Amino Acid Selectivity in the Aminoacylation of Coenzyme A and RNA Minihelices by Aminoacyl-tRNA Synthetases

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Coenzyme A (CoASH), a cofactor in carboxyl group activation reactions, carries out a function in nonribosomal peptide synthesis that is analogous to the function of tRNA in ribosomal protein synthesis. The amino acid selectivity in the synthesis of aminoacyl-thioesters by nonribosomal peptide synthetases is relaxed, whereas the amino acid selectivity in the synthesis of aminoacyl-tRNA by aminoacyl-tRNA synthetases is restricted. Here I show that isoleuyl-tRNA synthetase aminoacylates CoASH with valine, leucine, threonine, alanine, and serine in addition to isoleucine. Valyl-tRNA synthetase catalyzes aminoacylations of CoASH with valine, threonine, alanine, serine, and isoleucine. Lysyl-tRNA synthetase aminoacylates CoASH with lysine, leucine, threonine, alanine, valine, and isoleucine. Thus, isoleucyl-, valyl-, and lysyl-tRNA synthetases behave as aminoacyl-S-CoA synthetases with relaxed amino acid selectivity. In contrast, RNA minihelices comprised of the acceptor-T\(\text{C}\) helix of tRNA\(\text{Ile}\) or tRNA\(\text{Val}\) were aminoacylated by cognate synthetases selectively with isoleucine or valine, respectively. These and other data support a hypothesis that the present day aminoacyl-tRNA synthetases originated from ancestral forms that were involved in noncoded thioester-dependent peptide synthesis, functionally similar to the present day nonribosomal peptide synthetases.

Aminoacyl-tRNA synthetases (AARSs)\(^1\) attach cognate amino acids to tRNAs with the specificity dictated by the rules of the genetic code. In addition, many AARSs possess editing functions that prevent attachment of incorrect amino acids to tRNA (1, 2). The nonprotein amino acid homocysteine is misactivated by MetRS (3), IleRS (3), LeuRS (4), ValRS (3, 5), and LysRS (6). An editing mechanism of these AARSs destroys the homocysteinyl-AMP intermediate with the formation of the thioester homocysteine thiolactone (3–6); this prevents utilization of homocysteine in protein synthesis (1, 2, 11). Thioester bond formation during editing of homocysteine is facilitated by a subsite that binds the side chain thiol of homocysteine. Such thiol binding subsites exist in the synthetic/editing active sites of MetRS (7), IleRS (8), and LysRS (6). Similar thiol binding sites also exist in active sites of AARSs that apparently do not need editing function, such as ArgRS (9), AspRS (6), and SerRS (6). The thiol binding site confers on each of these six AARSs the ability to catalyze aminoacylation of thiols, including CoASH, and to promote peptide bond formation (6–9). CoASH binds to an AARS at a site, separate from the tRNA and ATP binding sites, that includes the thiol-binding subsite (10). Since the CoASH-binding site provides a thiol-binding subsite that may potentially be utilized for editing of homocysteine, it has been suggested that the editing site for homocysteine has evolved from the thiol-binding portion of the CoASH site (10).

IleRS activates not only isoleucine but also homocysteine, cysteine, valine, threonine, alanine, serine, and leucine (1, 2). The editing mechanism of IleRS destroys the noncognate intermediate and assures that only isoleucine is transferred to tRNA\(\text{Ile}\). In contrast, RNA minihelix comprised of the acceptor-T\(\text{C}\) helix of tRNA\(\text{Ile}\) has been reported to be aminoacylated with valine, as well as with isoleucine, by IleRS (13). However, the amino acid selectivity of the CoASH aminoacylation reaction is not known. Here, I show that IleRS and ValRS exhibit relaxed amino acid selectivity in the CoASH aminoacylation reaction and transfer noncognate amino acids to the thiol group of CoASH. It is also shown that the amino acid selectivity of IleRS and ValRS in the aminoacylation of RNA minihelix\(\text{Ile}\) and RNA minihelix\(\text{Val}\), respectively, is much greater than in the aminoacylation of S-CoASH. In addition, it is shown that LysRS catalyzes aminoacylation of CoASH, but not of RNA minihelix\(\text{Lys}\), with lysine and other amino acids.

**MATERIALS AND METHODS**

Aminoacyl-tRNA Synthetases—Escherichia coli IleRS, ValRS (8, 9), and LysRS (6, 11) were purified to homogeneity as described previously. Aminoacylation assays confirmed that preparations of IleRS, ValRS, and LysRS did not contain any other AARS activities.

Radiolabeled Amino Acids—Uniformly labeled \[^{14}\text{C}\]Leu (308 Ci/mol), \[^{14}\text{C}\]Ile (308 Ci/mol), \[^{14}\text{C}\]Val (246 Ci/mol), \[^{14}\text{C}\]Ser (151 Ci/mol), \[^{14}\text{C}\]Ala (150 Ci/mol), \[^{14}\text{C}\]Lys (316 Ci/mol), \[^{14}\text{C}\]Orn (56 Ci/mol), and \[^{14}\text{C}\]Thr (13,100 Ci/mol) were obtained from Amersham Pharmacia Biotech. \[^{14}\text{C}\]Thr (208 Ci/mol) was obtained from PerkinElmer Life Sciences.

Enzymatic Aminoacylation of CoASH—Reactions were carried out at 37 °C in mixtures containing, 10 mm CoASH (Sigma), \[^{13}\text{C}\]-labeled amino acid, 2 mm ATP, 0.1 mm K-HEPES (pH 7.4), 10 mm MgCl\(_2\), 0.1 mm EDTA, 5 units/ml yeast inorganic pyrophosphatase (Sigma), and 0.2 μM IleRS or ValRS. The formation of aminoacyl-S-CoA thioesters was monitored by TLC. Rates, \(v\), determined at different amino acid concentrations, [S], were used to calculate \(k_{\text{cat}}\) and \(K_{\text{m}}\) values from Eadie-Hofsette plots (v against [S]) according to \(v = \frac{\text{[E]}}{k_{\text{cat}} + [S]}\), where \([E]_0\) is the concentration of IleRS or ValRS.

RNA Substrates—RNA minihelices comprised of the acceptor-T\(\text{C}\) helix of tRNA\(\text{Ile}\), tRNA\(\text{Val}\), or tRNA\(\text{Lys}\) were chemically synthesized by Dr. Robert Donnelly using \(5^\prime\)-phenoxyacetyl-protected ribonucleoside phosphoramidates (Chem-Genes, Waltham, MA) on an Applied Biosystems 392 DNA synthesizer.

RNA Aminocylations—Reactions were carried out for 2 h at 37 °C in mixtures containing 0.1 mm K-HEPES (pH 7.4), 10 mm MgCl\(_2\), 0.2 mm EDTA, 1 mm diithiothreitol, 5 units/ml yeast inorganic pyrophosphatase.
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RESULTS

IleRS, ValRS, and LysRS Acylate CoA-SH with Noncognate Amino Acids—IleRS was incubated with 14C-labeled amino acids, ATP, and CoA-SH. Aliquots of the reaction mixtures were analyzed using two TLC systems. The PEI-cellulose TLC system separates amino acids, which migrate in the upper quarter of the TLC plate, from aminoacyl-S-CoA thioesters, which migrate in the middle of the TLC plate (10). In the cellulose TLC system aminoacyl-S-CoA thioesters stay at the origin, whereas free amino acids migrate with Rf values of 0.2–0.8 (10). New 14C-labeled compounds, migrating in the middle of a PEI-cellulose TLC plate (Fig. 1A) and staying at the origin of a cellulose TLC plate (Fig. 1B), were formed in reaction mixtures containing [14C]leucine (lane 1), [14C]isoleucine (lane 3), [14C]valine (lane 5), [14C]alanine (lane 7), and [3H]threonine (lane 11). These compounds were absent in reaction mixtures without CoA-SH, ATP, or IleRS (not shown). Addition of unlabeled isoleucine to reaction mixtures reduced formation of [14C]isoleucyl-S-CoA (lane 4) and abolished formation of other [14C]aminoacyl-S-CoA thioesters (lanes 2, 6, 8, 10, and 12). The inhibition by isoleucine confirms that aminoacyl-S-CoAs were formed by IleRS.

When ValRS was incubated with 14C-labeled amino acids, CoA-SH, and ATP, new 14C-labeled compounds, migrating in the middle of a PEI-cellulose TLC plate (Fig. 2A) and staying at the origin of a cellulose TLC plate (Fig. 2B), were formed in reaction mixtures containing [14C]isoleucine (lane 3), [14C]valine (lane 5), [14C]alanine (lane 7), [14C]serine (lane 9), and [3H]threonine (lane 11). These compounds were absent in reaction mixtures without CoA-SH, ATP, or ValRS (not shown). The incorporation of radiolabeled amino acids into CoA-SH was abolished when unlabeled valine was included in the ValRS-dependent aminoacylation mixtures (lanes 4, 6, 8, 10, and 12). The inhibition by valine confirms that aminoacyl-S-CoAs were formed by ValRS.

The identity of [14C]isoleucyl-S-CoA was established previously (10). To confirm the identity of other [14C]aminoacyl-S-CoAs, each thioester was isolated from reaction mixtures and subjected to chemical reactivity tests. Like [14C]isoleucyl-S-CoA, other [14C]aminoacyl-S-CoAs were sensitive to NaOH and dithiothreitol treatments. A corresponding free amino acid was a product of [14C]aminoacyl-S-CoA hydrolysis by NaOH (not shown). This further proves that the synthesis of [14C]aminoacyl-S-CoAs is not due to contamination of 14C-labeled amino acids by [14C]isoleucine or [14C]valine.

Kinetics of CoA-SH Aminoacylation—Examples of time courses of CoA-SH aminoacylations catalyzed by IleRS and ValRS. Reaction mixtures contained 10 mM CoA-SH, 2 mM ATP, 20 mM 14C-labeled amino acid, and 0.2 mM IleRS (A) or 100 mM 14C-labeled amino acid and 0.2 mM ValRS (B). Incorporation of the following radiolabeled amino acids into CoA-SH was determined by TLC. A, isoleucine ( ), alanine ( ), leucine ( ), and valine ( ); B, threonine (+), alanine ( ), valine ( ), and isoleucine ( ).
Selectivity in Aminoacylation of CoA and RNA Minihelix

Kinetic indices for aminoacylation of CoA-SH catalyzed by IleRS and ValRS

Table I

| Amino acid | IleRS | ValRS |
|------------|-------|-------|
|            | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
| Isoleucine | 1.1  | 2.5 | 440000 | >0.01 | >200 | 115 |
| Valine     | 1.7  | 38  | 45000  | 0.02  | 6   | 3300 |
| Leucine    | >1.2 | >200 | 7700   | <0.001 | <5  | <5 |
| Threonine  | >0.2 | >100 | 1200   | 0.18  | 130 | 1400 |
| Serine     | >0.4 | >320 | 1300   | >0.04 | >200 | 170 |
| Alanine    | >0.4 | >320 | 1300   | >0.14 | >200 | 500 |

Courses of aminoacyl-S-CoA formation catalyzed by IleRS and ValRS are shown in Fig. 3. IleRS-dependent aminoacylation of CoA-SH with the cognate isoleucine was about 2-, 6-, and 20-fold faster than the aminoacylation with noncognate valine, leucine, or alanine, respectively (Fig. 3A). However, ValRS-dependent aminoacylation of CoA-SH with the cognate valine was about 3- and 10-fold slower than the aminoacylation with noncognate alanine and threonine, respectively, and about as fast as the aminoacylation of CoA-SH with isoleucine (Fig. 3B).

Isoleucine was the most efficient amino acid substrate in the IleRS-dependent CoA-SH aminoacylation reaction (Table I). Catalytic efficiencies ($k_{\text{cat}}/K_m$) for CoA-SH aminoacylation with valine, leucine, alanine, and threonine were 10-, 57-, 340-, and 370-fold lower, respectively, than the catalytic efficiency for the aminoacylation with isoleucine. The discrimination between amino acids was mostly due to variations in $K_m$ values.

In ValRS-dependent CoA-SH aminoacylation reactions, valine was the most efficient substrate (Table I). Catalytic efficiencies for threonine, alanine, serine, and isoleucine were 2.4-, 6.6-, 19.4-, and 28-fold lower, respectively, than the catalytic efficiency for the aminoacylation with valine. Like with IleRS, the discrimination between amino acids was mostly due to variations in $K_m$ values. However, with the exception of isoleucine, $k_{\text{cat}}$ values for noncognate amino acids were up to 9-fold higher than for the cognate valine. Overall, the amino acid selectivity of ValRS is somewhat lower than the selectivity of IleRS in the CoA-SH aminoacylation reaction.

As shown in Table II, LysRS catalyzed aminoacylation of CoA-SH with several amino acids. The highest rates of CoA-SH aminoacylation were observed with threonine and leucine (both 2.7 $\mu$M/h). Rates of CoA-SH aminoacylation with lysine, alanine, valine, and isoleucine were 1.4-, 1.6-, 4-, and 11-fold slower, respectively, than rates observed with threonine and leucine. With all tested amino acids, the formation of AA-S-CoA was abolished by the addition of unlabeled lysine, confirming that LysRS is responsible for their formation.

AARSs catalyze the synthesis of cognate aminoacyl-S-CoAs using either AARS-bound aminoacyl adenylate or aminoacyl-tRNA as a donor, suggesting the existence of a CoA-SH binding site on an AARS (10). The formation of noncognate aminoacyl-S-CoAs is unlikely to be due to a reaction of CoA-SH with noncognate AA-AMP dissociated from the enzyme. For example, upper limits of the dissociation rate constants for Ile-AMP and Val-AMP from corresponding complexes with IleRS are 0.12 min$^{-1}$ and 0.16 min$^{-1}$ (3), too low to account for the rate constants for IleRS-dependent formation of Ile-S-CoA (1.1 min$^{-1}$) and Val-S-CoA (1.7 min$^{-1}$) (Table I).

Comparison of Aminoacylation Rates of CoA-SH and tRNA—Aminoacylation of CoA-SH with cognate amino acids catalyzed by IleRS (1.1 min$^{-1}$, Table I), ValRS (0.02 min$^{-1}$, Table I), and LysRS (0.03 min$^{-1}$, recalculated from Table II) was 30- (8), 16,000- (3), and 10,000-fold slower (Table II; Ref. 11), respectively, than the aminoacylation of a cognate tRNA.

Although IleRS catalyzed the transfer of noncognate amino acids to CoA-SH (Table I), there was no transfer of the noncognate amino acids to tRNA$^{\text{Ile}}$ (not shown). ValRS catalyzed the transfer of noncognate amino acids to CoA-SH (Table I), but, under similar experimental conditions, no transfer of noncognate amino acids to tRNA$^{\text{Val}}$ could be detected (not shown). The lack of stable transfer of noncognate amino acids to native tRNA$^{\text{Ile}}$ and tRNA$^{\text{Val}}$ has been well documented by other investigators (1, 2). LysRS-dependent transfer of noncognate amino acids to tRNA$^{\text{Lys}}$ is >10,000-fold slower than the transfer of lysine to tRNA$^{\text{Lys}}$ (11). However, for most noncognate amino acids the rates of the transfer to CoA-SH are similar to the rate of lysine transfer to CoA-SH catalyzed by LysRS (Table II). Thus, for each examined AARS, the amino acid selectivity in the CoA-SH aminoacylation reaction is much lower than the selectivity in the tRNA aminoacylation reaction.

Aminoacylations of RNA Minihelices—RNA minihelices comprised of the acceptor-T$_{34}$C helix of tRNA$^{\text{Ile}}$ (13) and tRNA$^{\text{Val}}$ (14) are substrates for aminoacylations by cognate AARSs, albeit some 10$^6$-fold less efficient than native tRNA. Aminoacylations of minihelix$^{\text{Ile}}$ and minihelix$^{\text{Val}}$ were carried out under conditions identical to those used for the aminoacylation of CoA-SH. When incorporation of radiolabeled amino acids into trichloroacetic acid precipitable material was measured, similar low degrees of incorporation were observed for all radiolabeled amino acids tested (not shown), consistent with published data (13). However, trichloroacetic acid precipitation could also monitor nonspecific incorporation of radiolabeled amino acids into protein (15, 16), particularly at high concentrations of AARSs used in the minihelix aminoacylation mixtures. To circumvent this problem, reaction mixtures were analyzed by electrophoresis on acid polyacrylamide gels which specifically monitors aminoacyl-tRNA (12). As shown in Fig. 4, IleRS catalyzed aminoacylation of RNA minihelix$^{\text{Ile}}$ with isoleucine (lane 9) and, much less efficiently, with leucine (lane 10) and valine (lane 12), but not with threonine (lane 11), alanine (lane 8), or serine (lane 7). It is estimated that the rate of Ile-RNA minihelix$^{\text{Ile}}$ formation was about 1000-fold lower than the rate of Ile-S-CoA formation catalyzed by IleRS. The amounts of Leu-RNA minihelix$^{\text{Ile}}$ and Val-RNA minihelix$^{\text{Val}}$ were at least 10- and 100-fold, respectively, lower that the amount of Ile-RNA minihelix$^{\text{Ile}}$. ValRS catalyzed aminoacylation of RNA minihelix$^{\text{Val}}$ with leucine (lane 10) and, much less efficiently, with threonine (lane 11) and valine (lane 12), but not with alanine (lane 8) or serine (lane 7). It is estimated that the rate of Val-RNA minihelix$^{\text{Val}}$ formation was about 100-fold lower than the rate of Val-S-CoA formation catalyzed by ValRS.

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Fig. 4. Detection of aminoacyl-RNA minihelices by acid gel electrophoresis. ValRS-dependent aminoacylation of RNA minihelix$^{\text{Val}}$ (lanes 1–6) and IleRS-dependent aminoacylation of RNA minihelix$^{\text{Ile}}$ (lanes 7–12) with [3H]serine (lanes 1 and 7), [3H]alanine (lanes 2 and 8), [3H]isoleucine (lanes 3 and 9), [3H]leucine (lanes 4 and 10), [3H]threonine (lanes 5 and 11), and [3H]valine (lanes 6 and 12) were examined.
Aminoacylations of CoA-SH and tRNA<sup>lys</sup> catalyzed by LysRS

Reactions were carried out with 10 mM CoA-SH, 2 mM ATP, 240 μM 14C-labeled amino acid, 5 units/ml yeast inorganic pyrophosphatase, and 1 μM LysRS. Data for aminoacylations of tRNA<sup>lys</sup> were re-calculated from Ref. 11.

| Amino acid | AA-S-CoA | AA-tRNA<sup>lys</sup> |
|------------|----------|------------------------|
| Lysine     | 1.9      | 22,000                 |
| Leucine    | 2.7      | 0.6                    |
| Isoleucine | 0.24     | 0.0                    |
| Valine     | 0.67     | 1.2                    |
| Alanine    | 1.7      | 24                     |
| Threonine  | 2.7      | 0.3                    |
| Serine     | <0.1     | 0.1                    |
| Ornithine  | <0.1     | 0.0                    |

The thioester-dependent mechanism of peptide synthesis may have preceded the RNA-dependent mechanism in the development of life (17). The ability of the present day AARSs to aminoacylate RNA minihelices was suggested to be an evolutionary vestige from a stage of the development of the aminoacylation function in the RNA world (21). Because it is likely that relaxed amino acid selectivities were a feature of primitive aminoacylation systems, the present findings that AARSs transfer some noncognate amino acids to CoA-SH, but not to RNA-minihelices (e.g. threonine, alanine, and serine by IleRS or isoleucine, alanine, and serine by ValRS) is consistent with the notion that the aminoacylation of thioesters may have originated before the aminoacylation of RNA (17). That structurally diverse class I (e.g. IleRS, ValRS, MetRS) and class II (LysRS, AspRS, SerRS) synthetases have the ability to catalyze the aminoacylation of CoA-SH points to the possible ancient origin of this reaction. After the appearance of aminoacyl-RNA at later stages of evolution, the thioester-dependent and RNA-dependent peptide synthesizing systems may have been developing in parallel. Before the advent of coded ribosomal protein synthesis, ancestral AARSs may have facilitated the formation of both aminoacyl-thioesters and aminoacyl-RNAs for noncoded peptide assembly. Ancestral AARSs themselves may have been involved in the thiol-dependent assembly of peptides. A vestige of this peptide forming ability exists in the present day AARSs, which can promote the synthesis of peptides in vitro (6–10).

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REFERENCES

1. Jakubowski, H. & Goldman, E. (1992) Microbiol. Rev. 56, 412–429
2. Jakubowski, H. (2000) in Encyclopedia of Life Sciences, www.els.net, Nature Publishing Group, London
3. Jakubowski, H. & Fersht, A. R. (1981) Nucleic Acids Res. 9, 3105–3117
4. Engelsch, U., Engelsch, U., von der Haar, F. & Cramer, F. (1986) Nucleic Acids Res. 14, 7529–7539
5. Jakubowski, H. (1988) Biochemistry 19, 5071–5078
6. Jakubowski, H. (1997) Biochemistry 36, 11077–11085
7. Jakubowski, H. (1996) Biochemistry 35, 8522–8529
8. Jakubowski, H. (1996) Nucleic Acids Res. 24, 2987–2990
9. Jakubowski, H. (1995) Nucleic Acids Res. 23, 4682–4615
10. Jakubowski, H. (1998) Biochemistry 37, 5147–5153
11. Jakubowski, H. (1999) Biochemistry 38, 8088–8093
12. Varshney, U., Lee, C.-P. & RajBhandary, T. L. (1991) J. Biol. Chem. 266, 24712–24718
13. Nordin, B. E. & Schimmel, P. (1999) J. Biol. Chem. 274, 6535–6538
14. Prugger, M., Florentz, C. & Giege, R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3990–3994
15. Gillet, S., Hountondji, C., Schmitter, J. M. & Blanquet, S. (1997) Protein Sci. 6, 2426–2431
16. Martinis, S. A. & Schimmel, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 65–69
17. De Duve, C. (1994) Vital Dust 875–884
18. Klei, H. & von Dohren, H. (1990) Eur. J. Biochem. 236, 335–351
19. Keating, T. A., Sun, Z., Ehmann, D. E. & Walsh, C. (2000) Biochemistry 39, 2297–2306
20. Schimmel, P. & Henderson, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11283–11286