tRNA Recognition Site of *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: We have previously shown that anticodon bases are essential for specific recognition of tRNA substrates by *Escherichia coli* methionyl-tRNA synthetase (MetRS) [Schulman, L. H., & Pelka, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6755–6759] and that the enzyme tightly binds to C_{34} at the wobble position of *E. coli* initiator methionine tRNA ($tRNA^{fMet}$) [Pelka, H., & Schulman, L. H. (1986) *Biochemistry 25*, 4450–4456]. We have also previously demonstrated that an affinity labeling derivative of $tRNA^{fMet}$ can be quantitatively cross-linked to the tRNA binding site of MetRS [Valenzuela, D., & Schulman, L. H. (1986) *Biochemistry 25*, 4555–4561]. Here, we have determined the site in MetRS which is cross-linked to the anticodon of $tRNA^{fMet}$, as well as the location of four additional cross-links. Only a single peptide, containing Lys_{465}, is covalently coupled to C_{34}, indicating that the recognition site for the anticodon is close to this sequence in the three-dimensional structure of MetRS. The D loop at one corner of the tRNA molecule is cross-linked to three peptides, containing Lys_{402}, Lys_{439}, and Lys_{596}. The 5′ terminus of the tRNA is cross-linked to Lys_{640}, near the carboxy terminus of the enzyme. Since the 3′ end of $tRNA^{fMet}$ is positioned close to the active site in the N-terminal domain [Hountondji, C., Blanquet, S., & Lederer, F. (1985) *Biochemistry 24*, 1175–1180], this result indicates that the carboxy ends of the two polypeptide chains of native dimeric MetRS are folded back toward the N-terminal domain of each subunit.

Aminoacyl-tRNA synthetases participate in the first step in the pathway of protein biosynthesis. Each of 20 different synthetases catalyzes the activation of a specific amino acid and its transfer to the 3′ terminus of a corresponding isoacceptor tRNA. The selection of tRNAs by these enzymes involves both specific and nonspecific interactions (Schimmel & Soll, 1979). Nonspecific contacts are made between the negatively charged phosphate groups of the tRNA backbone and appropriately oriented amino acids on the surface of both cognate and noncognate enzymes. Additional contacts, presumably involving formation of specific hydrogen bonds between nucleotide bases and amino acid residues unique to each cognate tRNA–synthetase pair, are required for specific aminoacylation of the appropriate tRNAs. Little is known about this specific recognition process.

Ideally, the contact sites between tRNAs and aminoacyl-tRNA synthetases could be determined by analysis of well-defined cocrystals of each cognate pair. Only one tRNA–synthetase complex has been crystallized to date, however, and the available crystals diffract to only 7.5-Å resolution (Lorber et al., 1983). High-resolution X-ray crystallographic data have been reported for two aminoacyl-tRNA synthetases: *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (TyrRS)[1] and a biologically active proteolytic fragment of *Escherichia coli* methionyl-tRNA synthetase (MetRS) (Bhat et al., 1982; Zelwer et al., 1982). In the case of the methionine enzyme, the crystal structures of two tRNA substrates have also been determined (Woo et al., 1980; Schevitz et al., 1979).

A great deal is known about the structural requirements for aminoacylation of tRNAs by MetRS [for reviews, see Schulman (1979) and Schulman and Pelka (1977b, 1984)]. Specific tRNA recognition requires interaction of the enzyme with nucleotide bases in the anticodon sequence (Schulman & Goddard, 1973; Stern & Schulman, 1977; Schulman & Pelka, 1977a,b). Single base changes in the anticodon produce drastic reductions in amino acid acceptor activity, with substitutions at the wobble position rendering $tRNA^{fMet}$ inactive (Schulman et al., 1983; Schulman & Pelka, 1983, 1984). The available data suggest that most, if not all, of the positive contacts required for tRNA recognition occur within this sequence. The pyrimidine ring nitrogen of the wobble base, C_{34}, is believed to be an important ligand of MetRS (Schulman & Pelka, 1977b), and this site has been shown to be strongly protected from attack by dimethyl sulfate in the tRNA–enzyme complex (Pelka & Schulman, 1986). Discrimination of methionine tRNAs from closely related noncognate tRNAs is expected to depend on the existence of negative interactions with specific sequences in the noncognate species, as well as positive interactions with the anticodon bases of cognate tRNAs.

In an effort to determine the location of the amino acid sequences in MetRS which form the anticodon recognition site, we have developed procedures for attachment of protein affinity labeling groups to the wobble base of $tRNA^{fMet}$ (Sarkar & Schulman, 1978; Schulman et al., 1981a). A cleavable, lysine-reactive cross-linker has proven to be particularly useful for obtaining high-yield cross-linking reactions (Schulman et al., 1981b). Attachment of the cross-linker to the tRNA involves two steps. First, a short three-carbon side chain terminating in a reactive primary amino group is joined to the

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[1] Abbreviations: TyrRS, *Bacillus stearothermophilus* tyrosyl-tRNA synthetase; MetRS, *Escherichia coli* methionyl-tRNA synthetase; $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; DTSP, dithiobis(succinimidyl propionate); PDA, propane-1,3-diamine; DTSP/PDA-$tRNA^{fMet}$, $tRNA^{fMet}$ modified with PDA followed by coupling to DTSP; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; ODS, octadecylsilane; DEAE, *N*,*N*-diethylaminoethyl; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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N^4 position of the cytidine base by transamination with propane-1,3-diamine (PDA) in the presence of bisulfite. Second, dithiobis(succinimidyl propionate) (DTSP) radioactively labeled with ^{35}S in the disulfide bond of the cross-linker is coupled to the reactive PDA side chain.

Modification of tRNA^{fMet} with PDA/bisulfite results in attachment of side chains to single-stranded cytidine residues at three other sites in addition to the anticodon. These include the unpaired 5′-terminal cytidine and the cytidine residues in the D loop and 3′-terminal CCA sequence. By controlling the extent of reaction with PDA, tRNA having an average of one side chain per molecule can be prepared. Addition of excess DTSP results in formation of a mixture of [^{35}S]DTSP/PDA-tRNA^{fMet} molecules, each carrying one lysine-reactive cross-linker, distributed over the four structural regions of the tRNA. This modified tRNA has been shown to quantitatively cross-link to the tRNA binding site of MetRS (Schulman et al., 1981b; Valenzuela et al., 1984). Cleavage of the covalent tRNA–enzyme complex with DTT results in release of tRNA and leaves an ^{35}S-labeled lysine residue at the site of each cross-link. Four of the five major cross-linked peptides have recently been purified and sequenced, and the sites of the modified lysine residues have been identified in the known primary structure of MetRS (Valenzuela & Schulman, 1986). Here, we determine the peptide sequence which is cross-linked to the anticodon of tRNA^{fMet} and also identify the MetRS peptides in close proximity to the D loop and 5′ terminus of the tRNA. In addition, we report the sequence of the remaining major cross-linked peptide.

Experimental Procedures

Reagents. [^{35}S]DTSP was synthesized by the method of Lomant and Fairbanks (1976), and PDA was obtained from Aldrich Chemical Co. *E. coli* tRNA^{fMet} having a specific activity of 1650 pmol/A_{260} unit was purchased from Subriden. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* CSR603 harboring the plasmid pLC20-25 from the Clarke–Carbon library of *E. coli* K12 chromosomal DNA (Clarke & Carbon, 1976). This plasmid carries *metG*, the structural gene for MetRS (Neidhardt et al., 1983). The specific activity of the enzyme was 91 units/mg, and the protein was judged to be pure by SDS gel electrophoresis. TPCK-treated trypsin was obtained from Worthington Biochemical Corp. and further purified as described elsewhere (Valenzuela & Schulman, 1986). T_1 RNase was obtained from Calbiochem and calf intestinal alkaline phosphatase from Boehringer. Superfine Sephacryl S-200 was purchased from Pharmacia.

Cross-Linking of MetRS and Isolation of the Covalent tRNA^{fMet}–MetRS Complex. [^{35}S]DTSP/PDA-tRNA^{fMet} was prepared as previously described (Schulman et al., 1981a,b). The cross-linking reaction mixture contained 4 μM MetRS and 23 μM [^{35}S]DTSP/PDA-tRNA^{fMet} (500 cpm/pmol) in a total volume of 2 mL of 20 mM Hepes, pH 8, and 10 mM $MgCl_2$. The solution was incubated at 25 °C for 30 min. Glycine, pH 7, was added to a final concentration of 50 mM and the incubation at 25 °C continued for 30 min. Small aliquots (2 × 5 μL) were removed for measurement of residual enzyme activity and assay of the amount of covalent coupling by retention of radioactively labeled tRNA on nitrocellulose filters as described before (Schulman et al., 1981b). By these criteria, the yield of cross-linked complex was 76% of the input enzyme. The remainder of the reaction mixture was applied to a 0.9 × 220 cm column of Sephacryl S-200 presaturated with bovine serum albumin and equilibrated with 0.2 M potassium phosphate, pH 6.5, and 1 M NaCl at room temperature. The sample was eluted with the same buffer at a flow rate of 12 mL/h. Fractions (1.6 mL) were collected, and aliquots were removed for measurement of radioactivity. The peak containing the cross-linked complex was pooled, concentrated to 0.4 mL in a collodion bag, and dialyzed vs. 0.1 M ammonium acetate, pH 6.5, and 0.1 mM $CaCl_2$. The recovery of cross-linked complex was 39%. Radioactivity and absorbance measurements indicated that the sample contained an approximately equal amount of free and cross-linked tRNA.

Tryptic Digestion of the Covalent Complex and HPLC of the Cross-Linked Peptides. RNase-free trypsin was added to the cross-linked complex to a concentration of 3% (w/w) trypsin/protein, and the sample was incubated at 25 °C for 7 h. NaCl was added to a final concentration of 0.1 M, together with 17 A_{260} units of carrier-unmodified tRNA^{fMet}. Three volumes of ethanol were added, and the sample was chilled at −20 °C for 10 min and centrifuged. The pellet, containing the tRNA-bound peptides + free tRNA, was dissolved in 1.2 mL of 7 M urea, 50 mM sodium acetate, pH 6.5, and 0.1 M NaCl and chromatographed on a 7.5 × 75 mm HPLC ion-exchange column of Bio-Gel TSK-DEAE 5PW as described before (Valenzuela & Schulman, 1986). The radioactive peak containing the tRNA was pooled and dialyzed twice vs. 0.1 M sodium acetate, pH 6.5, and once vs. 25 mM sodium acetate, pH 6.5. The solution was concentrated 10-fold by evaporation in a siliconized tube, and a portion (10%) was removed for analysis of the cross-linked peptides by HPLC chromatography. The covalently bound peptides were released from this portion by incubation with 50 mM DTT at 37 °C for 90 min as described before (Valenzuela & Schulman, 1986). The peptides in 6 M guanidinium chloride, 40 mM sodium acetate, pH 6.5, and 10 mM DTT were mixed with 250 μg of a tryptic digest of BSA as carrier and applied to a 4.6 × 250 mm Altex PTH–amino acid ODS reverse-phase HPLC column equipped with a 4.6 × 45 mm precolumn (Rainin) and equilibrated with 0.1% TFA in water (v/v) (buffer A). Elution was carried out at a flow rate of 0.65 mL/min using a Rainin gradient HPLC system and the following gradient profile: buffer A from 0 to 10 min, followed by a linear gradient from 0 to 42% buffer B from 10 to 130 min (buffer B = 0.1% TFA in acetonitrile, 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. One-minute fractions were collected and aliquots (100 μL) taken for measurement of radioactivity.

T_1 RNase Digestion of tRNA^{fMet} and HPLC of the Oligonucleotide-Bound Peptides. The sample containing 1.9 A_{260} units of tRNA-bound peptides and 12.1 A_{260} units of carrier tRNA^{fMet} was adjusted to a concentration of 40 A_{260} units/mL in 0.1 M sodium acetate, pH 6.5, and incubated with 1500 units/mL T_1 RNase at 37 °C for 2 h. Calf alkaline phosphatase was added to a concentration of 28 units/mL, and the incubation at 37 °C was continued for 1 h. The sample was adjusted to 7 M urea, 20 mM sodium acetate, pH 6.5, and 10% acetonitrile and injected onto a 7.5 × 75 mm column of Bio-Gel TSK-DEAE 5 PW equilibrated with the same buffer. The oligonucleotides were eluted with a linear salt gradient from 0 to 0.3 M NaCl over 1 h at a flow rate of 0.94 mL/min. One-minute fractions were collected, and aliquots were taken for measurement of radioactivity. The absorbance at 260 nm was monitored with a Gilson Holochrome detector. The gradient was formed by using an Eldex Chromat-A-trol Model II controller and a Milton Roy minipump.

Analysis of Cross-Linked Oligonucleotides and Peptides. Fractions across the entire elution profile of the oligonucleotide-bound peptides were pooled and incubated with 25

mM DTT at 37 °C for 90 min. One aliquot of each pool was mixed with 250 μg of a tryptic digest of BSA as carrier and diluted with 7 M urea/20 mM sodium acetate, pH 6.5, to a final concentration of 0.1 M NaCl and 2.5% acetonitrile in a total volume of 1.6 mL. The sample was injected onto the reverse-phase HPLC column, and the peptides were eluted as described above. Fractions (0.65 mL) were collected and mixed with 5 mL of ACSII scintillation fluid, and the radioactivity was measured. A second aliquot was taken from those pools found to contain cross-linked peptides, was mixed with an authentic sample of the putative sequenced peptide, and was cochromatographed under the same conditions. A third aliquot from those pools that contained cross-linked peptides was digested with T_1 RNase and calf intestinal alkaline phosphatase in the presence of 1 A_{260} unit of carrier $tRNA^{fMet}$ as described above. The digest was injected onto a 7.5 × 75 mm TSK-DEAE 5 PW column equilibrated with 7 M urea, 20 mM sodium acetate, pH 6.5, and 1 mM DTT, and the oligonucleotides were eluted as described in the legend to Figure 1. The absorbance of the carrier oligonucleotides was monitored at 260 nm. Two-minute fractions (0.6 mL) were collected, and 0.2-mL aliquots were taken for measurement of radioactivity in 5 mL of ACSII.

Isolation and Sequencing of Peptide V. Large-scale affinity labeling of $tRNA^{fMet}$, cross-linking to MetRS, and isolation of the cross-linked peptides were carried out as described by Valenzuela and Schulman (1986). The peptides were purified by reverse-phase HPLC using the gradient profile described above. Peptide sequencing was carried out on the peak fraction of peptide V by automated N-terminal degradation with PITC on an Applied Biosystems gas phase sequenator at the Yale University Protein Sequencing Facility as described by Stone and Williams (1986).

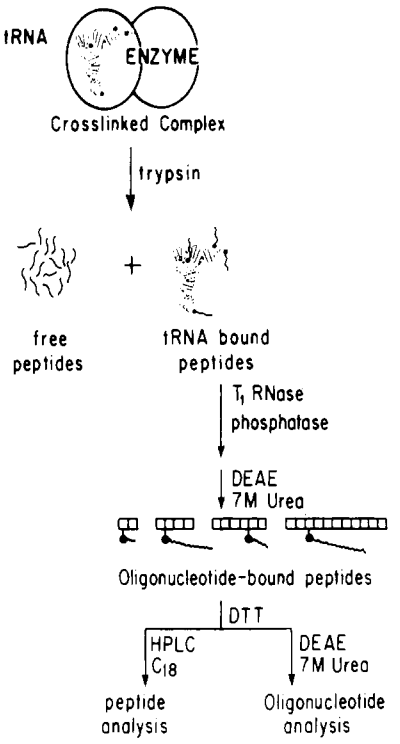
RESULTS

Isolation of MetRS Peptides Cross-Linked to $tRNA^{fMet}$. The cross-linking of [^{35}S]DTSP/PDA-$tRNA^{fMet}$ to MetRS was carried out as previously described (Valenzuela & Schulman, 1986). The reaction was quenched by addition of excess glycine, and the covalent tRNA–protein complex was separated from the bulk of the unreacted tRNA by gel filtration on Sephacryl S-200.

Fractions containing the cross-linked complex were pooled, dialyzed, concentrated, and digested with trypsin. The resulting tRNA-bound peptides were purified from excess free MetRS peptides by anion-exchange chromatography as described before (Valenzuela & Schulman, 1986). A portion of the purified tRNA–peptide fraction was treated with DTT, and the released ^{35}S-labeled peptides were separated by reverse-phase high-pressure liquid chromatography, yielding five major peptide products. Peptides I, II, III, and IV were identical with the four MetRS peptides previously isolated and sequenced, and shown to be cross-linked to $tRNA^{fMet}$ through lysine residues 640, 439, 402, and 465 in the primary sequence of the enzyme (Valenzuela & Schulman, 1986). The sequence of peptide V was determined in a separate experiment described below.

Identification of the Sites in $tRNA^{fMet}$ Cross-Linked to Specific Peptides. The procedure used for assignment of the sequenced peptides to specific cross-linking sites in $tRNA^{fMet}$ is illustrated in Scheme I. Each cross-linking site in the tRNA gives rise to an oligonucleotide of unique size when the tRNA is digested with T_1 RNase. These oligonucleotides are readily separated by anion-exchange chromatography on the basis of charge. Oligonucleotides covalently linked to specific peptides can similarly be separated by charge. The disulfide bond of

Scheme I: Procedure for the Assignment of MetRS Peptides to Specific Cross-Linking Sites on $tRNA^{fMet}$



the cross-linker can then be cleaved with DTT, and the identity of both the released peptide and the oligonucleotide from which it was derived can be determined.

The purified tRNA–peptide fraction was digested with T_1 RNase and phosphomonoesterase. The resulting ^{35}S-labeled oligonucleotide-bound peptides were separated by ion-exchange HPLC under denaturing conditions (not shown). Fractions across the entire elution profile were pooled, treated with DTT, and analyzed for the presence of peptides by reverse-phase HPLC. Only four regions of the profile (A, B, C, and D) were found to contain cross-linked peptides. The released peptides were identified by their retention times on reverse-phase HPLC and by comigration with the sequenced peptides when mixed and cochromatographed.

Additional aliquots were taken from pools A, B, C, and D for identification of the cross-linked oligonucleotide following cleavage of the cross-linker with DTT and anion-exchange chromatography of the released ^{35}S-labeled oligonucleotide in 7 M urea. The elution position of each oligonucleotide was compared with those of known oligonucleotides generated by T_1 RNase digestion of a control sample of DTT-treated [^{35}S]DTSP/PDA-$tRNA^{fMet}$ (Figure 1).

A summary of the analysis of the oligonucleotide–peptides is shown in Figure 2. Fractions eluting in region A contained the 5′C_pG dinucleotide cross-linked to peptide I. Regions B and C contained the tetranucleotide from the D loop cross-linked to peptides II, III, and V. Finally, region D contained the anticodon undecanucleotide cross-linked to peptide IV. Traces of minor peptides were found in the region containing the 3′-terminal CCA hexanucleotide.

Recoveries of the oligonucleotide-bound water-soluble peptides I, II, and III were in the range of 40–50%, while the oligonucleotide-bound hydrophobic peptides IV and V were recovered in only 10–20% yield. Omission of 10% acetonitrile from the T_1 RNase oligonucleotide elution buffer resulted in almost complete loss of these less soluble oligonucleotide-bound peptides.

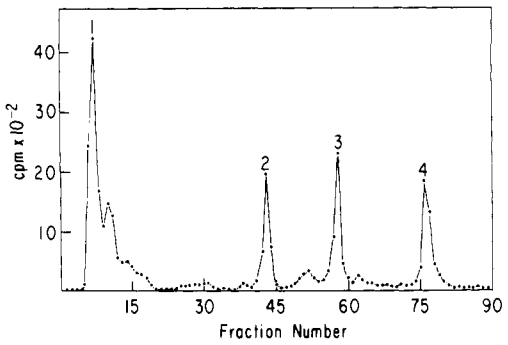


FIGURE 1: Separation of T_1 RNase oligonucleotides from DTT-treated [^{35}S]DTSP/PDA-tRNA^{fMet}. A control sample of [^{35}S]DTSP/PDA-tRNA^{fMet} was treated with DTT to cleave the disulfide bond of the cross-linker, and the released ^{35}S-labeled tRNA was digested with T_1 RNase and phosphomonoesterase as described under Experimental Procedures. The oligonucleotides were dissolved in 20 mM Tris-HCl, pH 7.5, 7 M urea, and 1 mM DTT and injected onto a TSK-DEAE 5-PW column equilibrated with the same buffer. The column was washed with starting buffer for 20 min at a flow rate of 0.3 mL/min. Oligonucleotides were eluted with a linear salt gradient from 0 to 0.3 M NaCl over 160 min. Two-minute fractions were collected and aliquots taken for measurement of radioactivity. Peak 1 is derived from the 5′-terminal CpG sequence, peak 2 from the D-loop oligonucleotide $C_pC_pU_pG$, peak 3 from the 3′-terminal $C_pA_pA_pC_pC_pA$ sequence, and peak 4 from the anticodon-containing sequence $C_p^mU_pC_pA_pU_pA_pA_pC_pC_pC_pG$. The origin of the shoulder on peak 1 is unknown.

Table I: Automated Degradation of Cross-Linked Peptide V: Val_{582}-Ala-Leu-Ile-Glu-Asn-Ala-Glu-Phe-Val-Glu-Gly-(Ser-Asp-Lys^*_{596}-Leu-Leu-Arg_{599})[a]

cycle	residue[b]	amino acid identified	yield (pmol)
1	582	Val	256
2	583	Ala	219
3	584	Leu	188
4	585	Ile	130
5	586	Glu	89
6	587	Asn, Asp	90, 29
7	588	Ala	112
8	589	Glu	69
9	590	Phe	96
10	591	Val	89
11	592	Glu	43
12	593	Gly	57

[a] Peptide peak V (295 pmol) was subjected to automated degradation on an Applied Biosystems gas-phase sequencer as described by Stone and Williams (1986). The amount of peptide was determined from the radioactivity of the sample. The portion of the peptide sequence not directly established is shown in parentheses. The site of cross-linking is indicated by an asterisk. [b] Residue number corresponding to the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984).

Purification and Sequencing of Cross-Linked Peptide V. Large-scale modification of tRNA^{fMet} and cross-linking to MetRS were carried out as described before (Valenzuela & Schulman, 1986). The cross-linked peptides were separated by reverse-phase HPLC using a gradient profile slightly different from that previously employed, which yields a sharp peak of pure peptide V. The peptide was found to comigrate on HPLC columns with a previously sequenced peptide obtained by cross-linking the elongator methionine tRNA to MetRS (Leon & Schulman, 1987). This peptide was cross-linked to $tRNA_m^{Met}$ by coupling the 3-(3-amino-3-carboxypropyl)uridine base in the tRNA to Lys_{596} in the primary sequence of MetRS. In order to confirm the identity of the peptide cross-linked to DTSP/PDA-tRNA^{fMet}, peptide V was subjected to automatic degradation on an Applied Biosystems gas phase sequencer (Stone & Williams, 1986). The results

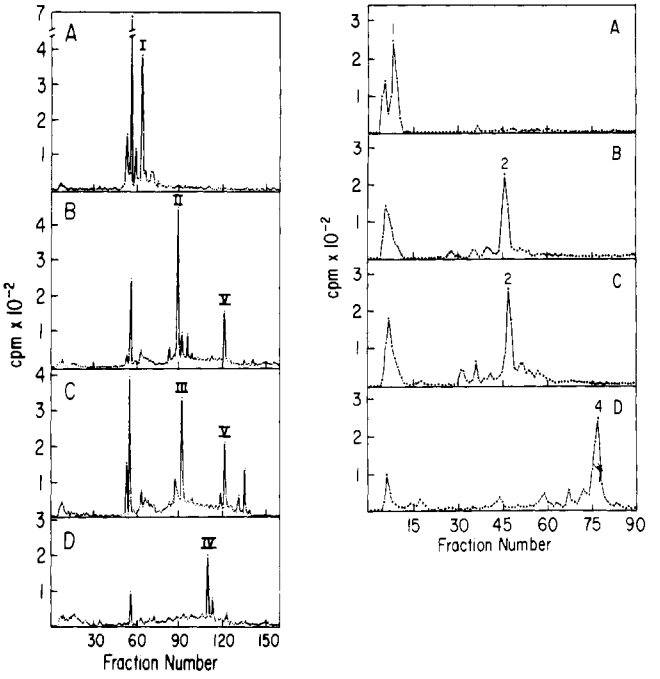


FIGURE 2: Summary of the analysis of oligonucleotide–peptide fractions by reverse-phase and ion-exchange HPLC. Fractions across the entire elution profile of the chromatogram of the oligonucleotide-bound peptides were treated with DTT. (Left) A portion of each DTT-treated sample was analyzed for peptides by reverse-phase HPLC. The elution profiles of the fractions found to contain peptides are shown in panels A–D. Samples in 6 M guanidinium chloride (0.5 mL) were injected onto a C_{18} column equilibrated with 0.1% trifluoroacetic acid in water (v/v), and the peptides were eluted with a gradient of acetonitrile as described under Experimental procedures. One-minute fractions were collected at a flow rate of 0.65 mL/min, and aliquots (100 μL) were taken for measurement of radioactivity. The numbering of the identified peptides corresponds to that given in Valenzuela and Schulman (1986). Radioactivity eluting prior to fraction 60 is nonpeptide material. (Right) A portion of each DTT-treated sample was analyzed for oligonucleotides by ion-exchange HPLC as described in the legend to Figure 1, except that the buffer contained 20 mM sodium acetate, pH 6.5.

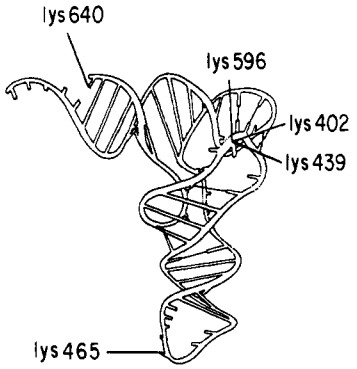


FIGURE 3: Location of the sites in tRNA^{fMet} cross-linked to specific lysine residues in MetRS. The attachment sites for cross-linking to the five identified peptide sequences in MetRS are indicated on a generalized three-dimensional tRNA structure based on the data of Kim et al. (1974) and Klug et al. (1974). The length of the lines extending from each attachment site approximates the length of the cross-linker side chain.

of 12 cycles of degradation (Table I) yield a sequence identical with the N terminus of an octadecapeptide cross-linked to tRNA^{fMet} through Lys_{596}.

Summary of the Covalent Attachment Sites between tRNA^{fMet} and MetRS. Table II summarizes the oligonucleotide sequences and peptide sequences which are covalently joined in the tRNA^{fMet}–MetRS complex. The location

Table II: Summary of the Sites of Cross-Linking between DTSP/PDA-tRNA^{fMet} and MetRS

tRNA^{fMet} oligonucleotide[a]			MetRS-cross-linked peptide[b]	
peak	sequence	location	peak	sequence
1	C_1G	5′ end	I	Lys_{640}-Met-Arg
2	$C_{16}C_{17}UG$	D loop	II	Glu-Phe-Gly-Lys_{439}-Ala-Val-Arg
			III	Asn-Ala-Gly-Phe-Ile-Asn-Lys_{402}-Arg
			V	Val-Ala-Leu-Ile-Glu-Asn-Ala-Glu-Phe-Val-Glu-Gly-Ser-Asp-Lys_{596}-Leu-Leu-Arg
3	$CAAC_{74}C_{75}A$	3′ end	–	no major lysine-cross-linked product
4	$C^mUC_{34}AUAACCCG$	anticodon	IV	Tyr-Val-Asp-Glu-Gln-Ala-Pro-Trp-Val-Val-Ala-Lys_{465}-Gln-Glu-Gly-Arg

[a] The oligonucleotides were derived by digestion of modified tRNA^{fMet} with T_1 RNase and alkaline phosphatase. Cytidine residues carrying cross-linking side chains are numbered according to Gauss et al. (1979), and their location in the structure of the tRNA is indicated. Oligonucleotide peaks are numbered according to Figure 1. The sequence of tRNA^{fMet} is from Dube et al. (1968). [b] Peptides were derived by tryptic digestion of modified MetRS. Cross-linked lysine residues are numbered by their position in the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). Peptide peaks are numbered according to Valenzuela and Schulman (1986).

of the cross-linking sites in the tRNA tertiary structure is shown in Figure 3.

Discussion

We have undertaken cross-linking experiments in order to determine the regions of protein and nucleic acid in close proximity in the MetRS–tRNA^{fMet} complex. Five peptides have been identified which are cross-linked to three different structural regions of the tRNA (Table II and Figure 3). This is the first instance in which a variety of sites in an RNA molecule have been coupled to known sites in a protein.

Others have previously cross-linked several tRNA^{fMet} substrates to MetRS using UV light (Rosa et al., 1979; Ackerman et al., 1985). Direct photoreaction has the advantage of a "zero-length" probe which identifies regions of a given tRNA in close contact with the enzyme surface. A disadvantage of this method is the difficulty in obtaining sufficient quantities of cross-linked peptides for determination of the corresponding contact sites on the enzyme. Chemical cross-linking of the 3′ terminus of periodate-oxidized tRNA^{fMet} has allowed isolation and sequencing of three MetRS peptides near the active site of the enzyme (Hountondji et al., 1985).

Native MetRS is a symmetrical dimer (M_r 2 × 76K) which binds tRNA in an anticooperative fashion (Blanquet et al., 1973). The enzyme can be converted to a biologically active monomeric form (64K) by removal of about 100 amino acid residues from the carboxy terminus by controlled proteolysis (Casio & Waller, 1971; Barker et al., 1982; Dardel et al., 1984). The monomeric form of the enzyme has been crystallized (Waller et al., 1971), and its structure at 2.5-Å resolution has been reported (Zelwer et al., 1982). The elongated molecule (90 Å × 52 Å × 44 Å) is organized in a biglobular structure composed of N-terminal and C-terminal domains. The N-terminal domain has been shown to contain the catalytic site of the enzyme (Risler et al., 1981) and the carboxy-terminal domain to contain the tRNA binding site (Valenzuela & Schulman, 1986). The monomeric enzyme aminoacylates methionine tRNAs with kinetic parameters similar to those of native MetRS, indicating that it contains all of the important contact sites for interaction with cognate tRNAs. The crystal structure has recently been refined to 1.8-Å resolution, and three of the lysine residues cross-linked to DTSP/PDA-tRNA^{fMet} have been located in the structure (S. Brunie, J. L. Risler, and C. Zelwer, unpublished results). Lysine-465 is found at the extreme periphery of the molecule, at a distance of 52 Å from the active site. This result indicates that the tRNA binds to MetRS without a significant distortion of its L-shaped conformation, with the anticodon positioned at one end of the C-terminal domain and the acceptor stem pointing toward the catalytic site in the N-terminal domain. The cross-linking of Lys_{640} to the 5′ terminus of tRNA^{fMet} indicates that the extreme carboxy end of each polypeptide chain in native dimeric MetRS folds back toward the N-terminal domain of the enzyme.

Lys_{402} and Lys_{439} are located between the anticodon binding site and the active-site cleft. These residues are cross-linked to the D loop of tRNA^{fMet}. It is unlikely that important contacts are made between the enzyme and nucleotide bases in the D loop since extensive sequence modifications in this region do not inactivate the tRNA. Bruton (1979) has shown that Lys_{402} is one of two lysine residues protected from acetylation following noncovalent binding of either tRNA^{fMet} or tRNA$_m^{Met}$ to MetRS. The other protected residue is Lys_{658}, which is part of the dispensible sequence not present in the monomeric enzyme. Two of the lysines we have cross-linked to tRNA^{fMet} also occur in this region: Lys_{640}, coupled to the 5′-terminal cytidine, and Lys_{596}, coupled to the D loop. The 5′-terminal cytidine of tRNA^{fMet} is known to be nonessential for aminoacylation since it can be enzymatically excised or chemically modified without inactivation of the tRNA (Schulman & Goddard, 1973; Seno et al., 1971). Thus, a dispensible region of the tRNA is aligned with a nonessential region of the protein in the cross-linked complex. Although not part of the tRNA binding site, the carboxy-terminal sequence of native MetRS must lie close to the bound tRNA, and the environment of amino acid residues within this sequence is influenced by tRNA binding. Protein–protein interactions within this region join the two subunits of the native enzyme. Previous neutron-scattering studies have suggested that tRNA binding produces subunit rearrangements affecting the orientation of the protomers at their hinge (Desson et al., 1978), and recent fluorescence studies have shown that a probe covalently attached to the intersubunit domain of MetRS undergoes a conformational change on tRNA binding (Ferguson & Yang, 1986).

No major MetRS peptide was found cross-linked to the 3′ end of DTSP/PDA-tRNA^{fMet}. There are several possible explanations for this result. Hountondji et al. (1985) cross-linked the 3′ terminus of periodate-oxidized tRNA^{fMet} to Lys_{335} and Lys_{61}. A low yield of cross-linking also occurred with Lys_{142}, Lys_{147}, and Lys_{149}, which are adjacent to two cysteine residues (Cys_{145} and Cys_{148}). The 3′ end of the bound tRNA therefore appears to be quite flexible, allowing reaction with at least five lysines, and is positioned close to several SH groups. The reactive ester of DTSP/PDA-tRNA^{fMet} can maximally extend a distance of 14 Å, allowing low-yield coupling to many different lysines. In addition, the disulfide bond of the cross-linker, located 10 Å from the 3′ end of the tRNA, could react with the nearby cysteine residues, decreasing the effective concentration of the lysine-reactive side chain at the CCA end. We have observed that DTSP/PDA-tRNA^{fMet} undergoes disulfide exchange with MetRS at a rate

similar to the rate of lysine coupling, leading to modification of an average of one cysteine residue per molecule of dimeric enzyme (Valenzuela et al., 1984). This exchange reaction is completely asymmetric, always leading to release of tRNA^{fMet}, implying a unique stereochemistry. Although no tRNA is cross-linked by exchange, the reaction does not occur in the absence of tRNA binding to the enzyme, since it can be blocked by prior cross-linking of tRNA^{fMet} with a nonexchangeable cross-linker (D. Valenzuela, unpublished results). The disulfide bond at the 3′ end of the bound tRNA is the most likely site of such exchange, since seven of the eight potentially available cysteines are located in the N-terminal domain of MetRS. Indeed, the same cross-linker attached to the variable loop of tRNA$_m^{Met}$ undergoes only a low level of exchange (Leon & Schulman, 1987).

The cross-linking experiments described here, together with earlier studies from the laboratories of Blanquet and Sigler (Hountondji et al., 1985; Rosa et al., 1979), provide the experimental basis for construction of a model of the interaction of tRNA^{fMet} with the crystallized fragment of MetRS (Zelwer et al., 1982). Future site-directed mutagenesis experiments will allow a high-resolution analysis of the functional groups in the tRNA and protein which are required for specific tRNA binding and aminoacylation.

Protein engineering techniques have already been used to investigate both the catalytic and tRNA binding sites of *B. stearothermophilus* TyrRS. The tyrosine enzyme is a dimer of 2 × 47 000 molecular weight which binds one tRNA per molecule of enzyme in solution. The N-terminal domain contains the active site for tyrosyl adenylate formation, while amino acid sequences in both the N-terminal and C-terminal domains are required for tRNA binding (Waye et al., 1983; Blow & Brick, 1985; Bedouelle & Winter, 1986; Carter et al., 1986). Construction of an extensive series of mutant enzymes has yielded a detailed description of the mechanism of amino acid activation by TyrRS (Fersht et al., 1985; Leatherbarrow et al., 1985). The sites of interaction of the tRNA backbone with positively charged residues on the surface of the enzyme have also recently been investigated by site-directed mutagenesis techniques and a model of the tRNA–protein interaction proposed (Bedouelle & Winter, 1986). Detailed modeling of tRNA binding to TyrRS is hampered by the fact that the C-terminal region of the enzyme is disordered in the crystal structure (Bhat et al., 1982).

The availability of a high-resolution map of the tRNA binding domain of MetRS greatly facilitates model-building studies in this system. In addition, since the anticodon is known to be the major site of recognition of tRNAs by MetRS, attention can immediately be directed to the peptide sequences near this region of the bound tRNA in the search for the corresponding essential amino acid residues in the protein.

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Sedimentation Equilibrium Measurements of Recombinant DNA Derived Human Interferon γ[†]

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ABSTRACT: Recombinant DNA derived human interferon γ (IFN-γ) from *Escherichia coli* was examined by equilibrium ultracentrifugation. Short-column equilibrium experiments at pH 6.9 in 0.1 M ammonium acetate buffer gave a *z*-average molecular weight of 33 500 ± 1400 at infinite dilution, corresponding to 1.98 ± 0.08 times the formula weight. Long- (2.6 mm) column experiments at pH 7.5 in 0.04 M imidazole buffer gave a molecular weight of 33 400 ± 500. Under the latter conditions IFN-γ behaves somewhat nonideally, with the departure from ideality accounted for by an effective (Donnan) charge of about 6+. No association of this dimer to form tetramer or higher polymers was observed, with the association constant for formation of tetramer from dimer K_{24} found to be less than 34 L mol^{-1}. Similarly, no dissociation to monomers was observable, with the dissociation constant to monomer K_{21} being less than 5×10^{-8} mol L^{-1}. At pH 3.55 in 0.02 M buffer (acetate plus acetic acid), there was virtually complete dissociation of the dimer to monomer. Extreme nonideality was seen in this low ionic strength system, and the effective charge on the protein was estimated to be about 11+. The reduced molecular weight $M(1 - \bar{v}\rho)$ of the monomer was found to be about 4.09 ± 0.20 kg mol^{-1}; this corresponds to a molecular weight of 16 410 ± 820, with the Scatchard definition of components. A small amount of a polymer with a molecular weight of about 0.5 $\times 10^6$ was detected under these conditions.

Although the amino acid sequence of human interferon γ (IFN-γ)[1] has been reported (Rinderknecht et al., 1984), there have been several conflicting reports on the molecular weight of IFN-γ. Values of 20 000 and 25 000 (along with a value of about 45 000 for a minor active component) have been observed by SDS-polyacrylamide gel electrophoresis for natural IFN-γ [see Kelker et al. (1983)]. The lower values are consistent with the expected monomer molecular weight for this glycoprotein. Treatment with glycosidases decreased the SDS gel estimates of sizes for these two components to values comparable to the sequence molecular weight with a possible C-terminal processing. Thus, the apparent subunit in SDS is a monomer. Gel filtration of either natural IFN-γ or recombinant *E. coli* derived IFN-γ in aqueous solution near neutral pH has indicated molecular weight values ranging from 34 000 to 70 000 [Rinderknecht et al., 1984; Devos et al., 1984; see Yip et al. (1982) for a review of earlier reports]. Target molecular weights ranging from 63 000 to 73 000 were found for similar IFN-γ preparations (Pestka et al., 1983) frozen at −135 °C in an unspecified solvent matrix when irradiated with 10-MeV electrons.

Accordingly, the size of the molecule in aqueous solution remains unknown, with the number of subunits variously es-

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[1] Abbreviations: IFN-γ, interferon γ; SDS, sodium dodecyl sulfate; DEC, Digital Equipment Co.; rpm, revolutions per minute; RMS, root mean square.

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