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# MODIFICATION OF SPECIFIC LYSINE RESIDUES IN E. COLI METHIONYL-tRNA SYNTHETASE BY CROSSLINKING TO E. COLI FORMYLMETHIONINE tRNA

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SUMMARY: A protein affinity labeling derivative of E. coli $tRNA^{fMet}$ has been prepared which carries an average of one reactive side chain per molecule, distributed over four structural regions. Each side chain contains a disulfide bond capable of reaction with cysteine residues and an N-hydroxysuccinimide ester group capable of coupling to lysine $\epsilon$-amino groups in proteins. Reaction of the modified tRNA with E. coli methionyl-tRNA synthetase leads to crosslinking only by reaction with lysine residues in the protein. Examination of the tRNA present in the crosslinked complex reveals that the enzyme is coupled to side chains attached to the 5' terminal nucleotide, the dihydrouridine loop, the anticodon and the CCA sequence. Digestion of the crosslinked enzyme with trypsin followed by peptide mapping reveals that the major crosslinking reactions occur at four specific lysine residues, with minor reaction at two additional sites. Native methionyl-tRNA synthetase contains 90 lysine residues, 45 in unique sequences of the dimeric $\alpha_2$ enzyme. Crosslinking of the protein to different regions in $tRNA^{fMet}$ thus occurs with the high degree of selectivity necessary for use in determining the peptide sequences which are near specific nucleotide sequences of tRNA bound to the protein.

Recent studies from this laboratory have involved the development of procedures for attachment of protein affinity labeling reagents of variable length and amino acid specificity to internal sites in $tRNA^{fMet}$ (1,2). One such crosslinking derivative containing a cleavable side chain has been used to couple $tRNA^{fMet}$ in a reversible manner to the tRNA binding site of E. coli methionyl-tRNA synthetase (EC 6.1.1.10) (3). In the present paper, we examine the suitability of this crosslinking reaction for use in mapping amino acid sequences at or near the tRNA binding site of this enzyme.

## MATERIALS AND METHODS

Dithiobis (succinimidylpropionate) (DTSP) was purchased from Pierce Chemical Co. and propane-1,3-diamine (PDA) from Aldrich Chemical Co. [$^{35}$S] DTSP was synthesized by the method of Lomant and Fairbanks (4). E. coli $tRNA^{fMet}$ having a specific activity of 1720 pmol/$A_{260}$ unit was obtained from Boehringer Mannheim. E. coli Met-tRNA synthetase was purified from E. coli $K_{12}$ strain EM20031 as described before (5). TPCK treated trypsin was obtained from Worthington Biochemical Corp.

### Attachment of protein affinity-labeling groups to $tRNA^{fMet}$ and crosslinking to Met-tRNA synthetase

$tRNA^{fMet}$ was treated with propane-1,3-diamine/bisulfite under conditions leading to incorporation of an average of one PDA side chain per molecule (2), and DTSP was

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coupled to each side chain as described before (3). Crosslinking reaction mixtures contained 1.22 μM Met-tRNA synthetase and excess DTSP/PDA-modified $tRNA^{fMet}$ in 20 mM HEPES, pH 8.0, 10 mM $MgCl_2$. Solutions were incubated at 25° and then quenched with 50 mM lysine prior to assay for residual methionine acceptor activity and/or binding of radioactively labeled tRNA to Millipore filters as described earlier (3).

Isolation of the crosslinked complex

Crosslinking was carried out using 1.22 μM Met-RNA synthetase and 4.43 μM [$^{35}$S] DTSP/PDA-$tRNA^{fMet}$ at 25° for 30 min. The sample (0.4 ml) was added to a 0.9 x 180 cm column of S-200 at room temperature and eluted with 1 M NaCl, 10 mM Tris, pH 8, 5 mM EDTA at a flow rate of 12 ml/hr. Fractions (1.7ml) were collected and chilled in ice. Fractions containing the crosslinked complex were pooled, dialyzed at 4° vs 0.1 M ammonium bicarbonate and then concentrated to 250 μl in a collodion bag with dialysis vs the same buffer in ice.

Sites in DTSP/PDA-$tRNA^{fMet}$ crosslinked to lysine residues in Met-tRNA synthetase

Enzyme-bound tRNA in the crosslinked complex isolated by S-200 chromatography was released by addition of DTT to a final concentration of 24 mM and incubation at 37° for 2 hrs. The solution was clarified by centrifugation and rechromatographed on the 0.9 x 180 cm S-200 column equilibrated with 1 M NaCl, 10 mM Tris, pH 8, 5 mM EDTA and 2 mM DTT. Fractions were collected and analyzed as before. Fractions containing the released $^{35}$S-labeled tRNA were pooled, dialyzed vs water, and evaporated to dryness. The tRNA was digested with $T_1$ RNase and phosphomonoesterase. Reaction mixtures containing 13$A_{260}$/ml tRNA and 500 units/ml $T_1$ RNase in 0.1 M Tris-HCl, pH 7.5 were incubated at 37° for 1-1/2 hrs. Intestinal calf phosphatase was added to a concentration of 2 units/ml and the incubation at 37° continued for 1 hr. 1 M DTT was added to a final concentration of 80 mM and the incubation at 37° continued for 30 min. The sample was chromatographed on a 0.5 x 100 cm column of DEAE cellulose equilibrated with 7 M urea, 20 mM Tris-HCl, pH 7.5 and 2 mM DTT. Elution was carried out with a linear gradient of 0-0.45 M NaCl/600 ml of the same buffer at a flow rate of 15 ml/hr.

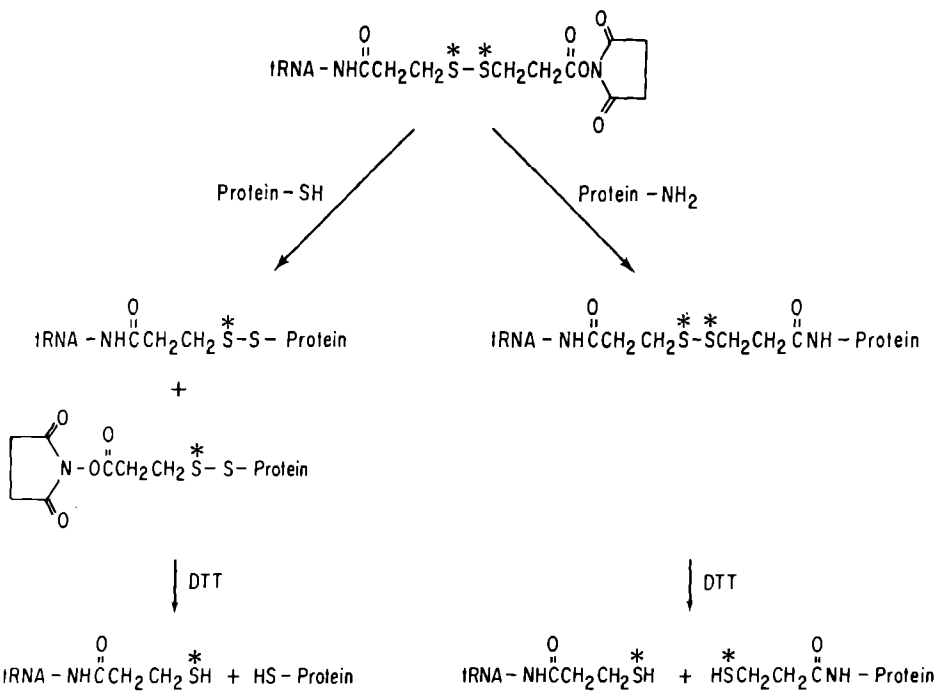
Determination of the number of lysine residues in Met-tRNA synthetase crosslinked to [$^{35}$S]DTSP/PDA-$tRNA^{fMet}$

Crosslinking was carried out as described above. Trypsin was added to a final ratio of 1:100 (w/w) trypsin/enzyme and the solution incubated for 7 hrs at 25°. The tryptic digest (0.5 ml) was loaded onto a 0.6 x 2 cm column of DEAE cellulose equilibrated with 0.1 M sodium acetate, pH 5.0. The column was washed with 5 ml of the same buffer, followed by 5 ml of 0.3 M NaCl, 0.1 M sodium acetate, pH 5.0. The tRNA fraction was eluted with 2 M NaCl, 0.1 M sodium acetate, pH 5.0., dialyzed exhaustively vs water and evaporated to dryness. The residue was dissolved in 100 μl of 75 mM 2-mercaptoethanol and incubated at 37° for 1 hr. The sample was mixed with the tryptic digest of 100 μg unlabeled Met-tRNA synthetase, lyophilized three times, dissolved in 10 μl of water, and spotted on a 0.1 mm, 20 x 20 cm cellulose thin layer plate (Merck). The $^{35}$S-labeled peptides were separated by electrophoresis in the first dimension and chromatography in the second dimension.

## RESULTS

### Crosslinking $tRNA^{fMet}$ to methionyl-tRNA synthetase

Protein affinity labeling groups are attached to $tRNA^{fMet}$ by a two step procedure. In the first step, short three-carbon side chains terminating in a reactive primary amino group are joined to the $N^4$-position of single stranded cytidine residues by transamination with propane-1,3-diamine (PDA) in the presence of bisulfite (2). This reaction is allowed to proceed until each tRNA molecule has incorporated an average of one side chain. The side chains are distributed over cytidine residues at positions 1, 16, 17, 34, 74 and 75 in the tRNA sequence (2). Dithiobis(succinimidylpropionate) (DTSP) is




Scheme I. Pathways for reaction of DTSP/PDA - $tRNA^{fMet}$ with proteins.

coupled to each side chain by incubation of the PDA-modified tRNA with excess diester (3). The resulting DTSP/PDA-$tRNA^{fMet}$ is isolated from free DTSP by repeated ethanol precipitation and dissolved in 10 mM $MgCl_2$ immediately before use in crosslinking reactions, since the lysine-reactive ester group is sensitive to hydrolysis.

The reactive tRNA derivative is capable of crosslinking to proteins by two pathways (Scheme I ). These potential pathways have been distinguished using [$^{35}$S] DTSP/PDA-$tRNA^{fMet}$. Coupling of the N-hydroxysuccinimide ester moiety to amino groups in the protein leads to attachment of 2 atoms of $^{35}$S per lysine reacted. Subsequent treatment of the covalent complex with dithiothreitol (DTT) leads to release of the tRNA, leaving one stable $^{35}$S per lysine reacted. Reaction of sulfhydryl groups in the protein with [$^{35}$S] DTSP/PDA-$tRNA^{fMet}$ leads to incorporation of one $^{35}$S per cysteine reacted, and subsequent treatment with DTT releases all of the bound radioactivity (Scheme I). Comparison of the amount of $^{35}$S covalently bound to Met-tRNA synthetase before and after treatment with DTT is shown in Figure 1. Approximately three atoms of $^{35}$S are incorporated per mole of native enzyme in a 30 min incubation at 25° in 20 mM HEPES buffer, pH 8, 10 mM $MgCl_2$ using a six-fold excess

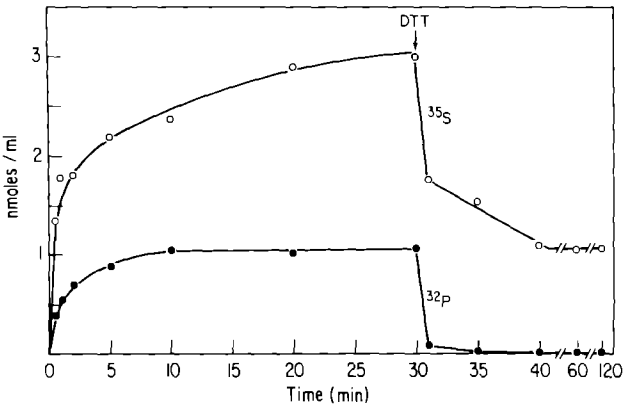



Figure 1. Reaction of radioactively labeled DTSP/PDA-tRNA$^{fMet}$ with methionyl-tRNA synthetase. E. coli Met-tRNA synthetase (1.2 μM) was incubated at 25° with 6.6 μM DTSP/PDA tRNA$^{fMet}$ labeled with $^{32}P$ at the 5' terminus of the tRNA or with $^{35}S$ in the disulfide bond of the crosslinker as described under Methods. Aliquots were withdrawn and assayed for radioactivity covalently bound to protein by Millipore filtration as described before (3). After 30 min. at 25°, DTT was added to a final concentration of 20 mM and additional aliquots taken.

of tRNA over enzyme. Treatment with DTT releases 2 atoms of $^{35}S$, and leaves one stable $^{35}S$ coupled to the protein. These results indicate that the original labeling occurred by reaction of an average of one lysine residue and one cysteine residue in the enzyme. Comparison of the amount of $^{35}S$ with the amount of 5'-$^{32}P$-labeled tRNA crosslinked in a 30 min incubation shows that only one mole of tRNA is coupled per mole of enzyme. Attachment of the tRNA therefore occurs exclusively by reaction with lysine residues. Reaction of cysteine residues in the enzyme with the disulfide bond of the crosslinker always leads to release of tRNA and covalent coupling to the ester side chain.

Sites of lysine-reactive groups in crosslinked DTSP/PDA-tRNA$^{fMet}$

In order to investigate the location of the side chains in DTSP/PDA-tRNA$^{fMet}$ which are crosslinked to Met-tRNA synthetase by reaction with lysine residues in the protein, the crosslinked complex was separated from excess unreacted tRNA by gel filtration on S-200 (not shown). The isolated crosslinked complex was treated with DTT to release the bound tRNA and the mixture rechromatographed on S-200 in the presence of sulfhydryl reagent (Figure 2). Modified enzyme and released tRNA were obtained in a 1:1 ratio. The $^{35}S$-labeled fraction of enzyme-bound tRNA (Peak 2, Figure 2) was digested with $T_1$ RNase and phosphomonoesterase and chromatographed on DEAE cellulose for analysis of the labeled oligonucleotides. Samples of DTT-treated control

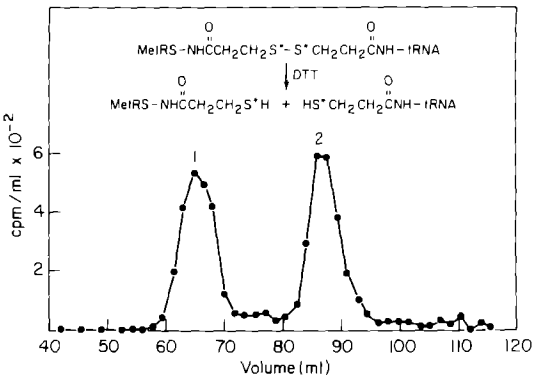



Figure 2. Gel filtration of the crosslinked complex of [$^{35}$S] DTSP/PDA-tRNA$^{fMet}$ and Met-tRNA synthetase on S-200 following treatment with DTT.

tRNA and unbound tRNA were similarly digested and chromatographed for comparison (Figure 3). Examination of the $T_1$ RNase profiles indicates that crosslinking occurs with side chains attached to all of the labeled sites in amounts roughly equal to the concentration of crosslinker at that site.

Since the exchange reaction generates tRNA derivatives having side chains terminating in sulfhydryl groups, it was necessary to determine whether such derivatives could displace DTSP/PDA-tRNA$^{fMet}$ previously crosslinked to lysine residues in the enzyme. If such displacement reactions occurred, it would not be possible to determine the site in the tRNA which was originally coupled to the enzyme, since it could be randomized by sulfhydryl-disulfide exchange of free and bound tRNA molecules. To examine this possibility, 5'-$^{32}$P-labeled DTSP/PDA-tRNA$^{fMet}$ was treated with excess DTT to completely reduce the disulfide bond of the crosslinker, generating the corresponding tRNA derivative with side chains terminating in sulfhydryl groups. The $^{32}$P-labeled SH derivative was incubated with the covalent complex prepared by coupling [$^{35}$S] DTSP/PDA-tRNA$^{fMet}$ to lysine residues in the protein and the amount of $^{32}$P subsequently coupled to the enzyme was measured by Millipore filtration. No incorporation of $^{32}$P was seen in incubations up to 30 min using concentrations of the reduced tRNA derivative in 3-fold excess over the amount known to be generated by exchange during the crosslinking reaction. Thus free tRNA is not capable of displacing the covalently bound tRNA.

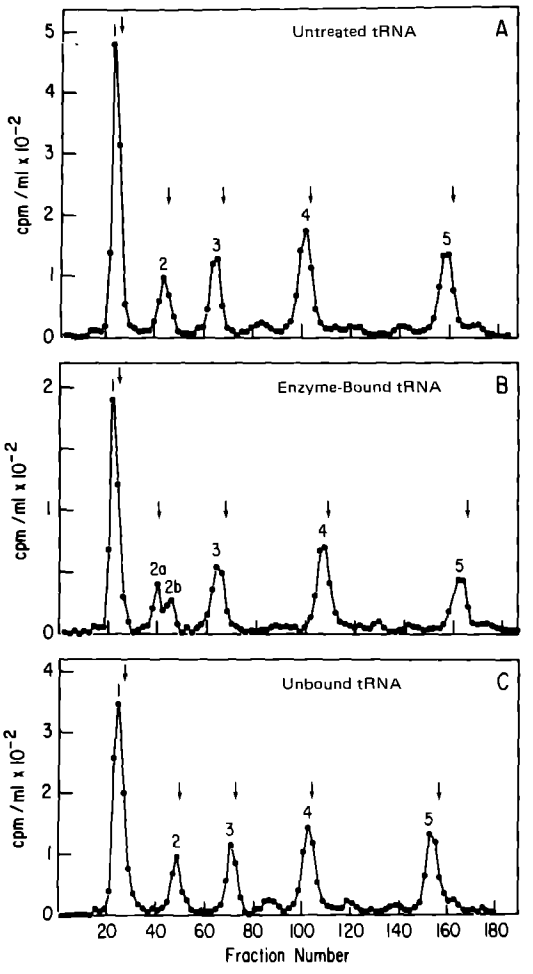



Figure 3. Chromatography of oligonucleotides obtained from digestion of [$^{35}$S] DTSP/PDA-tRNA$^{fMet}$ with $T_1$ RNase and phosphomonoesterase on DEAE cellulose in 7M urea. Arrows indicate the positions of unlabeled $A_{260}$ marker oligonucleotides derived from unmodified tRNA$^{fMet}$ and cochromatographed with the $^{35}$S-labeled tRNA. Radioactive peaks 1, 3, 4, and 5 correspond to $^{35}$S-labeled side chains attached to $C_1$, $C_{16,17}$ in the dihydrouridine loop, $C_{74,75}$ in the CCA sequence, and $C_{34}$ in the anticodon, respectively (2,3). The origin of peak 2 is presently unknown. A. Control sample of [$^{35}$S] DTSP-PDA-tRNA$^{fMet}$ incubated in the absence of enzyme. B. Fraction of [$^{35}$S] DTSP-PDA-tRNA$^{fMet}$ crosslinked to Met-tRNA synthetase on incubation with the enzyme. C. Fraction of [$^{35}$S] DTSP-PDA-tRNA$^{fMet}$ not crosslinked during incubation with Met-tRNA synthetase.

The overall data indicate that reactive lysine residues in the protein are located near four different structural regions of tRNA$^{fMet}$ at the tRNA binding site of the enzyme.

Complexity of crosslinked sites in MetRS

In order to quantitatively determine the number of modified peptides and their relative amounts, quenched crosslinking reaction mixtures were digested with trypsin and

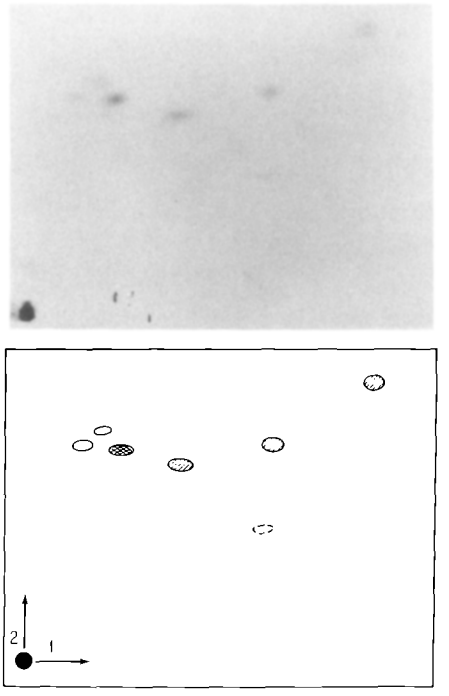



Figure 4. Map of $^{35}S$-labeled peptides obtained from crosslinked methionyl-tRNA synthetase. The enzyme was crosslinked to [$^{35}S$] DTSP/PDA-tRNA$^{fMet}$ as described under Methods. The crosslinked complex was digested with trypsin and the tRNA-bound peptides isolated by chromatography on DEAE cellulose. The $^{35}S$-labeled peptides were released by cleavage of the crosslinker with DTT and subjected to two dimensional thin layer chromatography and electrophoresis on cellulose plates according to ref. 6, except that the chromatography buffer was n-butanol/acetic acid/pyridine/water (15/3/10/12, v/v) and both buffers contained 2 mM DTT. Intact $^{35}S$-labeled tRNA remains at the origin under these conditions.

then chromatographed on a small DEAE cellulose column to separate free peptides from excess unreacted tRNA and tRNA covalently coupled to lysine residues. Modified $^{35}S$-labeled peptides were released from the isolated tRNA fraction by treatment with mercaptoethanol, mixed with a tryptic digest of unlabeled Met-tRNA synthetase and subjected to two dimensional electrophoresis and chromatography on cellulose thin layer plates (6). The radioactive peptides were detected by autoradiography. Four major peptides, two minor peptides and a trace peptide were observed (Figure 4), none of which comigrated with normal Met-tRNA synthetase peptides visualized by staining with ninhydrin.

## DISCUSSION

Reaction of [$^{35}S$]DTSP/PDA-tRNA$^{fMet}$ with native Met-tRNA synthetase followed by isolation of the crosslinked complex has shown that $^{35}S$-labeled enzyme and

$tRNA^{fMet}$ are present in a 1:1 ratio and that each crosslinking event leads to incorporation of one atom of DTT-stable $^{35}S$, in keeping with results expected for stoichiometric coupling of the modified tRNA to lysine residues in the enzyme.

Native methionyl-tRNA synthetase contains 90 lysine residues, 45 in unique sequences (7). Crosslinking groups attached to four different structural regions of the tRNA have been shown to couple to the protein, leading to significant modification of six tryptic peptides, with a majority of the reaction at four sites in the protein. These results indicate that the crosslinking reaction has the high degree of specificity necessary for isolation and sequencing of peptides at the tRNA binding site of the enzyme. Determination of the amino acid sequences close to a number of different regions of $tRNA^{fMet}$ bound to Met-tRNA synthetase will provide reference points for fitting the known crystal structures of the tRNA (8) and enzyme (9) into a three-dimensional picture of the complex. Such studies are now in progress.

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