Articles

Aminoacyl Thioester Chemistry of Class II Aminoacyl-tRNA Synthetases†

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ABSTRACT: Lysyl-tRNA synthetase, a class II enzyme, edits homocysteine by converting it into homocysteine thiolactone. In a similar reaction, the enzyme converts homoserine into homoserine lactone. Other class II enzymes, aspartyl-tRNA synthetase and seryl-tRNA synthetase, do not edit any of the amino acids tested. However, all three class II aminoacyl-tRNA synthetases catalyze AMP- and pyrophosphate-independent deacylation of cognate aminoacyl-tRNA in the presence of thiols, mimicking editing of homocysteine. Thiol-dependent deacylations exhibit saturation kinetics with respect to concentration of thiols, suggesting the presence of a thiol binding site on each enzyme. 3-Mercaptopropionate-, *N*-acetyl-L-cysteine-, and dithiothreitol-dependent deacylations of aminoacyl-tRNA yield corresponding aminoacyl thioesters. Cysteine-dependent enzymatic deacylations of aminoacyl-tRNA by these class II enzymes yield dipeptides, *N*-(aminoacyl)cysteine. The formation of *N*-(aminoacyl)cysteine involves thioester intermediates *S*-(aminoacyl)-L-cysteine, which are not observed because of the facile transacylation of the aminoacyl residue from the sulfur to the α-amino group of cysteine to form a stable peptide bond. These data indicate that class II aminoacyl-tRNA synthetases possess unique thiol-binding subsites within their active sites. That the thiol-binding subsite exists also in AspRS and SerRS, which do not need editing function, suggests that these class II enzymes possess *vestigial* editing functions.

Each of the 20 aminoacyl-tRNA synthetases (AARSs)[1] matches a cognate amino acid with its cognate tRNA, providing charged tRNAs for protein synthesis according to the rules of the genetic code. Accurate implementation of the genetic code by an AARS is achieved by a highly specific selection of cognate tRNA and amino acid. High accuracy of the selection of a cognate tRNA is determined by intrinsic binding energies of tRNAs to AARSs, which seem to be adequate to provide the required accuracy of translation. In contrast, in many cases the intrinsic binding energies of amino acids to AARSs are inadequate to give the required accuracy of translation. This has necessitated the evolution of a second determinant of specificity, proofreading or editing mechanisms that involve the expenditure of energy to remove errors in amino acid selection [reviewed in Fersht (1986), Jakubowski and Goldman (1992), and Jakubowski (1994)].

AARSs are divided into two classes according to primary (Hountondji et al., 1986; Eriani et al., 1990a; Brown & Doolittle, 1995) and tertiary (Brunie et al., 1990; Cusack et al., 1990, 1996) structural features. Class I enzymes are characterized by the existence of two signature peptides (HIGH and KMSKS; Hountondji et al., 1986) located within the Rossman fold in the active-site domain. Class II enzymes possess three common signature motifs within an antiparallel β fold of the active-site domain. These structural differences are manifested functionally: the CCA terminus of a tRNA approaches aminoacyl adenylate from different sides in class I and class II enzymes; thus, all class I enzymes attach amino acids to 2′ hydroxyl groups, whereas class II AARSs (with the exception of PheRS) attach their amino acid to the 3′ hydroxyl of the terminal adenosine (Eriani et al., 1990). Despite these structural differences, the aminoacylation mechanisms for class I and class II enzymes are similar (Cavarelli et al., 1994). However, it is not known whether proofreading mechanisms of class I and class II synthetases are similar.

Editing by AARSs is manifested by a deacylase activity that preferentially destroys incorrect aminoacyl adenylates or/and incorrect aminoacyl-tRNAs. In addition, at least some AARSs exhibit a weak deacylase activity toward cognate aminoacyl-tRNA (Schreier & Schimmel, 1972; Fersht & Dingwall, 1979). This weak deacylase activity is greatly enhanced by thiols, as has been shown for deacylations catalyzed by class I AARSs, such as ArgRS (Jakubowski, 1995), IleRS (Jakubowski, 1996a), and MetRS (Jakubowski, 1996b). Remarkably, these class I AARSs catalyze reactions of thiols with a cognate aminoacyl-tRNA that yield corresponding aminoacyl thioesters (Jakubowski, 1995a, 1996a,b). Thiol-dependent deacylations of cognate aminoacyl-tRNAs catalyzed by IleRS and MetRS mimic editing of homocysteine [which is a physiologically important function of these synthetases (Jakubowski, 1990, 1991, 1995b; Jakubowski & Goldman, 1994; Gao et al., 1994)], with a thiol acting as an analogue of the side chain of homocysteine (Jakubowski, 1996a,b). The ability of ArgRS [which does not have, or

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[1] Abbreviations: AARS, aminoacyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase (other synthetases are similarly abbreviated); DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol.

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need, editing function (Jakubowski, 1995a)] to catalyze thiol-dependent deacylation of Arg-tRNA indicates that ArgRS possesses a *vestigial* editing function.

It is unclear whether the ability to catalyze the thiolysis of aminoacyl-tRNA is limited to the class I AARSs studied so far (Jakubowski, 1995a, 1996a,b) or is a general property of all AARSs, including class II enzymes. This paper demonstrates that class II AARSs, such as AspRS, SerRS, and LysRS, catalyze thiolysis of the aminoacyl ester bond in corresponding aminoacyl-tRNA, which leads to formation of aminoacyl thioesters. With cysteine as a thiol, secondary acyl transfer reactions to the *cis* amino group yields dipeptides AA-Cys. Thus, thiol-binding subsites, which confer the ability to catalyze thioester formation, are present in the active sites of both class I and class II AARSs. It is also shown that LysRS possesses editing function, whereas AspRS and SerRS do not. Editing of homocysteine and homoserine by LysRS leads to formation of homocysteine thiolactone and homoserine lactone, respectively.

MATERIALS AND METHODS

Plasmids and Host Strain. Plasmids overexpressing AARSs were obtained from the following sources: pAspS1 (AspRS) from G. Eriani (Eriani et al., 1990b); pXLys5 (LysRS) from S. Blanquet (Brevet et al., 1986); pSerS2 (SerRS) from M. Härtlein (Härtlein et al., 1987). Plasmids were overexpressed in *Escherichia coli* strain JM101 (Yanisch-Perron et al., 1985) and used as a source of AARSs. Cells for enzyme purification were obtained from overnight cultures (usually 400 mL) grown at 37 °C in LB medium containing 0.1 mg/mL ampicillin.

Aminoacyl-tRNA Synthetases. The enzymes were purified to homogeneity from overexpressing strains as described in Jakubowski (1995a) with slight modifications. Briefly, bacterial cells (1.6 g) were disrupted by sonication in 2 × 5 mL of potassium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol and 10% glycerol (buffer A). Nucleic acids were precipitated from crude extracts (10 mL) with 3 mL of 6% streptomycin sulfate and discarded. Proteins were precipitated between 40% and 65% (AspRS) or between 50% and 65% (SerRS and LysRS) ammonium sulfate saturation, dissolved in 3 mL of buffer A, and loaded onto a 2.5 × 60 cm Sephacryl S-200 (Pharmacia) gel-filtration column equilibrated with buffer A. AspRS and LysRS were further purified on a 1.5 × 6 cm hydroxyapatite (Calbiochem) column equilibrated with buffer A. The column was washed with 6 volumes of buffer A and developed with a 300 mL linear gradient from 10 to 200 mM potassium phosphate, pH 7.5, containing 0.1 mM EDTA. Active fractions from the hydroxyapatite column were applied onto a 1.5 × 5 cm MonoQ (Pharmacia) ion-exchange column and eluted with a gradient from 0 to 400 mM KCl in buffer A. SerRS was further purified on a 1.5 × 8 cm DEAE-cellulose (Pharmacia) column equilibrated with buffer A and developed with a gradient from 0 to 400 mM KCl in buffer A. The enzymes were concentrated by ultrafiltration for storage in 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 50% glycerol. The synthetases were at least 95% pure as judged by SDS−PAGE.

Preparation of Radiolabeled Aminoacyl-tRNA. Reaction mixtures contained a standard buffer (0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, and 0.1 mM EDTA), 2 mM ATP, 20 μM $tRNA^{AA}$ (pure tRNAs with aminoacyl acceptor activity of 1400−1600 pmole/A_{260} were obtained from Subriden RNA), 25 μM radiolabeled amino acid (from Amersham), and an AARS. After 5 min at 37 °C, reaction mixtures were extracted with phenol (saturated with 0.1 M sodium acetate, pH 5) and charged tRNAs were recovered from aqueous layers by precipitation with ethanol. Residual low molecular weight components from aminoacylation mixtures were removed by repeated washes with 70% ethanol.

Thiol-Dependent Enzymatic Deacylation of Aminoacyl-tRNA. The radiolabeled aminoacyl-tRNA was incubated with an excess of a cognate AARS and varying concentrations of thiols at 37 °C in a standard buffer (0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, and 0.1 mM EDTA). The disappearance of the radiolabeled aminoacyl-tRNA was monitored by trichloroacetic acid precipitation. Apparent first-order rate constants for thiol-dependent enzymatic deacylation of AA-tRNA, k_{app}, were calculated from reaction half-lives, $t_{0.5}$, according to $k_{app} = (\ln 2)/t_{0.5}$. These rate constants were used to calculate k_{cat} ($=k_{app}$ at saturating concentration of a thiol) and apparent dissociation constant for a thiol·enzyme complex, K_m, values for thiols according to $k_{app} = k_{cat} - K_m \cdot k_{app}/[\text{thiol}]$. Under experimental conditions used ([AARS] > [AA-tRNA]; see legends to Figures 1 and 2 and Table 1), k_{app} was independent of both AARS and AA-tRNA concentrations, indicating that all AA-tRNA was present in the complex with AARS, as expected. To monitor all radiolabeled products of deacylation reactions, an aliquot of each reaction mixture was analyzed by TLC at the end of incubation.

Analysis of Aminoacyl Thioesters and Dipeptides by TLC. The TLC system [cellulose plates (Sigma) developed with 2-propanol:formic acid:water, 20:1:5, v/v/v] is the same as used before for analyses of deacylation products of Ile-$tRNA^{Ile}$, Val-$tRNA^{Ile}$, and Arg-$tRNA^{Arg}$ (Jakubowski, 1995a). The system adequately separates amino acids and their derivatives. Authentic aspartate, serine, and lysine (Sigma) were cochromatographed with samples and visualized by staining with ninhydrin. In some experiments authentic dipeptides Asp-Ala, Ser-Ala, and Lys-Ala (Sigma) were used as standards. The TLC plates were autoradiographed using Reflection (NEN) X-ray film. Exposure times were 1−3 days and 3−7 weeks for experiments with [^{14}C]aminoacyl-tRNA and [^{3}H]aminoacyl-tRNA, respectively.

Detection of Homocysteine Thiolactone and Homoserine Lactone by TLC. The TLC system (cellulose plates from Kodak developed in butanol:acetic acid:water, 4:1:1, v/v/v) for detection of homocysteine thiolactone and homoserine lactone is similar to that described previously (Jakubowski & Fersht, 1981). Reaction mixtures (10 μL) contained 0.1 M K-HEPES (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EDTA, 4 mM 2-mercaptoethanol, 5 mM ATP, 40 mM D,L-homocysteine (Sigma) or 20 mM homoserine (Sigma), and 1 μM LysRS. After 2−6 h at 30 °C, 1−2 μL aliquots were spotted onto the origin line of the cellulose plate. The chromatograms were developed for 1.5 h at room temperature. The spots were visualized under UV and after spraying with ninhydrin. Homocysteine ($R_f = 0.40$) and homoserine ($R_f = 0.17$) did not absorb UV light and stained red with ninhydrin. Homocysteine thiolactone ($R_f = 0.60$) was visible under UV light and gave a yellow color upon staining with ninhydrin. Homoserine lactone ($R_f = 0.42$) did not absorb UV light and stained yellow with ninhydrin.

Table 1: ATP-Pyrophosphatase Activities of LysRS[a]

amino acid (50 mM)	*V/E* (s^{-1}) +$tRNA^{Lys}$	*V/E* (s^{-1}) −$tRNA^{Lys}$
D,L-homocysteine	1.2[b]	0.4
L-cysteine	0.3	0.1
homoserine	0.24[b]	0.12
threonine		0.13
alanine	0.15	0.03
serine	0.034	
D-cysteine	0.034	
lysine	0.045	
arginine, histidine, or ornithine	0.034	
none	0.034	0.02
none, no enzyme	<0.01	

[a] Unless otherwise indicated, L-amino acids (50 mM) were used in the ATP pyrophosphatase assays (see Materials and Methods). [b] Non-saturation kinetics observed up to 50 mM amino acid.

ATP Pyrophosphatase Activity of AARSs. Amino acid-dependent AMP formation from 0.1 mM [^{3}H]ATP (1000 Ci/mol) in the presence of 50 mM amino acid and 0.2 μM LysRS or 1 μM AspRS or SerRS in a standard buffer was assayed by TLC on poly(ethylenimine)- (PEI-) cellulose as described previously (Jakubowski, 1980).

RESULTS

ATP Pyrophosphatase Activities of AspRS, SerRS, and LysRS. The ATP pyrophosphatase activity is a manifestation of an editing mechanism of an AARS (Jakubowski, 1980; Jakubowski & Fersht, 1981). This activity is measured as an amino acid-dependent hydrolysis of ATP to AMP when an amino acid (AA in eq 1) is continually activated and the product (an enzyme-bound aminoacyl adenylate) is then destroyed in the editing reaction.

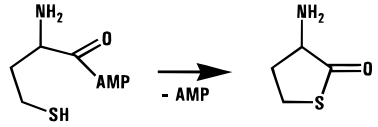
$$\mathrm{ATP} + \mathrm{AA} + \mathrm{AARS} \rightleftharpoons \mathrm{PP_i} + \mathrm{AARS{\cdot}AA\text{-}AMP} \rightarrow \mathrm{AARS} + \mathrm{AA} + \mathrm{AMP} \quad (1)$$

Although tRNA stimulates the ATP pyrophosphatase reaction in some cases, in general the reaction does not require tRNA (Fersht, 1986; Jakubowski & Goldman, 1992; Jakubowski, 1994). Here, the ATP pyrophosphatase activities of AspRS (1 μM), SerRS (1 μM), and LysRS (0.2 μM) were measured in the presence of excess cognate tRNA, 0.1 mM [^{3}H]ATP (0.1 mCi/mL), 5 units/mL yeast inorganic pyrophosphatase (Sigma) (added to prevent possible inhibition of the reaction by the accumulation of pyrophosphate), and 50 mM amino acid.

As shown in Table 1, LysRS catalyzed hydrolysis of ATP in the presence of D,L-homocysteine, homoserine, L-cysteine, threonine, and alanine. The activity was 2−5-fold higher in the presence of $tRNA^{Lys}$ than in its absence. The highest rate of ATP hydrolysis, $V/E = 1.2\ s^{-1}$, observed in the presence of D,L-homocysteine and $tRNA^{Lys}$, is comparable to the rates of homocysteine editing by MetRS, IleRS (Jakubowski & Fersht, 1981), and LeuRS (Englisch et al., 1986). The ATP pyrophosphatase activity in the presence of arginine, histidine, ornithine, lysine, or D-cysteine was not significantly different from a control without amino acid (Table 1). Thus, LysRS misactivates and edits homocysteine, homoserine, cysteine, threonine, and alanine.

To exclude a possibility that the observed homocysteine-dependent ATP pyrophosphatase is due to contamination of LysRS preparations by IleRS and/or MetRS [which are known to efficiently edit homocysteine; e.g., Jakubowski and Fersht (1981)], effects of isoleucine and methionine (which prevent homocysteine-dependent ATP hydrolysis by IleRS and MetRS, respectively; Jakubowski & Fersht, 1981; Jakubowski, 1990; Gao et al., 1994) , as well as lysine (which would prevent entry of a noncognate amino acid to the active site of LysRS, thereby inhibiting ATP hydrolysis), on the ATP pyrophosphatase activity were determined. These control experiments demonstrated that the homocysteine-dependent activity was completely inhibited by excess lysine but not by excess methionine or isoleucine. Homoserine-dependent activity was also inhibited by lysine (not shown). Thus, homocysteine- and homoserine-dependent ATP pyrophosphatase reactions are catalyzed by LysRS.

Scheme 1



On the other hand, for eight amino acids tested with AspRS and SerRS (cysteine, homocysteine, serine, homoserine, threonine, aspartate, asparagine, and alanine), ATP was hydrolyzed at ≤0.01 mol (mol of enzyme)$^{-1}$ s^{-1} (not shown), indicating essentially lack of the ATP pyrophosphatase activity. Thus, AspRS and SerRS do not seem to misactivate and edit any of the tested noncognate amino acids.

Editing of Homocysteine and Homoserine by LysRS Occurs by Cyclization to Thiolactone and Lactone, Respectively. Editing of homocysteine by MetRS, IleRS, and LeuRS occurs by cyclization to homocysteine thiolactone (Scheme 1), both *in vitro* (Jakubowski & Fersht, 1981, Englisch et al., 1986) and *in vivo* (Jakubowski 1990, 1991, 1995b, 1997; Jakubowski & Goldman, 1993; Gao et al., 1994). The thiolactone also formed when LysRS was incubated with homocysteine and ATP and was detected by TLC on cellulose plates. No thiolactone formed in the absence of ATP. The thiolactone cochromatographed with an authentic standard ($R_f = 0.62$), absorbed UV light, and gave the same yellow color upon staining with ninhydrin. Formation of the thiolactone was abolished by the presence of 10 mM lysine, the cognate amino acid, whereas methionine did not affect LysRS-dependent formation of the thiolactone. These characteristics of the conversion of homocysteine to the thiolactone indicate that homocysteine is first miscativated by LysRS to from Hcy-AMP. The side chain thiol of homocysteine then attacks the activated carboxyl group of Hcy-AMP displacing AMP and giving homocysteine thiolactone (Scheme 1).

Homoserine lactone formed in reaction mixtures containing LysRS, ATP, and homoserine. No lactone formed in the absence of ATP. The lactone cochromatographed with an authentic standard ($R_f = 0.38$) and gave the same yellow color with ninhydrin. Formation of the lactone was abolished by the presence of 10 mM lysine. These properties of the lactone formation reaction indicate that homoserine is first misactivated by LysRS to give Hse-AMP, which then undergoes conversion to the lactone by a mechanism analogous to the mechanism of the thiolactone formation (Scheme 2).

One Mole of ATP Is Used per Each Mole of Homocysteine Thiolactone Formed by LysRS. If formation of homocysteine

Scheme 2

Table 2: Stoichiometric Relationship between Hydrolysis of ATP to AMP and the Conversion of Homocysteine to the Thiolactone Catalyzed by LysRS[a]

time (min)	AMP (mM)	thiolactone (mM)	AMP/thiolactone
4	1.5	1.39	1.08
8	3.0	2.36	1.27
16	4.6	4.7	0.98
24	5.6	5.6	1.0
32	5.7	6.1	0.93

[a] Reactions were carried out at 37 °C in mixtures containing 10 mM [^{3}H]ATP (0.1 mCi/mL), 50 mM D,L-homocysteine, 0.1 M K-HEPES (pH 7.4), 20 mM $MgCl_2$, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 5 units/mL yeast inorganic pyrophosphatase (Sigma), and 2.5 μM LysRS. At indicated time intervals, reactions were quenched with an equal volume of 1 N formic acid. [^{3}H]AMP was determined by TLC on PEI-cellulose (Merck) (Jakubowski, 1980; Jakubowski & Fersht, 1981). Homocysteine thiolactone was determined by A_{240} measurements (Gao et al., 1994; Jakubowski, 1995) of 50× diluted quenched reaction mixtures. An $A_{240} = 0.35$ is equivalent to 0.1 mM thiolactone.

thiolactone is a major editing pathway, most, if not all, misactivated homocysteine (Hcy-AMP) should be converted into the thiolactone. Some of Hcy-AMP can possibly be hydrolyzed directly to free homocysteine and AMP. To determine the contribution of the two possible pathways of Hcy-AMP decomposition to overall editing of homocysteine by LysRS, measurements of AMP (generated from ATP, eq 1) and homocysteine thiolactone (generated from Hcy-AMP, Scheme 1) were carried out. To minimize errors of measurements, both AMP and the thiolactone were assayed in the same reaction mixtures. As shown in Table 2, similar amounts of AMP and homocysteine thiolactone formed in reaction mixtures containing ATP, homocysteine, and LysRS. The ratios AMP/thiolactone for individual measurements were 0.93−1.27 (Table 2). An average AMP/thiolactone ratio was 1.03, indicating that 1 mol of ATP is consumed per mol of homocysteine thiolactone formed by LysRS. Similar AMP/thiolactone ratios were also found for reactions catalyzed by MetRS and IleRS (not shown). Thus, conversion of homocysteine to the thiolactone is a major editing pathway.

Thiol-Dependent Enzymatic Deacylation of [^{3}H]Asp-tRNA^{Asp}. Under the experimental conditions used (pH 7.4, 37 °C), [^{3}H]Asp-tRNA^{Asp} in a complex with AspRS was relatively stable ($t_{0.5} = 20$ min). However, as shown in Figure 1A, addition of thiols such as L-cysteine or dithiothreitol (DTT), resulted in complete deacylation of [^{3}H]Asp-tRNA^{Asp} in ∼1 min. Other thiols, such as 2-mercaptoethanol, D-cysteine, and D,L-homocysteine, were less effective than L-cysteine or DTT (Figure 1).

Deacylation of free [^{3}H]Asp-tRNA^{Asp} in solution ($t_{0.5} =$ 40 min) was not affected by thiols (not shown). Serine, homoserine, aminoethanol, and *S*-methyl-L-cysteine did not affect the enzymatic deacylation of [^{3}H]Asp-tRNA^{Asp}, which indicates that a thiol group is important for the reaction. Addition of aspartate (5 mM) resulted in complete inhibition of the DTT-dependent deacylation (not shown). Exogenous aspartate most likely displaces from the enzyme the [^{3}H]-Asp residue in the AspRS·[^{3}H]Asp-tRNA^{Asp} complex, thereby preventing its reaction with thiols.

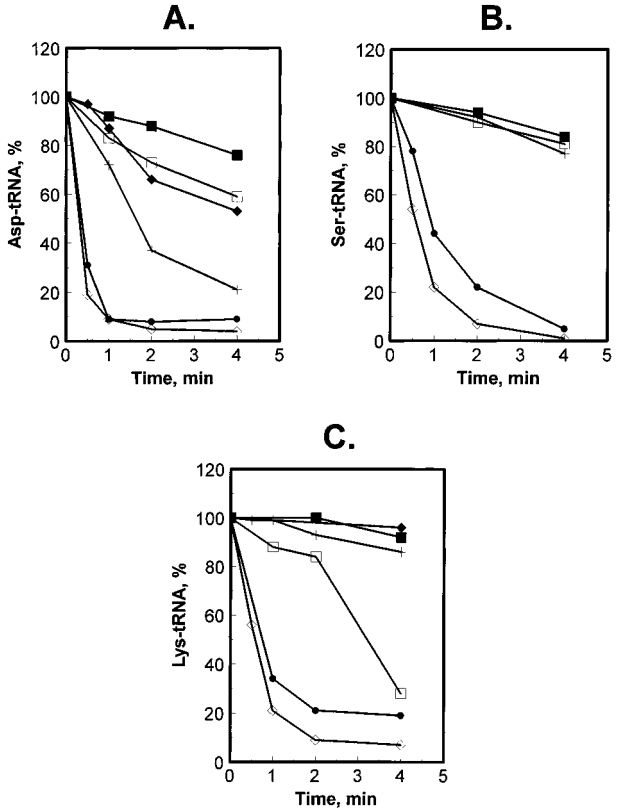


FIGURE 1: Time courses of thiol-dependent enzymatic deacylation of aminoacyl-tRNA by class II AARSs. Deacylation reactions were carried out in the presence of 50 mM (panels A and B) or 125 mM (panel C) thiol as described in Materials and Methods. (Panel A) 0.8 μM [^{3}H]Asp-tRNA^{Asp} and 1 μM AspRS; (panel B) 0.8 μM [^{3}H]Ser-tRNA^{Ser} and 1 μM SerRS; (panel C) 2.8 μM [^{14}C]Lys-tRNA^{Lys} and 3.5 μM LysRS. Enzymatic deacylations were followed in the absence (■) or in the presence of the following thiols: DTT (◇), L-cysteine (●), 2-mercaptoethanol (+), D-cysteine (◆), and D,L-homocysteine (□).

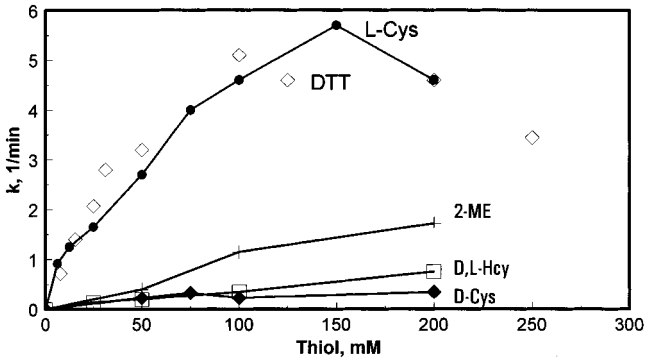


FIGURE 2: Apparent rate constants for thiol-dependent enzymatic deacylation of Asp-tRNA catalyzed by AspRS as a function of thiol concentration. Reactions were carried out as described in Materials and Methods. The apparent rate constants were determined from deacylations of 0.8 μM [^{3}H]Asp-tRNA^{Asp} by 1 μM AspRS in the presence of indicated concentrations of L-cysteine (●), D-cysteine (◆), 2-mercaptoethanol (+), DTT (◇), or D,L-homocysteine (□).

The relationship between the rate of enzymatic deacylation of [^{3}H]Asp-tRNA^{Asp} and thiol concentration is depicted in Figure 2. Saturation kinetics were observed with L-cysteine and DTT, indicating that these thiols bind to the AspRS·[^{3}H]-Asp-tRNA^{Asp} complex. Saturation kinetics were also observed with D-cysteine, but the reaction was some 10-fold

Table 3: Kinetic Indices for Thiols in the Thiol-Dependent Enzymatic Deacylation of Aminoacyl-tRNA by Class II AARSs[a]

	AspRS			LysRS			SerRS	
thiol	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})
L-cysteine	10.5	0.13	81			18	0.76	15
D-cysteine	0.6	0.07	8.6					
cysteamine			2.4	0.017			0.053	1.0
D,L-homocysteine	0.7	0.12	5.8			2.2	0.034	0.34
dithiothreitol	6.7	0.06	112	2.9	0.09	23	1.4	28
2-mercaptoethanol	5.2	0.26	20	0.017			0.086	1.7
glutathione			3.5	0.017			0.086	1.7
3-mercaptopropionate			0.92	0.017			0.047	0.6
none	0.034			0.017			0.017	
none, no enzyme	0.017			0.017			0.017	

[a] Deacylation reactions were carried out with 0.5 μM [^{3}H]Asp-tRNA^{Asp} (19.2 Ci/mmol) and 1 μM AspRS or 2.5 μM [^{14}C]Lys-tRNA^{Lys} (324 Ci/mol) and 3.5 μM LysRS, in the absence or presence of 10−200 mM indicated thiol. Deacylations of 0.8 μM [^{3}H]Ser-tRNA^{Ser} (36 Ci/mmol) with 1 μM SerRS were carried out in the absence and presence of 50 mM thiol. Kinetic indices for thiols were determined as described under Materials and Methods.

slower than with L-cysteine. Nonsaturating kinetics were observed with 2-mercaptoethanol and D,L-homocysteine in the tested range of concentrations (Figure 2). Kinetic indices calculated from these data are summarized in Table 3.

Up to 100-fold variation in catalytic efficiencies was observed between different thiols. The reaction was the fastest with cysteine (k_{cat} = 10.5 min^{-1}). Similar catalytic efficiencies were calculated for L-cysteine and DTT, 81 min^{-1} M^{-1} and 112 min^{-1} M^{-1}, respectively. 2-Mercaptoethanol was 4-fold less efficient than L-cysteine. The L-configuration, amino and carboxyl groups of L-cysteine, all contribute to its catalytic efficiency, as suggested by the observations that D-cysteine, D,L-homocysteine, cysteamine, and 3-mercaptopropionate were 10−100-fold less catalytically efficient than L-cysteine. Glutathione was a 23-fold less efficient substrate than L-cysteine.

Thiol-Dependent Enzymatic Deacylations of [^{3}H]Ser-tRNA^{Ser} and [^{14}C]Lys-tRNA^{Lys}. [^{3}H]Ser-tRNA^{Ser} and [^{14}C]-Lys-tRNA^{Lys} in complexes with a corresponding AARS exhibited stabilities identical to the stabilities of free [^{3}H]-Ser-tRNA^{Ser} and [^{14}C]Lys-tRNA^{Lys} in solution ($t_{0.5}$ = 40 min; Table 3). In the presence of thiols such as L-cysteine or DTT, the complexed forms of [^{3}H]Ser-tRNA^{Ser} and [^{14}C]-Lys-tRNA^{Lys} were completely deacylated within a few minutes (Figure 1B,C), whereas stabilities of free [^{3}H]Ser-tRNA^{Ser} and [^{14}C]Lys-tRNA^{Lys} were unaffected (not shown). Kinetic indices of thiol-dependent enzymatic deacylations of [^{3}H]Ser-tRNA^{Ser} and [^{14}C]Lys-tRNA^{Lys} are shown in Table 3.

Enzymatic deacylations of [^{3}H]Ser-tRNA^{Ser} in the presence of thiols were up to 12-fold less catalytically efficient than the deacylations of [^{3}H]Asp-tRNA^{Asp} (Table 3). Catalytic efficiencies for different thiols in enzymatic deacylation of [^{3}H]Ser-tRNA^{Ser} followed somewhat different patterns than those observed with the aspartate system. For example, catalytic efficiencies calculated for 2-mercaptoethanol and glutathione in the serine system were identical (1.7 min^{-1} M^{-1}), whereas in the aspartate system, 2-mercaptoethanol (20 min^{-1} M^{-1}) was 6-fold more catalytically efficient than glutathione (3.5 min^{-1} M^{-1}). However, in the serine system, like in the aspartate system, the highest catalytic efficiencies were observed with L-cysteine (15 min^{-1} M^{-1}) and DTT (28 min^{-1} M^{-1}). Other thiols were 9−44-fold less catalytically efficient than L-cysteine in enzymatic deacylation of [^{3}H]-Ser-tRNA^{Ser}.

In contrast to enzymatic deacylations of [^{3}H]Asp-tRNA^{Asp} and [^{3}H]Ser-tRNA^{Ser}, enzymatic deacylation of [^{14}C]Lys-tRNA^{Lys} was stimulated only by some thiols and not by others. For example, L-cysteine, DTT, and D,L-homocysteine, but not cysteamine, 2-mercaptoethanol, glutathione, or 3-mercaptopropionate, stimulated enzymatic deacylation of [^{14}C]Lys-tRNA^{Lys} (Table 3).

The data suggest the presence on these class II AARSs of a specific thiol-binding site (-SH subsite) that affects enzymatic deacylation of a cognate aminoacyl-tRNA. The thiol binding specificity of this site is unique for each AARS.

Identification of Products of Thiol-Dependent Enzymatic Deacylations of Asp-, Ser-, and Lys-tRNA. To analyze products of thiol-dependent enzymatic deacylations, aliquots of reaction mixtures were subjected to TLC. Figure 3 depicts autoradiograms exposed from the TLC separations. In reaction mixtures containing AspRS, [^{3}H]Asp-tRNA^{Asp}, and L-cysteine (lane 2), L-cysteine methyl ester (lane 3), cysteamine (lane 4), 3-mercaptopropionate (lane 5), *N*-acetyl-L-cysteine (lane 6), D,L-homocysteine (lane 8), or DTT (lane 10), new products formed (Figure 3A). Different mobilities of these products suggest that they were formed as a result of an enzymatic reaction between a thiol and [^{3}H]Asp-tRNA^{Asp}. A nonspecific product (migrating with about twice the mobility of aspartate) observed in control lane 1, as well as in lanes 4−9, is most likely a glycerol ester of [^{3}H]-aspartate (because stock solutions of AspRS are prepared in 50% glycerol, reaction mixtures contain 5% glycerol, which is known to react nonenzymatically with aminoacyl-tRNA; Jakubowski, 1996b). An additional control showed that *S*-methyl-L-cysteine did not react with [^{3}H]Asp-tRNA^{Asp} (lane 7, Figure 3A), indicating that a free thiol group of a substrate is essential for the reaction.

Figure 3C depicts similar TLC analyses of reaction mixtures containing SerRS, [^{3}H]Ser-tRNA^{Ser}, and L-cysteine (lane 2), L-cysteine methyl ester (lane 3), cysteamine (lane 4), 3-mercaptopropionate (lane 5), *N*-acetyl-L-cysteine (lane 6), D,L-homocysteine (lane 8), or DTT (lane 10). As in the aspartate system, with each of these thiols, new products were observed (Figure 3C), well separated from serine and a nonspecific product (see a spot above serine in lane 1 and similar spots in lanes 2−10; most likely glycerol esters of [^{3}H]serine).

To determine identity of products of enzymatic deacylations of [^{3}H]Asp-tRNA^{Asp} and [^{3}H]Ser-tRNA^{Ser}, several tests

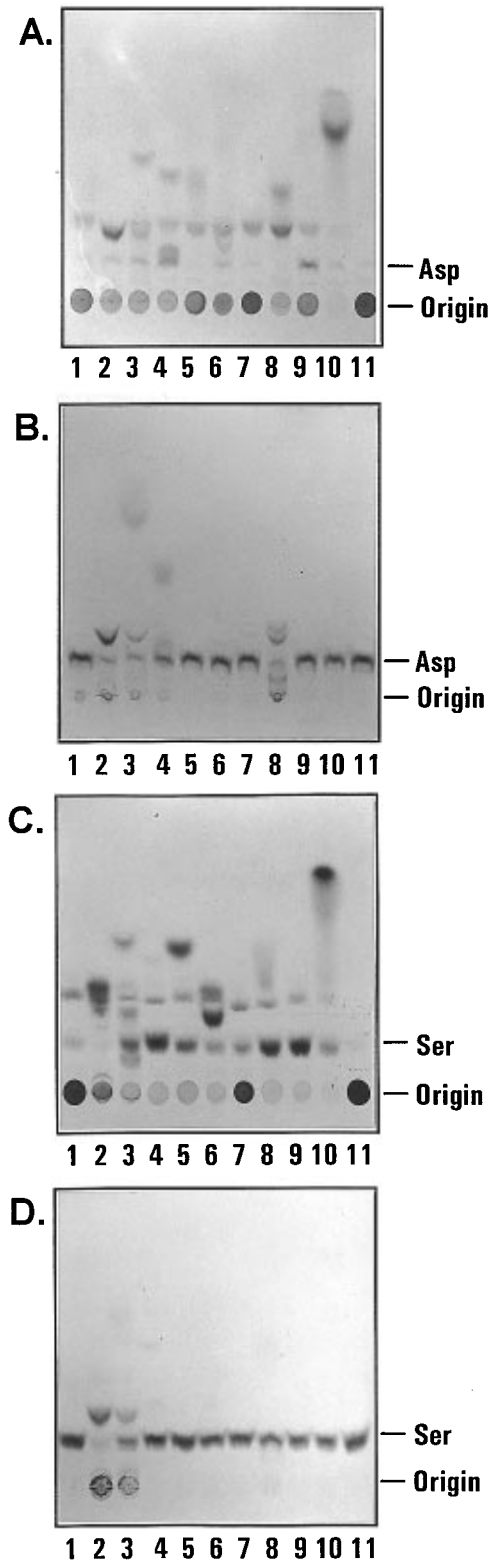


FIGURE 3: TLC analysis of products of enzymatic deacylation of aminoacyl-tRNA by class II AARSs. Reaction mixtures containing [^{3}H]Asp-tRNA^{Asp} and AspRS (panels A and B) or [^{3}H]Ser-tRNA^{Ser} and SerRS (panels C and D) and indicated thiols at 40 mM in a standard buffer were incubated at 37 °C for 30 min and analyzed by TLC. Reaction mixtures were analyzed directly (panels A and C) or after treatment with 0.1 N NaOH for 30 min followed by acidification with 1 N formic acid (panels B and D). (Panels A−D) Lanes 1 and 11, controls incubated at 37 and 0 °C, respectively; lane 2, L-cysteine; lane 3, L-cysteine methyl ester; lane 4, cysteamine; lane 5, 3-mercaptopropionate; lane 6, *N*-acetyl-L-cysteine; lane 7, *S*-methyl-L-cysteine; lane 8, D,L-homocysteine; lane 9, 2-mercaptoethanol; lane 10, DTT. Unreacted aminoacyl-tRNA stays at the origin.

were performed. Products formed from [^{3}H]Asp-tRNA^{Asp} in the presence of 3-mercaptopropionate (compare lanes 5 in Figure 3A,B), *N*-acetyl-L-cysteine (lanes 6 in Figure 3A,B), and DTT (lanes 10 in Figure 3A,B) yielded [^{3}H]-aspartate upon NaOH treatment. A nonspecific product observed in control lane 1, as well as in lanes 4−9, was also converted to [^{3}H]aspartate upon NaOH treatment. Residual [^{3}H]Asp-tRNA^{Asp} present in controls (lanes 1 and 11) and test samples (lanes 2−9) yielded [^{3}H]aspartate upon NaOH treatment, as expected. Although D,L-homocysteine-dependent products were sensitive to NaOH, the NaOH treatment did not yield [^{3}H]aspartate (lanes 8, Figure 3A,B). On the other hand, products formed during enzymatic deacyaltion of [^{3}H]Asp-tRNA^{Asp} in the presence of L-cysteine were not sensitive to NaOH (compare lanes 2 in Figure 3A,B). Similar lack of sensitivity to NaOH was exhibited by products formed from [^{3}H]Asp-tRNA^{Asp} in the presence of L-cysteine methyl ester (lanes 3 in Figure 3A,B) and cysteamine (lanes 4 in Figure 3A,B). These results indicate that products formed in the presence of 3-mercaptopropionate, *N*-acetyl-L-cysteine, and DTT are most likely corresponding thioesters of aspartate. Products of L-cysteine-, L-cysteine methyl ester-, and cysteamine-dependent deacylations are not thioesters. An apparent sensitivity to NaOH of products formed in the presence of D,L-homocysteine (compare lanes 8 in Figure 3A,B) is most likely due to their oxidation to disulfides.

Products formed from [^{3}H]Ser-tRNA^{Ser} in the presence of 3-mercaptopropionate (compare lanes 5 in Figure 3C,D), *N*-acetyl-L-cysteine (lanes 6 in Figure 3C,D), D,L-homocysteine (lanes 8 in Figure 3C,D), and DTT (lanes 10 in Figure 3C,D) yielded [^{3}H]serine upon NaOH treatment. A nonspecific product observed in control lane 1, as well as in lanes 3−10, was also converted to [^{3}H]serine upon NaOH treatment. Residual [^{3}H]Ser-tRNA^{Ser} present in controls (lanes 1 and 11) and test samples (lanes 2−10), yielded [^{3}H]-serine upon NaOH treatment, as expected. Although products formed during enzymatic deacyaltion of [^{3}H]Ser-tRNA^{Ser} in the presence of L-cysteine were sensitive to NaOH, the NaOH treatment did not yield [^{3}H]serine (compare lanes 2 in Figure 3C,D). This apparent sensitivity to NaOH of a major product of cysteine-dependent deacylation is most likely due to its oxidation to a disulfide. NaOH treatment of products formed during enzymatic deacylation of [^{3}H]-Ser-tRNA^{Ser} in the presence of L-cysteine methyl ester yielded two products: one was [^{3}H]serine and the other was similar to a product observed in lane 2, Figure 3D (compare lines 3 in Figure 3C,D). These results indicate that products of deacylation reactions formed in the presence of 3-mercaptopropionate, *N*-acetyl-L-cysteine, D,L-homocysteine, and DTT are most likely corresponding thioesters of serine. A major product of L-cysteine-dependent deacylation is not a thioester.

Additional tests were performed to determine the identity of a product of cysteine-dependent enzymatic deacylation of [^{3}H]Ser-tRNA^{Ser}. As shown in Figure 4, Raney nickel treatment, which desulfurizes cysteine to alanine, resulted in formation of a compound that comigrated with an authentic dipeptide Ser-Ala (lane 4). A product of cysteine-dependent deacylation was also sensitive to treatment with 5,5′-dithiobis(2-nitrobenzoate) (DTNB), a sulfhydryl group reagent (lane 6). Raney nickel treatment of a control deacylation mixture without cysteine did not yield Ser-Ala;

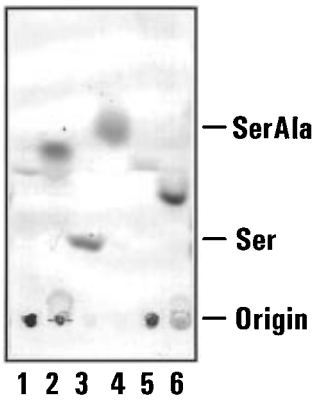


FIGURE 4: Identification of products formed during L-cysteine-dependent enzymatic deacylation of [^{3}H]Ser-tRNA^{Ser} by SerRS. [^{3}H]-Ser-tRNA^{Ser} was deacylated in the absence (lanes 1, 3, and 5) or presence of 40 mM L-cysteine (lanes 2, 4, and 6) and SerRS. Deacylation products were analyzed by TLC directly (lanes 1 and 2) and after treatment with Raney nickel (Sigma) (lanes 3 and 4) or 5,5′-dithiobis(2-nitrobenzoate) (lanes 5 and 6). An autoradiogram exposed from the TLC separation is shown. Positions of authentic serine and the dipeptide Ser-Ala on the TLC plate are indicated. Unreacted [^{3}H]Ser-tRNA^{Ser} stays at the origin.

instead [^{3}H]Ser-tRNA^{Ser} present in this control was deacylated to [^{3}H]serine (lane 3), as expected. DTNB-treated control deacylation mixture without cysteine (lane 5) was not different from a corresponding untreated sample (lane 1). These results indicate that a major product of enzymaticdeacylation of Ser-tRNA in the presence of cysteine is the dipeptide Ser-Cys.

TLC analyses of reaction mixtures containing LysRS, [^{14}C]-Lys-tRNA^{Lys}, and thiols were also performed. A new major distinct product formed in deacylation mixtures containing 20 mM DTT. With 80 mM cysteine and 40 mM 3-mercaptopropionate, new minor products, in addition to the major product lysine, were observed. With 20 mM cysteine, only lysine was formed during enzymatic deacylation of Lys-tRNA. NaOH treatment of new products formed in the presence of DTT and 3-mercaptopropionate yielded [^{14}C]-lysine, whereas a new product observed in the presence of cysteine was not sensitive to NaOH (not shown). The product formed in the presence of 3-mercaptopropionate was not sensitive to a thiol reagent, DTNB. These tests indicate that products formed in the presence of DTT and 3-mercaptopropionate, but not cysteine, are corresponding thioesters of lysine. The product formed in the presence of cysteine is a dipeptide, Lys-Cys.

Nonenzymatic Reactions of [^{3}H]Asp-tRNA^{Asp}, [^{14}C]Lys-tRNA^{Lys}, and [^{3}H]Ser-tRNA^{Ser} with Thiols. Although the rates of nonenzymatic deacylations were not affected by thiols, this does not exclude that thiols react nonenzymatically with aminoacyl-tRNA at rates slower than the rate of thiol-independent deacylation. To determine to what extent cysteine and other thiols react nonenzymatically with Asp-tRNA, Lys-tRNA, and Ser-tRNA, [^{3}H]Asp-tRNA^{Asp}, [^{14}C]-Lys-tRNA^{Lys}, and [^{3}H]Ser-tRNA^{Ser} were incubated with 100 mM thiol at pH 7.4, 37 °C, for 1 h and the products were analyzed by TLC. In addition to a corresponding radiolabeled amino acid as a major product, reactions of [^{3}H]Asp-tRNA^{Asp} and [^{14}C]Lys-tRNA^{Lys} with cysteine yielded ~2% Asp-Cys and Lys-Cys, respectively. [^{3}H]Ser-tRNA^{Ser} did not react with cysteine. All three aminoacyl-tRNAs reacted with DTT yielding ~2% aminoacyl-DTT, in addition to a radiolabeled amino acid as a major product. These extents of nonenzymatic thiolysis of Asp-tRNA, Lys-tRNA, and Ser-tRNA are about 10 times lower that those observed with Arg-tRNA (Jakubowski, 1995a) but similar to those observed previously with Met-tRNA (Jakubowski, 1996b).

Coupling relative amounts of Asp-X, Lys-X, and Ser-X with an overall first-order rate constant for nonenzymatic deacylation (k = 0.017 min^{-1}, Table 3), one can estimate that the apparent rate constants for the reactions of the three aminoacyl-tRNAs with thiols were ~0.0003 min^{-1} (at 100 mM thiol). Thus, nonenzymatic reactions of Asp-tRNA, Lys-tRNA, and Ser-tRNA with thiols were up to 30 000 times slower than the enzymatic reactions (see Table 3).

DISCUSSION

This work demonstrates the following: (1) LysRS, a class II enzyme, edits homocysteine by converting it into homocysteine thiolactone. In a similar reaction, LysRS converts homoserine into homoserine lactone. (2) Two other class II enzymes, AspRS and SerRS, do not seem to possess editing function. (3) Thiol-dependent deacylations of aminoacyl-tRNA catalyzed by AspRS, LysRS, and SerRS yield aminoacyl thioesters, mimicking thioester bond formation during editing of homocysteine. (4) Enzymatic reactions between cysteine and an aminoacyl-tRNA catalyzed by AspRS, LysRS, and SerRS yield dipeptides Asp-Cys, Lys-Cys, and Ser-Cys, respectively.

The finding of this work that class II LysRS possesses editing function, particularly efficient with homocysteine and cysteine, supports a hypothesis that a major selectivity problem for AARSs is with thioamino acids. Hcy is misactivated and edited *in vitro* by five AARSs: MetRS, IleRS, ValRS (Jakubowski & Fersht, 1981), LeuRS (Englisch et al., 1986), and LysRS (this work); of these, MetRS, IleRS, and LeuRS edit homocysteine *in vivo* (Jakubowski, 1990, 1991, 1995b; Jakubowski & Goldman, 1993; Gao et al., 1994). Cysteine is misactivated and edited *in vitro* by three AARS: ValRS (Igloi et al., 1978; Jakubowski, 1980; Jakubowski & Fersht, 1981), IleRS (Jakubowski & Fersht, 1981), and LysRS (this work). No other amino acids are known to be misactivated and edited by so many AARSs.

Conversion of homocysteine to a cyclic thioester, homocysteine thiolactone, by LysRS as described in this work suggests that the side chain of homocysteine directly participates in the editing reaction by a nucleophilic attack on the activated carboxyl group of Hcy-AMP, a mechanism similar to that described previously for editing of homocysteine by MetRS, IleRS, ValRS (Jakubowski & Fersht, 1981), and LeuRS (Englisch et al., 1986).

The *in vitro* cyclization of homoserine to homoserine lactone by LysRS, described in this work, most likely occurs by a similar mechanism (Scheme 2). Although homoserine lactone has been shown to be an inducer of stationary gene expression in *E. coli*, the mechanism of the lactone formation *in vivo* is not known (Huisman & Colter, 1994). It remains to be established whether LysRS is involved in the synthesis of homoserine lactone *in vivo*.

Formation of the dipeptides Asp-Cys, Lys-Cys, and Ser-Cys catalyzed by AspRS, LysRS, and SerRS, respectively, is consistent with the following mechanism (Figure 5). The ester bond in Asp-tRNA, Lys-tRNA, or Ser-tRNA undergoes thiolation by the sulfhydryl group of cysteine. The resulting

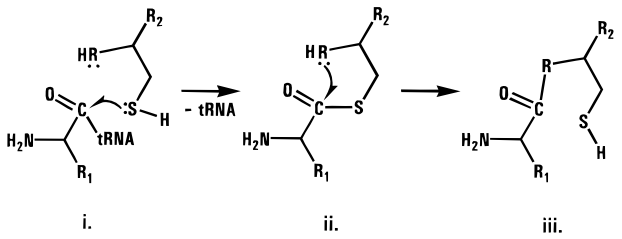


FIGURE 5: Proposed mechanism for reactions of thiols with aminoacyl-tRNA. Formation of aminoacyl thioester (**ii**) from Asp-, Lys-, or Ser-tRNA and a thiol (**i**) is catalyzed by AspRS, LysRS, or SerRS, respectively. When R = NH, facile rearrangement of the thioester **ii** to form a peptide bond in **iii** occurs spontaneously (Wieland & Pfeiderer, 1957). R_1 is the side chain of aspartate, lysine, or serine. Cysteine, R = NH and R_2 = COOH; *N*-acetylcysteine: R = acetyl-N and R_2 = COOH; cysteamine, R = NH and R_2 = H; 3-mercaptopropionate, R is absent and R_2 = COOH; DTT, R = O and R_2 = $CH(OH)CH_2SH$.

thioester (**ii** in Figure 5) is not observed as an intermediate because of the rapid rearrangement to form a stable peptide bond (**iii** in Figure 5). Thioesters of cysteine are known to undergo facile intramolecular reaction resulting from the favorable geometric arrangement of the α-amino group of cysteine with respect to the thioester bond (Wieland & Pfleiderer, 1957). This mechanism is supported by the following observations. First, the -SH group is required for the reaction (serine and *S*-methylcysteine do not react with Asp-tRNA nor with Ser-tRNA). Second, aspartate, lysine, and serine thioesters do indeed form with thiols that do not have an additional amino group, for example with 3-mercaptopropionate (**ii** in Figure 5). A similar mechanism accounts for synthesis of the dipeptides Arg-Cys, Val-Cys (Jakubowski, 1995a), Ile-Cys (Jakubowski, 1995a, 1996a), and Met-Cys (Jakubowski, 1996b) by corresponding class I AARSs.

The data presented in this work support the conclusion that reactions between thiols and aminoacyl-tRNA involve binding of a thiol to a specific site on an aminoacyl-tRNA·AARS complex and not simple binary collisions between a thiol and the complex. Although it is possible that the aminoacyl ester bond in an aminoacyl-tRNA·AARS complex is in general more chemically reactive than the same bond in free aminoacyl-tRNA, the data seem to indicate otherwise. For example, the aminoacyl ester bond in an aminoacyl-tRNA·AARS complex is not more susceptible to hydrolysis than the same bond in free aminoacyl-tRNA (Table 3). However, hydroxylamine, a potent nucleophile, was found to be much less effective than thiols in promoting enzymatic deacylation of Asp-tRNA, Ser-tRNA, and Lys-tRNA. Actually, in the presence of 125 mM hydroxylamine, free Lys-tRNA was deacylated 4 times faster ($t_{0.5}$ = 9 min) than Lys-tRNA in a complex with LysRS ($t_{0.5}$ = 40 min). In contrast, thiols did not accelerate nonenzymatic deacylation of aminoacyl-tRNA (not shown). Thus, there is a thiol-specific, but not general, increase in the reactivity of the aminoacyl ester bond upon binding of an aminoacyl-tRNA to an AARS.

At least in the AspRS and LysRS systems, kinetics of the enzymatic reactions of thiols with aminoacyl-tRNA are also consistent with binding of a thiol to a specific site on an aminoacyl-tRNA·AARS complex and not simple binary collisions between a thiol and the complex. For example, dependencies of rates of thiolysis of aminoacyl-tRNA on concentrations of DTT (in the AspRS and LysRS systems), cysteine, and homocysteine (in the AspRS system) exhibit saturation kinetics typical of enzymatic reactions involving enzyme−substrate complexes. In addition, thiolysis of aminoacyl-tRNA exhibits substrate (thiol) specificity (Table 3), which is a hallmark of enzymatic reactions involving substrate binding.

The data presented in this work with class II AARSs, in conjunction with previous data obtained with class I AARSs, indicate that each of the examined AARSs possesses unique thiol- (cysteine-) binding subsites within their active sites. With MetRS (Jakubowski, 1996b), IleRS (Jakubowski, 1996a), ValRS (Jakubowski, 1995a), and LysRS (this work), a thiol-binding subsite participates in rejecting homocysteine from the synthetic/editing active site. That a similar thiol-binding sub-site exists also in AARSs that do not need editing function, such as class I ArgRS (Jakubowski, 1995a) and class II AspRS and SerRS (this work), indicates that these synthetases possess a *vestigial* editing function. The existence of the *vestigial* editing function in some of the present-day AARSs suggests that ancestral forms of these AARSs may have been less accurate in initial selection of their cognate amino acids and needed editing function. Considering that homocysteine, unlike any other amino acid, is edited by five present-day AARSs, this thioamino acid may have presented a major selectivity problem for most, if not all, ancestral AARSs. Alternatively, the thioester chemistry may have been responsible for the origin of aminoacylation reactions in the "thioester world" (de Duve, 1994) and may have been used to achieve aminoacylation of tRNA by ancestral AARSs. Thioesters of amino acids form spontaneously under acidic conditions, which could have provided substrates for ancestral tRNA aminoacylation in the absence of ATP. However, so far my attempts to reverse the aminoacyl thioester bond formation have been unsuccessful.

Finally, facile formation of aminoacyl-Cys dipeptides (Jakubowski, 1995a, 1996a,b; this work) and aminoacyl-Cys-Gly tripeptides (Jakubowski, 1996b) by AARSs is reminiscent of nonribosomal peptide bond formation utilized in the thiotemplate synthesis of peptide antibiotics (Kleinkauf & van Dören, 1996) and suggests a functional similarity between the two amino acid activating systems (despite the lack of structural similarities). In the multienzyme thiotemplate systems, activated amino acids are condensed to peptides via thioester intermediates. In the AARS systems activated amino acids are condensed with cysteine (or Cys-Gly) to form dipeptides (or tripeptides), also via thioester intermediates.

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