The Synthetic/Editing Active Site of an Aminoacyl-tRNA Synthetase: Evidence for Binding of Thiols in the Editing Subsite†

Hieronim Jakubowski*

Department of Microbiology & Molecular Genetics, UMDNJ−New Jersey Medical School, Newark, New Jersey 07103

Received February 13, 1996; Revised Manuscript Received April 18, 1996⊗

ABSTRACT: The active site of methionyl-tRNA synthetase (MetRS) possesses two functions: synthetic, which provides Met-tRNA for protein synthesis, and editing, which rejects inadvertently misactivated homocysteine. During editing, the side chain -SH group of homocysteine reacts with its activated carboxyl group forming a cyclic thioester, homocysteine thiolactone. As shown here, the side chain -SH and the activated carboxyl groups do not need to be present on the same molecule for the editing to occur. Thioester formation occurs when a thiol and activated methionine, in the form of Met-tRNA, are incubated with MetRS. Depending on the structure of thiols, methionine thioesters may undergo secondary acyl transfer reactions to *cis* amino, hydroxy, or carboxyl groups which yield methionine dipeptides, esters, or anhydrides, respectively. At saturating thiol concentrations, formation of some thiol derivatives of methionine is as fast as formation of homocysteine thiolactone. Thiol specificity of the reaction and noncompetitive inhibition by the cognate methionine, as well as structure−function studies of active site MetRS mutants, all indicate that there is a specific -SH binding subsite, distinct from the methionine binding subsite, in the synthetic/editing active site of MetRS.

Editing reactions are an essential component of biological information transfer processes, including translation. The editing in amino acid selection for protein synthesis by aminoacyl-tRNA synthetases, the first proofreading process discovered in the flow of genetic information (Norris & Berg, 1964), prevents attachment of incorrect amino acids to tRNA (reviewed in Jakubowski & Goldman, 1992; Jakubowski, 1994a). A major selectivity problem for aminoacyl-tRNA synthetases is with thio-amino acids such as homocysteine and cysteine. Homocysteine is misactivated by MetRS[1] (Old & Jones, 1977; Fersht & Dingwall, 1979; Smith & Cohen, 1981; Jakubowski & Fersht 1981), IleRS (Jakubowski & Fersht, 1981), and LeuRS (Englisch et al., 1986) at frequencies exceeding the frequency of tranaslational errors *in vivo* (reviewed in Parker, 1989; Jakubowski & Goldman, 1992). Two other synthetases, ValRS (Jakubowski, 1980; Jakubowski & Fersht, 1981) and LysRS (H. Jakubowski, unpublished), misactivate homocysteine less efficiently. Cysteine is also misactivated by IleRS (Jakubowski & Fersht, 1981), ValRS (Jakubowski, 1980; Jakubowski & Fersht, 1981), and LysRS (H. Jakubowski, unpublished). Misactivated thio-amino acids are efficiently edited *in vitro* (Jakubowski, 1980; Jakubowski & Fersht, 1981; Englisch et al., 1986). Editing of homocysteine has also been shown to occur *in vivo* (Jakubowski, 1990, 1991, 1993a, 1995a; Jakubowski & Goldman, 1993; Gao et al., 1994). The side chain thiol group of homocysteine directly participates in the editing reaction by a nucleophilic attack on the activated carboxyl group of Hcy-AMP to give homocysteine thiolactone (Jakubowski & Fersht, 1981).

A fundamental question is how the structure of the active site determines the accuracy of the aminoacylation reaction. Are the synthetic and editing sites physically separated or are they subsites of a single active site? What are the structural elements of the active site that prevent a cognate substrate from being edited and a noncognate substrate from completing the synthetic pathway? We have begun to answer these questions with MetRS (Kim et al., 1993), a class I aminoacyl-tRNA synthetase whose crystallographic structure is known (Brunie et al., 1990). Our molecular model proposes that a single active site of MetRS partitions an amino acid substrate between synthetic and editing pathways. Hydrophobic and hydrogen bonding interactions direct the cognate methionine through the synthetic pathway and prevent it from entering the editing pathway. Some of these interactions are missing in the case of the noncognate homocysteine, which therefore enters the editing pathway (Kim et al., 1993). The chemistry of homocysteine editing, involving thioester bond formation, requires the existence of a subsite (-SH subsite) that specifically binds the side chain -SH group of homocysteine in the active site. The existence of such an -SH subsite is documented in this paper.

MATERIALS AND METHODS

Plasmids and Host Strain. A plasmid containing the full length gene for *Escherichia coli* MetRS was obtained from S. Blanquet (Brevet et al., 1989). Plasmids encoding mutant MetRSs were obtained as previously described (Ghosh et al., 1991; Kim et al., 1993). Plasmids were maintained in *E. coli* strain JM101 and used as a source of MetRS. Cells for enzyme purification were obtained from overnight cultures (usually 400 mL, yielding about 2 g of cells) grown at 37 °C in LB medium containing 100 μg/mL ampicillin.

† This research was supported by grants from the American Cancer Society (NP-904) and the National Science Foundation (MCB-9513127).

* Phone: (201) 982-4679; Fax: (201) 982-3644; E-mail: jakubows@umdnj.edu.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: MetRS, methionyl-tRNA synthetase; other synthetases similarly abbreviated; DTNB, 5,5'-dithiobis(2-nitrobenzoate).

S0006-2960(96)00344-3 CCC: $12.00

Methionyl-tRNA Synthetases. MetRSs were purified to homogeneity from the overproducing strains as described in (Gao et al., 1994). Full length MetRS676 dimer (Brevet et al., 1989) was used for most experiments. Truncated, but active MetRS547 monomer (Ghosh et al., 1991; Gao et al., 1994) and its derivatives (Kim et al., 1993) were used in experiments described in Table 2.

Preparation of [^{3}H]Met-tRNA^{Met} and [^{35}S]Met-tRNA^{Met}. 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^{fMet} (Subriden RNA), 14.5 μM [^{3}H]methionine (1.25 mCi/mL, 86 Ci/mmol) or 50 μM [^{35}S]methionine (0.50 mCi/mL, 10 Ci/mmol) (Amersham), and 0.5 μM MetRS. 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 methionine, dissolved in 0.2 mL of glass-distilled water, and stored at −20 °C. Alternatively, radiolabeled Met-tRNA was prepared using 10 mg/mL unfractionated *E. coli* tRNA (Schwartz Mann) (10 mg/mL tRNA contains 8 μM tRNA^{Met} + tRNA^{fMet}).

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

TLC Analysis. TLC separations were carried out on cellulose plates from Kodak using butanol/acetic acid/water (4:1:1 v/v) as solvent (Jakubowski & Fersht, 1981; Jakubowski, 1993b, 1994b, 1995b). An authentic methionine (Sigma) standard was cochromatographed with samples and visualized by staining with ninhydrin. TLC plates were autoradiographed using Reflection (NEN) autoradiography film. Exposure times were 1−3 days and 4 weeks for experiments with [^{35}S]methionine and [^{3}H]methionine, respectively. Quantitation of spots from TLC separations in some experiments was carried out using a Molecular Dynamics phosphorimager.

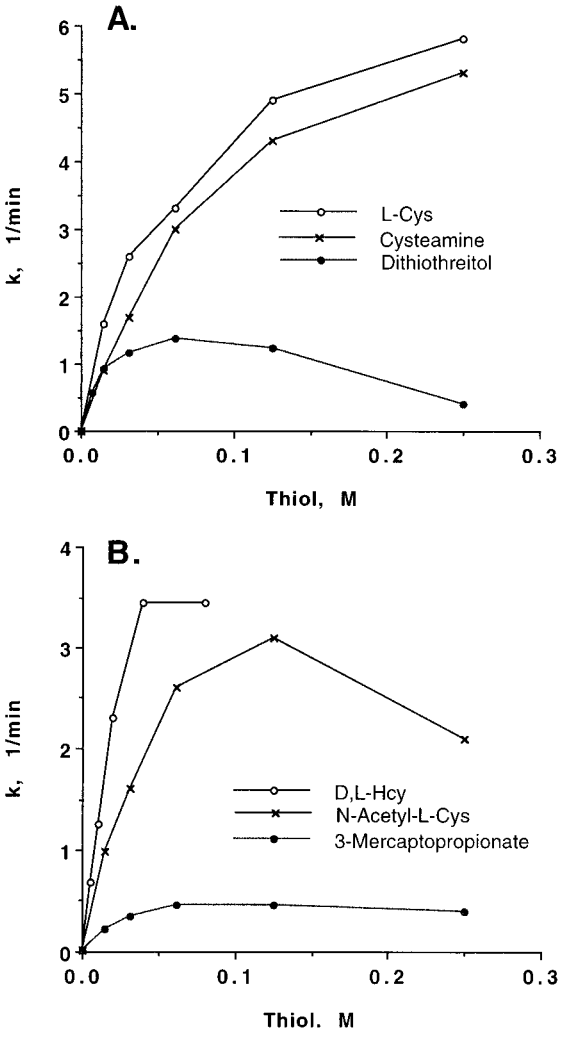

FIGURE 1: The rate constants for enzymatic deacylation of Met-tRNA^{fMet} as a function of thiol concentration. Reactions were carried out at 37 °C in mixtures containing 0.5 μM [^{35}S]Met-tRNA^{fMet}, 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 5−250 mM indicated thiol, and 0.05 μM MetRS (panel A) or 1 μM MetRS (panel B). Panel A: L-Cysteine (○), cysteamine (×), and dithiothreitol (●). Panel B: 3-Mercaptopropionate (●), *N*-acetyl-L-cysteine (×), and D,L-homocysteine (○). The rate constants, *k*, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln 2/t_{0.5}$.

RESULTS

Thiol-Dependent Enzymatic Deacylation of Met-tRNA: Kinetic Indices. At pH 7.4, 37 °C, [^{35}S]Met-tRNA^{Met} in a complex with MetRS is relatively stable ($t_{0.5} \sim 60$ min). However, addition of thiols (10 mM) such as cysteine, cysteamine, Cys-Gly, 2-mercaptoethanol, or dithiothreitol resulted in essentially complete deacylation of [^{35}S]Met-tRNA^{Met} within <1 min. Serine and homoserine did not accelerate deacylation of Met-tRNA. Systematic measurements of steady state kinetics of thiol-dependent deacylations of [^{35}S]Met-tRNA^{Met} showed that the reactions exhibited saturation kinetics with respect to thiol concentration for all thiols tested (Figure 1). Kinetic indices are summarized in Table 1.

Up to 200-fold variation in catalytic efficiencies was observed between different thiols. The reaction was the fastest with cysteine and cysteamine; $k_{cat} = 1.12$ s^{-1} and 1.23 s^{-1}, respectively, were essentially identical to the value of $k_{cat} = 1.2$ s^{-1} for editing of homocysteine by MetRS (Jakubowski & Fersht, 1981; Kim et al., 1993). The data presented in Table 1 indicate that the -SH group is the major determinant of catalytic efficiency of cysteine. The carboxyl group of cysteine is not required for the reaction: cysteine derivatives in which the carboxyl group has been removed (cysteamine), methylated (cysteine methyl ester), or amidated (Cys-Gly) were almost as efficient substrates as cysteine. The amino group of cysteine, although not absolutely required, is important. Cysteine derivatives in which the amino group has been removed (3-mercaptopropionate) or acetylated (*N*-acetyl-L-cysteine) stimulated the reaction less efficiently than cysteine by 66- or 17-fold, respectively. Glutathione, in which both amino and carboxyl groups of cysteine are blocked, was 200-fold less efficient than cysteine. These observations indicate that the effects of thiols are not merely due to reactivation of essential sulfhydryl groups on the enzyme and that thiols bind to the MetRS·Met-tRNA^{Met} complex.

The K_m values for thiols in the thiol-dependent enzymatic deacylation of Met-tRNA are high, from 18 mM to 100 mM (Table 1), suggesting relatively weak binding of thiols to

Table 1: Kinetic Indices for Thiol-Dependent Enzymatic Deacylation of Met-tRNA[a]

thiol	k_{cat} (min^{-1})	K_m (K_i) (M)	k_{cat}/K_m (min^{-1} M^{-1})
none	0.010		
none, no enzyme	0.017		
L-cysteine (1−250 mM)	69	0.05	1380
cysteamine (15−250 mM)	74	0.10	740
3-mercaptopropionate (10−250 mM)	0.74	0.035 (0.17)	21
D,L-homocysteine (10−250 mM)	23	0.10	230
L-cysteine methyl ester (20 mM)			1060
N-acetyl-L-cysteine (15−250 mM)	5.7	0.068 (0.065)	83
L-Cys-Gly (5−10 mM)			1380
glutathione (10−20 mM)			6.8
thioglycolate (10 mM)			150
dithiothreitol (1−250 mM)	17.2	0.018 (0.12)	960
2-mercaptoethanol (1 mM)			460

[a] Reactions were carried out at 37 °C in mixtures contained 0.5 μM [^{3}H]Met-tRNA^{fMet}, 0.025−1 μM MetRS, 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, and 0.2 mM EDTA. 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 $k = \ln(2)/t_{0.5}$, correcting for enzyme concentration where necessary. These rate constants were used to calculate k_{cat}, K_m, and k_{cat}/K_m values for indicated thiols according to $k = k_{cat} - K_m k/S$, where S is thiol concentration. In cases where inhibition was observed, K_i values were obtained from slopes of k *vs* k/S plots at highest thiol concentrations.

MetRS. For comparison, the K_m value for homocysteine is 5 mM in both the synthetic reaction (measured in the homocysteine-dependent ATP−PP_i exchange reaction; Jakubowski & Fersht, 1981) and the editing reaction (measured in the homocysteine-dependent ATP−pyrophosphatase reaction; not shown).

Comparison of Enzymatic and Nonenzymatic Deacylation of Met-tRNA^{fMet}. To prove that stimulation of the enzymatic deacylation by cysteine is mediated by MetRS, effects of cysteine on a nonenzymatic deacylation of Met-tRNA^{fMet} were also determined. The slow rate of nonenzymatic deacylation ($k = 0.017$ min^{-1}, Table 1) was not affected by 20 or 125 mM cysteine, D,L-homocysteine, and dithiothreitol. However, these thiols at high concentrations reacted nonenzymatically with Met-tRNA^{Met} to a small extent (see below).

Identification of Products of Thiol-Dependent Deacylations of Met-tRNA. [^{35}S]Met-tRNA^{Met} was enzymatically deacylated in the absence and presence of various thiols as described in the legend to Figure 2. Reaction mixtures were analyzed by TLC, and the products were visualized by autoradiography. In the presence of cysteine (lane 2), cysteine methyl ester (lane 4), 3-mercaptopropionate (lane 5), 2-mercaptoethanol (lane 10), and dithiothreitol (lane 11), a distinct major product, well separated from the methionine standard, was formed in each reaction. Products formed in the presence of cysteamine (lane 3), *N*-acetyl-L-cysteine (lane 6), and D,L-homocysteine (lane 9) were less well separated from the methionine standard. Further analyses, including separation of these products on silica gel TLC plates, proved that no methionine was formed in reactions with cysteamine and *N*-acetyl-L-cysteine (not shown). Different mobilities of these new products (lanes 2, 4, 5, 10, and 11) suggest that they are formed as a result of a reaction between a thiol and Met-tRNA. Reaction products formed in the presence

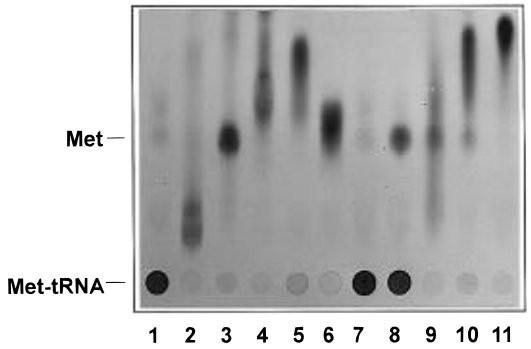

FIGURE 2: TLC analysis of products of enzymatic deacylation of Met-tRNA^{fMet}. Reactions were carried out at 37 °C for 5 min in mixtures containing 0.5 μM [^{35}S]Met-tRNA, 1 μM MetRS, 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, and no thiols (lane 1) or 20 mM cysteine (lane 2), cysteamine (lane 3), cysteine methyl ester (lane 4), 3-mercaptopropionate (lane 5), *N*-acetyl-L-cysteine (lane 6), *S*-methyl-L-cysteine (lane 7), methionine (lane 8), D,L-homocysteine (lane 9), 2-mercaptoethanol (lane 10), and dithiothreitol (lane 11). The position of an authentic methionine standard on the TLC plate is indicated. [^{35}S]Met-tRNA^{Met}, where present, stays at the origin.

of *S*-methyl-L-cysteine (lane 7) were not different from control (lane 1). More radiolabeled methionine was formed in the presence of exogenous unlabeled methionine (lane 8) than in its absence (lane 1). Radiolabeled methionine (somewhat obscured in lane 9, Figure 2, but clearly visible in lanes 6, Figure 3) was also one of the products of the D,L-homocysteine-dependent deacylation.

To determine identity of these products, several tests were performed. Some of these analyses are shown in Figure 3. Products formed in the presence of cysteine (lane 2) were not sensitive to NaOH treatment (compare lanes 2 in Figure 3A and 3B). Similar lack of sensitivity to NaOH treatment was exhibited by products formed in the presence of cysteamine (lanes 4 in Figure 3A and 3B) and D,L-homocysteine (lanes 6 in Figure 3A and 3B), Cys-Gly, and cysteine methyl ester (not shown). On the other hand, products formed in the presence of 3-mercaptopropionate (compare lanes 3 in Figure 3A and 3B) yielded methionine upon NaOH treatment. Similar sensitivity to NaOH treatment was exhibited by products formed in the presence of *N*-acetyl-L-cysteine, dithiothreitol, and 2-mercaptoethanol (not shown). A product formed in the absence of thiols (compare lanes 1 in Figure 3A and 3B) was also sensitive to NaOH treatment. All products of thiol-dependent deacylations that were sensitive to NaOH treatment were also found to be sensitive to hydroxylamine treatment, yielding methionine hydroxamate (not shown). These results suggest that products of deacylation reactions formed in the absence of thiols and in the presence of 3-mercaptopropionate, *N*-acetyl-L-cysteine, dithiothreitol, and 2-mercaptoethanol are thioesters of methionine. Products of cysteine-, Cys-Gly-, cysteine methyl ester-, cysteamine-, and D,L-homocysteine-dependent deacylations are not thioesters.

Tests for the presence of the -SH group were also performed. A product formed in the presence of cysteine was sensitive to DTNB treatment (compare lanes 2 in Figure 3C and 3A). Similar sensitivity to DTNB was exhibited by products formed in the presence of cysteamine (compare lanes 4 in Figure 3C and 3A), D,L-homocysteine (lanes 6 in Figure 3C and 3A), Cys-Gly, and cysteine methyl ester (not shown). The slower migrating product formed in the

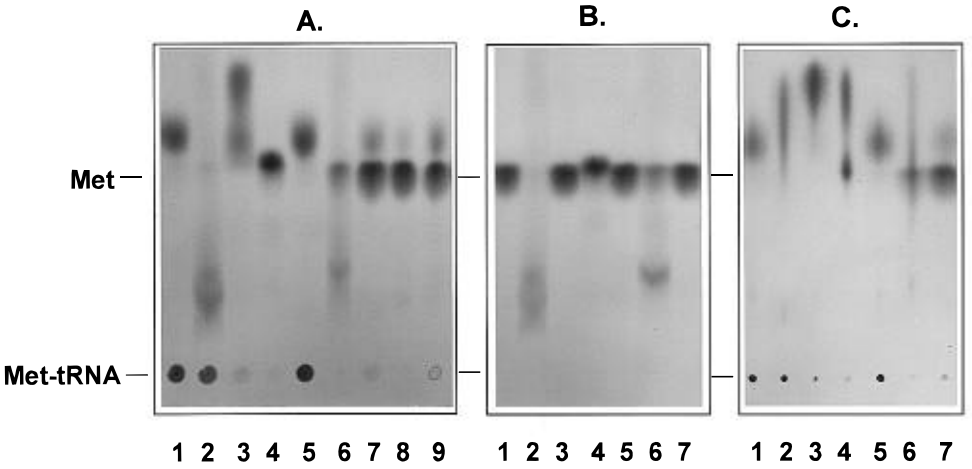

FIGURE 3: TLC analysis of products of enzymatic deacylation of Met-tRNA^{fMet}: sensitivity to NaOH and DTNB. Reactions were carried out at 37 °C for 15 min in mixtures containing 0.5 μM [^{35}S]Met-tRNA, 1 μM MetRS, 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, and 20 mM cysteine (lane 2), 3-mercaptopropionate (lane 3), cysteamine (lane 4), *S*-methyl-L-cysteine (lane 5), D,L-homocysteine (lane 6), methionine (lane 7), cysteine and methionine (lane 8), and D,L-homocysteine and methionine (lane 9) or no additions (lane 1). Reaction mixtures were subjected to TLC without any further treatment (panel A) or after 20-min treatment with 180 mM NaOH (panel B) or 35 mM 5,5′-dithiobis(2-nitrobenzoate) (DTNB) (pH 7.4) (panel C). The position of an authentic methionine standard on the TLC plate is indicated. [^{35}S]Met-tRNA^{Met} present in some reaction mixtures stays at the origin.

presence of 3-mercaptopropionate was sensitive to DTNB (compare lanes 3 in Figure 3C and 3A) and iodoacetate (not shown) treatments. However, the faster migrating product formed in the presence of 3-mercaptopropionate was not sensitive to DTNB (compare lanes 3 in Figure 3C and 3A) nor to iodoacetate (not shown). Similar lack of sensitivity to DTNB and iodoacetate was exhibited by products of *N*-acetyl-L-cysteine- and 2-mercaptoethanol-dependent deacylations (not shown). Thus, products of thiol-dependent enzymatic deacylations of Met-tRNA formed in the presence of cysteine, cysteine methyl ester, cysteamine, Cys-Gly, and D,L-homocysteine are methionine derivatives containing free -SH groups. Taken together, sensitivity to thiol reagents and lack of sensitivity to NaOH suggest that these products are the corresponding amides of methionine. Products formed in the presence of 3-mercaptopropionate, *N*-acetyl-L-cysteine, and 2-mercaptoethanol do not contain free -SH groups, but are sensitive to NaOH and hydroxylamine, which suggestes that they are corresponding thioesters of methionine.

With some thiols, such as 3-mercaptopropionate (lane 5, Figure 2; lane 3, Figure 3A) and 2-mercaptoethanol (lane 10, Figure 2), two new products were formed. Based on the tests described above, faster migrating products are corresponding thioesters of methionine. In both cases, the second, slower migrating product, in addition to being NaOH-sensitive, was also iodoacetate-sensitive. Thus, the slower migrating products are concluded to be a mixed anhydride of methionine and 3-mercaptopropionic acid, and a 2-mercaptoethanol ester of methionine, respectively.

As shown in control lanes of Figure 2 (lane 1) and Figure 3A (lane 1), a product different from methionine formed also during thiol-independent deacylation of Met-tRNA. This product was sensitive to NaOH (compare lanes 1 in Figure 3B and 3A) and resistant to thiol reagents, DTNB (compare lanes 1 in Figure 3C and 3A) and iodoacetate (not shown). Formation of this product was largely inhibited by exogenous methionine (compare lanes 7 and 1 in Figure 3A). Because MetRS preparations are stored routinely in 50% glycerol, up to 2.5% glycerol can be present in reaction mixtures containing MetRS. At this concentration, glycerol slowly reacts with Met-tRNA in the absence of MetRS. In fact, the product seen in lane 1 (Figure 3A) was also formed in the absence of MetRS when glycerol was included in the reaction mixture (not shown). Thus, the product of thiol-independent enzymatic deacylation of Met-tRNA is concluded to be an ester of methionine and glycerol. It has been confirmed that intact, fully chargeable tRNA^{Met} was always recovered from reaction mixtures.

Enzymatic deacylation of Met-tRNA, under conditions including the presence of dithiothreitol and glycerol, has yielded a thioester of methionine thought to be *S*-methyl-homocysteine thiolactone (Jakubowski, 1993a). In the light of the data presented above, that product was most likely a (thio)ester of methionine and dithiothreitol.

Nonenzymatic Reactions of Met-tRNA^{Met} with Thiols. To determine whether cysteine and other thiols can react nonenzymatically with Met-tRNA^{Met}, [^{35}S]Met-tRNA^{Met} 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. Reactions in the presence of cysteine, homocysteine, and dithiothreitol yielded about 2% of Met-Cys, Met-Hcy, and Met-dithiothreitol, respectively, in addition to the major product methionine (not shown).

Coupling relative amounts of Met-X and methionine formed during deacylation with an overall first order rate constant for deacylation (k = 0.017 min^{-1}; Table 1), one can estimate that rate constants for the reactions of Met-tRNA^{Met} with cysteine, homocysteine, and dithiothreitol were about 0.0003 min^{-1} (at 125 mM thiol). Thus, nonenzymatic reactions of Met-tRNA^{Met} with thiols are up to 10^6 times slower than the enzymatic reactions (see Table 1).

Thiols Inhibit tRNA^{Met} Aminoacylation. Possible effects of thiols on aminoacylation reaction were also tested. Thiols such as cysteine, dithiothreitol, and 2-mercaptoethanol somewhat inhibited the rate of tRNA^{Met} aminoacylation: 25−56% inhibition was observed in the presence of 125−250 mM thiol. D,L-Homocysteine was more inhibitory, with 78−84% inhibition observed at 125−250 mM of the thiol. These inhibitory effects are most likely due to competition between a thiol and tRNA^{Met} for the Met-AMP intermediate (see

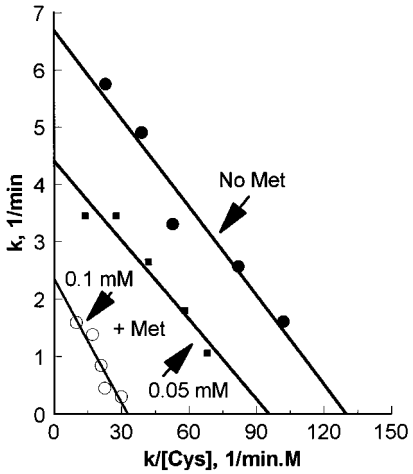

FIGURE 4: Methionine is a noncompetitive inhibitor of cysteine-dependent enzymatic deacylation of Met-tRNA. Incubation mixtures contained 0.5 μM [^{3}H]Met-tRNA^{fMet}, 0.05 μM MetRS, 0.05 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 10−250 mM L-cysteine, and 0 (●), 0.05 (■), or 0.1 (○) mM methionine. Rate constants, k, were calculated from reaction half-lives, $t_{0.5}$, according to $k = \ln(2)/t_{0.5}$. These rate constants were plotted *vs* k/[Cys]. The slopes of k *vs* k/[Cys] plots give K_m values for cysteine.

below). In addition, homocysteine, a noncognate substrate of MetRS, inhibits tRNA^{Met} aminoacylation by competing with methionine for the synthetic subsite.

Reactions of Thiols with MetRS·Met-AMP. Thiols also reacted with enzyme-bound Met-AMP. Reactions were carried out in mixtures containing MetRS·[^{35}S]Met-AMP (prepared *in situ* by incubating 1 μM MetRS, 5 μM [^{35}S]-Met, 2 mM ATP, and 5 units/mL inorganic pyrophosphatase) and 20 mM thiol, and products were analyzed by TLC. With dithiothreitol and 3-mercaptopropionate, corresponding methionine thioesters were formed. With cysteine and D,L-homocysteine, dipeptides Met-Cys and Met-Hcy, respectively, were formed. However, reactions of thiols with MetRS·[^{35}S]Met-AMP were much slower than with MetRS·[^{35}S]Met-tRNA. For example, at 20 mM cysteine rate constants for the enzymatic thiolysis of Met-AMP and Met-tRNA were about 1 min^{-1} and 30 min^{-1}, respectively.

Thiols Bind at a Distinct Site of MetRS. To determine whether thiol and methionine binding sites coincide, effects of methionine on cysteine- and homocysteine-dependent enzymatic deacylation of Met-tRNA^{Met} were determined. As shown in Figure 4, methionine was a noncompetitive inhibitor of the cysteine-dependent reaction. The slopes of the Eadie−Hofstee plots, corresponding to K_m values for cysteine, were essentially the same in the the absence and presence of 0.05 or 0.1 mM methionine. Methionine was also a noncompetitive inhibitor of the D,L-homocysteine-dependent deacylation reaction (not shown). In another set of experiments, inhibition of cysteine-dependent deacylation by increasing concentrations of methionine was found to be the same at 2 and 22 mM cysteine (not shown), with K_i for methionine being 35 μM. These data indicate that thiols and methionine bind at separate sites of MetRS.

Products of cysteine- and D,L-homocysteine-dependent enzymatic deacylation in the presence of exogenous methionine were also analyzed. As shown in Figure 3A, methionine prevented formation of [^{35}S]Met-Cys (compare lanes 8 and 2) and [^{35}S]Met-Hcy (compare lanes 9 and 6) from [^{35}S]Met-tRNA and a corresponding thiol; [^{35}S]Met was a major product. Although the results shown in Figure 3A were obtained with the dimeric MetRS676, similar results were also obtained with the monomeric MetRS547 enzyme. These results indicate that exogenous methionine physically displaces the [^{35}S]Met residue from the methionine binding subsite in the [^{35}S]Met-tRNA·MetRS complex, thus rendering the radiolabeled methionine unavailable to react with thiols.

Table 2: Cysteine-Dependent Met-tRNA Deacylase Activity of Methionyl-tRNA Synthetases[a]

MetRS	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})
wild type	0.05	70	1400
D52A	0.10	0.13	1.2
R233A			0.2
R233Q	0.11	0.023	0.2
W305A	0.035	20	570
Y15A	0.025	52	2080
Y15F	0.022	22	1000

[a] Reaction mixtures contained 0.5 μM [^{35}S]Met-tRNA, 5 μM (for D52A, R233A, and R233Q enzymes) or 0.05 μM (for wild type, W305A, Y15A, and Y15F enzymes) indicated MetRS, 0.1 M K-HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, and 10−250 mM L-cysteine.

Surprisingly, in the presence of excess exogenous methionine, the charged tRNA in the [^{35}S]Met-tRNA·MetRS complex was deacylated *faster* (k = 0.23 min^{-1}) than free [^{35}S]Met-tRNA (k = 0.017 min^{-1}, Table 1). This suggests that, even when the [^{35}S]Met residue in the [^{35}S]Met-tRNA·MetRS complex is displaced from the active site by exogenous methionine, the aminoacyl ester bond is still accessible to hydrolysis by the enzyme. Because the charged tRNA in the complex with MetRS is more stable in the absence of exogenous methonine (Table 1) than in its presence, it is concluded that the hydrolytic activity of MetRS toward [^{35}S]Met-tRNA is suppressed when [^{35}S]Met residue in the [^{35}S]Met-tRNA·MetRS complex is bound in the active site.

Substitutions of D52 and R233 in MetRS Affect k_{cat} for Cysteine-Dependent Deacylation of Met-tRNA. Active site residues of MetRS that interact with α-amino (D52) and carboxyl (R233) groups of methionine (Ghosh et al., 1991) and homocysteine (Kim et al., 1993) and contribute to catalysis of the synthetic (Ghosh et al., 1991) and editing reactions (Kim et al., 1993) were also found to be important for catalysis of the thiol-dependent deacylation of Met-tRNA. For example, D52A, R233A, and R233Q MetRSs were 1200- to 7000-fold less catalytically efficient than wild type MetRS in cysteine-dependent deacylation of Met-tRNA (Table 2). For each substitution, most of the effect was on k_{cat}; effects on K_m values for cysteine were relatively minor, about 2-fold (Table 2). On the other hand, substitutions of residues W305 and Y15 that interact with the side chain of methionine substrate affected k_{cat}/K_m for cysteine-dependent deacylation only up to 2.5-fold. Thus, active site mutations examined in this work have a relatively minor effect on binding of cysteine by MetRS, which further supports the existence of a distinct thiol (cysteine) binding subsite in the active site of MetRS. As previously demonstrated, effects of these mutations are limited to interactions with the amino acid substrates (methionine and homocysteine); K_m values for ATP and tRNA^{fMet} were not affected (Ghosh et al., 1991; Kim et al., 1993). The similarity of mutational effects on the thiol-dependent deacylation of Met-tRNA (Table 2) and

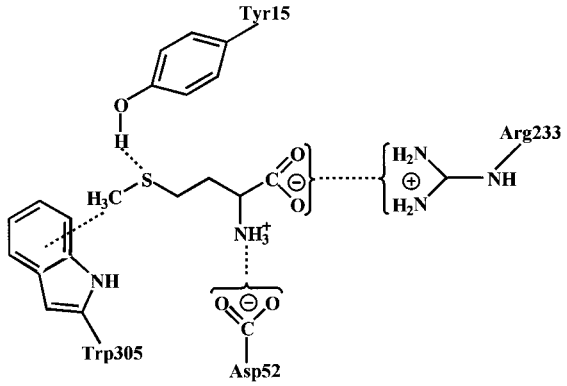

FIGURE 5: Schematic drawing of the interactions between methionine and MetRS. The interactions between methionine and indicated active site residues of MetRS are symbolized by a broken line. The roles of Asp52, Arg233 (Ghosh et al., 1991), Trp305 (Ghosh et al., 1991; Fourmy et al., 1991), and Tyr15 (Kim et al., 1993) were deduced from site directed mutagenesis studies. Interactions with Trp305 and Tyr15 are important for keeping methionine on the synthetic pathway (Kim et al., 1993). Misactivated homocysteine, unable to interact with Trp305 and Tyr15 as strongly as methionine does, cannot complete the synthetic pathway and enters the editing pathway (Kim et al., 1993).

on synthetic and editing functions of MetRS (Kim et al., 1993) indicates that these activities reside in a single active site.

DISCUSSION

In our previous structure−function work, the molecular basis of the editing function and its relation to the synthetic function of *E. coli* MetRS were analyzed. These studies led to a model of a single active site that partitions amino acid substrates between synthetic and editing pathways (Kim et al., 1993). The model was supported by observations that several specific active site mutations that affect synthetic activity similarly affect editing activity. In particular, it was shown that active site residues D52 and R233, which interact with α-amino and carboxyl groups, respectively, of methionine substrate (Figure 5), were also essential for activation and editing of noncognate homocysteine. This suggests that the portion of the active site that binds α-amino and carboxyl groups of an amino acid substrate is shared by the synthetic and editing subsites. The present work expands the model by documenting the presence of a subsite that binds the side chain -SH group of homocysteine in the editing subsite of MetRS.

The synthetic pathway involves intermolecular reaction of the activated carboxyl group of methionine with the 2′-hydroxyl of the terminal adenosine of tRNA. The editing pathway involves intramolecular reaction of the activated carboxyl group of homocysteine with the sulfur of its side chain. Whether an amino acid completes the synthetic or editing pathway is determined by the competition for its activated carboxyl group between the side chain of the amino acid and the terminal adenosine of tRNA. Methionine completes the synthetic pathway because, we believe, its side chain is firmly bound in the active site by hydrophobic and hydrogen bonding interactions with Trp305 and Tyr15 (Figure 5; see also Kim et al., 1993), respectively, preventing the sulfur atom of methionine from competing with the 3′-terminal adenosine of tRNA for the carboxyl carbon of methionine. Consistent with these functions, side chains of

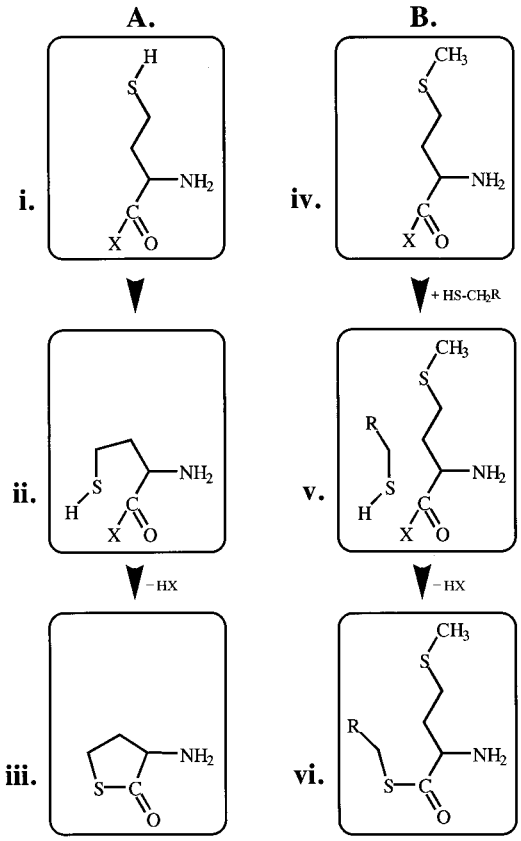

FIGURE 6: A model for single site editing by MetRS. The active site is proposed to have two partially overlapping subsites, synthetic and editing. The α-carbon, carboxyl, and amino groups, common to α-amino acids, bind to the overlapping region common to the synthetic and editing subsite. The side chain of an amino acid can bind to nonoverlapping portions of either synthetic or editing subsite. Thus, an amino acid binds in either the synthetic or editing mode. Amino acid substrates of MetRS bind initially in the synthetic mode. Binding of the noncognate amino acid homocysteine in the editing mode is induced at some point of the synthetic pathway. Panel A: (**i**) The noncognate homocysteinyl adenylate is formed in the synthetic subsite. (**ii**) The side chain of homocysteine moves to the editing subsite. Nucleophilic attack of the side chain thiolate on activated carboxyl carbon yields homocysteine thiolactone (**iii**). Panel B: (**iv**) Methionyl-tRNA (or Met-AMP) is formed in the synthetic subsite. (**v**) An organic thiol, analogue of the side chain of homocysteine, binds in the editng subsite. The thiol reacts with Met-tRNA to form a thioester of methionine (**vi**) in a reaction mimicking editing of homocysteine. X is AMP (in panel A and B) or tRNA (in panel B).

Trp305 and Tyr15 are located on opposite sides of the cavity forming a pocket observed in the three-dimensional structure of MetRS (Brunie et al., 1990); methionine can be easily docked in this pocket (Figure 2 in Kim et al., 1993). Homocysteine, missing the methyl group of methionine on its side chain, cannot interact with Trp305 and Tyr15 as strongly as methionine does (Kim et al., 1993). Therefore, the activated carboxyl of homocysteine reacts intramolecularly with the sulfur of its side chain (Figure 6A) instead of the 3′-terminal adenosine of tRNA. Since intramolecular reactions are more favored than intermolecular reactions, this explains why homocysteine is not transferred to tRNA but is cyclized to homocysteine thiolactone. As expected from this model, methionine enters the editing pathway when its side chain cannot firmly bind to the active site, which is indeed the case with W305A, Y15F, and Y15A MetRSs (Kim et al., 1993).

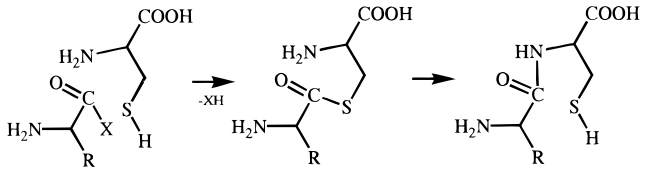

FIGURE 7: Proposed mechanism of peptide bond formation catalyzed by MetRS. Formation of the thioester *S*-(L-methionyl)-L-Cys (**ii**) from Met-tRNA and L-cysteine (**i**) is catalyzed by MetRS. Subsequent rearrangement of **ii** to the dipeptide Met-Cys (**iii**) may occur spontaneously (Wieland & Pfleider, 1957). R is the side chain of Met, X denotes tRNA. Similar transacylations will occur to other nucleophilic groups, such as hydroxy or carboxyl, if present, yielding corresponding ester or anhydride bonds.

Another prediction of this model, substantiated by the data described in the present paper, is that activated methionine, which binds mostly in the synthetic mode (Figure 6B), will enter the editing pathway even with wild type MetRS if the subsite that binds the -SH group of the side chain of homocysteine (Figure 6A.ii) is occupied by a thiol (Figure 6B.v). When misactivated homocysteine is bound in the editing mode in the active site, its -SH group occupies a subsite (an -SH subsite) next to its carboxyl carbon (Figure 6A.ii). In contrast, with activated cognate amino acid, for example, in the form of Met-tRNA, in the active site, the -SH subsite is empty (Figure 6B.iv) and can bind organic thiols (analogues of the side chain of homocysteine, $HSCH_2$-R) (Figure 6B.v). This leads to MetRS-dependent reactions of thiols with enzyme-bound Met-tRNA to yield thioesters Met-SCH_2-R (Figure 6B.vi), mimicking homocysteine thiolactone formation (Figure 6A.iii).

As expected, MetRS catalyzes reactions between thiols and Met-$tRNA^{Met}$ which yield methionine thioesters as products. With thiols having other nucleophilic groups (such as amino, hydroxy, or carboxy) on a carbon next to the carbon bearing the -SH group, subsequent facile transacylation (Wieland & Pfleider, 1957) yields peptides (Figure 7), esters, and anhydrides. For example, Cys-dependent deacylation of Met-tRNA yields the dipeptide, Met-Cys (lanes 2 in Figure 3A and 3B). A similar, but less efficient, reaction with homocysteine yields the dipeptide Met-Hcy (lanes 6 in Figure 3A and 3B). That these reactions occur between Met-tRNA, bound in the synthetic subsite, and an organic thiol, an analogue of the side chain of Hcy, bound in the editing subsite, indicates that the two subsites are intimately close on the surface of the enzyme, forming a synthetic/editing active site (Figure 6).

This model would also explain editing of homocysteine by other synthetases. Indeed, cysteine-dependent deacylation reactions are catalyzed by other class I aminoacyl-tRNA synthetases: IleRS and ValRS (Jakubowski, 1995b), both of which need and possess editing function (reviewed in Jakubowski & Goldman, 1992). Moreover, aminoacyl thioesters were formed during deacylation reactions catalyzed by IleRS and ValRS in the presence of 3-mercaptopropionate, *N*-acetyl-L-cysteine, and dithiothreitol (H. Jakubowski, unpublished), suggesting that a model for single site editing (Figure 6) applies also to editing of homocysteine by IleRS and ValRS. However, editing of valine by IleRS has been suggested to involve a separate site (Schmidt & Schimmel, 1994).

Another class I synthetase, ArgRS, is also able to catalyze synthesis of thioesters from thiols and the cognate charged tRNA (Jakubowski, 1995b). Because ArgRS does not seem to need editing function (Jakubowski, 1995b), its ability to catalyze thiolysis of Arg-tRNA suggests that this syntheatse possesses a *cryptic* editing function. However, there are significant differences in both catalytic efficiencies and thiol specificities between thiolysis reactions catalyzed by ArgRS and MetRS, consistent with a functional role of the thiol-binding subsite in the editing function of MetRS. First, MetRS, is at least 10-fold more efficient than ArgRS in catalyzing the thiolysis of the aminoacyl ester bond in cognate aminoacyl-tRNA. For example, k_{cat}/K_m for cysteine with ArgRS is 120 min^{-1} M^{-1} (Jakubowski, 1995b) and, with MetRS, 1380 min^{-1} M^{-1} (Table 1). Second, MetRS exibits a rather broad thiol specificity whereas the specificity of ArgRS for thiols is more narrow. For example, MetRS utilizes efficiently cysteine, cysteine methyl ester, Cys-Gly, cysteamine, dithiothreitol, and 2-mercaptoethanol; k_{cat}/K_m for these thiols differ only by a factor <3 (Table 1). In contrast, ArgRS utilizes preferentially cysteine; other thiols, such as cysteine methyl ester, Cys-Gly, and cysteamine, are 15−30-fold less efficient than cysteine, whereas ditihiothreitol and 2-mercaptoethanol are 5 and >100 less efficient than cysteine (Jakubowski, 1995b).

The present data point to a close chemical relationship between aminoacylation and editing and are also relevant for understanding coevolution of aminoacylation and editing reactions. Since 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 existence of thioester chemistry involving cognate amino acids in present-day aminoacyl-tRNA synthetases, documented in this work and elsewhere (Jakubowski, 1995b), opens up an interesting possibility that thioester chemistry may have been responsible for the origin of aminoacylation reactions, perhaps in the "thioester world" (de Duve, 1994), and may have been used more extensively by ancestral aminoacyl-tRNA synthetases. Thioesters of amino acids, which form spontaneously under acidic conditions, may have been immediate substrates for ancestral tRNA aminoacylation in the absence of ATP. With the emergence of ATP-dependent and more accurate aminoacylation, thioester chemistry was primarily used to weed out misactivated thio-amino acids, which constitutes a major editing function of aminoacyl-tRNA synthetases in present-day biological systems.

ACKNOWLEDGMENT

I thank Sylvain Blanquet for the full length MetRS plasmid. I am grateful to Manny Goldman for his critical discussions.

REFERENCES

Brevet, A., Chen, J., Leveque, F., Plateau, P., & Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8275−8279.
Brunie, S., Zelwer, Z., & Risler, J. L. (1990) *J. Mol. Biol. 216*, 411−424.
de Duve, C. (1994) *Vital Dust*, pp 43−45, Basic Books, New York.
Englisch, S., Englisch, U., von der Haar, F., & Cramer, F. (1986) *Nucleic Acids Res. 14*, 7529−7539.
Fersht, A. R., & Dingwall, C. (1979) *Biochemistry 18*, 1245−1249.
Fourmy, D., Mechulam, Y., Brunie, S., Blanquet, S., & Fayat, G. (1991) *FEBS Lett. 292*, 259−263.

Gao, W., Goldman, E., & Jakubowski, H. (1994) *Biochemistry 33*, 11528−11533.
Ghosh, G., Pelka, H., Schulman, L. H., & Brunie, S. (1991) *Biochemistry 30*, 9569−9575.
Jakubowski, H. (1980) *Biochemistry 19*, 5071−5078.
Jakubowski, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4504−4508.
Jakubowski, H. (1991) *EMBO J. 10*, 593−598.
Jakubowski, H. (1993a) *FASEB J. 7*, 168−172.
Jakubowski, H. (1993b) *J. Biol. Chem. 268*, 6549−6553.
Jakubowski, H. (1994a) *Ann. N.Y. Acad. Sci. 745*, 4−20.
Jakubowski, H. (1994b) *Nucleic Acids Res. 22*, 1155−1160.
Jakubowski, H. (1995a) *J. Biol. Chem. 270*, 17672−17673.
Jakubowski, H. (1995b) *Nucleic Acids Res. 23*, 4608−4615.
Jakubowski, H., & Fersht, A. R. (1981) *Nucleic Acids Res. 9*, 3105−3117.
Jakubowski, H., & Goldman, E. (1992) *Microbiol. Rev. 56*, 412−429.
Jakubowski, H., & Goldman, E. (1993) *FEBS Lett. 317*, 237−240.
Kim, H. Y., Ghosh, G., Schulman, L. S., Brunie, S., & Jakubowski, H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11553−11557.
Norris, A. T., & Berg, P. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 330−337.
Old, J. M., & Jones, D. S. (1977) *Biochem. J. 165*, 367−373.
Parker, J. (1989) *Microbiol. Rev. 53*, 273−298.
Schmidt, E., & Schimmel, P. (1994) *Science 264*, 265−267.
Smith, L. T., & M. Cohn. (1981) *Biochemistry 20*, 385−391.
Wieland, T., & Pfleiderer, G. (1957) *Adv. Enzymol. 19*, 235−266.

BI960344V