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Identification of Peptide Sequences at the tRNA Binding Site of *Escherichia coli* Methionyl-tRNA Synthetase†

Dario Valenzuela‡ and LaDonne H. Schulman*
Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: Four different structural regions of *Escherichia coli* $tRNA^{fMet}$ have been covalently coupled to *E. coli* methionyl-tRNA synthetase (MetRS) by using a tRNA derivative carrying a lysine-reactive cross-linker. We have previously shown that this cross-linking occurs at the tRNA binding site of the enzyme and involves reaction of only a small number of the potentially available lysine residues in the protein [Schulman, L. H., Valenzuela, D., & Pelka, H. (1981) *Biochemistry 20*, 6018–6023; Valenzuela, D., Leon, O., & Schulman, L. H. (1984) *Biochem. Biophys. Res. Commun. 119*, 677–684]. In this work, four of the cross-linked peptides have been identified. The tRNA–protein cross-linked complex was digested with trypsin, and the peptides attached to the tRNA were separated from the bulk of the tryptic peptides by anion-exchange chromatography. The tRNA-bound peptides were released by cleavage of the disulfide bond of the cross-linker and separated by reverse-phase high-pressure liquid chromatography, yielding five major peaks. Amino acid analysis indicated that four of these peaks contained single peptides. Sequence analysis showed that the peptides were cross-linked to $tRNA^{fMet}$ through lysine residues 402, 439, 465, and 640 in the primary sequence of MetRS. Binding of the tRNA therefore involves interactions with the carboxyl-terminal half of MetRS, while X-ray crystallographic data have shown the ATP binding site to be located in the N-terminal domain of the protein [Zelwer, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81]. The methods developed for the present studies should be applicable to determination of peptide sequences at the tRNA binding sites of other proteins.

The highly specific interaction of transfer RNAs with aminoacyl-tRNA synthetases represents an attractive model system for study of the molecular basis of RNA–protein recognition. The interaction of *Escherichia coli* initiator methionine tRNA ($tRNA^{fMet}$)[1] with *E. coli* methionyl-tRNA synthetase (MetRS) has been actively investigated in this laboratory for a number of years [for reviews, see Schulman (1979) and Schulman & Pelka (1977a, 1984)]. These studies have provided considerable information on the role of specific nucleotide bases in $tRNA^{fMet}$ in recognition by MetRS. In contrast, little is known about the peptide sequences which comprise the tRNA binding site of the enzyme. Covalent cross-linking of $tRNA^{fMet}$ to MetRS provides an approach to determining the relative orientation of the two macromolecules in solution. High-resolution X-ray crystallographic data for the tRNA (Woo et al., 1980) and for a biologically active

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‡ Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

[1] Abbreviations: $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; DTSP, dithiobis(succinimidyl propionate); PDA, propane-1,3-diamine; DTSP/PDA-$tRNA^{fMet}$, $tRNA^{fMet}$ modified with PDA followed by coupling to DTSP; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; DMF, dimethylformamide; TFA, trifluoroacetic acid; GdmCl, guanidinium chloride; TPCK, tosylphenylalanine chloromethyl ketone; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; DABITC, 4-(dimethylamino)azobenzene-4′-isothiocyanate; DABS or dabsyl, 4-(dimethylamino)azobenzene-4′-sulfonyl; ODS, octadecylsilane; DEAE, *N*,*N*-diethylaminoethyl.

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monomeric form of MetRS (Zelwer et al., 1982) have been reported, providing a unique opportunity to interpret the results of cross-linking experiments in light of the known three-dimensional structures of the two macromolecules.

A prerequisite to such studies is the availability of cross-linking methods suitable for obtaining high-yield coupling reactions. Direct UV irradiation has been the most commonly used method to cross-link tRNA–aminoacyl-tRNA synthetase complexes (Schoemaker & Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975; Rosa et al., 1979; Renaud et al., 1979; Baltzinger et al., 1979; Ackerman et al., 1985); however, in only two cases have specific cross-linked nucleotides on the tRNA been located with this technique (Ackerman et al., 1985), and no photo-cross-linked peptides have been identified to date. Chemically reactive groups have been attached to the amino acid moiety of aminoacyl-tRNAs (Bruton & Hartley, 1970; Santi et al., 1973; Santi & Cunnion, 1974; Lavrik & Khutoryanskaya, 1974; Bartmann et al., 1974; Gorshkova & Lavrik, 1975; Gorshkova et al., 1975; Akhverdyan et al., 1977; Knorre & Kisselev, 1980), to 4-thiouridine residues (Budker et al., 1974; Gorshkova et al., 1976; Wetzel & Soll, 1977), and to internal guanosine residues (Vlasov et al., 1980). Although covalent coupling to aminoacyl-tRNA synthetases could be observed, no peptide sequences have yet emerged from these studies. Only two examples are presently known of successful identification of aminoacyl-tRNA synthetase peptides by affinity labeling with tRNAs. Both of these cases involve the coupling of tRNAs having periodate-oxidized 3′ termini to lysine residues at the active sites of the enzymes (Renaud et al., 1982; Hountondji et al., 1985).

In order to obtain high-yield cross-linking to other regions of $tRNA^{fMet}$, we have developed new methods to attach protein affinity labeling reagents of variable length and amino acid specificity to internal sites in the tRNA (Sarkar & Schulman, 1978; Schulman et al., 1981a). We have previously shown that a $tRNA^{fMet}$ derivative carrying a cleavable lysine-reactive cross-linker can be quantitatively coupled to the tRNA binding site of MetRS (Schulman et al., 1981b; Valenzuela et al., 1984). In the present paper, we report the sequences of four peptides covalently coupled to this modified tRNA.

Materials and Methods

Materials

Dithiobis(succinimidyl propionate) (DTSP), sequanal-grade trifluoroacetic acid, ethyl acetate, butyl acetate, pyridine, and heptane were purchased from Pierce Chemical Co. Propane-1,3-diamine (PDA), *N,N′*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, sodium sulfide nonahydrate, and spectrophotometric-grade dimethyl sulfoxide were obtained from Aldrich Chemical Co. Amino acid standards for high-sensitivity protein hydrolysis, DTT, grade I sodium bisulfite, and β-propiolactone were from Sigma Chemical Co. Sublimed sulfur was from Mallinckrodt. HPLC-grade acetonitrile was from Waters Associates, Inc. Highly purified water was obtained from a Milli-Q reagent-grade water system (Millipore Corp). Dabsyl chloride and DABITC were purchased from Fluka Chemical Corp. and purified further by recrystallization from boiling acetone. [^{35}S]DTSP was synthesized by the method of Lomant and Fairbanks (1976). ^{35}S-Labeled elemental sulfur and [^{35}S]methionine were obtained from Amersham. Superfine Sephacryl S-200 and SP-Sephadex C-50 were obtained from Pharmacia Fine Chemicals and 8-mL capacity collodion bags (25 000 molecular weight cutoff) from Schleicher & Schuell. *E. coli* $tRNA^{fMet}$ having a specific activity of 1720 pmol/A_{260} unit was purchased from Boehringer Mannheim. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K12 strain EM20031 as described before (Schulman & Pelka, 1977b). TPCK-treated trypsin was obtained from Worthington Biochemical Corp. and further purified as described by Beeley and Neurath (1968), except that the chromatography was carried out on SP-Sephadex C-50.

Methods

Large-Scale Affinity Labeling of $tRNA^{fMet}$ and Cross-Linking to MetRS. PDA-$tRNA^{fMet}$ was prepared by treatment of $tRNA^{fMet}$ with propane-1,3-diamine/bisulfite as described before (Schulman et al., 1981a,b), and the extent of modification was determined by the method of Sarkar and Schulman (1978). [^{35}S]DTSP (15.8 mg, 225 cpm/pmol) was dissolved in 2.25 mL of Me_2SO in a 50-mL Sorvall Tefzel centrifuge tube, followed by addition of 75 A_{260} units of PDA-$tRNA^{fMet}$ (1 mol of PDA/mol of tRNA) in 10 mL of 0.2 M Hepes, pH 7.8. The tube was vortexed vigorously and incubated at 25 °C for 15 min. Two volumes of cold ethanol (−20 °C) were added, and the sample was chilled at −80 °C for 5 min. After centrifugation in a Sorvall SS-34 rotor for 10 min at 15 000 rpm, the tRNA pellet was redissolved in 3.8 mL of 0.1 M sodium acetate, pH 6, transferred to a new 50-mL centrifuge tube, and precipitated with cold ethanol as before. The tRNA was precipitated for an additional 3 times until a constant cpm/A_{260} ratio was obtained. Under these conditions, 71% of the tRNA was radioactively labeled with DTSP. The modified tRNA was redissolved in 10 mM $MgCl_2$ immediately before use in the cross-linking reaction.

Cross-linking was carried out in a reaction mixture containing 4 μM MetRS and 7–10 μM [^{35}S]DTSP/PDA-$tRNA^{fMet}$ in a total volume of 10.2 mL of 20 mM Hepes, pH 8, and 10 mM $MgCl_2$ at 25 °C for 30 min. The reaction was quenched by addition of 1 mL of 0.5 M glycine, pH 7. Small aliquots (2 × 5 μL) were removed for measurement of residual enzyme activity and binding of radioactively labeled tRNA to nitrocellulose filters as described before (Schulman et al., 1981b).

Isolation of Cross-Linked Peptides. The cross-linking reaction mixture was concentrated to 5.9 mg of protein/mL in a collodion bag and dialyzed vs. 0.1 M ammonium acetate, pH 6.5, and 0.1 mM $CaCl_2$. The sample was transferred to a 12-mL Pyrex centrifuge tube and digested with RNase-free trypsin at a ratio of 3/100 (w/w) trypsin/protein for 7 h at 25 °C. The tryptic digest was adjusted to 85 mM NaCl by the addition of 4 M NaCl. Three volumes of cold ethanol were added, and the sample was chilled at −20 °C for 10 min, followed by centrifugation for 10 min in an IEC Model CL clinical centrifuge. The pellet containing the tRNA-bound peptides was redissolved in 7 M urea, 0.1 M NaCl, and 0.05 M ammonium acetate, pH 6.5 (buffer A), and injected onto a 75 × 7.5 mm Bio-Gel TSK-DEAE 5 PW ion-exchange HPLC column (Bio-Rad) equilibrated with the same buffer. Peptides not attached to the tRNA were removed from the column with a 0–25% buffer B gradient in 5 min followed by a 20-min wash with 25% buffer B at a flow rate of 0.5 mL/min (buffer B = 7 M urea, 1 M NaCl, and 0.05 M ammonium acetate, pH 6.5). After 25 min, a second gradient from 25% to 100% buffer B was run over 35 min. This was followed by a 100% buffer B isocratic wash for 30 min. One-minute fractions were collected, and aliquots were taken for measurement of radioactivity. The radioactive peak from the second gradient was pooled and dialyzed 4 times for 2 h vs. 300 volumes of 0.1 M sodium acetate, pH 6.5. The dialysate was concentrated to 17 A_{260} units/mL and 0.3 M sodium acetate. Two volumes of ethanol were added, and the sample

was chilled at −80 °C for 10 min, followed by centrifugation for 10 min as described above. The pellet was redissolved in 0.1 M sodium acetate, pH 6.5, and reprecipitated 3 times.

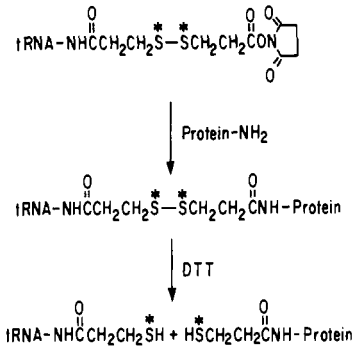
The pellet was dissolved in 0.1 M ammonium acetate, pH 6.5, 0.1 M NaCl, and 50 mM DTT and incubated at 37 °C for 1.5 h. Two volumes of ethanol were added, and the sample was chilled for 10 min at −20 °C and centrifuged for 10 min. The supernatant, containing the peptides released from the tRNA, was transferred to a glass centrifuge tube. Guanidinium chloride was added, and the ethanol was completely removed by evaporation. The sample was concentrated down to a volume of 0.5 mL, giving final concentrations of 0.2 M NaCl, 0.2 M ammonium acetate, and 6 M GdmCl. To ensure retention of the peptides on the reverse-phase HPLC column used in the following chromatographic step, the concentration of NaCl should not be higher than 0.35 M.

The peptides were applied to a 250 × 4.6 mm Altex PTH-amino acid ODS reverse-phase HPLC column equipped with a 45 × 4.6 mm precolumn (Rainin) and preequilibrated with 0.1% TFA in water (v/v) (buffer A). The peptides were eluted at a flow rate of 1.0 mL/min by using a Rainin gradient HPLC system and the following binary gradient profile: buffer A from 0 to 20 min, followed by a linear gradient from 0% to 42% buffer B in 130 min (buffer B = 0.1% TFA in CH_3CN v/v). A second linear gradient from 42% to 70% buffer B was run from 130 to 150 min, followed by a 10-min wash with 70% buffer B. The absorbance of the effluent at 280 nm was monitored by using an Altex Model 153 detector. One-minute fractions were collected, and 10-μL aliquots were taken for measurement of radioactivity.

Amino Acid Composition Analysis and Peptide Sequencing. (*A*) *Amino Acid Analysis Using Dabsyl Chloride.* The precolumn derivatization procedure of Chang et al. (1982) was used. Polypeptide (0.1–0.6 nM) was placed at the bottom of a heat-cleaned glass tube (3 mm × 10 cm) and dried in a vacuum heater at 40 °C. Twenty microliters of 6 M HCl (Pierce, sequanal grade) was added, and the tube was sealed under vacuum (2 cm from the top). After 24-h incubation at 110 °C, the tube was gently centrifuged and opened at a point 5 cm from the tube bottom. The sample was vacuum evaporated over NaOH pellets and subjected to dabsylation. Ten microliters of 0.1 M sodium bicarbonate buffer, pH 9.0, was added to the dried hydrolysate followed by 20 μL of DABS-Cl solution (4 nmol/μL in acetone). The tube was sealed with parafilm and aluminum foil and heated at 70 °C for 12 min, with occasional shaking. After derivative formation, the sample was dried under vacuum and redissolved in 110 μL of 70% v/v ethanol, and aliquots were subjected to HPLC analysis. Amino acid standards (100–500 pmol, Sigma A9781) were also subjected to acid hydrolysis under conditions identical with those for the unknown sample, followed by dabsylation.

Standard DABS-amino acids were separated by gradient elution using an Altex PTH-amino acid ODS HPLC column and a precolumn (Rainin) by a modification of the procedure of Chang et al. (1981). Chromatography was carried out at 50 °C at a flow rate of 0.85 mL/min. Buffer A was 13 mM KH_2PO_4, pH 6.5, and 2% v/v DMF; buffer B was 4% v/v DMF in CH_3CN. The column was preequilibrated with 20% buffer B. After injection of the sample, a linear gradient from 20% to 37% buffer B was run from 0 to 30 min. This was followed by a second linear gradient from 37% to 70% buffer B from 30 to 40 min and an isocratic wash of 70% buffer B from 40 to 47 min. The amino acid derivatives were detected by their absorbance at 436 nm, using an Altex Model 153 detector. Under these conditions, all of the normal DABS-amino acids were separated.

Scheme I: Pathway of Cross-Linking [^{35}S]DTSP/PDA-tRNA^{fMet} to MetRS



(*B*) *Amino Acid Analysis Using Phenyl Isothiocyanate.* The phenyl isothiocyanate precolumn derivatization procedure was carried out in a PICO-TAG work station (Waters Associates) at the Protein Sequencing Facility at Yale University as described by Stone and Williams (1986). Norleucine was added to each sample prior to hydrolysis to serve as an internal standard.

(*C*) *Automated Degradation of Cross-Linked Peptides.* Peptides were subjected to automated N-terminal degradation with PITC on an Applied Biosystems gas phase sequencer at Yale University as described by Stone and Williams (1986). High-sensitivity phenylthiohydantoin-amino acid detection was carried out as described by Merrill et al. (1984). Aliquots were taken at each cycle for measurement of radioactivity in order to determine the position of the lysine residue labeled with ^{35}S by cross-linking.

Results

Cross-Linking of a Lysine-Reactive Derivative of tRNA^{fMet} to MetRS. Lysine-reactive cross-linking groups were attached to *E. coli* tRNA^{fMet} by using a previously described two-step procedure (Schulman et al., 1981a,b). In the first step, three-carbon side chains terminating in a reactive primary amino group were joined to the N^4-position of single-stranded cytidine residues by transamination with propane-1,3-diamine (PDA) in the presence of bisulfite. ^{35}S-Labeled dithiobis-(succinimidyl propionate) (DTSP) was then coupled to each side chain by addition of excess diester to the PDA-modified tRNA. The resulting [^{35}S]DTSP/PDA-tRNA^{fMet} contained an average of one lysine-reactive side chain per molecule of tRNA distributed over four different structural regions: the 5′ terminus, the dihydrouridine loop, the anticodon, and the 3′-terminal CCA end. The side chain of the cross-linker contains a disulfide bond potentially capable of reacting with cysteine residues in MetRS. Previous studies have shown, however, that the tRNA is covalently coupled to the enzyme only through reaction with lysine residues (Schulman et al., 1981b; Valenzuela et al., 1984) as illustrated in Scheme I. Treatment of the cross-linked complex with DTT results in release of the tRNA and leaves an ^{35}S-labeled modified lysine residue at the site of each cross-link in the protein (Scheme I).

Cross-linking reaction mixtures contained 4 μM MetRS and 7–10 μM [^{35}S]DTSP/PDA-tRNA^{fMet} in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$. Samples were incubated at 25 °C for 30 min, and the reaction was quenched by addition of excess glycine. Assays of residual enzyme activity and measurement of DTT-stable ^{35}S coupled to MetRS by nitrocellulose filter binding showed that 60–70% of the enzyme was covalently

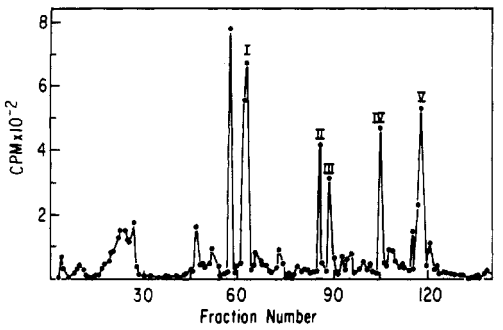


FIGURE 1: Reverse-phase chromatography of MetRS peptides cross-linked to [^{35}S]DTSP/PDA-tRNA^{fMet}. The sample in 0.5 mL of 6 M GdmCl was injected onto a reverse-phase HPLC column equilibrated with 0.1% TFA in water (v/v), and the peptides were eluted with a gradient of CH_3CN as described under Materials and Methods. One-minute fractions were collected at a flow rate of 1.0 mL/min, and 10-μL aliquots were taken for radioactivity measurements. The radioactivity eluting prior to fraction 60 is nonpeptide material.

cross-linked to tRNA under these conditions.

Purification of Peptides Cross-Linked to [^{35}S]DTSP/PDA-tRNA^{fMet}. The peptides cross-linked to [^{35}S]DTSP/PDA-tRNA^{fMet} were purified by using a protocol that takes advantage of the difference in properties of free and tRNA-bound peptides. The cross-linked enzyme, containing one tRNA molecule per dimer of native MetRS (Schulman et al., 1981b), and unreacted enzyme were digested with 3% trypsin (w/w) for 7 h at 25 °C. Un-cross-linked tRNA and the tRNA-bound peptides were precipitated with ethanol, and the supernatant containing the soluble peptides was removed. The tRNA pellet was dissolved in 7 M urea, 0.1 M NaCl, and 50 mM ammonium acetate, pH 6.5, and separated from free insoluble peptides by chromatography on a Bio-Gel TSK DEAE 5PW ion-exchange HPLC column under denaturing conditions (not shown). The tRNA fraction was pooled, dialyzed, and concentrated. The tRNA-bound peptides were released by cleavage of the disulfide bond of the cross-linker following incubation of the sample with 50 mM DTT at 37 °C for 1.5 h. The free tRNA was precipitated with ethanol, and the supernatant containing the released ^{35}S-labeled peptides was concentrated and taken up in 6 M GdmCl. The sample was injected onto a reverse-phase HPLC column, and the peptides were eluted with a CH_3CN/H_2O gradient in 0.1% TFA (Figure 1). The radioactive peaks eluting from the column prior to fraction 60 were also present in control samples of [^{35}S]DTSP/PDA-tRNA^{fMet} carried through the same procedure in the absence of MetRS and are degradation products of the cross-linker side chain released from the uncoupled tRNA (data not shown).

The supernatant obtained after ethanol precipitation of the tryptic digest of the cross-linked complex and the tRNA fraction obtained after incubation of the tRNA-bound peptides with DTT and ethanol precipitation were also analyzed by reverse-phase HPLC. No lysine-modified peptides were found in these two fractions. In addition, control experiments showed that omission of the ion-exchange step did not change the radioactive peptide profile; however, the latter step was essential for the purification of the ^{35}S-labeled peptides. These results indicate that no preferential losses of cross-linked peptides occurred during the procedure. From a number of experiments, we have determined that this procedure gives an overall recovery of peptides equal to approximately 20% of the initial amount of cross-linked enzyme.

Amino Acid Analysis and N-Terminal Sequencing of Cross-Linked Peptides. The amino acid compositions of the major peptide peaks were determined in this laboratory by using the highly sensitive method of precolumn derivatization with dabsyl chloride (Chang et al., 1982). The dabsyl-amino acids were separated by gradient elution using reverse-phase HPLC chromatography (Chang et al., 1981). Quantitative analysis of the derivatized amino acids was achieved by integration of the peak areas of the absorbance profile at 436 nm. Absorbance detection using a full scale of 0.005 optical unit at 436 nm allows 5 pmol of dabsyl-amino acids to be determined with reliability. In our hands, the main drawback of this technique was the failure to detect the bis(dabsyl) derivatives of tyrosine, histidine, and lysine. This was due to the presence of dabsyl chloride byproducts that comigrated with these derivatives (data not shown). Several of the peptides were also analyzed for amino acid composition at Yale University by using a recently developed method based on precolumn derivatization with phenyl isothiocyanate (Stone & Williams, 1986).

Table I: Amino Acid Composition of Purified Cross-Linked Peptides[a]

amino acid	peptide peak I[b]	II[b]	III[c]	IV[c,d]
Asp	- (0)	0.1 (0)	2.3 (2)	1.1 (1)
Thr	- (0)	0.2 (0)	- (0)	- (0)
Ser	- (0)	0.2 (0)	- (0)	- (0)
Glu	- (0)	1.0 (1)	- (0)	3.4 (4)
Pro	- (0)	0.2 (0)	0.1 (0)	1.0 (1)
Gly	- (0)	1.5 (1)	1.8 (1)	1.3 (1)
Ala	0.1 (0)	1.0 (1)	1.2 (1)	2.0 (2)
Val	- (0)	0.9 (1)	0.1 (0)	2.4 (3)
Met	0.4 (1)	- (0)	- (0)	- (0)
Ile	- (0)	0.2 (0)	0.9 (1)	0.1 (0)
Leu	- (0)	0.2 (0)	- (0)	0.1 (0)
Phe	- (0)	0.9 (1)	1.0 (1)	0.1 (0)
Arg	1.0 (1)	0.8 (1)	0.9 (1)	1.0 (1)
Lys	1.2 (1)	0.9 (1)	ND[e] (1)	ND (1)
Tyr	- (0)	0.1 (0)	ND (0)	ND (1)
His	- (0)	- (0)	ND (0)	ND (0)
pmol of peptide according to				
radioact.	221	166	100	184
amino acid anal	190	189	120	145

[a] Values are represented as moles of amino acid per mole of peptide. The integer values shown in parentheses represent the theoretical composition for lysine-containing tryptic peptides derived from the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). Dashes signify that the value was less than 0.1 mol (mol of peptide)$^{-1}$. [b] PITC method. In each case, about one lysine residue is present per mole of peptide. This is what is predicted, since the amide bond of the modified lysine should be labile under the conditions used for acid hydrolysis of the peptides. [c] Dabsyl chloride method. [d] This peptide has strong absorbance at 280 nm. [e] ND not determined.

Five major peaks were obtained by reverse-phase HPLC chromatography of the cross-linked peptides. Peak IV (Figure 1) was the only one that had strong absorbance at 280 nm, indicating the presence of tryptophan in this peptide (not shown). Amino acid analysis of the main fraction of each peak showed that peaks I–IV were sufficiently pure for direct N-terminal sequence analysis. The amino acid composition of peak V did not correspond to that of any single peptide present in the known primary sequence of MetRS, indicating that this peak contains more than one peptide.

The amino acid analysis data for peptide peaks I–IV are shown in Table I. The amino acid compositions corresponded to those expected from tryptic peptides containing cross-linked lysines at positions 640, 439, 402, and 465, respectively, in the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). These peptides were partially sequenced manually by using the DABITC/PITC double coupling method (Chang,

Table II: Automated Degradation of Cross-Linked Peptides[a]

cycle	residue[b]	amino acid identified	amount (pmol)
	Peptide I, 125 pmol[c]		
1	Lys_{640}	Lys*[d]	19
2	Met_{641}	Met	85
3	Arg_{642}	Xxx[e]	
	Peptide II, 100 pmol[c]		
1	Glu_{436}	Glu	41
2	Phe_{437}	Phe	62
3	Gly_{438}	Gly	30
4	Lys_{439}	Lys*[d]	12
5	Ala_{440}	Ala	42
6	Val_{441}	Val	36
7	Arg_{442}	Xxx[e]	
	Peptide III, 83 pmol[c]		
1	Asn_{396}	Asn, Asp	39, 11
2	Ala_{397}	Ala	63
3	Gly_{398}	Gly	45
4	Phe_{399}	Phe	26
5	Ile_{400}	Ile	34
6	Asn_{401}	Asn, Asp	30, 8
7	Lys_{402}	Lsy*[d]	14
8	Arg_{403}	Xxx[e]	
	Peptide IV,[f] 55 pmol[c]		
1	Tyr_{454}	Tyr	17
2	Val_{455}	Val	16
3	Asp_{456}	Asp	10
4	Glu_{457}	Glu	11
5	Gln_{458}	Xxx[e]	

[a] The peptides were submitted to automated degradation on an Applied Biosystems gas phase sequencer (Stone & Williams, 1986). [b] Residue number corresponding to the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). [c] The amount of peptide applied to the sequencer was determined from the radioactivity of the sample. [d] Lys* represents the chemically modified lysine. The amount released was determined by radioactive counting (Figure 2). [e] Xxx represents a cycle in which no identifiable amino acid derivative was detectable. [f] No amino acid derivative was identified after the fourth cycle.

1981) and were also subjected to automated degradation on an Applied Biosystems gas phase sequencer (Stone & Williams, 1986). The sequencing results (Table II) clearly confirm that the peptides have the sequences predicted from the amino acid composition analyses. No normal phenylthiohydantoin-amino acid derivative was observed at the position corresponding to the cross-linked lysine; however, a peak of radioactivity was released which coincided with the position of the modified lysine in the sequence of peptides I, II, and III (Figure 2). Only the first four N-terminal amino acids of peptide IV were identified; however, the amino acid composition analysis and N-terminal sequence data are unique, indicating that this peptide contains modified lysine residue 465 in the primary sequence of MetRS. The presence of both tyrosine and tryptophan in the sequence of this peptide accounts for its strong absorbance at 280 nm.

The complete sequence and position of the cross-linked peptides in the primary structure of MetRS are shown in Figure 3.

Discussion

New cross-linking methods developed in this laboratory have been used to prepare a lysine-reactive derivative of $tRNA^{fMet}$ carrying a cleavable cross-linker attached to single-stranded cytidines in four structural regions of the tRNA. Cross-linking of this derivative to native MetRS has previously been extensively characterized (Schulman et al., 1981b; Valenzuela et al., 1984). In the presence of excess modified tRNA, a maximum of 1 mol of tRNA is cross-linked per mole of MetRS, in keeping with the known anticooperative tRNA

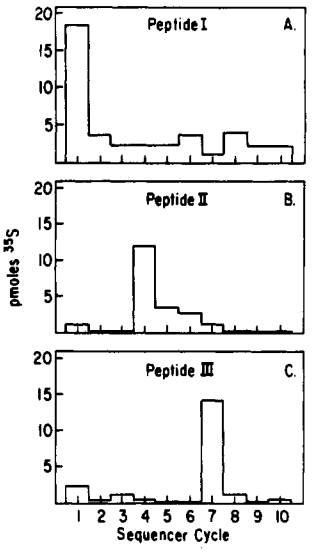


FIGURE 2: Release of ^{35}S during automatic degradation of cross-linked peptides. The peptides radioactively labeled by cross-linking to [^{35}S]DTSP/PDA-$tRNA^{fMet}$ were subjected to automated degradation on an Applied Biosystems gas phase sequencer. The amount of ^{35}S released was determined by radioactive counting of aliquots of the phenylthiohydantoin fraction. Aliquots of each cycle were also analyzed by reverse-phase HPLC. As expected, no normal phenylthiohydantoin derivatives were found at the position corresponding to the peak of released radioactivity.

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ThrGlnValAlaLysLysIleLeuValThrCysAlaLeuProTyrAlaAsnGlySerIleHisLeuGlyHisMet
          5  6
LeuGluHisIleGlnAlaAspValTrpValArgTyrGlnArgMetArgGlyHisGluValAspPheIleCysAla
AspAspAlaHisGlyThrProIleMetLeuLysAlaGlnGlnLeuGlyIleThrProGluGlnMetIleGlyGlu
                          61
MetSerGlnGluHisGlnThrAspPheAlaGlyPheAspIleSerTyrAspAsnTyrHisSerThrHisSerGlu
GluAsnArgGlnLeuSerGluLeuIleTyrSerArgLeuLysGluAsnGlyPheIleLysAsnArgThrIleSer
                                  114          120
GlnLeuTyrAspProGluLysGlyMetPheLeuProAspArgPheValLysGlyThrCysProLysCysLysSer
          132                      142      147  149
ProAspGlnTyrGlyAspAsnCysGluValCysGlyAlaThrTyrSerProThrGluLeuIleGluProLysSer
                                                      174
ValValSerGlyAlaThrProValMetArgAspSerGluHisPhePhePheAspLeuProSerPheSerGluMet
LeuGlnAlaTrpThrArgSerGlyAlaLeuGlnGluGlnValAlaAsnLysMetGlnGluTrpPheGluSerGly
                              217
LeuGlnGlnTrpAspIleSerArgAspAlaProTyrPheGlyPheGluIleProAsnAlaProGlyLysTyrPhe
                                                  248
TyrValTrpLeuAsnAlaProIleGlyTyrMetGlySerPheLysAsnLeuCysAspLysArgGlyAspSerVal
                          265          270
SerPheAspGluTyrTrpLysLysAspSerThrAlaGluLeuTyrHisPheIleGlyLysAspIleValTyrPhe
          282 283                      295
HisSerLeuPheTrpProAlaMetLeuGluGlySerAsnPheArgLysProSerAsnLeuPheValHisGlyTyr
                              316
ValThrValAsnGlyAlaLysMetSerLysSerArgGlyThrPheIleLysAlaSerThrTrpLeuAsnHisPhe
          332  335          342
AspAlaAspSerLeuArgTyrTyrTyrThrAlaLysLeuSerSerArgIleAspAspIleAspLeuAsnLeuGlu
                362
AspPheValGlnArgValAspAlaAspIleValAsnLysValValAsnLeuAlaSerArgAsnAlaGlyPheIle
                          388
AsnLysArgPheAspGlyValLeuAlaSerGluLeuAlaAspProGlnLeuTyrLysThrPheThrAspAlaAla
402                                   419
GluValIleGluGluAlaTrpGluSerArgGluPheGlyLysAlaValArgGluIleMetAlaLeuAlaAspLeu
                                  439
AlaAsnArgTyrValAspGluGlnAlaProTrpValValAlaLysGlnGluGlyArgAspAlaAspLeuGlnAla
                          465
IleCysSerMetGlyIleAsnLeuPheArgValLeuMetThrTyrLeuLysProValLeuProLysLeuThrGlu
                              492          497
ArgAlaGluAlaPheLeuAsnThrGluLeuThrTrpAspGlyIleGlnGlnProLeuLeuGlyHisLysValAsn
                                                  523
ProPheLysAlaLeuTyrAsnArgIleAspMetArgGlnValGluAlaLeuValGluAlaSerLysGluGluVal
      528                                         547
LysAlaAlaAlaAlaProValThrGlyProLeuAlaAspAspProIleGlnGluThrIleThrPheAspAspPhe
551
AlaLysValAspLeuArgValAlaLeuIleGluAsnAlaGluPheValGluGlySerAspLysLeuLeuArgLeu
  577                                         596
ThrLeuAspLeuGlyGlyGluLysArgAsnValPheSerGlyIleArgSerAlaTyrProAspProGlnAlaLeu
                608
IleGlyArgHisThrIleMetValAlaAsnLeuAlaProArgLysMetArgPheGlyIleSerGluGlyMetVal
                                  640
MetAlaAlaGlyProGlyGlyLysAspIlePheLeuLeuSerProAspAlaGlyAlaLysProGlyHisGlnVal
                658                               670
Lys-COOH
676
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FIGURE 3: Location of cross-linked peptides in the primary structure of MetRS. The figure shows the complete amino acid sequence of *E. coli* MetRS, as determined by Barker et al. (1982) and Dardel et al. (1984). The 40 lysine residues of the enzyme are in bold print and numbered. Residue 1 is the NH_2-terminal amino acid. The cross-linked peptides identified in this study are underlined. They contain the modified lysines at positions 402, 439, 465, and 640.

binding properties of the native dimeric synthetase. Nonspecific proteins are unreactive, and the specific coupling reaction is effectively inhibited by unmodified $tRNA^{fMet}$, but not by noncognate tRNAs.

The data presented in this paper provide further evidence of the specificity of the cross-linking reaction. Cross-linkers attached to different regions of the tRNA couple to the enzyme roughly in proportion to the concentration of the cross-linker at each site (Valenzuela et al., 1984). Each tRNA carries an average of only one side chain per molecule; thus, each site reacts independently. Figure 3 shows the distribution of lysine residues in MetRS. The native enzyme contains 80 lysines, 40 in unique sequences, located throughout the structure. The small number of peptides obtained following cross-linking of MetRS to DTSP/PDA-$tRNA^{fMet}$ indicates that cross-linkers at each site in the tRNA are capable of extensive reaction with only one or two lysine residues in the protein.

Native MetRS is a symmetrical dimer of molecular weight 152 000 (Dardel et al., 1984). The enzyme can be converted to a biologically active monomeric form by removal of approximately 130 amino acids from the carboxy terminus by controlled proteolysis (Waller et al., 1971). The monomeric form of the enzyme has been crystallized and its structure determined at 2.5-Å resolution (Zelwer et al., 1982). The monomeric enzyme is an elongated molecule (90 Å 52 × Å × 44 Å) organized in a biglobular structure composed of N-terminal and C-terminal domains. The structural division of the enzyme may also be a functional one, since the ATP binding site has been located in the N-terminal domain (Risler et al., 1981; Zelwer et al., 1982) and the present data place the tRNA binding site in the carboxy-terminal region.

The identified cross-linked peptides contain modified lysine residues 402, 439, 465, and 640 in the primary sequence of MetRS (Figure 3). Bruton (1979) has also studied the binding of $tRNA^{Met}$ substrates to MetRS using the competitive labeling technique of Kaplan et al. (1971). This method involves comparing the chemical reactivity of ϵ-amino groups in the free enzyme and the enzyme/substrate complex with acetic anhydride. Lysine residues in only 2 of the 60 tryptic peptides examined showed a decrease in the extent of acetylation following noncovalent binding of either $tRNA^{fMet}$ or $tRNA_m^{Met}$. These peptides contained lysine residues 402 and 658.

It is known that amino acid residues between positions 550 and 676 are not required for tRNA binding since the kinetic parameters for aminoacylation by the MetRS fragment missing this sequence and the native dimeric enzyme are very similar (Lawrence et al., 1973). Lysine residue 640 is cross-linked to the cytidine residue at the 5′ terminus of $tRNA^{fMet}$.[2] This nucleotide is known not to be required for methionine acceptor activity since it can be chemically modified or enzymatically excised without inactivation of the tRNA (Schulman & Goddard, 1973; Seno et al., 1971). The data therefore indicate that a dispensable region of the tRNA is in close proximity to a dispensable region of the enzyme. Protein sequences in the vicinity of lysine residues 402, 439, and 465 are believed to be essential for biological activity, however. Dardel et al. (1984) have deleted the carboxy-terminal region of the cloned MetRS gene and produced a polypeptide product of molecular weight 52 000. The protein, consisting of 475 amino acids of MetRS sequence and 9 amino acids derived from pBR322, was unable to complement a structural mutant of *metG* and was inactive when assayed in crude cell extracts. These results are consistent with the idea that amino acid residues near lysine-465 are important for tRNA binding.

Studies on several other aminoacyl-tRNA synthetases indicate a role for sequences in the carboxy-terminal region of these enzymes in tRNA binding. *E. coli* AlaRS is a tetrameric enzyme composed of identical subunits of 875 amino acids (Putney et al., 1981). Monomeric forms of the enzyme containing the N-terminal 385 or 404 amino acids are active in adenylate synthesis but unable to transfer the activated amino acid to $tRNA^{Ala}$ (Jasin et al., 1983). Extending the length of the polypeptide to 461 amino acid residues allows specific aminoacylation of the cognate tRNA; however, the activity is reduced 5-fold compared to the native enzyme, suggesting that additional sequences stabilize the interaction with the tRNA. X-ray crystallographic studies of *Bacillus stearothermophilis* TyrRS have shown that the N-terminal domain of the native dimeric enzyme (419 amino acids/subunit) contains the binding sites for tyrosine, ATP, and tyrosyl adenylate (Monteilhet & Blow, 1978; Bhat et al., 1982). Deletion of carboxyl-terminal sequences from the cloned TyrRS gene has produced a truncated dimeric enzyme containing only 319 amino acids/subunit (Waye et al., 1983). This truncated enzyme catalyzes formation of tyrosyladenylate with unchanged k_{cat} and K_m values but no longer binds or aminoacylates $tRNA^{Tyr}$. These results suggest that amino acid activation by the N-terminal domain and tRNA binding by the carboxy-terminal domain may be a widespread feature of aminoacyl-tRNA synthetases.

Hountondji et al. (1985) have recently cross-linked periodate-oxidized $tRNA^{fMet}$ to the biologically active monomeric fragment of MetRS and identified three cross-linked peptides. The peptide found in highest yield was coupled to the 3′ terminus of the tRNA through lysine-335. Another peptide obtained in significant amounts was derived by cross-linking to lysine-61. These lysine residues are expected to be near the active site of the enzyme, since the 3′ terminus of the tRNA must be suitably positioned for the transfer step of the reaction. It has not yet been possible to determine the exact positions of the lysine residues cross-linked to $tRNA^{fMet}$ in the carboxy-terminal domain of the 2.5-Å crystal structure; however, higher resolution data (1.8 Å) have recently been obtained (S. Brunie, J. L. Risler, and C. Zelwer, unpublished results). Analysis of these data is expected to allow the positions of these amino acids to be identified. Experiments are presently in progress in this laboratory to identify the specific sites in $tRNA^{fMet}$ which are coupled to each of the cross-linked lysine residues. A combination of these data with the refined crystal structure of the enzyme is expected to allow construction of the first model of a tRNA–synthetase complex.

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Registry No. MetRS, 9033-22-1.

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