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Synthesis of cysteine-containing dipeptides by aminoacyl-tRNA synthetases

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ABSTRACT

Arginyl-tRNA synthetase (ArgRS) catalyses AMP- and PP_i-independent deacylation of Arg-tRNA^{Arg} in the presence of cysteine. A dipeptide, Arg–Cys, is a product of this deacylation reaction. Similar reaction with homocysteine yields Arg–Hcy. Arginine is a noncompetitive inhibitor of the cysteine-dependent deacylation which indicates that cysteine binds to the enzyme–Arg-tRNA^{Arg} complex at a site separate from the arginine binding site. In the presence of arginine, [^{14}C]Arg-tRNA^{Arg} is deacylated at a rate similar to the rate of its spontaneous deacylation in solution and [^{14}C]arginine is a product. Experiments with cysteine derivatives indicate that the -SH group is essential for the reaction whereas -NH_2 and -COOH groups are not. Thioesters of arginine are formed with 3-mercaptopropionic acid, *N*-acetyl-L-cysteine and dithiothreitol. These data suggest that formation of the dipeptide Arg–Cys involves a thioester intermediate, *S*-(L-arginyl)-L-cysteine, which is not observed because of the rapid rearrangement to form a stable peptide bond. Facile intramolecular reaction results from the favorable geometric arrangement of the α-amino group of cysteine with respect to the thioester formed in the initial reaction. Similar reactions, yielding Ile–Cys and Val–Cys, are catalyzed by isoleucyl- and valyl-tRNA synthetases, respectively.

INTRODUCTION

Most, if not all, aminoacyl-tRNA synthetases (AARSs) exhibit a deacylase activity toward cognate aminoacyl-tRNAs. With synthetases that exhibit low initial selectivity (such as ValRS, IleRS and MetRS), this activity is a manifestation of an editing function which contributes to high accuracy of tRNA aminoacylation by destroying noncognate intermediates or products (reviewed in 1). Because of their high initial selectivity, some AARSs, such as CysRS (2), do not need an editing function to reject noncognate amino acids. Yet, CysRS also possesses deacylase activity, which might be a remnant of chemistry used by an ancestral synthetase (3). To shed more light on the nature of the deacylase activity, systematic studies of deacylation reactions catalyzed by purified AARSs were undertaken. As shown in this paper, ArgRS exhibits cysteine-dependent deacylase activity which leads to formation of the dipeptide Arg–Cys from Arg-tRNA^{Arg} and cysteine. The mechanism of dipeptide formation appears to involve nucleophilic attack of the thiolate group of cysteine on the ester bond in Arg-tRNA^{Arg} to yield a thioester, *S*-(L-arginyl)-L-cysteine. Facile intramolecular reaction, resulting from favorable arrangement of the α-NH_2 group of cysteine with respect to the thioester bond, would then yield Arg–Cys. Similar cysteine-dependent deacylations of cognate aminoacyl-tRNA catalyzed by IleRS and ValRS yield dipeptides Ile–Cys and Val–Cys, respectively.

MATERIALS AND METHODS

Plasmids and host strain

Plasmids containing genes for *Escherichia coli* AARSs were obtained from the following sources: pArgRS from G. Eriani (4), pIleRS from P. Schimmel (5) and pValRS from S. Blanquet (6). Plasmids were overexpressed in *E.coli* strain JM101 and used as a source of AARSs. Cells for enzyme purification were obtained from overnight cultures (usually 400 ml, yielding ~2 g cells) grown at 37°C in LB medium containing 100 μg/ml ampicillin.

Arginyl-tRNA synthetase

Cells (2.2 g) of *E.coli* strain JM101 harboring plasmid pArgRS were disrupted by sonication in 2 × 5 ml 10 mM potassium phosphate buffer, pH 7.5, 0.2 mM EDTA (buffer A). The lysate was cleared by centrifugation successively at 15 000 *g* for 10 min and 105 000 g for 2 h. Clear supernatant was loaded on a hydroxyapatite (Calbiochem) column (1.5 × 7 cm) equilibrated with buffer A. The column was washed with 6 vol (75 ml) of buffer A and developed with a 300 ml linear gradient from 10 to 50 mM potassium phosphate, pH 7.5, containing 0.1 mM EDTA. Fractions containing ArgRS activity (eluted at ~25 mM phosphate) were concentrated by ultrafiltration and loaded onto a Sephacryl S-300 (Pharmacia) column (1.5 × 70 cm) equilibrated and developed with buffer A. Active fractions were further purified on a 1.5 × 5 cm Mono Q (Pharmacia) column with a gradient from 0 to 0.4 M KCl in buffer A. The enzyme activity eluted as a single peak corresponding to a major protein peak at 0.25 M KCl. ArgRS was concentrated by ultrafiltration to 0.1 mM in 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol (DTT), 50% glycerol and stored at –20°C. The enzyme was >95% pure as judged by SDS–PAGE. The concentration of ArgRS was calculated from A_{280} using a mol. wt of 67 000 and a value of 1 $cm^{-1}mg^{-1}$. Measurements of the rate of tRNA^{Arg} aminoacylation at pH 7.4, 37°C in reaction mixtures with 25 μM [^{3}H]arginine (0.1 mCi/ml), 5 μM tRNA^{Arg} (1300 pmol/A_{260}; Subriden RNA), 2.5 mM ATP and 6 nM ArgRS yielded k_{cat} = 3.0 s^{-1}.

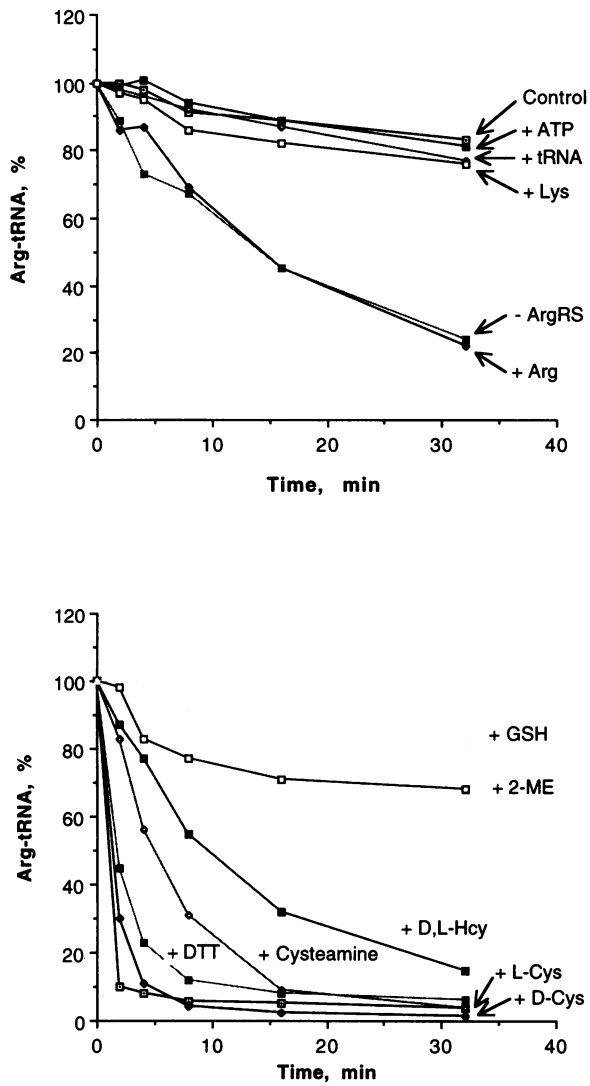

Figure 1. Time courses of enzymatic deacylation of Arg-tRNA^{Arg}. Reactions were carried out at 37°C in mixtures containing 0.2 μM [^{3}H]Arg-tRNA^{Arg}, 2.5 μM ArgRS, 0.05 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA and indicated additions. When present, ATP and tRNA^{Arg} were used at 2 mM and 20 μM, respectively. Other indicated compounds were used at 20 mM. % remaining [^{3}H]Arg-tRNA^{Arg} was determined by trichloroacetic acid precipitation at indicated time intervals. The upper panel shows effects of substrates Arg (solid diamonds), ATP (solid squares), tRNA^{Arg} (stippled diamonds) and lysine (empty squares) on enzymatic deacylation of Arg-tRNA^{Arg} as well as a control (dotted empty squares). Time course of nonenzymatic deacylation of Arg-tRNA^{Arg} (– ArgRS, stippled squares) is also shown in the upper panel. The lower panel shows effects of the following thiols on enzymatic deacylation of Arg-tRNA^{Arg}: L-cysteine (dotted empty squares), D-cysteine (solid diamond), dithiothreitol (DTT, stippled squares), cysteamine (stippled diamonds), D,L-homocysteine (solid squares); 2-mercaptoethanol (2-ME, empty squares) and glutathione (GSH, stippled triangles).

Isoleucyl- and Valyl-tRNA synthetases

The procedure for purification of IleRS was similar to that described above for ArgRS, except that the Sephacryl S-300 gel filtration step was omitted. ValRS was purified from crude extracts of the overproducing strain by ammonium sulfate fractionation (45–60% fraction collected), gel filtration on high resolution Sephacryl S-300 column, and ion exchange chromatography on Mono Q column. Both synthetases were at least 95% pure as judged by SDS–PAGE.

Preparation of [^{14}C]Arg-tRNA^{Arg}, [^{3}H]Arg-tRNA^{Arg}, [^{14}C]Ile-tRNA^{Ile} and [^{14}C]Val-tRNA^{Val}

Aminoacylation mixtures (0.2 ml) contained 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM ATP, 10 μM tRNA^{Arg}, 29.2 μM [^{14}C]arginine (0.01 mCi/ml, 342 Ci/mol; 1 Ci = 37 GBq) or 10.9 μM [^{3}H]arginine (0.68 mCi/ml, 62 Ci/mmol) (Amersham), and 0.5 μM ArgRS. After 5 min at 37°C the charged tRNA was purified by phenol extraction, and recovered by precipitation with ethanol. The precipitate was washed several times with 70% ethanol to remove traces of free radiolabeled arginine, dissolved in 0.2 ml glass-distilled water and stored at –20°C. Alternatively, radiolabeled Arg-tRNA was prepared using 10–20 mg/ml unfractionated *E.coli* tRNA (Schwartz Mann) (10 mg/ml tRNA contains 8 μM tRNA^{Arg}). [^{14}C]Ile-tRNA^{Ile} and [^{14}C]Val-tRNA^{Val} were prepared by similar procedures using purified tRNA^{Ile} and tRNA^{Val} (Subriden RNA).

Enzymatic deacylation of radiolabeled aminoacyl-tRNA

The reactions were carried out at 37°C in 0.1 M K–HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA. In one set of experiments, the disappearance of radiolabeled Arg-tRNA was monitored by trichloroacetic acid precipitation. In another set of experiments in which all forms of radioactive arginine, isoleucine and valine were followed, the aliquots were analyzed by TLC.

TLC analysis

TLC separations were carried out on cellulose plates from Merck. The developing solvent, butanol:acetic acid:water, 4:1:1, v/v) was the same as used before for determination of aminoacyl adenylates (7) and homocysteine thiolactone (8), and for analyses of deacylation products of Met-tRNA^{fMet} (9) and Cys-tRNA^{Cys} (3). Some TLC separations were carried out with 2-propanol:formic acid:water (20:1:5) as a solvent. An authentic arginine (Sigma) standard was co-chromatographed with samples and visualized by staining with ninhydrin. In some experiments authentic dipeptides Arg–Ala, Ile–Ala and Val–Ala (Sigma) were used as standards. In experiments with [^{14}C]Arg-tRNA^{Arg}, [^{14}C]Ile-tRNA^{Ile} and [^{14}C]Val-tRNA^{Val} the TLC plates were autoradiographed using XAR-5 (Kodak) or Reflection^{TM} (NEN) autoradiography film. Quantitation of spots from TLC separations in some experiments was carried out using a Molecular Dynamics PhosphorImager. Significant oxidation of thiols to disulfides occurred during these experiments, in particular when samples were stored (at –20°C) before TLC analysis. However, this did not interfere with interpretation of results.

RESULTS

Stimulation of enzymatic deacylation of Arg-tRNA^{Arg} by cysteine

As shown in the upper panel of Figure 1, [^{3}H]Arg-tRNA^{Arg} in a complex with ArgRS ('control') is deacylated slower (k = 0.003 min^{-1}) than free [^{3}H]Arg-tRNA^{Arg} ('- ArgRS') in solution (k = 0.035 min^{-1}) (pH 7.4, 37°C). Arginine, but not ATP nor tRNA^{Arg}, accelerated the deacylation of [^{3}H]Arg-tRNA^{Arg} in the

complex to a rate observed for free [^{3}H]Arg-tRNA^{Arg}. Unexpectedly, whereas some amino acids, such as lysine (upper panel in Fig. 1), serine and methionine (not shown), tested as controls did not affect the deacylation reaction, L-cysteine greatly accelerated enzymatic deacylation of Arg-tRNA^{Arg} (lower panel in Fig. 1). Several derivatives of cysteine and other thiols such as D-cysteine, cysteamine, D,L-homocysteine and DTT, shown in the lower panel of Figure 1, also accelerated the reaction, but less effectively than L-cysteine. Deacylation reactions in the presence of glutathione and 2-ME were not much different from the control.

Table 1. Kinetic indices for thiol-dependent enzymatic deacylation of Arg-tRNA

Thiol	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m ($min^{-1} \cdot M^{-1}$)
None	0.003		
None, no enzyme	0.035		
L-Cysteine (6–250 mM)	18	0.15	120
D-Cysteine (20–250 mM)	4.6	0.23	20
Cysteamine (20–250 mM)			9.2
3-Mercaptoprpionate (10–160 mM)			3.6
D,L-Homocysteine (12.5–250 mM)	0.6	0.1	5.7
L-Cysteine methyl ester (20 mM)			4.0
N-Acetyl-L-cysteine (20 mM)			4.0
L-Cys–Gly (20–167 mM)			7.9
Glutathione (20–167 mM)			0.8
Thioglycolate (125 mM, 250 mM)			7.8
Dithiothreitol (12.5–250 mM)	2.0	0.075	27

Reactions were carried out at 37°C in mixtures containing 4 μM [^{14}C]Arg-tRNA^{Arg}, 5 μM ArgRS, 0.1 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, and 5–250 mM thiol. Rate constants for reactions in the presence of indicated (in parentheses) concentrations of thiols, k, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln 2/t_{0.5}$. These rate constants were used to calculate k_{cat}, K_m, and k_{cat}/K_m values for indicated thiols according to $k = k_{cat} - K_m \cdot k/S$, where S is thiol concentration.

Cysteine-dependent deacylation exhibits saturation kinetics

The relationship between the rate of enzymatic deacylation of Arg-tRNA^{Arg} and L-cysteine concentration at pH 7.4 and 6.6 is depicted in the upper panel of Figure 2. At low L-cysteine concentrations, the rate increases proportionately to the increase in concentration of L-cysteine. At high L-cysteine concentrations, the increases in rate were progressively smaller at both pH 7.4 and 6.6. This type of relationship indicates that L-cysteine binds to ArgRS–Arg-tRNA^{Arg} complex. Saturation kinetics were also observed with DTT (middle panel, Fig. 2), D-cysteine and D,L-homocysteine (lower panel, Fig. 2). The reaction with L-cysteine was 6–24-fold faster than with any other thiol. Non-saturating kinetics were observed with cysteamine and 3-mercaptopropionate in the tested range of concentrations.

Kinetic indices for L-cysteine and other thiols in enzymatic deacylation of Arg-tRNA^{Arg} are summarized in Table 1. Up to 150-fold variation in catalytic efficiencies was observed between

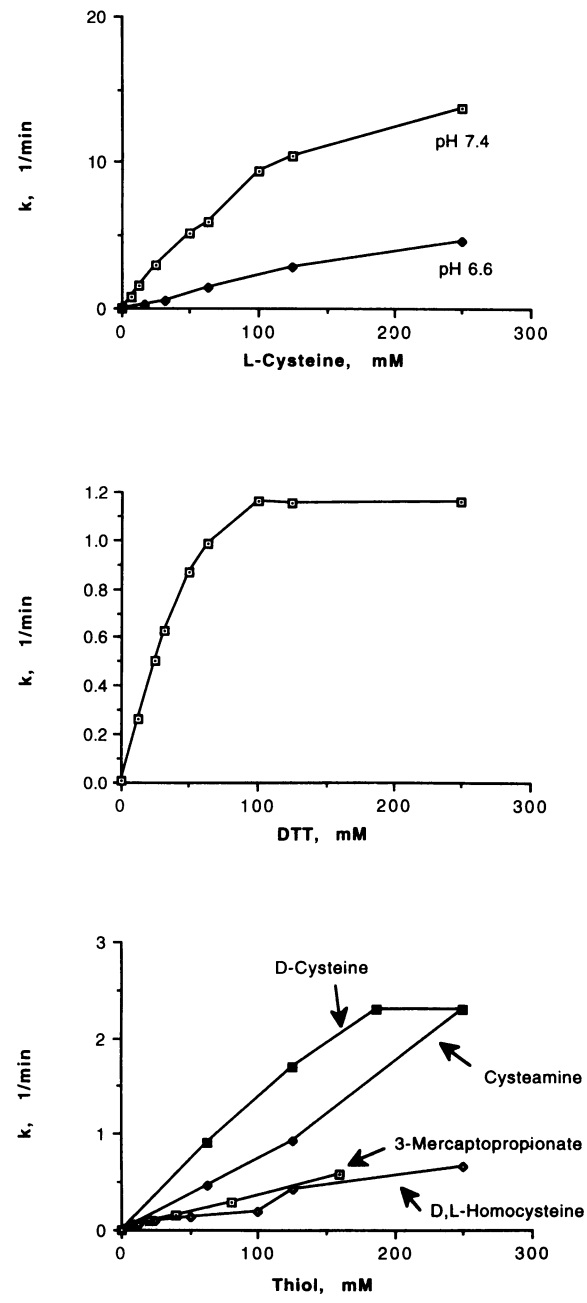

Figure 2. The rate constants for enzymatic deacylation of Arg-tRNA^{Arg} as a function of thiol concentration. Reactions were carried out at 37°C in mixtures containing 4 μM [^{14}C]Arg-tRNA^{Arg}, 5 μM ArgRS, 0.1 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA and 5–250 mM L-cysteine (upper panel), dithiothreitol (DTT, middle panel), or, shown in the lower panel, D-cysteine (solid squares), cysteamine (solid diamonds), 3-mercaptopropionate (empty squares), D,L-homocysteine (empty diamonds). Cysteine-dependent reactions were also carried out at pH 6.6 (solid diamonds in upper panel). The rate constants, k, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln 2/t_{0.5}$.

different thiols; L-cysteine was the most efficient thiol in promoting the deacylation reaction. The data indicate that the -SH group is the major determinant of catalytic efficiency of L-cysteine. Both carboxyl and amino groups of cysteine are not required for the reaction: L-cysteine derivatives in which the carboxyl group has

been removed (cysteamine) or methylated (L-cysteine methyl ester), as well as derivatives in which the amino group has been removed (3-mercaptopropionate) or acetylated (*N*-acetyl-L-cysteine) still stimulated the reaction, albeit much less efficiently than L-cysteine. Glutathione, in which both amino and carboxyl groups of L-cysteine are blocked, was the least effective thiol. The difference in extents of Arg-tRNA^{Arg} deacylation reactions in the presence of DTT and 2-ME (lower panel, Fig. 1) may be due to different equilibria for the two reactions.

Cysteine binds at a distinct site of Arg•RSArg-tRNA^{Arg}

To determine whether cysteine and arginine binding sites coincide, effects of arginine on enzymatic deacylation of Arg-tRNA^{Arg} were determined. As shown in Table 2, arginine affected the k_{cat} but not the K_m for cysteine, indicating that arginine is a noncompetitive inhibitor of the L-cysteine-dependent reaction. The inhibition constant for arginine, K_i, is calculated to be 0.23 mM.

Table 2. Arginine is a noncompetitive inhibitor of L-cysteine-dependent enzymatic deacylation of Arg-tRNA

[Arginine] (mM)	k_{cat} (min^{-1})	K_m (mM)
0	13.1	95
0.2	2.9	75
1.0	2.0	82

Incubation mixtures contained 0.2 µM [^{3}H]Arg-tRNA^{Arg}, 2.5 µM ArgRS, 0.05 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 10–100 mM L-cysteine, and 0, 0.2 or 1.0 mM arginine. Rate constants, k, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln 2/t_{0.5}$. These rate constants were used to calculate k_{cat} and K_m values for L-cysteine according to $k = k_{cat} - K_m \bullet k/[\text{L-Cys}]$.

Comparison of enzymatic and nonenzymatic deacylation of Arg-tRNA^{Arg}

To prove that stimulation of the enzymatic deacylation by L-cysteine is mediated by ArgRS, effects of L-cysteine on a nonenzymatic deacylation of Arg-tRNA^{Arg} were also determined. At pH 7.4, the nonenzymatic deacylation was not affected by 10 or 125 mM L-cysteine, whereas the enzymatic reaction was accelerated 470- and 3300-fold, respectively (Table 3). At pH 9.0, the rate of the nonenzymatic deacylation was not affected by 10 mM L-cysteine (k = 0.07 min^{-1}) and was stimulated 1.3-fold by 125 mM L-cysteine (k = 0.09 min^{-1}). At pH values in the range from 6.6 to 9.0, the enzymatic reaction in the presence of 10 and 125 mM L-cysteine was 17.5–52.5-fold and 150–250-fold, respectively, faster than the nonenzymatic reaction (Table 3). Thus, L-cysteine did not significantly affect the rate of the nonenzymatic deacylation of Arg-tRNA^{Arg}. However, L-cysteine at high concentrations does react nonenzymatically with Arg-tRNA^{Arg} (see below).

Identification of a product of cysteine-dependent deacylation of Arg-tRNA^{Arg}

[^{14}C]Arg-tRNA^{Arg} was enzymatically deacylated in the presence of L-cysteine as described in the legend to Figure 3. Reaction mixtures were analyzed by TLC and the products were visualized by autoradiography. For comparison, similar analyses were performed for reactions in the presence of D-cysteine, cysteamine, D,L-homocysteine, DTT and 2-ME, as well as in the absence of thiols. As shown in Figure 3A, a distinct major product, well separated from an arginine standard, was formed in each thiol-dependent reaction. Different mobilities of these products suggest that they are formed as a result of a reaction between a thiol and Arg-tRNA^{Arg}. Minor products were also formed, most clearly seen in lane 5 (the fastest migrating spot), lane 6 (a faint spot barely migrating from the origin) and lane 7 (a spot co-migrating with arginine standard; absent in panel D).

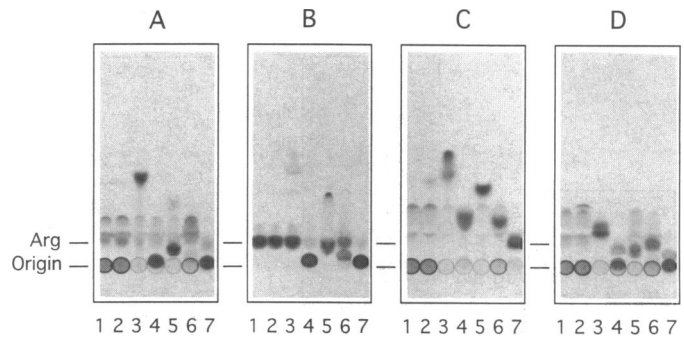

Figure 3. TLC analysis of products of enzymatic deacylation of Arg-tRNA^{Arg}. Reactions were carried out at 37°C for 20 min in mixtures containing 4 µM [^{14}C]Arg-tRNA^{Arg}, 5 µM ArgRS, 0.1 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA and 20 mM 2-ME (lane 2), DTT (lane 3), L-cysteine (lane 4), cysteamine (lane 5), D,L-homocysteine (lane 6), D-cysteine (lane 7) or no additions (lane 1). Reaction mixtures were subjected to TLC without any further treatment (**A**) or after 20-min-treatment with 180 mM NaOH (**B**), 35 mM DTNB (pH 7.4) (**C**) or 35 mM iodoacetate (pH 7.4) (**D**). TLC separations were carried out on soft layer cellulose plates (Merck) with butanol:acetic acid:water (4:1:1, v/v) as a solvent. The position of an authentic arginine standard on the TLC plate is indicated. [^{14}C]Arg-tRNA^{Arg} present in some reaction mixtures stays at the origin. The differences in chromatographic mobilities of L- and D-cysteine derivatives [most pronounced after DTNB treatment, compare lanes 4 and 7 in (D)] are general properties of derivatized L and D-amino acids which are utilized for separation of the L- and D-enantiomers (22).

Table 3. Comparison of pH effects on nonenzymatic and L-cysteine-dependent enzymatic deacylation of Arg-tRNA

pH	k (min^{-1})	
	+ ArgRS	– ArgRS
6.6	0.14	0.008
7.4	1.4	0.04
7.4, no L-Cys	0.003	0.04
7.4 + 125 mM L-Cys	10	0.04
8.0	2.1	0.04
8.5	2.1	0.07
9.0	1.7	0.07
9.0 + 125 mM L-Cys	14	0.09

Incubation mixtures for nonenzymatic deacylation contained 4 µM [^{14}C]Arg-tRNA^{Arg}, 0.1 M K-Hepes (pH 6.6, 7.4 or 8.0) or Tris-HCl (pH 7.4, 8.5 or 9.0), 10 mM $MgCl_2$, 0.2 mM EDTA. Unless indicated otherwise, enzymatic deacylations were carried out in the presence of 10 mM L-cysteine and 5 µM ArgRS. Rate constants for reactions, k, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln 2/t_{0.5}$. L-Cysteine at 10 mM did not affect the rate of nonenzymatic deacylation of Arg-tRNA.

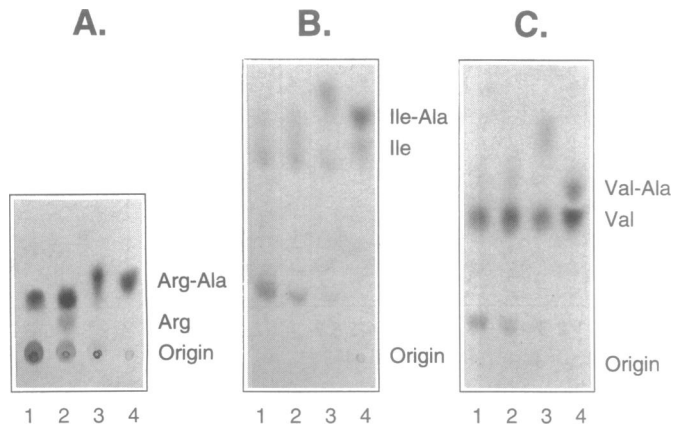

Figure 4. Identification of products formed during L-cysteine-dependent enzymatic deacylation of AA-tRNA^{AA}. [^{14}C]AA-tRNA^{AA} were deacylated in the presence of 20 mM L-cysteine and cognate AARS. Deacylation products were treated with NaOH (lane 2), DTNB (lane 3) or Raney nickel (Sigma) (lane 4). Lane 1, untreated reaction mixture. TLC separations were carried out on a hard layer cellulose plate (Merck) with 2-propanol:formic acid:water (20:1:5, v/v) as a solvent. Positions of authentic arginine, isoleucine, valine, dipeptides Arg–Ala, Ile–Ala and Val–Ala on the TLC plate are indicated. Unreacted [^{14}C]AA-tRNA^{AA} stays at the origin. (**A**), (**B**) and (**C**) show analyses of products formed from Arg-tRNA, Ile-tRNA and Val-tRNA, respectively.

To determine identity of these products, several tests were performed. The analyses were slightly complicated by oxidation of thiols to disulfides which leads to formation of slower migrating spots on TLC plates. Disulfides generally migrate slower on TLC plates. They would also react with 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) in the presence of 5-thio-2-nitrobenzoate (which forms when the acid form of DTNB is neutralized with NaOH) due to sulfide disulfide interchange.

A product formed in the presence of L-cysteine was not sensitive to NaOH treatment (compare lanes 4 in Fig. 3A and B). Similar lack of sensitivity to NaOH treatment was exhibited by products formed in the presence of cysteamine (lanes 5 in Fig. 3A and B) and D-cysteine (lanes 7 in Fig. 3A and B). On the other hand, a major product formed in the presence of DTT (compare lanes 3 in Fig. 3A and B) yielded arginine upon NaOH treatment. A minor product formed in the presence of 2-ME (compare lanes 2 in Fig. 3A and B) was also sensitive to NaOH treatment. These results indicate that products of deacylation reactions formed in the presence of L-cysteine, D-cysteine and cysteamine are not thioesters. Products of DTT- and 2-ME-dependent deacylation reactions are most likely corresponding (thio)esters of arginine. An apparent sensitivity to NaOH of a product formed in the presence of D, L-homocysteine (compare lanes 6 in Fig. 3A and B) is due to its oxidation to a disulfide.

Tests for the presence of the -SH group were also performed. A product formed in the presence of L-cysteine was sensitive to DTNB treatment (compare lanes 4 in Fig. 3A and C). Similar sensitivity to DTNB was exhibited by products formed in the presence of cysteamine (compare lanes 5 in Fig. 3A and C), D,L-homocysteine (lanes 6 in Fig. 3A and C), and D-cysteine (lanes 7 in Fig. 3A and C). Products formed in the presence of DTT and 2-ME were sensitive to iodoacetate (lanes 2 and 3, in Fig. 3D and A, respectively). An apparent lack of sensitivity of these products to DTNB (lanes 2 and 3 in Fig. 3C and A) is most likely due to similar chromatographic mobilities of these products and their 5-thio-2-nitrobenzoate derivatives. Iodoacetate treatment did not affect mobilities of slower migrating major spots in lanes 4, 5 and 7, but it affected mobilities of faster migrating minor spots in these lanes (compare lanes 4, 5 and 7 in panels D and A). Together with DTNB reactivity data, iodoacetate treatments indicate that the slower migrating spots are disulfide forms of the faster migrating spots in lanes 4, 5 and 7. A product formed in the presence of D,L-homocysteine was sensitive to DTNB and iodoacetate (compare lanes 6 in panels C, D and A). These results indicate that products of thiol-dependent deacylations are arginine derivatives containing free -SH groups.

Products of deacylation reactions in the presence of 3-mercaptopropionate and *N*-acetyl-L-cysteine were also analyzed by TLC. In both cases distinct products were formed. These products yielded arginine upon NaOH treatment and were not sensitive to DTNB and iodoacetate (not shown). Thus, products of 3-mercaptopropionate- and *N*-acetyl-L-cysteine-dependent reactions are most likely corresponding thioesters of arginine.

Raney nickel treatment, which desulfurizes cysteine to alanine, was also employed to determine the identity of a product of cysteine-dependent deacylation. As shown in Figure 4A, Raney nickel treatment resulted in formation of a compound that co-migrated with an authentic dipeptide Arg–Ala (lane 4). This indicates that a major product of enzymatic deacylation of Arg-tRNA^{Arg} in the presence of cysteine is the dipeptide Arg–Cys. For comparison, effects of NaOH (lane 2) and DTNB (lane 3) treatments are also shown.

IleRS and ValRS catalyze synthesis of Ile–Cys and Val–Cys, respectively

Incubations of other AARSs with cognate aminoacyl-tRNA and cysteine also led to formation of dipeptides. For example, TLC analysis showed that, in addition to isoleucine, two new major products were formed in incubation mixtures containing IleRS, [^{14}C]Ile-tRNA^{Ile}, and cysteine (lane 1, Fig. 4B). These products were not sensitive to NaOH treatment (lane 2, Fig. 4B) but were sensitive to DTNB treat–ment (lane 3, Fig. 4B). Raney Nickel treatment of these new products resulted in formation of Ile–Ala (lane 4, Fig. 4B). Thus, the new product is Ile–Cys, which gradually oxidizes to a slower migrating disulfide. Figure 4C shows similar analyses of enzymatic reaction of Val-tRNA^{Val} with cysteine which yields Val–Cys as a product. As can be seen in Figure 4B and C, cysteine-dependent deacylation reactions catalyzed by IleRS and ValRS, in contrast to ArgRS (Fig. 4A), have also yielded significant amounts of corresponding cognate amino acids. This reflects high editing activity (in this case directed toward cognate products) of these two AARSs. Formation of cognate amino acids can be abolished with higher concentrations of cysteine which shifts the reaction to formation of dipeptides (not shown).

Cysteine and other thiols do not affect the rate of tRNA^{Arg} aminoacylation

Possible effects of cysteine and other thiols on aminoacylation reaction were also tested. No thiol, tested at 50 mM, affected aminoacylation activity of ArgRS. However, prolonged incubations of aminoacylation mixtures containing cysteine resulted in progressive lowering of Arg-tRNA^{Arg} levels and concomitant formation of the dipeptide Arg–Cys (not shown).

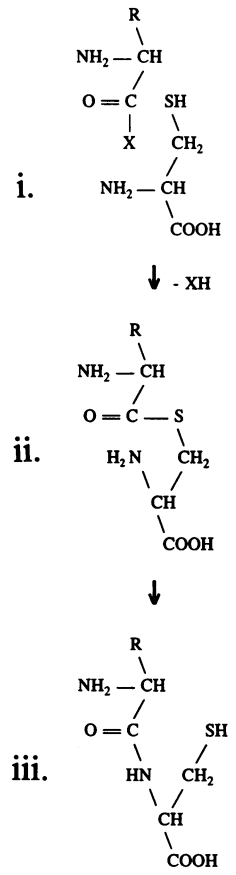

Figure 5. Proposed mechanism of synthesis of the dipeptide Arg–Cys from Arg-tRNA and cysteine. Formation of the thioester *S*-(L-arginyl)-L-cysteine (**ii.**) is catalyzed by ArgRS. Subsequent rearrangement of **ii.** to Arg–Cys (**iii.**) may occur spontaneously (21). R is the side chain of arginine, X denotes tRNA.

ArgRS does not have ATP pyrophosphatase activity

The ATP pyrophosphatase reaction is characteristic of those synthetases that possess an editing mechanism (1). The reaction measures the total consumption of ATP as an amino acid is first activated in the synthetic reaction and the product (an enzyme-bound aminoacyl adenylate and/or aminoacyl-tRNA) is then destroyed in the editing reaction that yields AMP. Here, the ATP pyrophosphatase activity of the enzyme (2 μM) has been measured in the presence of 6 μM $tRNA^{Arg}$, 0.1 mM [^{3}H]ATP (0.1 mCi/ml), 1 U/ml inorganic pyrophosphatase and 25 mM L-amino acid. For eight amino acids tested (Arg, Lys, His, citrulline, ornithine, homoserine, Hcy and Cys) ATP was hydrolyzed at 0.01 mol/mol enzyme/s, indicating essentially lack of the ATP pyrophosphatase activity. Thus, ArgRS does not seem to misactivate and edit any of these noncognate amino acids.

Nonenzymatic reactions of Arg-$tRNA^{Arg}$ with thiols

To determine whether cysteine and other thiols can react nonenzymatically with Arg-$tRNA^{Arg}$, [^{14}C]Arg-$tRNA^{Arg}$ was incubated with 125 mM thiol at pH 7.4, 37°C for 1 h and the products were analyzed by TLC and quantitated by phosphorimaging. As shown in Table 4, reactions in the presence of cysteine, homocysteine and DTT yielded ~20% of Arg–Cys, Arg–Hcy and Arg–DTT, respectively, in addition to arginine as products. Reaction in the presence of 2-ME yielded ~3% of Arg-2-ME in addition to the major product arginine. Control reactions with Ala, Ser, homoserine and Arg have shown only [^{14}C]arginine as a product (Table 4).

Table 4. Quantitation of products formed during nonenzymatic deacylation of Arg-$tRNA^{Arg}$ in the presence and absence of thiols

Additions	Relative amounts (%) of		
(125 mM)	Arg-$tRNA^{Arg}$	Arg	Arg-X
None	23	77	0
L-Cysteine	24	55	21
D-Cysteine	24	64	12
D,L-Homocysteine	16	60	24
Dithiothreitol	17	67	16
2-Mercaptoethanol	18	79	3
None	19	81	0
Alanine, serine, homoserine, arginine	20	80	0

Reactions were carried out at 37°C for 1 h in mixtures containing 4 μM [^{14}C]Arg-$tRNA^{Arg}$, 0.1 M K–Hepes pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA and 125 mM thiol or amino acid as indicated. TLC separations were carried out on hard layer cellulose plates (Merck) with 2-propanol:formic acid:water (20:1:5, v/v) as a solvent. Spots from TLC plates were quantitated with Molecular Dynamics PhosphorImager. Arg-X is a product of reaction of an indicated thiol with Arg-$tRNA^{Arg}$. Zero, 0, means that no other spot, except those for Arg-tRNA and arginine, was present on TLC plates.

Coupling relative amounts of Arg-X and arginine formed during deacylation with an overall first order rate constant for deacylation ($k = 0.04$ min^{-1}; Table 3), one can estimate that rate constants for the reactions of Arg-$tRNA^{Arg}$ with cysteine, homocysteine and DTT were ~0.01 min^{-1} (at 125 mM thiol). Thus, nonenzymatic reactions of Arg-$tRNA^{Arg}$ with thiols are up to 1000 times slower than the enzymatic reactions (see Table 1). Also, most of the selectivity toward cysteine observed in the enzymatic deacylation is lost in the nonenzymatic deacylation in which cysteine, homocysteine and DTT are about equally reactive. However, 2-ME is a very poor substrate in both enzymatic and nonenzymatic deacylation of Arg-$tRNA^{Arg}$.

DISCUSSION

This work demonstrates a novel chemistry of cysteine-dependent enzymatic deacylation of aminoacyl-tRNA. The evidence presented above demonstrates that ArgRS, a class I aminoacyl-tRNA synthetase (10–13), catalyzes a reaction between cysteine and Arg-$tRNA^{Arg}$ which yields the dipeptide Arg–Cys as a product. A similar, but less efficient, reaction occurs with homocysteine which yields the dipeptide Arg–Hcy. The reaction is not limited to ArgRS since similar reactions are also catalyzed by two other class I aminoacyl-tRNA synthetases, IleRS and ValRS, yielding Ile–Cys and Val–Cys, respectively.

The data are consistent with the mechanism shown in Figure 5. The ester bond in Arg-$tRNA^{Arg}$ undergoes a nucleophilic attack

by the thiolate of cysteine (Fig. 5i). The resulting thioester *S*-arginyl-cysteine (Fig. 5ii) is not observed as a discrete intermediate because of the rapid rearrangement to form a stable peptide bond (Fig. 5iii). Facile intramolecular reaction results from the favorable geometric arrangement of the α-amino group of cysteine with respect to the thioester bond. This mechanism is supported by the observation that thioesters of arginine do indeed form with cysteine derivatives that do not have free amino group, such as 3-mercaptopropionate and *N*-acetyl-L-cysteine.

Catalysis of an aminoacyl-thioester bond formation may involve an increase in the reactivity of the aminoacyl ester bond between arginine and the 3′-terminal ribose of tRNA in an ArgRS–Arg-tRNA^{Arg} complex, compared to the reactivity of the same ester bond in free Arg-tRNA^{Arg} in solution. Reaction of cysteine with ArgRS–Arg-tRNA^{Arg} would be due to binary collisions between cysteine and the complex without intermediate binding of cysteine. However, this seems unlikely since ArgRS-bound Arg-tRNA^{Arg} does not exhibit increased reactivity toward water; in fact the aminoacyl ester bond in ArgRS-bound Arg-tRNA^{Arg} is protected against hydrolysis (Table 1). Other properties of the cysteine-dependent deacylation reaction are consistent with a mechanism which involves binding of cysteine to the complex. First, the reaction exhibits saturation kinetics with respect to cysteine and some other thiols (Fig. 2). Secondly, the deacylation reaction exhibits thiol specificity with cysteine being the most efficient thiol in promoting the reaction (Table 1). Saturation kinetics and specificity are characteristic properties of enzymatic reactions involving substrate binding. Two other class I aminoacyl-tRNA synthetases, IleRS and ValRS, which have been shown to catalyze formation of cysteine-containing dipeptides in this work, are also known from previous work to bind cysteine (8).

A possible explanation of the existence of a cysteine binding site on ArgRS may come from consideration of editing mechanisms of aminoacyl-tRNA synthetases. A major selectivity problem of aminoacyl-tRNA synthetases is with thio amino acids such as homocysteine and cysteine. Homocysteine is misactivated by MetRS, IleRS and LeuRS at frequencies exceeding the frequency of translational errors *in vivo* (1,14–17). Two other synthetases, ValRS (8,18) and LysRS (H. Jakubowski, unpublished) misactivate Hcy less efficiently. Cysteine is also misactivated by IleRS, ValRS (8,18) and LysRS (H. Jakubowski, unpublished). Misactivated thio amino acids are efficiently edited *in vitro* (8,18) and *in vivo* (14–17) which prevents their misincorporation to tRNA and protein. The side chain thiol group of Hcy directly participates in the editing reaction by a nucleophilic attack on the activated carboxyl group of Hcy-AMP to give Hcy thiolactone (8). Thus, thiols should be able to mimic a function of the SH group of Hcy and react with the activated carboxyl group in an aminoacyl-tRNA. This would explain why cysteine reacts with Ile-tRNA^{Ile} and Val-tRNA^{Val} in the presence of IleRS and ValRS, respectively. ArgRS does not seem to need an editing function but it exhibits structural homology with IleRS and ValRS, which need and possess editing functions, indicating common evolutionary origin of the three synthetases which they share with other class I aminoacyl-tRNA synthetases (10–13). This work shows that ArgRS shares additional functional homologies with IleRS and ValRS in that it catalyzes reaction of cysteine with cognate aminoacyl-tRNA. The ability of ArgRS to catalyze the reaction between Arg-tRNA and cysteine may indicate that the present day ArgRS possesses a cryptic, thiol-dependent, editing function. It follows that an ancestral ArgRS, presumably less accurate in initial selection of the correct substrate than the present-day ArgRS, may have had an editing function. An editing sub-site of an ancestral ArgRS has been preserved as a cysteine binding site in the present-day ArgRS. The reaction of cysteine with Arg-tRNA^{Arg} ($k_{cat}/K_m = 2\ s^{-1}\bullet M^{-1}$, $k_{cat} = 0.3\ s^{-1}$) is several orders of magnitude less efficient than arginylation of tRNA^{Arg} (k_{cat}/K_m is $\sim 10^6\ s^{-1}\bullet M^{-1}$, $k_{cat} = 3\ s^{-1}$) and, therefore, is expected to be without adverse functional effects on the aminoacylation function of ArgRS.

The reaction between Arg-tRNA and cysteine is reminiscent of non-ribosomal peptide bond formation utilized in the synthesis of peptide antibiotics (19). In the multi-enzyme thio-template system, activated amino acids are condensed to peptides via thioester intermediates. The thiol group of active site pantetheine serves as an intermediate acceptor of an aminoacyl moiety from an aminoacyl adenylate in each domain. Peptide bond formation occurs as a result of transfer of the acyl group from *S*-[aminoacyl (or peptidyl)]-pantetheine in one domain to the amino group of a pantetheine-bound amino acid in another domain (20). The evidence presented here indicates that in the synthesis of Arg–Cys catalyzed by ArgRS, activated arginine is transferred from Arg-tRNA to the SH group of cysteine. The resulting thioester *S*-(arginyl)-cysteine undergoes intramolecular transacylation from the sulfur to the amino group of cysteine, yielding the dipeptide Arg–Cys. It should be noted that the unique thioester chemistry of cysteine has recently been successfully utilized in chemical synthesis of proteins (21).

Synthesis of arginyl-thioesters from Arg-tRNA^{Arg} and thiols may also be relevant for understanding evolution of aminoacylation reactions. Since at least one aminoacyl-tRNA reacts with thiols to form aminoacyl-thioesters, then, by the principle of microscopic reversibility, tRNA must also react with aminoacyl-thioesters to form aminoacyl-tRNA. The equilibrium of the reaction of aminoacyl-tRNA with an organic thiol will depend on the structure of the thiol. This may account for much lower efficiency of reactions of Arg-tRNA^{Arg} with 2-ME, compared with DTT. The existence of thioester chemistry in present- day aminoacyl-tRNA synthetases, documented in this work, suggests that thioester chemistry might be responsible for the origin of aminoacylation reactions, perhaps in the 'thioester world' (23), and may have been used more extensively by ancestral aminoacyl-tRNA synthetases.

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