Affinity Labeling of Aminoacyl-tRNA Synthetases with Adenosine Triphosphopyridoxal: Probing the Lys-Met-Ser-Lys-Ser Signature Sequence as the ATP-Binding Site in *Escherichia coli* Methionyl- and Valyl-tRNA Synthetases†

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ABSTRACT: Pyridoxal 5′-triphospho-5′-adenosine (AP_3-PL), the affinity labeling reagent specific for lysine residues in the nucleotide-binding site of several enzymes [Tagaya, M., & Fukui, T. (1986) *Biochemistry 25*, 2958–2964; Yagami, T., Tagaya, M., & Fukui, T. (1988) *FEBS Lett. 229*, 261–264], was used to identify the ATP-binding site of *Escherichia coli* methionyl-tRNA synthetase (MetRS). Incubation of this enzyme with AP_3-PL followed by reduction with sodium borohydride resulted in a rapid inactivation of both the $tRNA^{Met}$ aminoacylation and the methionine-dependent ATP–PP_i exchange activities. Complete inactivation corresponded to the incorporation of 0.98 mol of AP_3-PL/mol of monomeric trypsin-modified MetRS. ATP or MgATP protected the enzyme from inactivation. The labeling with AP_3-PL was also applied to *E. coli* valyl-tRNA synthetase (ValRS). Both the $tRNA^{Val}$ aminoacylation and the valine-dependent ATP–PP_i exchange activities were abolished by the incorporation of 0.91 mol of AP_3-PL/mol of monomeric ValRS. AP_3-PL was found attached to lysine residues 335, 402, and 528 in the primary structure of MetRS. In the case of ValRS, the AP_3-PL-labeled residues corresponded to lysines 557, 593, and 909. We therefore conclude that these lysines of MetRS and ValRS are directed toward the ATP-binding site of these synthetases, more specifically at or close to the subsite for the γ-phosphate of ATP. AP_3-PL-labeled Lys-335 of MetRS and Lys-557 of ValRS belong to the consensus tRNA CCA-binding Lys-Met-Ser-Lys-Ser sequence [Hountondji, C., Dessen, P., & Blanquet, S. (1986) *Biochimie 68*, 1071–1078]. This indicates that, in the case of the two studied enzymes, the ATP-dependent activation of the amino acid and its subsequent transfer to the 3′-adenosine of tRNA take place in the same region. Furthermore, comparison of the primary structure of MetRS around Lys-402 to that of ValRS around Lys-593 reveals little significant similarity, with four identities and two conservative replacements out of 11 amino acid residues.

During protein biosynthesis, aminoacyl-tRNA synthetases activate their specific amino acid at the expense of ATP and ensure the subsequent charging of the aminoacyl moiety onto the 3′-end of specific isoacceptor tRNAs (Schimmel & Söll, 1979). The specific recognition of tRNAs by aminoacyl-tRNA synthetases makes these enzymes attractive model systems for studying protein–nucleic acid interaction. High-resolution crystal structures are available for three aminoacyl-tRNA synthetases: a trypsin-modified monomeric form of methionyl-tRNA synthetase from *Escherichia coli* (Zelwer et al., 1982; Brunie et al., 1987, 1990), tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (Bhat et al., 1982), and *E. coli* glutaminyl-tRNA synthetase complexed with both ATP and $tRNA^{Gln}$ (Rould et al., 1989).

To probe the structure–activity relationships of aminoacyl-tRNA synthetases, chemical modifications and site-directed mutagenesis are being currently used. In particular, several methods dealing with the covalent labeling of the tRNA binding site of aminoacyl-tRNA synthetases have already been developed. These methods were based on chemical cross-linking between synthetases and tRNAs induced either by ultraviolet irradiation (Schimmel, 1977) or with the help of reactive groups introduced into the tRNA molecule (Akhverdyan et al., 1977; Valenzuela & Schulman, 1986; Leon & Schulman, 1987). Another strategy involved the use of periodate-oxidized tRNA as an affinity label for basic amino acid residues within the CCA-binding site of tRNA on aminoacyl-tRNA synthetases (Fayat et al., 1979; Hountondji et al., 1979, 1985, 1986a, 1987). Comparison of the amino acid sequences of the peptides labeled by oxidized tRNA in *E. coli* methionyl- and tyrosyl-tRNA synthetases has shown significant similarities (Hountondji et al., 1985, 1986a). From this, the consensus sequence KMSKS[1] could be proposed that serves now as a signature sequence for aminoacyl-tRNA synthetases specific for the aliphatic hydrophobic class of amino acids (Hountondji et al., 1986b).

The lysine residues of the KMSKS sequence must be close to the active center of aminoacyl-tRNA synthetases, since the 3′-end of tRNA has to react with the anhydride bond of the enzyme-bound aminoacyl adenylate. As discussed in Hountondji et al. (1980), these lysines may serve either to guide the tRNA CCA arm or to help adenylate formation by interacting with the ATP substrate as suggested by the following data: the tRNA-labeled lysine residues (Lys-229, -234, and -237) of *E. coli* tyrosyl-tRNA synthetase (Hountondji et al., 1986a)

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[1] Abbreviations: AP_3-PL, pyridoxal 5′-triphospho-5′-adenosine (or adenosine triphosphopyridoxal). Aminoacyl-tRNA synthetases are abbreviated as a three-letter code of their specific amino acid followed by RS; the one- and three-letter amino acid codes are those suggested by the IUPAB–IUB Commission on Biochemical Nomenclature.

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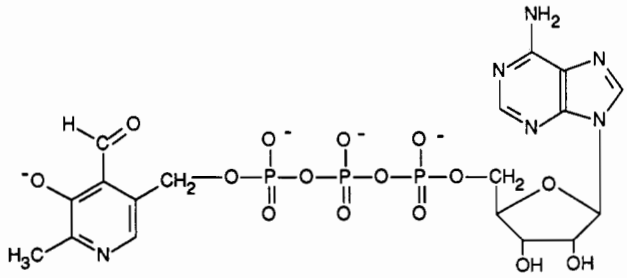

FIGURE 1: Structure of pyridoxal 5′-triphospho-5′-adenosine (AP_3-PL) (Tagaya & Fukui, 1986; Yagami et al., 1988).

are found conserved in the homologous enzyme from *B. stearothermophilus* as Lys-225, -230, and -233. Two of the latter lysines have been shown by mutagenesis to be involved in the stabilization of the transition state during the synthesis of tyrosyl adenylate (Fersht et al., 1988). Moreover, in the case of MetRS, many observations have evidenced the importance of positive charges in the active site of this enzyme on the way from methionine and ATP to methionyl adenylate [reviewed in Blanquet et al. (1979)]. In particular, the occurrence of a cationic locus at the catalytic center of MetRS was deduced from the demonstration of synergistic couplings between the sites for methionine and adenosine 5′-triphosphate in the methionine activation reaction (Fayat et al., 1977).

Several attempts have been made to affinity label the ATP-binding site of various aminoacyl-tRNA synthetases (Ankilova et al., 1975; Akhverdyan et al., 1977), including the use of periodate-oxidized ATP (Fayat et al., 1978). However, these approaches did not succeed in identifying the corresponding reactive amino acid residues. In this study, we take advantage of pyridoxal 5′-triphospho-5′-adenosine (Figure 1) to explore the ATP-binding site of *E. coli* methionyl- and valyl-tRNA synthetases. These enzymes share the KMSKS signature sequence.

Pyridoxal 5′-triphospho-5′-adenosine (or adenosine triphosphopyridoxal) is an adenine nucleotide analogue with an adenosine diphosphate moiety and a pyridoxal phosphate moiety capable of covalently modifying amino groups in or near the phosphate binding site. Nucleotide polyphosphopyridoxals have been recently used as affinity labeling reagents of lysyl residues at the nucleotide binding site of various proteins (Tagaya & Fukui, 1986; Tagaya et al., 1985, 1987; Yagami et al., 1988; Rao et al., 1988). In particular, the structural similarities between pyridoxal 5′-triphospho-5′-adenosine and ATP allowed Yagami et al. (1988) to specifically label and identify the ATP-binding site in rabbit muscle adenylate kinase.

Materials and Methods

Materials

Native methionyl-tRNA synthetase was purified to homogeneity from the overproducing strain PAL 1803.5 carrying recombinant plasmid pX1 (Dardel et al., 1984). Trypsin-modified methionyl-tRNA synthetase was derived from the native enzyme as already described (Cassio & Waller, 1971). Valyl-tRNA synthetase was prepared according to Brevet et al. (1989). The concentration of methionyl-tRNA synthetase was determined from its absorbance at 280 nm by using a specific extinction coefficient of 1.47 units·mg^{-1}·cm^2 and a molecular ratio of 64K (Blanquet et al., 1973). For valyl-tRNA synthetase, the molecular ratio and optical extinction coefficient at 280 nm were 108K and 1.53 units·mg^{-1}·cm^2, respectively, as deduced from the amino acid composition (Härtlein et al., 1987; Heck & Hatfield, 1988).

Pyridoxal 5′-triphospho-5′-adenosine (AP_3-PL) (Figure 1) was synthesized and purified as already described (Tagaya & Fukui, 1986). Its molar absorption coefficient at 393 nm in 0.1 N NaOH was 5550 M^{-1}·cm^{-1}. The following materials were purchased from the sources indicated: L-methioninol from Fluka, ATP and AMP from Boehringer (Mannheim), and TPCK-treated trypsin from Serva.

For all the HPLC analyses, data acquisition and processing were made with a Nelson Analytical Model 762S interface linked to an IBM AT computer with Nelson Analytical Series 3000 software.

Methods

Inactivation Kinetics. Inactivation of 6 μM methionyl-tRNA synthetase (or valyl-tRNA synthetase) was carried out in the presence of 0.3 mM AP_3-PL at 37 °C in 50 μL of 50 mM HEPES (pH 7.8) containing 1 mM EDTA and 0.1 mM 2-mercaptoethanol. At different times, 5-μL portions of the incubation mixture were withdrawn and quenched by the addition of 15 μL of $NaBH_4$ (0.1 M) in 10 mM NaOH. After 2 min of reduction, portions were diluted with 1 mL of Tris-HCl buffer (pH 7.6) containing 10 mM 2-mercaptoethanol and 200 μg/mL bovine serum albumin. The diluted enzyme solutions were allowed to stand in an ice bath, and their residual tRNA aminoacylation and amino acid dependent isotopic [^{32}P]ATP–PP_i exchange activities were measured (Lawrence et al., 1973; Blanquet et al., 1974). Control experiments without AP_3-PL or with 0.3 mM ATP instead of AP_3–PL were also carried out.

Stoichiometry of Labeling. Incorporation of AP_3-PL into trypsin-modified MetRS or native ValRS was monitored by incubation of the enzyme (6 μM) in the presence of various concentrations of AP_3-PL at 37 °C in the same buffer conditions as above. It was verified that the inactivation reaction was completed within 10 min at any concentration of the reagent examined. After 15 min, a 35-μL aliquot of each incubation mixture was withdrawn and quenched with $NaBH_4$; then 5 μL of each $NaBH_4$-quenched sample was diluted and assayed for the amino acid dependent isotopic [^{32}P]ATP–PP_i exchange activity, while the remaining 30 μL was chromatographed through a TSK 4000 PW column (300 × 7.5 mm) equilibrated with 0.1 M ammonium acetate (pH 7.5). Elution was monitored by the absorbance at 325 nm and by fluorescence (excitation at 280 nm and emission recorded at 330 nm). The absorbance at 325 nm reflects the pyridoxal phosphate moiety of AP_3-PL. A molar absorption coefficient of 8800 M^{-1}·cm^{-1} at 325 nm and pH 7.5 was used to calculate the amount of AP_3-PL bound to the enzyme (Forrey et al., 1971; Tagaya & Fukui, 1986; Colanduoni & Villafranca, 1985). Enzyme concentration in the eluate was calculated from the area of the tryptophan fluorescence peak.

Trypsin Digestion. The AP_3-PL-labeled synthetase (6 μM in a 10-mL incubation mixture) was dialyzed overnight at 4 °C against 2 L of 0.1 M NH_4HCO_3 (pH 8). Dialysis was continued for 8 h at 4 °C after the bath was changed. The protein was digested overnight at 37 °C with TPCK-treated trypsin, at a protease to substrate ratio of 1:50 (w/w). Then, a second equal addition of trypsin was made and digestion was continued during 3 h at 37 °C. The tryptic digest was frozen and lyophilized.

Peptide Purification. Tryptic peptides were purified by three consecutive chromatographic steps. In step 1, the tryptic digest was applied to a Vydac C4 reverse-phase column (150 mm × 2.1 mm) equilibrated with 10 mM ammonium formate buffer (pH 7.2) (solvent A). Linear gradients of acetonitrile were developed from 0% to 40% B during 80 min (solvent B:

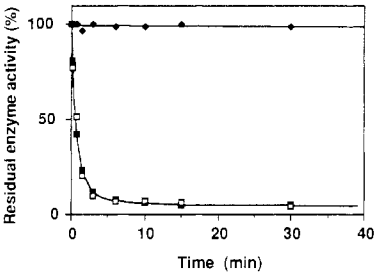

FIGURE 2: Time course of inactivation of methionyl-tRNA synthetase by AP_3-PL. The enzyme (6 μM) was incubated with 0.3 mM AP_3-PL at 37 °C in the presence of HEPES buffer (50 mM, pH 7.8), 2-mercaptoethanol (0.1 mM), and EDTA (1 mM). At the indicated times, aliquots were quenched by reduction with $NaBH_4$, diluted, and assayed for $tRNA^{Met}$ aminoacylation (□) or isotopic ATP–PP_i exchange (■) activities. The control experiments, assayed for $tRNA^{Met}$ aminoacylation or isotopic ATP–PP_i exchange activities (◆), were without AP_3-PL or with ATP instead of AP_3-PL.

20% solvent A in acetonitrile). The flow rate was 0.25 mL/min. The absorbance of peptides was monitored at 215 nm (spectromonitor D, LDC). The label was detected by the fluorescent emission at 395 nm with excitation at 335 nm (fluorescence spectrophotometer F1000, Merck).

In step 2, fractions containing the label were pooled, lyophilized, and applied to a column of TSK 4000 PW coupled to a TSK 2000 SW column. The eluent was 0.2 M ammonium bicarbonate (pH 8). The flow rate was 0.5 mL/min. Both absorbance at 215 nm and fluorescence at 395 nm were monitored.

In step 3, fluorescent material was loaded on a Vydac C18 reverse-phase column (150 mm × 2.1 mm) equilibrated with 10 mM ammonium formate (pH 7.2) (solvent A). Solvent B was 20% solvent A in acetonitrile. Peptides were eluted with a linear gradient of solvent B, from 0% to 75%, over 60 min. The flow rate was 0.25 mL/min. Dual detection (fluorescence and absorbance) was as above.

N-Terminal Sequencing. Automated Edman degradation was carried out on a gas-phase sequencer (Applied Biosystems, Model 470A). Peptides samples (30 μL, 100–500 pmol) were loaded on glass fiber filters treated with 2.5 mg of Biobrene (Applied Biosystems) and previously submitted to three precycles. PTH-amino acids were identified as already described (Beauvallet et al., 1988).

RESULTS

Inactivation of Methionyl-tRNA Synthetase by Pyridoxal 5′-Triphospho-5′-adenosine. Both the ATP–PP_i exchange and tRNA aminoacylation activities of methionyl-tRNA synthetase (MetRS) (6 μM) were abolished by more than 95% within 10 min upon incubation with 0.3 mM pyridoxal 5′-triphospho-5′-adenosine (AP_3-PL) (Figure 2). The addition of 10 mM $MgCl_2$ to the incubation mixture resulted in an acceleration of the inactivation reaction (not shown).

Prior reduction of AP_3-PL with sodium borohydride prevented the inactivation and the labeling of the enzyme (Figure 3, lane h). Moreover, inactivation could be fully reversed by 200-fold dilution of the sample in the incubation buffer, provided that the borohydride reduction step was omitted (not shown). These behaviors suggest that inactivation occurred well through the formation of a reversible Schiff base between the aldehyde group of the reagent and amino group(s) of the synthetase.

Stoichiometry of Labeling of MetRS by AP_3-PL. The stoichiometry of labeling was established by first incubating MetRS with AP_3-PL at several concentrations and then separating the enzyme-bound reagent from the unbound one by

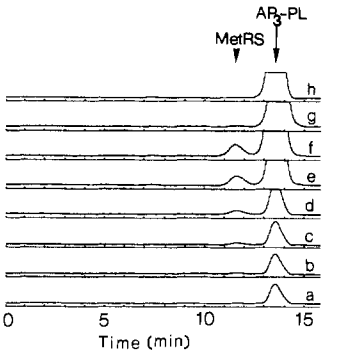

FIGURE 3: TSK 4000 chromatography of methionyl-tRNA synthetase incubated with increasing concentrations of AP_3-PL. The enzyme (6 μM) was incubated with AP_3-PL at the following concentrations: 3 μM (a), 6 μM (b), 15 μM (c), 30 μM (d), 300 μM (e), or 600 μM (f). Protection (g) by the combination of L-methioninol (8 mM) and MgATP (8 mM) was carried out in the presence of 600 μM AP_3-PL. Control experiments (h) were performed with 600 μM AP_3-PL that had been reduced with 75 mM $NaBH_4$ (30 min at room temperature) prior to enzyme addition. After incubation at 37 °C for 10 min, $NaBH_4$ (75 mM) was added to each sample, and a 30-μL aliquot of each incubation mixture was applied to a TSK 4000 PW column (300 mm × 7.5 mm) and eluted as described under Methods. The covalently bound and the unbound $NaBH_4$-reduced AP_3-PL thus separated were detected by the absorbance at 325 nm. The elution times of MetRS and AP_3-PL (indicated by the arrows) were determined by TSK analysis of each MetRS and $NaBH_4$-reduced AP_3-PL.

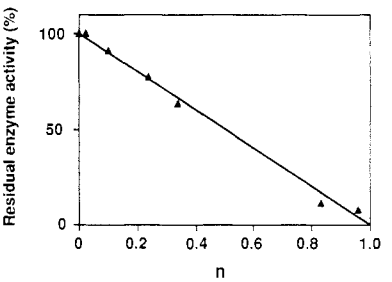

FIGURE 4: Stoichiometry of labeling of MetRS by AP_3-PL. The incubation mixtures were the same as in Figure 3. In parallel to the TSK 4000 analysis of 30-μL aliquots (Figure 3), 5-μL aliquots were diluted and assayed for isotopic ATP–PP_i exchange activity. The residual enzyme activity is plotted against the molar fraction of AP_3-PL incorporated per mole of enzyme (n) (calculated from Figure 3).

size-exclusion chromatography on a TSK 4000 PW column (Figure 3). For each sample, the residual methionine-dependent ATP–PP_i exchange activity was measured and plotted as a function of the ratio of bound AP_3-PL to enzyme. Complete inactivation corresponded to the incorporation of 0.98 ± 0.02 mol of AP_3-PL/mol of the monomeric trypsin-modified MetRS (Figure 4). This result agrees with the known presence of one binding site for ATP or MgATP on trypsin-modified MetRS (Fayat & Waller, 1974; Hyafil et al., 1976). The data from Figures 3 and 4 were also used to plot the fraction of inactivated MetRS at equilibrium versus increasing reagent concentrations. Figure 5 shows the saturation curve obtained. It indicates at K_d value of AP_3-PL in the inactivation process of about 600 μM.

Protection of MetRS against Inactivation by AP_3-PL. The effects of substrates on the inactivation of MetRS by 0.3 mM AP_3-PL were examined (Figure 6). The presence of 8 mM ATP afforded full protection. The protection was slightly decreased if 8 mM $MgCl_2$ was also present. This slight difference might be related to the lower affinity of ATP–Mg^{2+} to the enzyme, compared to that of ATP (K_d = 4 mM vs 0.2 mM) (Fayat et al., 1977).

When combined with L-methioninol (8 mM), the analogue of methionine in which the carboxylate is replaced by a

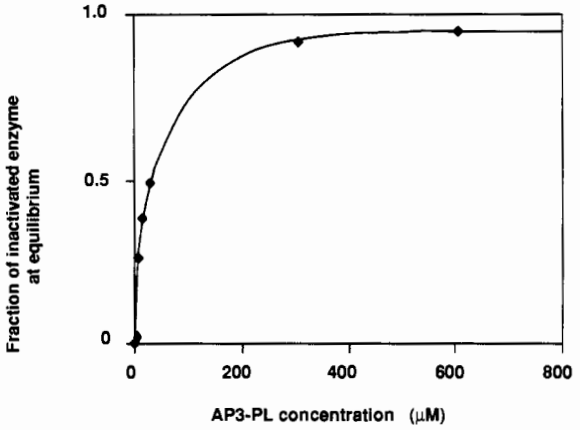

FIGURE 5: Saturation of MetRS by AP_3-PL. The data were from Figures 3 and 4. Analysis of the extent of inactivation of MetRS measured at equilibrium as a function of AP_3-PL concentration was performed according to the following reaction scheme (Fayat et al., 1979) with $K_d = [E][AP_3\text{-PL}]/[E{\cdot}AP_3\text{-PL}]$, the equilibrium constant

$$E + AP_3\text{-PL} \overset{K_d}{\rightleftharpoons} E{\cdot}AP_3\text{-PL} \overset{K}{\rightleftharpoons} E\text{-}AP_3\text{-PL}$$

of the enzyme with the reagent, and $K = [E\text{-}AP_3\text{-PL}]/[E{\cdot}AP_3\text{-PL}]$, the Schiff base equilibrium. The apparent equilibrium constant for inactivation $K_d/K = [E][AP_3\text{-PL}]/[E\text{-}AP_3\text{-PL}]$, as deduced from the saturation curve, is equal to 30 μM. Calculating $K = 19$ from the percentage of maximum inactivation at saturating AP_3-PL enables the deduction of a K_d value of 600 μM.

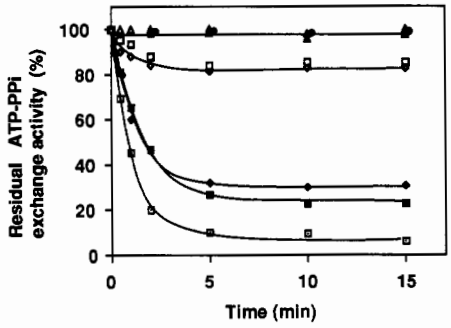

FIGURE 6: Substrate protection of methionyl-tRNA synthetase against inactivation by AP_3-PL. The enzyme (6 μM) was preincubated in 50 mM HEPES (pH 7.8) for 5 min, without addition (⊡) or with 8 mM AMP (■), 8 mM MgATP (◇), 8 mM ATP (▲), 8 mM L-methioninol (◆), 8 mM L-methioninol + 8 mM AMP (□), or 8 mM L-methioninol + 8 mM MgATP (●). The reaction was started by addition of 0.3 mM AP_3-PL, and aliquots were withdrawn and assayed for enzyme activity as described under Methods. Control experiments with or without ATP (△) were also performed. Conditions were as described in Figure 2.

CH_2OH group, ATP–Mg^{2+} (8 mM) completely protected the enzyme from inactivation. Combination of L-methioninol (8 mM) and AMP (8 mM) provided substantial protection, whereas each of these ligands alone was less effective in protecting the enzyme from inactivation by AP_3-PL (Figure 6).

L-Methioninol is known to couple synergistically with (i.e., to reinforce) ATP or AMP binding within the site of amino acid adenylation (Fayat et al., 1977; Blanquet et al., 1975). Therefore, protection by L-methioninol in the presence of ATP–Mg^{2+} or AMP reflects the synergistic coupling and suggests at the same time that labeling by AP_3-PL occurs well at the enzyme active site.

In order to prove that protection of the enzyme activity by the synergistic couple L-methioninol/MgATP (Figure 6) precludes any incorporation of AP_3-PL into the enzyme, incubation mixtures containing 6 μM MetRS, 0.6 mM AP_3-PL with or without 8 mM ATP, 8 mM $MgCl_2$, and 8 mM L-methioninol were analyzed by gel filtration. The comparison

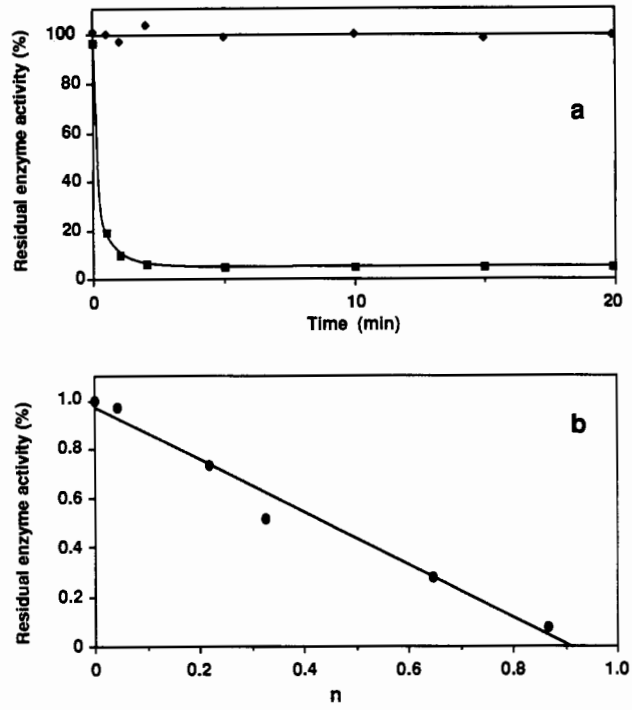

FIGURE 7: Inactivation and labeling of ValRS by AP_3-PL. The experimental procedure was identical with that for MetRS (see Figures 2–4). (a) Time course of inactivation of 6 μM ValRS by 0.3 mM AP_3-PL; (b) stoichiometry of labeling.

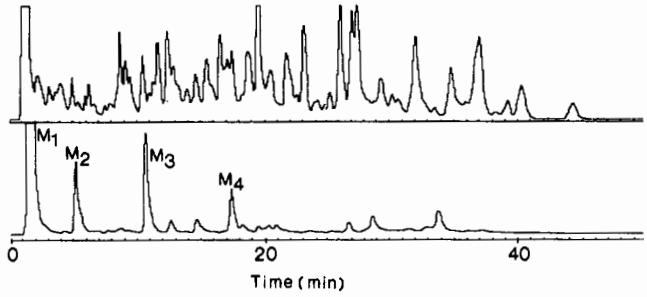

FIGURE 8: High-performance liquid chromatography of the AP_3-PL-labeled peptides of MetRS. The tryptic digest was applied to a C4 reverse-phase column, as described under Methods. The tryptic peptides were separated with an 80-min linear gradient from 0% to 30% CH_3CN. Absorbance at 215 nm (upper panel) and fluorescence (lower panel) were monitored. The four main peaks containing fluorescent material were numbered M_1–M_4. Each of the peaks was further purified to homogeneity by HPLC.

of lanes g and f in Figure 3 shows that incorporation of AP_3-PL into MetRS in the presence of L-methioninol + MgATP was made negligible. A stoichiometry of 0.07 mol of reagent incorporated per mole of MetRS was calculated, indicating that at least 90% of the ATP-binding site is protected by the methioninol/ATP couple.

Inactivation and Labeling of Valyl-tRNA Synthetase by AP_3-PL. Similarly to MetRS, valyl-tRNA synthetase (6 μM) was rapidly and completely inactivated upon incubation with 0.3 mM AP_3-PL (Figure 7a). Both the $tRNA^{Val}$ aminoacylation and the valine-dependent ATP–PP_i exchange activities were abolished by the labeling. Incorporation of 0.91 mol of AP_3-PL completely inactivated 1 mol of ValRS (Figure 7b). This result is in agreement with the known 1:1 ValRS:ATP active stoichiometry (Berthelot & Yaniv, 1970; Fersht & Dingwall, 1979).

AP_3-PL-Labeled Peptides of MetRS. The AP_3-PL-labeled MetRS was digested with trypsin, and the tryptic peptides were fractionated by three consecutive chromatographic steps. In the first step, the tryptic digest was applied to a Vydac C4

Table I: Amino Acid Sequences of AP_3-PL-Labeled Peptides of MetRS and ValRS[a]

peptide	amino acid sequence
	MetRS
M_2	333 * 337 Met-Ser-Lys-Ser-Arg
M_3	396 * 403 Asn-Ala-Gly-Phe-Ile-Asn-Lys-Arg
M_4	524 * 533 Val-Asn-Pro-Phe-Lys-Ala-Leu-Tyr-Asn-Arg
	ValRS
V_2	555 * 559 Met-Ser-Lys-Ser-Lys
V_3	590 * 595 Leu-Ala-Asp-Lys-Ile-Arg
V_4	906 * 915 Ile-Glu-Asn-Lys-Leu-Ala-Asn-Glu-Gly-Phe

[a] A solid arrow indicates residues identified as PTH-amino acids. An asterisk indicates presumed AP_3-PL-labeled lysine residues (see Results).

reverse-phase column. Fluorescent material was eluted as four major peaks, numbered M_1, M_2, M_3, and M_4 (Figure 8). Each of these peaks was purified further by size-exclusion chromatography followed by RPLC on a Vydac C18 column. Peak M_1, which was eluted in the void volume of the C4 reverse-phase column, was the label itself, as revealed by control RPLC analysis of $NaBH_4$-reduced AP_3-PL and by sequence analysis of peak M_1. In fact, it appeared that the reagent was not totally eliminated by dialysis prior to tryptic digestion and peptide purification. Peptides M_2, M_3, and M_4 represented 25%, 32%, and 15% of the labeled synthetase, respectively. Other peaks, each accounting for less than 4% of the loaded peptidic material, were ignored. Altogether, these unidentified peaks represented 20% of the labeled synthetase.

Automated Edman degradation allowed peptides M_2, M_3, and M_4 to be identified in the primary structure of *E. coli* methionyl-tRNA synthetase (Dardel et al., 1984). The expected phenylthiohydantoin (PTH) derivative of the AP_3-PL-labeled lysines could not be detected, however (Table I) (Tagaya et al., 1987). As reported by Sejlitz et al. (1990), it is much probable that, after it was cleaved in the presence of trifluoroacetic acid (TFA), the anilinothiazolinone (ATZ) derivative of the AP_3-PL-labeled lysine could not be extracted and transferred from the filter cartridge to the conversion flask of the gas-phase sequencer because of its strong adsorption to the polybrene-treated glass-fiber filter and/or its low solubility in the extraction solvent (butyl chloride). Peptides M_2, M_3, and M_4 corresponded respectively to positions 333–337 (Met-Ser-Lys-Ser-Arg), 396–403 (Asn-Ala-Gly-Phe-Ile-Asn-Lys-Arg), and 524–533 (Val-Asn-Pro-Phe-Lys-Ala-Leu-Tyr-Asn-Arg) in the sequence of MetRS (Dardel et al., 1984). Thus, the AP_3-PL-labeled amino acid residues of peptides M_2, M_3, and M_4 were identified as Lys-335, Lys-402, and Lys-528, respectively (Table I).

The lysine residues that were protected by substrates (Figure 6) were identified as follows: Either MgATP or ATP (8 mM) or MgATP (8 mM) + L-methioninol (8 mM) was incubated with 6 μM MetRS prior to the addition of 0.3 mM AP_3-PL. At time 15 min, the incubation mixtures were treated with 75 mM $NaBH_4$, dialyzed against NH_4HCO_3, and treated with trypsin as described under Methods. The tryptic digests were analyzed by HPLC on a Vydac C4 reverse-phase column (results not shown). In the case of MgATP-protected MetRS, the three peaks corresponding to Lys-335, Lys-402, and Lys-528 were decreased to 8%, 5%, and 7% of the total protein, respectively, indicating that all these lysines were protected to similar extents by the presence of MgATP. In the enzyme protected with ATP or MgATP + L-methioninol, none of the three lysine residues could be found labeled.

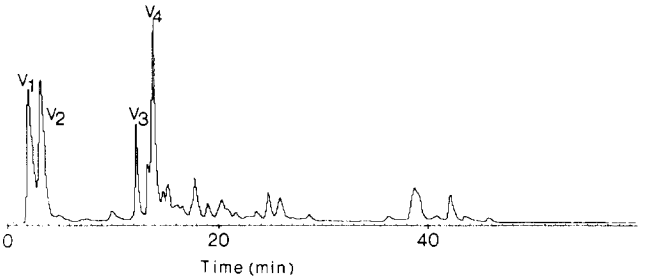

FIGURE 9: High-performance liquid chromatography of the AP_3-PL-labeled peptides of ValRS. The experimental procedure was identical with that in Figure 8. Only the monitoring by fluorescence is shown. Four main peaks containing fluorescent material (numbered V_1–V_4) were obtained.

AP_3-PL-Labeled Peptides of ValRS. Figure 9 shows separation of the AP_3-PL-labeled tryptic peptides of ValRS. As in the case of MetRS, the peak (V_1) in the void volume of the C_4 column was the label itself. The peptidic material eluted as peaks V_2, V_3, and V_4, which represented 32%, 11%, and 30% of the labeled synthetase, respectively. The amino acid sequences of tryptic peptides V_2, V_3, and V_4 were compared to the primary structure of *E. coli* ValRS (Härtlein et al., 1987; Heck & Hatfield, 1988). Peptides V_2, V_3, and V_4 coincide with positions 555–559 (Met-Ser-Lys-Ser-Lys), 590–595 (Leu-Ala-Asp-Lys-Ile-Arg), and 906–915 (Ile-Glu-Asn-Lys-Leu-Ala-Asn-Glu-Gly-Phe), respectively, in the sequence of ValRS. The AP_3-PL-labeled lysine residues of ValRS are Lys-557, Lys-593, and Lys-909 (Table I).

Discussion

Inactivation and Labeling of Methionyl-tRNA Synthetase by AP_3-PL. Several observations suggest that AP_3-PL (Figure 1) is an affinity labeling reagent for methionyl-tRNA synthetase. (i) First, incubation of this reagent with MetRS resulted in the rapid loss of enzymatic activity. Both the methionine-dependent isotopic [^{32}P]PP_i–ATP exchange and $tRNA^{Met}$ aminoacylation activities were destroyed in parallel by the labeling. Inactivation of MetRS by AP_3-PL occurred through the formation of a Schiff base between the aldehyde group of the reagent and amino groups of the protein, since, in particular, inactivation could be reversed by dilution if the borohydride reduction step was omitted and since reduction of AP_3-PL by $NaBH_4$ prior to incubation with the enzyme prevented the labeling. (ii) Second, the stoichiometry of labeling corresponded to 1 mol of AP_3-PL incorporated per mole of enzyme, consistent with the 1:1 trypsin-modified MetRS:ATP active stoichiometry. (iii) Third, AP_3-PL incorporation into MetRS, as well as the resulting enzyme inactivation, showed saturation behavior with respect to the reagent concentration, indicating that AP_3-PL binds reversibly to the enzyme. The plot of the fraction of inactivated MetRS at equilibrium versus increasing reagent concentrations indicated a K_d value of AP_3-PL in the inactivation process of about 600 μM, suggesting that the affinity of AP_3-PL for MetRS remains comparable to that of ATP (K_d = 180 ± 20

μM), as measured by equilibrium dialysis (Fayat et al., 1977). (iv) Finally, ATP or MgATP protected the enzyme from inactivation by AP_3-PL. Moreover, L-methioninol, the analogue of methionine in which the carboxylate is replaced by a CH_2OH group, formed with MgATP or AMP a synergistic couple that strongly protected the enzyme from inactivation and labeling, as expected. Altogether, these results support the view that AP_3-PL, when bound to MetRS, is well fitted to the ATP-binding site.

AP_3-PL-Labeled Peptides of MetRS. Three labeled peptides (M_2, M_3, and M_4) were isolated that represented 25%, 32%, and 15% of the total AP_3-PL-labeled MetRS, respectively. Peptides M_2, M_3, and M_4 encompassed lysines 335, 402, and 528, respectively, in the primary structure of MetRS. Thus, even though affinity labeling of MetRS with AP_3-PL yields a covalent 1:1 complex, consistent with the existence of one ATP-binding site (Fayat et al., 1977), more than one peptide were found to carry the label. This result strongly suggests that, when bound to the ATP site, the label may react with any of the three above lysines in a mutually exclusive fashion. Moreover, covalent modification of each of the lysines appeared to equally block the enzyme activity.

Lysines 335 and 528 have been located in the crystallographic structure of methionyl-tRNA synthetase, which is solved at 2.5-Å resolution (Brunie et al., 1987, 1990). The MetRS polypeptide chain is folded into two domains. The amino-terminal 360 residues comprise the N-terminal domain, which contains the $\beta\alpha\beta$ units of the "mononucleotide binding fold" (Risler et al., 1981; Blow et al., 1983). The second domain is the C-terminal one, which is composed of residues 361–551 and is dominated by α-helices. The active-site cleft is located at the junction of the two domains. The C-terminal end folds back toward the N-terminal domain, so that the segment 520–537 recovers the catalytic crevice (Brunie et al., 1987, 1990).

Lys-335 is located at the end of the fifth β-strand of the nucleotide binding fold in a loop that forms one surface of the MetRS active site [color plate 1 of Brunie et al. (1987)]. Previous chemical cross-linking of the 3′-adenosine of periodate-treated $tRNA^{Met}$ to MetRS has demonstrated that Lys-335 is an essential residue which participates in the tRNA CCA-binding site (Hountondji et al., 1985). Further support for an essential role of Lys-335 of MetRS comes from site-directed mutagenesis, since replacement of this residue with a glutamine residue resulted in the loss of both the ATP–PP_i exchange and tRNA aminoacylation activities (Brunie et al., 1987; Méchulam et al., 1990). The Lys-Met-Ser-Lys-Ser sequence surrounding the catalytically essential Lys-335 of MetRS was observed in a number of aminoacyl-tRNA synthetases, providing additional support for the importance of this residue (Hountondji et al., 1986b).

Several roles are possible for Lys-335. As suggested by previous affinity labeling studies, this residue could serve as a ligand for the CCA arm of tRNA through electrostatic interactions between a phosphate group of the nucleic acid and an ϵ-amino group (Hountondji et al., 1980, 1986b). However, the present labeling of Lys-335 with AP_3-PL suggests that this residue could be involved in the binding of ATP. Indeed, the presence of a positively charged amino acid side chain in the adenylating site of MetRS was predicted from the study of the coupling of methionine binding with that of ATP (Fayat et al., 1977; Hyafil et al., 1976). Lys-335 is a likely candidate for this cationic locus that is supposed to interact with either the carboxylate of the amino acid or the α-phosphate of the nucleotide. However, a proximity between the α-phosphate of ATP and the Lys-335 side chain in the enzyme·ATP complex is ruled out by the fact that the reactive pyridoxal moiety of AP_3-PL is attached to the γ-phosphate. Thus, alternatively, Lys-335 might contact and help orient the γ-phosphate of bound ATP in such a way as to facilitate pyrophosphate liberation. At this stage the possibility remains, however, that during the reaction of adenylate formation the conformation of the enzyme changes, making the Lys-335 residue closer to the α-phosphate of ATP. Evidence for such conformational change during aminoacyl adenylate synthesis is provided by the rather large associated variations of the fluorescence of the tryptophan residues of the enzyme (Blanquet et al., 1972; Hyafil et al., 1976).

Lys-528 is located in the middle of the protruding C-terminal tail of the enzyme [color plate 1 of Brunie et al. (1987)] with its ϵ-amino group pointing toward the catalytic crevice, about 4 Å apart from the ϵ-NH_2 of Lys-335 (S. Brunie, personal communication). This places the pyridoxal-linked γ-phosphate of ATP at the top of the active-site crevice, while the adenine moiety should lie in the bottom. It is possible that the labeling of Lys-528 is only due to its proximity to Lys-335 and that this residue is not involved in ATP binding or adenylate formation. Note that Lys-335 and Lys-528 may come even closer to each other, owing to the flexibility of the Lys-528 region as suggested by site-directed mutagenesis studies (Mellot et al., 1989). Mellot et al. (1989) proposed also that the Lys-528 region is involved in the binding and the guiding of the 3′-acceptor end of tRNA toward the active-site crevice. Thus, it is tempting to suppose that, like Lys-335, Lys-528 may interact with either the γ-phosphate of ATP or the acceptor arm of tRNA. Consistently, the synergistic couple methioninol + MgATP, which is shown here to block AP_3-PL incorporation into MetRS, was also shown to completely block the incorporation of periodate-oxidized $tRNA^{Met}$ (Hountondji, 1985). Altogether, these data suggest that the ATP-dependent activation of methionine and its subsequent transfer to the 3′-adenosine of $tRNA^{Met}$ take place in the same restricted area of MetRS.

In the available X-ray structure of MetRS, Lys-402 is not located in the active-site crevice. Rather, it is located in a loop situated in the C-terminal helical domain, about 30 Å apart from the enzyme active site (Brunie et al., 1990). Thus, it was surprising to find that modification of Lys-402 could block enzyme activity. Indeed, the enzyme is inactivated by 95%, while 32% of the enzyme is labeled at the level of lysine 402 only.

The possibility that Lys-402 was labeled in an unspecific manner seems to be excluded by the protection afforded by the synergistic couple L-methioninol/MgATP or L-methioninol/AMP. In the presence of these ligands, the binding of which is directed toward the active site of the enzyme, covalent incorporation of the reagent into the enzyme is inhibited. Indeed, the percentage of incorporation of AP_3-PL into MetRS is by far less in the presence of L-methioninol + MgATP than in its absence (7% vs 95%), especially to Lys-402 (0% vs 32%). Moreover, in the enzyme protected with MgATP, lysines 335, 402, and 528 were equally protected, while in the presence of ATP or MgATP + L-methioninol, none of the three lysine residues was found labeled. Therefore, the three AP_3-PL-labeled lysines, including Lys-402, appear to belong to the same ATP-binding site.

The possibility may be considered that the enzyme has two distinct nucleotide-binding sites, with Lys-402 representing one of these sites. Previous studies using equilibrium dialysis (Fayat & Waller, 1974) have indicated the existence in each

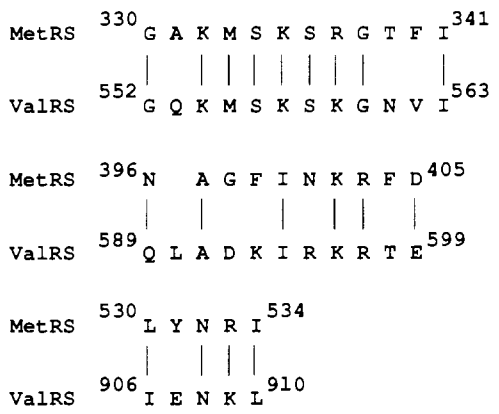

FIGURE 10: Alignment of the AP_3-PL-labeled M_2, M_3, and M_4 peptides of MetRS with the corresponding ones (V_2, V_3, and V_4) of ValRS. Solid vertical lines indicate identical or chemically similar residues (K = R, Q = N, D = E, L = I). Sequence information was taken from Dardel et al. (1984) and Heck and Hatfield (1988).

promoter of the native dimeric MetRS of a low-affinity ATP-binding site, in addition to the high-affinity one. However, no such situation was reported for the monomeric tryptic fragment that we used in this study. Furthermore, the presence of two distinct ATP-binding sites in the tryptic fragment of MetRS is ruled out by the 1:1 enzyme:AP_3-PL stoichiometry of labeling. Another rather unlikely possibility would be the occurrence of two mutually exclusive ATP sites per monomer.

In fact, Leon and Schulman (1987) have shown that Lys-402 can be cross-linked to a DTSP/PDA reactive arm attached to the D-loop of $tRNA^{fMet}$. In another work, Bruton (1979) has demonstrated that lysines 402, 609, and 659 of the native dimeric MetRS were shielded from acetylation upon binding $tRNA^{Met}$. These reports suggest that Lys-402 participates in the binding site of the tRNA polynucleotide and, as such, could correspond to a subsite capable of nonspecifically binding a nucleotide. Further studies are necessary to resolve this contradiction.

AP_3-PL-Labeled Peptides of ValRS. The affinity-labeling method was also applied to valyl-tRNA synthetase. The latter synthetase was shown to be covalently bound to AP_3-PL, with total loss of both the $tRNA^{Val}$ aminoacylation and the isotopic ATP–PP_i exchange activities. The stoichiometry of labeling was 1 mol of AP_3-PL incorporated per mole of ValRS, consistent with the presence of one ATP-binding site on the synthetase. These results, together with the demonstration that AP_3-PL is an affinity-labeling reagent for MetRS, suggest that AP_3-PL may represent a tool for the exploration of the ATP-binding site on other aminoacyl-tRNA synthetases.

From the tryptic digest of the AP_3-PL-labeled ValRS, three peptides, V_2, V_3, and V_4, were isolated that encompassed lysines 557, 593, and 909, respectively, in the primary structure of the enzyme. Lys-557 belongs to a sequence (^{554}Lys-Met-Ser-Lys-Ser-Lys^{559}) that closely resembles the above cited Lys-Met-Ser-Ly-Ser-Arg active-site sequence of MetRS (Figure 10). The latter six residues long stretch was observed among the aminoacyl-tRNA synthetase family (Hountondji et al., 1986b), especially in a subfamily of synthetases including isoleucyl-, leucyl-, and valyl-tRNA synthetases (Webster et al., 1984; Härtlein & Madern, 1987; Härtlein et al., 1987; Heck & Hatfield, 1988; Borgford et al., 1987; Jordana et al., 1987). In particular, the Lys-Met-Ser-Lys-Ser sequence represents one of the most conserved regions in the primary structures of three valyl-tRNA synthetases of prokaryotic and eukaryotic origins (Härtlein et al., 1987; Heck & Hatfield, 1988; Borgford et al., 1987; Jordana et al., 1987). The presence of the Lys-Met-Ser-Lys-Ser sequence in the ATP-binding site of ValRS demonstrates that this amino acid sequence has also a functional significance in ValRS and suggests at the same time that it may be considered as a signature sequence for the active site of aminoacyl-tRNA synthetases (Hountondji et al., 1986b). Consistent with this is the fact that the KMSKS-like sequences of TyrRS and GlnRS (Hountondji et al., 1986a,b) were recently shown to be involved in ATP binding. Indeed, Fersht et al. (1988) have demonstrated that lysines 230 and 233 of the KFGKT sequence of *B. stearothermophilus* TyrRS contribute to the stabilization of the transition state during the synthesis of tyrosyl adenylate. Recently, crystal structure resolution of the complex of GlnRS with ATP and $tRNA^{Gln}$ has revealed that Lys-270, which belongs to the sequence MSK of GlnRS, interacts with ATP phosphates (Rould et al., 1989). Thus, the above cited lysine residues of TyrRS and GlnRS as well as Lys-335 of MetRS and Lys-557 of ValRS might participate to the transition-state complex stabilization through electrostatic interaction with the pyrophosphate moiety of ATP.

Finally, comparison of peptide M_3 of MetRS to peptide V_3 of ValRS has revealed little significant primary structure similarity, with four identities and two conservative replacements out of 11 amino acid residues (Figure 10). There is no sequence similarity around M_4 and V_4. However, we noted that residues 530–534 in MetRS show one identity and three conserved substitutions with residues 906–910 in ValRS (Figure 10).

A striking feature of the active-site sequences of MetRS and ValRS is the location of the critical lysine residues in the C-terminal half of the primary structures of these synthetases. Lys-335 and Lys-528 of MetRS are located in two regions essential for the structuring of the active site of this synthetase: Lys-335 lies near the $\beta\alpha\beta$ units of the mononucleotide binding fold (Risler et al., 1981), while Lys-528 belongs to segment 528–537 that overhangs the catalytic crevice (Brunie et al., 1987, 1990; Mellot et al., 1989). These observations, together with the primary structure similarities between the labeled peptides of MetRS and ValRS, argue for a similar folding of the C-terminal region of these synthetases.

It should be noted that primary structure similarities between MetRS and IleRS from *E. coli* (Webster et al., 1984) along with the structural modeling of IleRS (Starzyk et al., 1987) previously suggested that a similar fold occurs in the N-terminal parts of these two synthetases. Since IleRS also shares extensive primary structure similarities with ValRS and LeuRS (Heck & Hatfield, 1988; Tzagoloff et al., 1988), it is likely that IleRS, LeuRS, ValRS, and MetRS present similar structural modules all along their 3-D models. IleRS from *Saccharomyces cerevisiae* also shows significant primary structure similarities to MetRS, LeuRS, and ValRS (Englisch et al., 1987). Furthermore, deletion by mutagenesis of a small part of the C-terminus of yeast IleRS results in the loss of enzyme activity (Englisch-Peters & Cramer, 1989). This finding indicates that, similarly to MetRS (Mellot et al., 1989), the C-terminus of IleRS participates in shaping an active-site region otherwise formed by the N-terminal domain. These observations agree with the proposition by Wetzel (1978) that the four synthetases (IleRS, LeuRS, MetRS, ValRS) are members of a particular subfamily of synthetases that may have close evolutionary relation.

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Registry No. ATP, 56-65-5; L-Lys, 56-87-1; aminoacyl-transfer RNA synthetase, 9028-02-8; methionyl-transfer RNA synthetase, 9033-22-1; valyl-transfer RNA synthetase, 9023-47-6; adenosine triphosphopyridoxal, 101418-63-7.

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