. Based on several reports, we expect that the β-amino acids will not be translated (56–58). Conversely, at least some of the *N*-Me amino acid AARS substrates should be efficient translation substrates (8, 58–60). Several α,α-disubstituted analogs have been tested in translation after chemoenzymatic charging onto either a suppressor tRNA (61) or a modified $tRNA^{Asn}$ (58). The results from these studies conflict, perhaps suggesting that the tRNA context of the unnatural α,α-disubstituted amino acid influences its ability to be translated. The wide variety of AARS substrates that we identified will facilitate studies of the specificity of the translation apparatus.

In conclusion, we have identified AARS substrate analogs for 17 of the 20 canonical amino acids (all but Gly, Ser, and Cys), corresponding to 80% of the 61 sense codons (see Fig. 19, which is published as supporting information on the PNAS web site). Considering only side-chain analogs, 70% of the code (all of the above codons except those for Asn and Ala) can be reassigned at the tRNA/AARS level. Additional screening and AARS engineering should further increase the number of building blocks that can be introduced into the translation machinery by enzymatic tRNA aminoacylation. This expanded set of unnatural building blocks will facilitate the fully enzymatic preparation of large libraries of highly modified peptides, which then can be used for the *in vitro* selection of high-affinity ligands to important biological targets.

Materials and Methods

Amino Acids. Aqueous solutions of amino acids were prepared at 10 mM concentration or their maximum solubility (always >1 mM) and were stored at −20°C. Supplier information for the amino acids is listed in Table 1, which is published as supporting information on the PNAS web site. Some of the amino acids tested were purchased as a racemic mixture; no stereochemistry is denoted in the structures of these analogs.

Cloning and Protein Purification. All of the recombinant AARS listed in this work were cloned and purified as described in refs. 1 and 4 except for AlaRS, which was amplified from *E. coli* K12 genomic DNA by using two primers: AlaRSFWD, 5′-GGGAATTCCATATGAGCAAGAGCACCGCTGAG-3′, and AlaRSREV, 5′-CCGCTCGAGT TATTGCAATT-TCGCGCTGACC-3′. The amplified DNA was digested (NdeI and XhoI) and ligated into an NdeI- and XhoI-digested pET28a vector. The resulting clone contained a single mutation, E777K, in a nonconserved region of the protein.

MS Analysis. MS analysis was carried out on an Applied Biosystems Voyager DE by using delayed extraction operating in the positive mode. All spectra were calibrated internally to the 4-formylphenoxypropyl triphenylphosphonium AMP derivative with a mass of 756.23. The detected peaks were always within 0.1 mass unit of the theoretical m/z.

Screening Assay General Procedure. Each assay contained 40 mM Hepes-KOH (pH 7.4), 17 mM $MgCl_2$, 45 mM KCl, 3.4 mM 2-mercaptoethanol, 6 mM ATP, 6% glycerol, 0.02 units/ml yeast PP_iase, 350 μM *E. coli* total tRNA (Roche), 0.09 mg/ml BSA, 550 nM ArgRS, 3.1 μM CysRS, 3.0 μM GluRS, 280 nM IleRS, 390 nM LeuRS, 910 nM MetRS, 640 nM TyrRS, 1.8 μM TrpRS, 560 nM ValRS, 470 nM AlaRS, 2.1 μM AspRS, 770 nM AsnRS, 270 nM GlyRS, 1.3 μM HisRS, 600 nM LysRS, 220 nM PheRS, 230 nM ProRS, 77 nM SerRS, 460 nM ThrRS, 6.2 μM GlnRS, and amino acids in the 100–1,000 μM range. The assay was initiated by addition of the mixture of AARS and was incubated for 30 min at 37°C. A solution of 3 M NaOAc (pH 5.2; 0.1 vol, 2.5 μl) was added, and the assay solution was extracted with unbuffered phenol:$CHCl_3$:isoamyl alcohol (25:24:1) and then $CHCl_3$. The final aqueous layer was precipitated with EtOH (3 vol) and 3 M NaOAc (pH 5.2; 0.1 vol) at −20°C. The resulting pellet was washed twice with 70% EtOH, allowed to dry at room temperature (≈5 min), and dissolved in 200 mM NaOAc (pH 5.0; 12.5 μl). Half of the solution was frozen at −20°C, and the remaining half was added to water (3.75 μl), freshly prepared 4-formylphenoxypropyl triphenylphosphonium bromide [Aldrich (62)] (69 mM in MeOH; 12.5 μl), and freshly prepared $NaCNBH_3$ (200 mM in 50 mM NaOAc, pH 5.0; 2.5 μl). The solution was placed in a tumbler at 37°C [occasionally additional MeOH (1–2 μl) was required for complete dissolution].

After 2 h, 4.4 M NH_4OAc (pH 5.0; 0.1 vol, 2.5 μl) was added, and the solution was precipitated with EtOH (3 vol, 75 μl). The pellet was washed with 70% EtOH (2×) and 100% EtOH (2×) and was allowed to dry at room temperature. The dry pellet was then dissolved in 200 mM NH_4OAc, pH 5.0 (2.25 μl), and ≈0.25 μl of 1 unit/μl Nuclease P1 (in 200 mM NH_4OAc, pH 5.0) was added. After 20 min at room temperature, a 1-μl aliquot was removed and added to a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid) matrix in 1:1 MeCN:1% trifluoroacetic acid (9 μl). A 1-μl aliquot of the resulting suspension was added to a MALDI plate and analyzed. For further details, see *Supporting Experimental Procedures*, which is published as supporting information on the PNAS web site.

Figure Assays. The general screening procedure was followed with the noted exceptions.

Natural Amino Acids (Fig. 1*B*). Each of the amino acids was present at 200 μM except for Tyr (20 μM).

***N*-Me Amino Acids (Fig. 2*A*).** The *N*-Me amino acids were assayed (1 h) at 667 μM, and the assay included only ValRS (1.9 μM), LeuRS (1.3 μM), AspRS (2.0 μM), LysRS (3.5 μM), HisRS (4.1 μM), and TrpRS (6.1 μM).

β-Amino Acids No. 1 (Fig. 2*B*). **β1**, **β2**, **β3**, and **β5** were assayed (1 h) at 667 μM; **β4** and **β6** were at 450 μM, and the assay included only ValRS (2.2 μM), AsnRS (3 μM), LeuRS (1.2 μM), PheRS (0.32 μM), and TyrRS (2.6 μM).

β-Amino Acids No. 2 (Fig. 2*C*). Each of the β-amino acids was assayed (1 h) at 800 μM, and the assay contained only MetRS (3.6 μM), TyrRS (2.6 μM), ValRS (2.2 μM), LysRS (4.9 μM), and PheRS (0.32 μM).

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