Aminoacylation of Coenzyme A and Pantetheine by Aminoacyl-tRNA Synthetases: Possible Link between Noncoded and Coded Peptide Synthesis†

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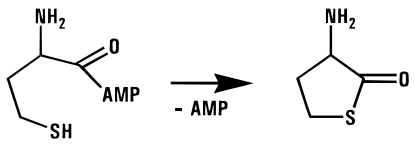
ABSTRACT: Isoleucyl-tRNA synthetase (IleRS) catalyzes transfer of isoleucine from the enzyme-bound Ile-AMP and Ile-tRNA to the thiol group of coenzyme A, forming a thioester, Ile-S−CoA. Identity of Ile-S−CoA has been confirmed by several enzymatic and chemical tests. The synthesis of Ile-S−CoA, like the synthesis of other isoleucyl thioesters, is strongly shifted toward products. Other aminoacyl-tRNA synthetases, such as MetRS, AspRS, and SerRS also use CoA-SH as an acceptor for their cognate amino acids. Pantetheine also serves as an amino acid acceptor in reactions catalyzed by AspRS, IleRS, and MetRS, forming corresponding aminoacyl-S-pantetheine thioesters. It appears that CoA-SH reacts with activated amino acids by binding to each synthetase at a site, separate from the tRNA and ATP binding sites, that includes the thiol-binding subsite. These and other data support a hypothesis that the present-day aminoacyl-tRNA synthetases have originated from ancestral forms that were involved in noncoded thioester-dependent peptide synthesis, functionally similar to the present-day nonribosomal peptide synthesis by multi-enzyme thiotemplate systems.

Aminoacyl-tRNA synthetases (AARSs)[1] attach cognate amino acids to tRNAs with the specificity dictated by the rules of the genetic code. In addition, many AARSs possess proofreading or editing functions that remove occasionally misactivated incorrect amino acids, thereby preventing their attachment to tRNA (*1*).

A major selectivity problem for AARSs appears to be with the nonprotein amino acid homocysteine, an obligatory precursor of methionine in all cells. Homocysteine is misactivated in vitro by MetRS (*2*), IleRS (*2*), and LeuRS (*3*) at a frequency exceeding the frequency of translational errors in vivo. ValRS (*2*, *4*) and LysRS (*5*) misactivate homocysteine less efficiently (*6*). These five enzymes possess an efficient editing mechanism that destroys the Hcy-AMP intermediate. The editing reaction involves nucleophilic attack of the side chain thiol group of homocysteine on its activated carboxyl group, forming a cyclic thioester, homocysteine thiolactone (*2*, *5*) (eq 1). Editing of homocysteine occurs in vivo and is enhanced in microbial (*6*−*9*) and mammalian, including human (*10*, *11*), cells in which homocysteine metabolism is deregulated.



(1)

The chemistry of homocysteine editing, involving thioester bond formation, requires the existence of a subsite that specifically binds the side chain thiol of homocysteine in the active site of a synthetase. Such thiol-binding subsites indeed exist in the synthetic/editing active sites of MetRS (*12*), IleRS (*13*), and LysRS (*5*). Similar thiol-binding sites exist also in active sites of enzymes that do not seem to need editing function, such as ArgRS (*14*), AspRS (*5*), and SerRS (*5*). The thiol-binding site confers on a synthetase the ability to catalyze aminoacylation of thiols according to

$$\text{AA-tRNA}\{\text{AMP}\} + \text{HS-CH}_2\text{-CH-(R)(R}_1) \rightarrow \text{AA-S-CH}_2\text{CH-(R)(R}_1) + \text{tRNA}\{\text{AMP}\} \quad (2)$$

For thiols with R = NH_2, secondary acyl transfer from the sulfur to the nitrogen of the amino group yields a peptide (*5*, *12*−*14*). The chemical nature of substituents R and R_1 affects catalytic efficiencies of thiols. For example, cysteine (R = NH_2, R_1 = COOH) is the best substrate, dithiothreitol (R = OH, R_1 = $CH(OH)CH_2$ SH) is the second best, whereas 3-mercaptopropionate (R = H, R_1 = COOH) and *N*-acetyl-cysteine (R = acetyl-NH, R_1 = COOH) are poor substrates for all AARSs tested. Although cysteamine (R = NH_2, R_1 = H) and 2-mercaptoethanol (R = OH, R_1 = H) are good substrates for MetRS, they are poor substrates for ArgRS, IleRS, AspRS, LysRS, and SerRS in the thiol aminoacylation reaction. Glutathione (R = Glu-NH-, R_1 = −C(=O)−Gly) is not a substrate for LysRS and is a very poor substrate for MetRS, AspRS, and SerRS.

It is not clear why AARSs that do not have editing function still possess a thiol-binding site. A plausible explanation is that the thiol-binding site is a vestige from a thioester world (*15*) in which ancestral AARSs provided (and possibly utilized) aminoacyl thioesters for noncoded peptide synthesis similar to that of the present-day thiotemplate RNA-

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[1] Abbreviations: AARS, aminoacyl-tRNA synthetase, e.g., IleRS, isoleucyl-tRNA synthetase (other synthetases are similarly abbreviated).

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Table 1: Kinetic Indices for Aminoacylation of CoA-SH and Other Thiols Catalyzed by AspRS and MetRS[a]

	AspRS			MetRS		
thiol	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} M^{-1})
CoA-SH	0.25 ± 0.01	2.1 ± 0.2	119	1.3 ± 0.09	0.82 ± 0.15	1650
pantetheine	1.8 ± 0.3	17.5 ± 5.5	135	0.84 ± 0.07	11.3 ± 2.3	74
cysteamine			0.77 ± 0.04[b]	80.4 ± 6.5	100 ± 14	690
cysteine	7.4 ± 1.6	77 ± 32	96	67.2 ± 4.7	51 ± 7	1318
glutathione			0.65 ± 0.38			3.1 ± 0.3[c]

[a] Reactions were carried out with 0.5 μM [^{3}H]Asp-tRNA^{Asp} (19.2 Ci/mmol) and 1 μM AspRS or 0.5 μM [^{35}S]Met-tRNA^{fMet} and 0.050−1 μM MetRS, in the presence of 0.5−250 mM indicated thiol. [b] Nonsaturating kinetics up to 250 mM. [c] Nonsaturating kinetics up to 60 mM. Slow nonenzymatic deacylations of Asp-tRNA and Met-tRNA ($k = 0.017$ min^{-1}) were not affected by thiols.

independent synthesis of microbial peptide antibiotics (*16*, *17*). Thioesters are immensely important in the present-day biological systems and may have been even more important in early biological systems (*15*). For example, CoA-SH and pantetheine participate in carboxyl group activation reactions, with the notable exception of coded protein synthesis (*16*, *17*). The nonribosomal peptide synthesis systems utilize as a cofactor phosphopantetheine, whose thiol group serves as an acceptor for amino acids (*16*, *17*). The ability of the present-day AARSs to utilize CoA or pantetheine as acceptors for amino acids would support the hypothesis that the present-day AARSs originated from ancestral forms that utilized thioester chemistry to provide activated precursors for peptide assembly and would shed a new light on the origin of the thiol-binding subsites of the present-day AARSs.

Here, it is shown that CoA-SH (R = NH-C(O)CH_2CH_2-NHC(O)CH(OH)C(CH_3)$_2$$CH_2$O-$PO_3$-(5′,3′-ADP), R_1 = H) binds to the active site of IleRS and reacts with activated isoleucine (Ile-AMP or Ile-tRNA) to form the thioester Ile-S−CoA. Corresponding aminoacyl-S−CoA thioesters are also formed by MetRS, AspRS, and SerRS.

MATERIALS AND METHODS

Plasmids and Host Strain. Plasmids containing the genes for *Escherichia coli* IleRS (*18*), MetRS (*19*), AspRS (*20*), and SerRS (*21*) 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.

Aminoacyl-tRNA Synthetases. E. coli IleRS (*13*), MetRS (*9*, *12*, *22*), AspRS (*5*), and SerRS (*5*) were purified to homogeneity from the overproducing strains using standard procedures.

Aminoacylation of Thiols by AARS·AA-AMP. Enzyme-bound radiolabeled aminoacyl adenylates were prepared and incubated with thiols in the same mixture. Unless stated otherwise, reactions were carried out at 37 °C in mixtures containing AARS, radiolabeled amino acid, 1 mM ATP, 0.1 M K-HEPES (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EDTA, 5 units/mL yeast inorganic pyrophosphatase (Sigma), and a thiol. The aminoacyl derivatives of thiols were separated from amino acids by TLC. Kinetic parameters were derived from initial velocity measurements at variable substrate (thiol) concentrations. The K_m and k_{cat} values and their standard errors for CoA-SH were derived from at least two independent experiments.

Preparation of Radiolabeled Aminoacyl-tRNA. Reaction mixtures contained 0.1 M K-HEPES (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM ATP, 20 μM tRNA (pure tRNAs with aminoacyl acceptor activities of 1400−1600 pmol/A_{260} were obtained from Subriden RNA), 25 μM radiolabeled amino acid, 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 Deacylaction of Radiolabeled Aminoacyl-tRNA. The radiolabeled aminoacyl-tRNA was incubated with a thiol and a cognate AARS at 37 °C in 0.1 M K-HEPES (pH 7.4), 10 mM $MgCl_2$, and 0.1 mM EDTA. The disappearance of the radiolabeled aminoacyl-tRNA as a function of time was monitored by trichloroacetic acid precipitation. Apparent first-order 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 as well as their standard errors (see Table 1), according to $k = k_{cat} - K_m \cdot k/[\text{thiol}]$ (*5*, *12*−*14*). Individual measurements, repeated at least twice, agreed within ±20%. To monitor all products of deacylation reactions, aliquots of reaction mixtures were analyzed by TLC.

TLC Analyses. Products of the reactions of CoA-SH with activated amino acids were analyzed by TLC on polyethyleneimine(PEI)−cellulose (from Merck or Sigma) using 0.8 M LiCl as a solvent. Radiolabeled aminoacyl-S−CoA derivatives exhibited chromatographic mobilities similar or slightly greater than the mobility of CoA-SH ($R_f \sim 0.5$) and were well separated from the corresponding radiolabeled amino acids that migrated close to the solvent front. Products of reactions of other thiols with activated amino acids were separated on TLC cellulose plates (from Kodak) using butanol:acetic acid:water (4:1:1, v/v) as a solvent. Radiolabeled aminoacyl-thiol derivatives migrated faster than the corresponding radiolabeled amino acids in this system. TLC plates were autoradiographed using Kodak BioMax MR-1 film.

Preparation of [^{14}C]Ile-S−CoA and [^{35}S]Met-S−CoA. Incubation mixtures (50 μL) contained 50 mM K-HEPES (pH 7.4), 20 mM $MgCl_2$, 3 mM ATP, 10 mM CoA-SH (from Sigma; sodium salt adjusted to pH 7.4 with NaOH), 5 units/mL yeast inorganic pyrophosphatase, 158 μM [^{14}C]isoleucine (306 Ci/mol, 5 μCi; from NEN) or 200 μM [^{35}S]methionine (5000 Ci/mol, 50 μCi; from Amersham), and 0.5 μM IleRS or 25 μM MetRS, respectively. After 2 h (with IleRS) or 15 min (with MetRS) at 37 °C, radiolabeled aminoacyl-S−CoA was purified by two-dimensional TLC on 10 × 10 cm

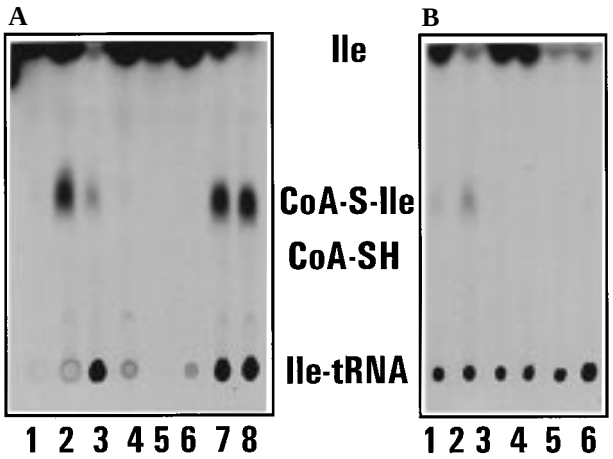

FIGURE 1: Requirements for the synthesis of Ile-S−CoA. Reactions were carried out with 8.15 μM [^{14}C]isoleucine (306 Ci/mol, Amersham) and 5 mM ATP (A) or 2 μM [^{14}C]Ile-tRNA^{Ile} (306 Ci/mol) (B) for 20 min at 37 °C in mixtures containing 5 mM CoA-SH, 0.1 M K-HEPES, 20 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.4 μM IleRS. Panel A: lane 1, −ATP; lane 2, complete reaction mixture; lane 3, + 5 μM tRNA^{Ile}; lane 4, −CoA-SH; lane 5, −IleRS; lane 6, −ATP, + 5 μM tRNA^{Ile}; lanes 7 and 8, complete reaction mixture incubated for 20 min, then 5 μM tRNA^{Ile} was added, and the incubation was continued for additional 1 and 10 min, respectively. Panel B: lane 1, complete reaction mixture; lane 2, +5 mM ATP; lane 3 +0.3 mM isoleucine; lane 4, −CoA-SH; lane 5, −enzyme; lane 6, +5 μM tRNA^{Ile}. 2 μL aliquots were analyzed by TLC on PEI−cellulose. An autoradiogram exposed from TLC separations is shown. Positions of CoA-SH (visualized under UV light) and [^{14}C]isoleucine derivatives are indicated.

cellulose plates (Kodak). The plates were developed with pyridine:formic acid:water (1:1:1, v/v) in the first dimension and with 2-propanol:formic acid:water (20:1:5) in the second dimension. After separation, radiolabeled aminoacyl-S−CoA, visualized by autoradiography, was eluted from TLC plates with water on ice and stored at − 20 °C. About 10% of the radiolabeled amino acid was recovered as radiolabeled aminoacyl-S−CoA. Half-lives for spontaneous hydrolysis of Ile-S−CoA and Met-S−CoA were 135 min (0.1 M K-HEPES, 10 mM $MgCl_2$, and 0.1 mM EDTA, pH 7.4, 37 °C).

RESULTS

CoA-SH Is a Substrate for IleRS, MetRS, AspRS, and SerRS. AARSs were incubated with ATP, radiolabeled amino acid, and CoA-SH, and the products were analyzed by TLC. In reactions catalyzed by IleRS, MetRS, AspRS, and SerRS, adducts between a corresponding cognate amino acid and CoA-SH, migrating just above CoA-SH on TLC PEI−cellulose plates, were detected. Aminoacylations of CoA-SH catalyzed by IleRS and MetRS were studied in detail, and the results are described below.

IleRS was incubated with [^{14}C]isoleucine, ATP, and CoA-SH. Aliquots of the reaction mixtures were subjected to TLC separation on PEI−cellulose. As shown in Figure 1A, a major new [^{14}C]spot, migrating just above CoA-SH, as expected of putative [^{14}C]Ile-S−CoA, was formed in a complete reaction mixture (lane 2). This new spot was absent in reaction mixtures without CoA-SH (lane 4), ATP (lanes 1 and 6), or IleRS (lane 5). When tRNA^{Ile} was added at the beginning of incubation, lower amounts of the putative [^{14}C]Ile-S−CoA were formed (lane 3). This suggests that tRNA^{Ile} is a more efficient acceptor than CoA-SH for isoleucine from IleRS·Ile-AMP. However, addition of tRNA^{Ile} at the end of incubation did not affect the amount of putative [^{14}C]Ile-S−CoA formed (lanes 7 and 8), suggesting that there is no significant transfer of [^{14}C]isoleucine from putative [^{14}C]Ile-S−CoA to tRNA^{Ile}.

As shown in Figure 1B, putative [^{14}C]Ile-S−CoA also formed as a minor product (in addition to the major product [^{14}C]isoleucine) when [^{14}C]Ile-tRNA^{Ile} was deacylated by IleRS (*13*) in the presence of CoA-SH (lane 1). Addition of ATP led to an increase in putative [^{14}C]Ile-S−CoA (lane 2), most likely due to the formation of [^{14}C]Ile-AMP. Formation of putative [^{14}C]Ile-S−CoA was abolished by the addition of isoleucine (lane 3) or tRNA^{Ile} (lane 6) to complete reaction mixtures. No putative [^{14}C]Ile-S−CoA formed in the absence of CoA-SH (lane 4) or IleRS (lane 5). These data indicate that IleRS catalyzes transfers of [^{14}C]isoleucine to CoA-SH from both IleRS-bound [^{14}C]Ile-tRNA^{Ile} and [^{14}C]-Ile-AMP.

Similar results (not shown) were obtained with MetRS: a [^{35}S]Met−CoA adduct was formed when MetRS was incubated with CoA-SH, ATP, and [^{35}S]methionine. When MetRS was incubated with CoA-SH and [^{35}S]Met-tRNA, the [^{35}S]Met−CoA adduct was a major product. An analogue of CoA-SH without the thiol, desulfoCoA, was not a substrate for aminoacylation (not shown). These results indicate that MetRS catalyzes the transfer of methionine both from Met-AMP and Met-tRNA to CoA-SH and that the thiol of CoA-SH is essential for its aminoacylation.

Identification of Ile-S−CoA and Met-S−CoA. To confirm that the product of enzymatic reactions of CoA-SH with [^{14}C]-Ile-AMP or [^{14}C]Ile-tRNA is indeed the thioester [^{14}C]Ile-S−CoA, [^{14}C]Ile-S−CoA was purified from reaction mixtures and subjected to chemical and enzymatic tests. Products of these tests were separated by TLC on PEI−cellulose (Figure 2A) and cellulose (Figure 2B). Nuclease P_1 treatment converted putative [^{14}C]Ile-S−CoA into a faster migrating compound (compare lanes 2 and 1), most likely [^{14}C]Ile-S−(3′-dephospho)-CoA, as expected. [Nuclease P_1 removes the 3′-phosphate group from the 5′,3′-ADP moiety of CoA-SH, converting it into less polar, and therefore faster migrating on PEI−cellulose TLC plates, 3′-dephospho-CoA-SH (*23*).] Actually, authentic 3′-dephospho-CoA-SH itself when incubated with IleRS, [^{14}C]isoleucine, and ATP formed an adduct with [^{14}C]isoleucine that comigrated with the product of nuclease P_1 treatment of putative [^{14}C]Ile-S−CoA (not shown). Putative [^{14}C]Ile-S−CoA was sensitive to mild NaOH (lanes 3) and hydroxylamine (lanes 4) treatments, a property of thioesters. The presence of a thioester bond is further supported by the sensitivity of putative [^{14}C]Ile-S−CoA to treatments with thiols, such as cysteine (lanes 8 and 9) or 2-mercaptoethanol (lanes 6 and 7). Treatment of a putative thioester, [^{14}C]Ile-S−CoA, with an excess of a thiol leads to a thioester interchange that results in the transfer of [^{14}C]isoleucine from [^{14}C]Ile-S−CoA to the thiol (see products distinct from [^{14}C]isoleucine and [^{14}C]Ile-S−CoA in lanes 6−9 in panel B). Putative [^{14}C]Ile-S−CoA was not sensitive to treatment with the sulfhydryl group reagent 5,5′-dithiobis(2-nitrobenzoate) (lanes 5), indicating that the free thiol group of CoA-SH is the site of attachment for isoleucine. These tests confirm that the adduct between isoleucine and CoA-SH is a thioester, Ile-S−CoA.

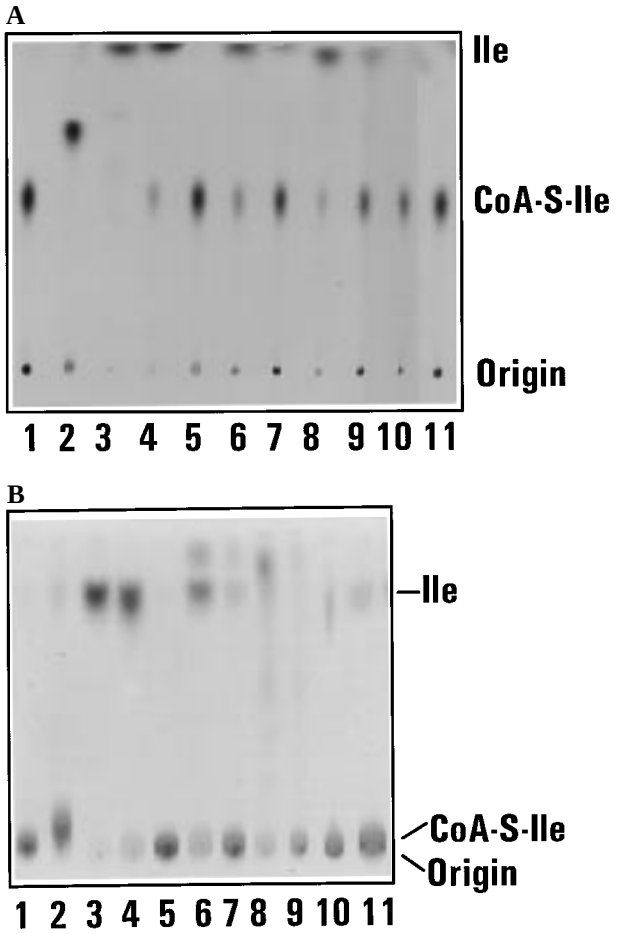

FIGURE 2: Properties of Ile-S−CoA. Reactions were carried out in mixtures containing 0.7 μM [^{14}C]Ile-S−CoA (1400 cpm/3 μL) 10 mM K-HEPES (pH 7.4), 1 mM $MgCl_2$, and no additions (lane 1), 0.5 unit nuclease P_1 (lane 2), 80 mM NaOH (lane 3), 150 mM hydroxylamine (lane 4), 3 mM 5,5′-dithiobis(2-nitrobenzoate) (lane 5), 2-mercaptoethanol (200 mM, lane 6; 20 mM, lane 7), cysteine (200 mM, lane 8; 20 mM, lane 9), 5% glycerol (lane 10), or 0.4 μM IleRS and 5 μM tRNA^{Ile} (lane 11). After incubation at 37 °C for 15 min, samples were analyzed by TLC on PEI−cellulose (B) and cellulose (A). Autoradiograms exposed from these separations are shown.

Similar tests, shown in Figure 3, were carried out with an adduct of [^{35}S]methionine and CoA-SH, purified from reaction mixtures containing MetRS, CoA-SH, ATP, and [^{35}S]methionine (see Materials and Methods). Like the Ile−CoA adduct, the Met−CoA adduct was sensitive to treatments with NaOH (lane 3), hydroxylamine (lane 4), and thiols such as 2-mercaptoethanol (lane 6) and cysteine (lane 7), but was not sensitive to a treatment with a sulfhydryl group reagent, iodoacetate (lane 5). These tests indicate that the free thiol group of CoA-SH is the site of attachement for methionine. The Met−CoA adduct was also sensitive to treatment with nuclease P_1 (lane 2), a property expected of the thioester Met-S−CoA.

Reversibility of Thiol Aminoacylation Reactions. To determine whether aminoacylation of CoA-SH and other thiols is reversible, thiols were incubated with [^{14}C]isoleucine, ATP, and IleRS. As a positive control, tRNA^{Ile} was also aminoacylated with [^{14}C]isoleucine. After 30 min, 3.5 mM AMP + PPi was added, and the incubation was continued for another 30 min. Addition of these concentrations of AMP + PPi to aminoacylation mixtures containing [^{14}C]-

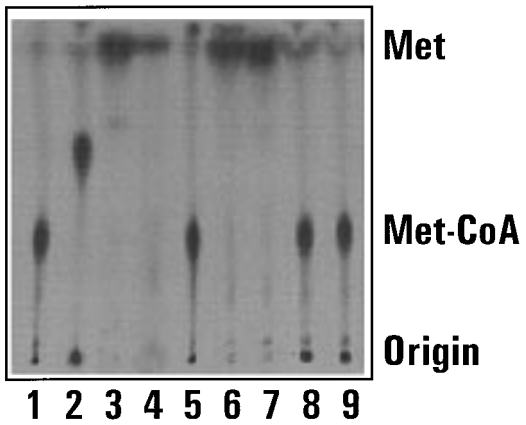

FIGURE 3: Properties of Met-S−CoA. Reactions were carried out in mixtures containing 1.3 μM [^{35}S]Met-S−CoA (34 000 cpm/4 μL), 25 mM K-HEPES (pH 7.4), 2.5 mM $MgCl_2$, and no additions (lane 1), 0.5 unit nuclease P_1 (lane 2), 100 mM NaOH (lane 3), 200 mM hydroxylamine (lane 4), 5 mM iodoacetate (lane 5), 0.2 M 2-mercaptoethanol (lane 6), 0.2 M cysteine (lane 7), 2.5% glycerol (lane 8), or 1 μM MetRS and 12.5 μM tRNA^{Ile} (lane 9). After incubation at 37 °C for 15 min, samples were analyzed by TLC on PEI−cellulose (Sigma). An autoradiogram exposed from these separations is shown.

Ile-tRNA^{Ile} completely reversed the aminoacylation (95% drop in the level of Ile-tRNA within 5 min after addition of AMP + PPi; not shown). [^{14}C]Isoleucine thioesters were determined before and after the addition of AMP + PPi. As shown in Figure 4, aminoacylation of CoA-SH, pantetheine (panel A), and dithiothreitol (DTT) (panel B) was essentially completed within 30 min. Aminoacylation of 2-mercaptoethanol with isoleucine reached a plateau at about 40% of that observed during aminoacylations of CoA-SH, pantetheine, and DTT. This suggests that the equilibria of aminoacylations of CoA-SH, pantetheine, and DTT with isoleucine are shifted more toward the products than the equlibrium of the isoleucylation of 2-mercaptoethanol. Following addition of AMP + PPi, the levels of Ile-S−CoA and Ile−pantetheine declined only slightly (panel A), whereas levels of Ile-DTT, Ile-(3-mercaptopropionate), and Ile-(2-mercaptoethanol) declined by 50%, 25%, and 90%, respectively (panel B). In most cases, the decline occurred within 10 min following the addition of AMP + PPi. The declines in the thioester levels after addition of AMP + PPi are not due to spontaneous hydrolysis of the thioesters; the half-lives of Ile-S−CoA and Ile-DTT are 135 min (see Materials and Methods) and 120 min (*13*), respectively, and other isoleucyl thioesters are expected to be as stable. Thus, the reversibility of the thioester formation reaction depends on the nature of the thiol. For some thiols, such as CoA-SH and pantetheine, the equilibrium of the aminoacylation reaction is shifted far toward the products, and the reaction is difficult to reverse. The equilibrium of aminoacylation of other thiols, such as 2-mercaptoethanol, must apparently lie not as far toward the products and the reaction is relatively easy to reverse. Purified Ile-S−CoA did not transfer isoleucine onto tRNA^{Ile} in the presence of IleRS (Figure 2, lane 11). Similarly, purified Met-S−CoA did not appreciably transfer methionine onto tRNA^{Met} in the presence of MetRS (Figure 3, lane 9). These results suggest that the equilibria of reactions of CoA-SH with aminoacyl-tRNA are shifted far toward products (aminoacyl-S−CoA and tRNA).

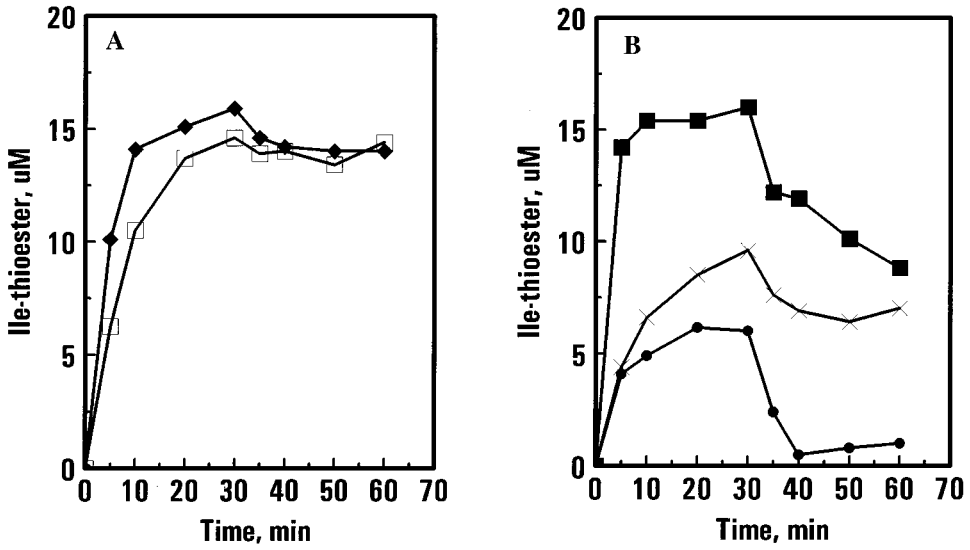

FIGURE 4: Reversibility of the thioester formation reaction. Reactions were carried out at 37 °C in mixtures containing 15 μM [^{14}C]-isoleucine (306 Ci/mol), 1 mM ATP, 0.1 M K-HEPES, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 μM IleRS, and a thiol. After 30 min, 3 mM AMP and PP_i were added, and incubations were continued for another 30 min. [^{14}C]Isoleucyl thioesters, separated by TLC and visualized by autoradiography, were quantitated by scintillation counting. Time courses of the formation of isoleucine thioesters with the following thiols are shown. Panel A: 10 mM CoA-SH (□), 40 mM pantetheine (♦); Panel B: 40 mM DTT (●), 50 mM 2-mercaptoethanol (■), or 3-mercaptopropionate (×).

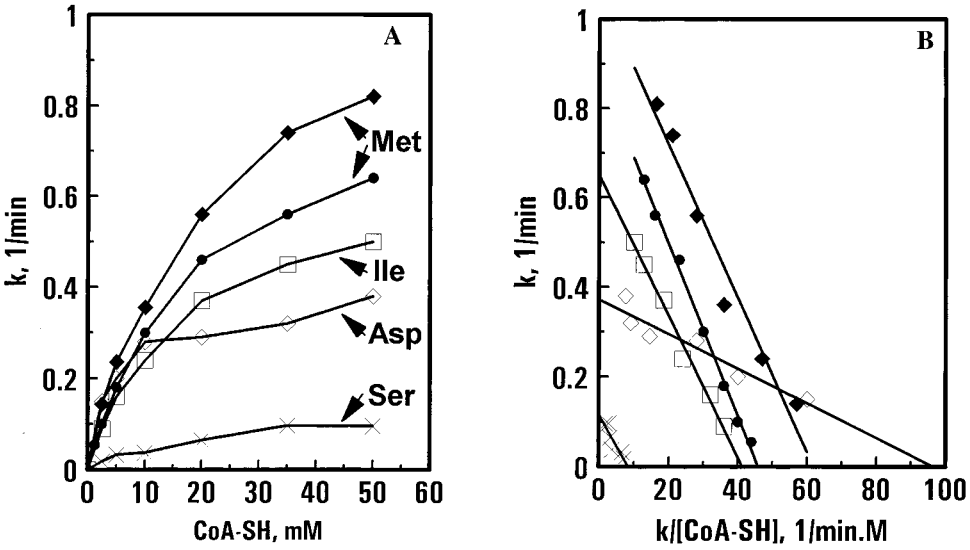

FIGURE 5: Kinetics of aminoacyl-S−CoA synthesis by IleRS, MetRS, AspRS, and SerRS. Reactions were carried out at 37 °C for 7.5−30 min in mixtures containing 15 μM [^{14}C]isoleucine (306 Ci/mol, from NEN), 20 μM [^{35}S]methionine (5000 Ci/mol, from Amersham), 10.4 μM [^{3}H]aspartate (19300 Ci/mol, from Amersham), or 11.2 μM [^{3}H]serine (36000 Ci/mol, from Amersham), 2 mM ATP, 0.1 M K-HEPES (pH 7.4), 70 mM $MgCl_2$, 0.5 mM EDTA, 0.5 μM IleRS, MetRS, AspRS, or SerRS, and 0−50 mM CoA-SH (sodium salt, adjusted to pH 7.4 with NaOH). Radiolabeled aminoacyl thioesters, separated by TLC and visualized by autoradiography, were quantitated by scintillation counting. Panel A shows apparent rate constants, *k*, for aminoacyl-S−CoA formation as a function of CoA-SH concentration. Panel B shows Eadie−Hofstee plots of the data. Kinetics of aminoacyl-S−CoA formation catalyzed by IleRS (□), native MetRS (♦), truncated but biologically active MetRS547 (e.g., ref *9*) (●), AspRS (◊), and SerRS (×) are shown.

Chemical reactivity tests, similar to those described in the preceding section for aminoacyl−CoA adducts, determined that the Ile−pantetheine adduct was sensitive to NaOH, hydroxylamine, but resistant to iodoacetate treatments (not shown), thus confirming that the thiol group of pantetheine is the site of attachment for isoleucine.

Formation of Ile-S−CoA, Met-S−CoA, Asp-S−CoA, and Ser-S−CoA Exhibits Saturation Kinetics with Respect to the Concentration of CoA-SH. Rates of formation of aminoacyl-S−CoA catalyzed by IleRS, MetRS, AspRS, and SerRS were measured as a function of CoA-SH concentration in reaction mixtures containing an AARS, radiolabeled amino acid, and ATP. As shown in Figure 5A, aminoacylation of CoA-SH with isoleucine, methionine, aspartate, and serine catalyzed by IleRS, MetRS, AspRS, and SerRS, respectively, followed saturation kinetics typical of enzymatic reactions, indicating that CoA-SH binds to each of the four enzymes. Eadie−Hofstee plots of the data (Figure 5A) yield K_m values for CoA-SH of 15.7 ± 0.8, 17.1 ± 1.6, 3.9 ± 0.6, and 13.9 ± 3.6 mM with IleRS, MetRS, AspRS, and SerRS, respectively. The fastest aminoacylation of CoA-SH was observed with MetRS (k_{cat} = 1.06 ± 0.06 min^{-1}) and the slowest with SerRS (k_{cat} = 0.12 ± 0.017 min^{-1}). However, the reaction catalyzed by AspRS exhibited the highest catalytic efficiency

with the k_{cat}/K_m value of 95 min^{-1} M^{-1}. Catalytic efficiencies of the reactions catalyzed by MetRS, IleRS, and SerRS were 1.6-, 2.4-, and 12.6-fold lower, respectively.

CoA-SH Is an Efficient Acceptor for Amino Acid from an Aminoacyl-tRNA·AARS Complex. Kinetic indices for the aminoacylation of CoA-SH and other thiols were also determined using radiolabeled aminoacyl-tRNAs as donors. The progress of reactions was monitored by measurements of the amount of remaining radiolabeled aminoacyl-tRNA by trichloracetic acid precipitation (see Materials and Methods). CoA-SH appears to be a 28-fold better acceptor for methionine from MetRS·Met-tRNA (Table 1) than from MetRS·Met-AMP (Figure 5), mostly due to lower K_m value for CoA-SH in its reaction with MetRS·Met-tRNA than in the reaction with MetRS·Met-AMP. However, CoA-SH is about as efficient acceptor for aspartate from AspRS·Asp-tRNA as from AspRS·Asp-AMP, with k_{cat}/K_m values of 119 (Table 1) and 95 min^{-1} M^{-1} (Figure 5), respectively. It is likely that aminoacyl-tRNA induces better binding of CoA-SH to an AARS in the methionine system, but not in the aspartate system. In the aspartate system, catalytic efficiency for CoA-SH was similar to that for pantetheine (k_{cat}/K_m = 119 min^{-1} M^{-1}, Table 1), somewhat greater that that for cysteine (k_{cat}/K_m = 96 min^{-1} M^{-1}, Table 1), but 155-fold greater that that for cysteamine. Glutathione was essentially not a substrate for aspartylation by AspRS (Table 1). In the methionine system, catalytic efficiency for CoA-SH (k_{cat}/K_m = 1600 min^{-1} M^{-1}, Table 1) was similar to that for cysteine (k_{cat}/K_m = 1318 min^{-1} M^{-1}, Table 1). On the other hand, catalytic efficiency for CoA-SH was 2.3-, 22-, and 516-fold greater, respectively, than that for cysteamine (k_{cat}/K_m = 690 min^{-1} M^{-1}), pantetheine (k_{cat}/K_m = 74 min^{-1} M^{-1}), and glutathione (k_{cat}/K_m = 3.1 ± 0.3 min^{-1} M^{-1}). This appears to be due to much better binding of CoA-SH (K_m = 0.82 ± 0.15 mM) than of any other thiol, such as pantetheine (K_m = 11.3 ± 2.3 mM), cysteamine (K_m = 51 ± 7 mM), cysteine (K_m = 116 ± 14 mM), or glutathione (K_m > 60 mM) to MetRS·Met-tRNA. 3′-Dephosphorylated CoA-SH (10 mM) was aminoacylated 70% and 40% as fast as CoA-SH by MetRS and IleRS, respectively (not shown). Aminoacylation of other thiols, such as 3-mercaptopropionate, *N*-acetyl-L-cysteine, and D,L-homocysteine catalyzed by MetRS (*12*) and AspRS (*5*) was also less efficient than aminoacylation of CoA-SH by each of these AARSs. Collectively, these data suggest a certain degree of specificity for CoA-SH in the thiol aminoacylation reactions.

DISCUSSION

CoA-SH and pantetheine are biologically important thiols that participate in carboxyl group activation reactions, with the exception of coded protein synthesis (*16*, *17*). This paper demonstrates that CoA-SH and pantetheine are also substrates for AARSs whose major biological function is to provide aminoacyl-tRNAs for coded protein synthesis. Aminoacyl-S−CoA and aminoacyl-S−pantetheine thioesters, respectively, are the products of these reactions.

The data presented in this paper suggest that enzymatic aminoacylation of CoA-SH involves its binding to each of the four AARSs examined: class I IleRS and MetRS and class II AspRS and SerRS. For example, aminoacylation of CoA-SH catalyzed by each of these enzymes exhibits saturation kinetics with respect to the concentration of CoA-SH, a hallmark of enzymatic reactions involving substrate binding. One may argue that Michaelis−Menten kinetics in the enzymatic aminoacylation of CoA-SH arise because AARS·AA-tRNA (or AA-AMP) complex reacts with CoA-SH in a second-order reaction to give the products and an inactive form of the AARS that slowly reverts to the active form. In such a case, the apparent k_{cat} would be the rate constant for the enzyme reactivation, independent of the nature of a thiol. However, this is unlikely because, as shown in Table 1, the apparent k_{cat} values for aminoacylation of thiols vary depending on the nature of thiol substrates.

Because the thiol group of CoA-SH chemically reacts with activated amino acid to form a thioester, AA-S−CoA, the thiol group of CoA-SH must bind in the active site of an AARS right next to the activated carboxyl of an amino acid substrate. Since aminoacyl-S−CoA can be synthesized from both AA-tRNA and AA-AMP, the binding site for CoA-SH must be separate from the tRNA and ATP binding sites on these enzymes. Most likely, the thiol group of CoA-SH enters the thiol-binding subsite of the synthetic/editing active site of MetRS (*12*) and IleRS (*13*) or a vestigial thiol-binding subsite of the active site of AspRS and SerRS (*5*). It appears that CoA-SH binds better than its smaller precursors, pantetheine and cysteamine, to AspRS and MetRS (Table 1), consistent with the possibility that all portions of the CoA-SH molecule contribute to its binding. Thus, it appears that the thiol-binding subsite is a part of a larger CoA-SH binding site. The observations that glutathione and several other thiols are aminoacylated much less efficiently than CoA-SH suggest that substrate specificities of AARSs in these reactions are similar to those of other CoA-SH-utilizing enzymes rather than to those of glutathione-utilizing enzymes (*24*).

If we assume that the thiol-binding subsite of MetRS or IleRS evolved to accept a relatively small side chain of homocysteine, then it is suprising that such thiol-binding subsite would also accept the bulky molecule of CoA-SH. However, if we assume that the thiol-binding subsite is a part of CoA-SH-binding site, as the data presented in this paper suggest, then it is easier to imagine how this thiol-binding portion of the CoA-SH binding site would accept the side chain of homocysteine. Both the side chain of homocysteine and the thiol portion of the CoA-SH molecule have identical chemical structures, $HS\text{-}CH_2\text{-}CH_2$-, that undergo similar thiol aminoacylation reactions. The presence of CoA-SH-binding sites in AspRS and SerRS (AARSs that do not possess editing functions) also acounts for the presence of thiol-binding sites in these AARSs. A CoA-SH-binding site provides an AARS with a thiol-binding subsite that may potentially be utilized for editing.

The aminoacylations of CoA-SH and pantetheine catalyzed by AARSs, reported in this paper, are important because they are reminiscent of the transient formation of aminoacyl-S−pantetheine thioesters in the multienzyme thiotemplate nonribosomal peptide synthesis (*16*, *17*) and, despite the lack of similarities in primary structures (*17*), suggest a functional link between these amino acid activating systems (Figure 6). Thus, the ability of AARSs to bind and aminoacylate CoA-SH and panthetheine could be a vestige from a thioester world (*15*) in which ancestralAARSs provided aminoacyl-thioesters for noncoded peptide synthesis.

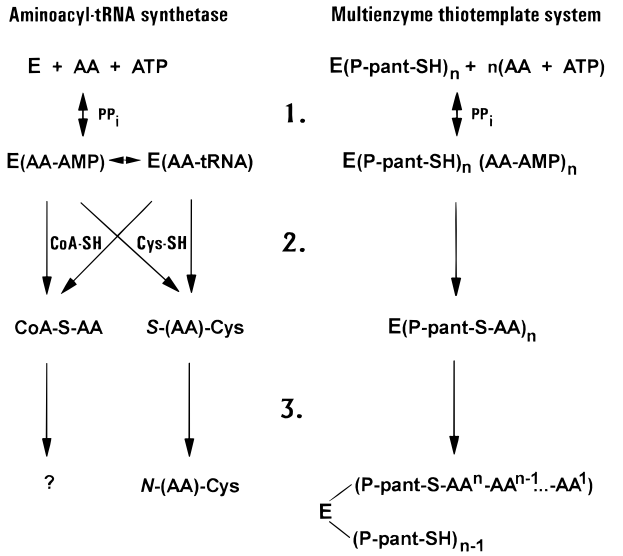

FIGURE 6: Functional analogies between aminoacyl-tRNA synthetases (AARSs) and multienzyme thiotemplate systems (MTSs). Similar steps in reactions are indicated: 1, amino acid activation leads to formation of enzyme-bound aminoacyl adenylates, E(AA-AMP); 2, transfer of an activated amino acid to the thiol group of an acceptor leads to thioester bond formation; 3, transacylation from sulfur to amino group results in peptide bond formation. In the MTSs, activated amino acids are condensed to peptides via thioester intermediates. The thiol group of active site pantetheine serves as an intermediate acceptor of an amino acid moiety from an aminoacyl adenylate in each domain. Peptide bond formation occurs as a result of intermolecular 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 (*17*). In each domain, pantetheine cofactor is attached to a serine residue via phosphate. The '*n*' denotes the number of domains (and the length of synthesized peptide) in the MTSs. With AARSs, CoA-SH and pantetheine can serve as acceptors for activated amino acids, and corresponding thioesters are formed (this work). When cysteine [or cysteamine] is an acceptor, the thioesters *S*-(AA)-Cys [or *S*-(AA)-cysteamine] are not directly observed because of facile intramolecular transacylation from the sulfur to the amino group, which results in the formation of a peptide bond (*5*, *12*−*14*, *26*).

It has been proposed that the thioester-dependent mechanism of peptide synthesis may have preceded the RNA-dependent mechanism in the development of life (*16*). This proposal was taken further by suggesting that thiols were part of the early organic molecules that seeded the development of life on the prebiotic Earth (*15*). Prebiotic conditions may have favored formation of thioesters from thiols and amino acids or other acids. For example, thioesters form easily under acidic conditions. Thioesters would have provided protometabolism with catalysis and energy. Remnants of this thioester world are still present: thioesters are immensely important in the present-day metabolism (*15*). The ability of the present-day AARSs to synthesize aminoacyl-S−CoA, described in this paper, could be another remnant of the thioester world that may have preceded the RNA world (*15*). It has been speculated that the ability of the present-day AARSs to aminoacylate RNA minihelixes and tetraloops is an evolutionary vestige from a stage of the development of the aminoacylation function in the RNA world (*25*). It is plausible that the thioester-dependent and RNA-dependent peptide synthesizing systems were developing in parallel, possibly via some common stages. Peptides that eventually led to ancestral AARSs could have been equally well formed from aminoacyl-RNA and aminoacyl-thioesters (*26*). Before the development of coded protein synthesis, ancestral AARSs could have facilitated formation of aminoacyl-RNA and aminoacyl-thioesters for noncoded peptide assembly. The abilities of present-day AARSs to catalyze formation of aminoacyl-RNA (*25*, *27*) and aminoacyl-S−CoA and aminoacyl-S−pantetheine (this work) are consistent with this hypothesis. Ancestral AARSs themselves may have carried out thiol-dependent assembly of peptides. A vestige of this peptide-forming ability exists in the present-day AARSs, which indeed can promote the synthesis of cysteine-containing di- and tripeptides (*5*, *12*−*14*). It would be interesting to determine if a high molecular weight complex of AARSs, which is present in mammalian cells but whose function is not clear (*28*), is a vestige of an ancestral multienzyme noncoded peptide synthesis system and, like the present-day multienzyme thiotemplate systems, may have the ability to synthesize peptides.

REFERENCES

1. Jakubowski, H., and Goldman, E. (1992) *Microbiol. Rev. 56*, 412−429.
2. Jakubowski, H., and Fersht, A. R. (1981) *Nucleic Acids Res. 9*, 3105−3117.
3. Englisch, S., Englisch, U., von der Haar, F., and Cramer, F. (1986) *Nucleic Acids Res. 14*, 7529−7539.
4. Jakubowski, H. (1980) *Biochemistry 19*, 5071−5078.
5. Jakubowski, H. (1997) *Biochemistry 36*, 11077−11085.
6. Jakubowski, H. (1995) *J. Biol. Chem. 270*, 17672−17673.
7. Jakubowski, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4504−4508.
8. Jakubowski, H. (1991) *EMBO J. 10*, 593−598.
9. Gao, W., Goldman, E., and Jakubowski, H. (1994) *Biochemistry 33*, 11528−11535.
10. Jakubowski, H., and Goldman, E. (1993) *FEBS Lett. 317*, 237−240.
11. Jakubowski, H. (1997) *J. Biol. Chem. 272*, 1935−1942.
12. Jakubowski, H. (1996) *Biochemistry 35*, 8252−8259.
13. Jakubowski, H. (1996) *Nucleic Acids Res. 24*, 2505−2510.
14. Jakubowski, H. (1995b) *Nucleic Acids Res. 23*, 4608−4615.
15. de Duve, C. (1994) *Vital Dust*, pp 43−45, Basic Books, New York.
16. Lipmann, F. (1971) *Science 173*, 875−884.
17. Kleikauf, H., and von Döhren, H. (1996) *Eur. J. Biochem. 236*, 335−351.
18. Webster, T., Tsai, H., Kula, M., Mackie, G. A., and Schimmel, P. (1984) *Science 226*, 1315−1317.
19. Brevet, A., Chen, J., Leveque, F., Plateau, P., and Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8275−8279.
20. Eriani, G., Dirheimer, G., and Gangloff, J. (1990) *Nucleic Acids Res. 18*, 7109−7117.
21. Härtlein, M., Madern, D., and Leberman, R. (1987) *Nucleic Acids Res. 15*, 1005−1017.
22. Kim, H. Y., Ghosh, G., Schulman, L. H., Brunie, S., and Jakubowski, H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11553−11557.
23. Bochner, B. R., and Ames, B. N. (1982) *J. Biol. Chem. 257*, 9759−9769.
24. Racker, E. (1953) *Fed. Proc. 12*, 711−714.
25. Schimmel, P., and Henderson, B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11283−11286.
26. Wieland, T., and Pfeiderer, G. (1957) *Adv. Enzymol. Relat. Subj. Biochem. 19*, 235−266.
27. Frugier, M., Florentz, C., and Giege, R. (1994) *EMBO J. 13*, 2219−2226.
28. Mirande, M. (1991) *Prog. Nucleic Acids Res. Mol. Biol. 40*, 95−142.

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