Reversible Inactivation of *Escherichia coli* Methionyl-tRNA Synthetase by Covalent Attachment of Formylmethionine tRNA to the tRNA Binding Site with a Cleavable Cross-Linker†

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ABSTRACT: Protein affinity labeling groups have been attached to single-stranded cytidine residues in four structural regions of $tRNA^{fMet}$. Modification of the tRNA with an average of one cross-linking group per molecule is achieved with retention of 75% of the original methionine acceptor activity. Incubation of the modified tRNA with methionyl-tRNA synthetase (MetRS) results in covalent coupling of the protein and nucleic acid by reaction of *N*-hydroxysuccinimide ester groups attached to the tRNA with lysine residues in the enzyme. In the presence of excess MetRS, approximately 30% of the input tRNA can be covalently bound to protein, indicating that lysine residues are appropriately oriented for reaction with cross-linking groups attached to certain sites in the tRNA but not to others. The cross-linking reaction results in loss of aminoacylation activity of MetRS equal to the amount of covalently bound tRNA. Enzyme activity is restored by release of bound tRNA following cleavage of the disulfide bond of the cross-linker with a sulfhydryl reagent. The data indicate that cross-linking occurs at the tRNA binding site of the enzyme. In the presence of excess modified $tRNA^{fMet}$, a maximum of 1 mol of tRNA is cross-linked per mol of MetRS, in keeping with the known anticooperative tRNA binding properties of the native dimeric synthetase. In addition, the coupling reaction is effectively inhibited by unmodified $tRNA^{fMet}$, but not by noncognate tRNAs.

A variety of experimental approaches have yielded considerable information on the structural requirements of specific tRNAs for aminoacylation by their cognate aminoacyl-tRNA synthetases (Schimmel & Söll, 1979). In contrast, little is known about which amino acid sequences in these enzymes are required for substrate binding or catalytic activity. Covalent cross-linking of substrates to aminoacyl-tRNA synthetases provides an approach to identifying the structural regions at or near these functional sites. A number of laboratories have carried out such experiments by using chemically or photochemically reactive analogues of amino acids (Frolova et al., 1973; Silver & Laursen, 1974; Kovaleva et al., 1974; Rainey et al., 1976) and of ATP (Budker et al., 1974; Ankilova et al., 1975; Akhverdyan et al., 1977; Wetzel & Söll, 1977; Fayat et al., 1978; Bulychev et al., 1980; Madoyan et al., 1981). Similar experiments have been carried out with aminoacyl-tRNAs containing chemically reactive groups attached to the amino acid moiety (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). More recently, periodate-treated tRNAs have been covalently coupled to aminoacyl-tRNA synthetases by reaction of the oxidized 3′-terminal adenosine residue with the ϵ-amino group of a lysine residue at the active site of the enzymes (Fayat et al., 1979; Hountondji et al., 1979; Gerlo & Charlier, 1979; Baltzinger et al., 1979). To date, only a limited amount of peptide sequence information has emerged from these studies. Covalent coupling of isoleucyl bromomethyl ketone to *Escherichia coli* isoleucyl-tRNA synthetase (IleRS)[1] has lead to determination of the sequence of fifteen amino acids at the catalytic site of this enzyme (Rainey et al., 1976). Reaction of *p*-nitrophenylcarbamoylmethionyl-$tRNA^{Met}$ with *E. coli* MetRS has resulted in isolation and sequencing of a cross-linked octapeptide (Bruton & Hartley, 1970), and direct UV irradiation of a complex of ATP and *E. coli* IleRS has yielded the sequence of a pentapeptide at the ATP binding site of this protein (Yue & Schimmel, 1977).

Direct UV irradiation has also been used 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). Such studies have allowed determination of specific regions of the nucleic acid which are in contact with the protein and have assisted in construction of a general model for the topography of such complexes (Rich & Schimmel, 1977). Other methods which have been used to cross-link aminoacyl-tRNA synthetases to internal sites in tRNAs have involved attachment of photoactive groups to 4-thiouridine residues (Budker et al., 1974; Gorshkova et al., 1976; Wetzel & Söll, 1977) or guanosine residues (Vlasov et al., 1980). To date, such experiments have not yielded information on the structural regions of protein at the tRNA binding sites of these enzymes or on the relative orientation of specific nucleotide and peptide sequences within these macromolecular complexes.

Previous studies from this laboratory have concentrated on identifying the structural features of the *E. coli* methionine tRNAs which are required for recognition by *E. coli* methionyl-tRNA synthetase (Schulman, 1970, 1971, 1972, 1979; Schulman & Goddard, 1973; Schulman & Pelka, 1977a,b; Stern & Schulman, 1977). More recently, we have developed procedures for attachment of protein affinity labeling reagents of variable length and amino acid specificity to internal sites in $tRNA^{fMet}$ (Sarkar & Schulman, 1978; Schulman et al., 1981). It is our goal to use such tRNA derivatives to obtain

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[1] Abbreviations used: $tRNA^{fMet}$, the *E. coli* initiator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; IleRS, *E. coli* isoleucyl-tRNA synthetase; PheRS, yeast phenylalanyl-tRNA synthetase; PDA, propane-1,3-diamine; DTSP, dithiobis(succinimidylpropionate); DTSP/PDA-$tRNA^{fMet}$, $tRNA^{fMet}$ modified with PDA followed by coupling to DTSP; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; Me_2SO, dimethyl sulfoxide; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin.

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high-yield cross-linking reactions which will allow us to map the amino acid sequences close to a number of different regions of tRNA bound to protein. In the present paper, we describe the quantitative cross-linking of tRNA^{fMet} to the tRNA binding site of *E. coli* methionyl-tRNA synthetase by using a cleavable cross-linker.

Materials and Methods

Materials. Dithiobis(succinimidylpropionate) (DTSP) was purchased from Pierce Chemical Co., and propane-1,3-diamine (PDA) was obtained from Aldrich Chemical Co. [^{35}S]DTSP was synthesized by the method of Lomant & Fairbanks (1976). [γ-^{32}P]ATP and [^{14}C]methionine were purchased from New England Nuclear Corp. *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* K_{12} strain EM20031 as described before (Schulman & Pelka, 1977b). Polynucleotide kinase was purified from T_4-bacteriophage-infected *E. coli* by the procedure of Panet et al. (1973), as modified by Efstratiadis et al. (1977). Crystalline bovine serum albumin was purchased from Miles Laboratories, and RNase-free bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp. Yeast tRNA^{Phe}, *E. coli* tRNA$_1^{Val}$, and *E. coli* tRNA^{Tyr} having specific activities of 1400, 1085, and 1000 pmol/A_{260} units, respectively, were purchased from Boehringer Mannheim.

Preparation of DTSP/PDA-Modified tRNA^{fMet}. Reaction mixtures for modification of *E. coli* tRNA^{fMet} with propane-1,3-diamine contained 16 A_{260} units/mL tRNA in 1 M PDA, 2 M sodium bisulfite, pH 7.0, and 10 mM $MgCl_2$. Solutions were incubated at 25 °C for a given amount of time, diluted with 2 volumes of water, and dialyzed once vs. 0.15 M NaCl and 10 mM Tris, pH 7.0, and twice vs. 0.05 M NaCl and 10 mM Tris, pH 7.0. One-tenth volume of 1 M Tris-HCl, pH 9.2, was added, and the solution was incubated at 37 °C for 8 h to reverse uridine bisulfite adducts formed during the PDA/bisulfite reaction. Samples were then dialyzed overnight vs. 10 mM Tris-HCl, pH 7, and 5 mM $MgCl_2$, concentrated by evaporation to 20 A_{260} units/mL, and precipitated by addition of 2 volumes of 95% ethanol. The number of N^4-substituted cytidine residues per mole of tRNA was determined by attachment of fluorescein to the reactive amino groups as described elsewhere (Schulman et al., 1981). Assays for methionine acceptor activity were carried out as described before (Schulman & Pelka, 1977b).

All DTSP coupling reactions were carried out in siliconized polypropylene Eppendorf centrifuge tubes. Unlabeled DTSP or [^{35}S]DTSP (60 cpm/pmol) was dissolved in fresh Me_2SO at a concentration of 2.57 mg/mL immediately before use. PDA-modified tRNA^{fMet} was dissolved in 0.2 M Hepes buffer, pH 7.8, at a concentration of 25 A_{260} units/mL. The tRNA and DTSP solutions were mixed in a 1:0.75 ratio and incubated at 25 °C for 15 min, and the reaction was stopped by addition of 2 volumes of ethanol. Samples were chilled for 10 min at −20 °C and centrifuged. The modified tRNA was reprecipitated twice from 0.1 M sodium acetate, pH 6.0. The extent of reaction of N^4-substituted cytidine residues in tRNA^{fMet} with DTSP was determined as described before (Sarkar & Schulman, 1978). The precipitate of DTSP/PDA-modified tRNA was dissolved in 10 mM $MgCl_2$ just before use in cross-linking reactions.

Hydrolysis of the reactive *N*-hydroxysuccinimide ester groups of DTSP/PDA-modified tRNA^{fMet} was carried out by incubation of the tRNA in 0.1 M Hepes, pH 9, at 37 °C for 4 h followed by addition of 0.1 volume of 1 M sodium acetate, pH 6, and precipitation with 2 volumes of ethanol.

Preparation of 5′-^{32}P-Labeled tRNA^{fMet}. Unmodified tRNA^{fMet} was dephosphorylated by incubation with bacterial alkaline phosphatase at 65 °C as described before (Schulman et al., 1974a). Phosphatase was removed by adsorption of 10 A_{260} units of dephosphorylated tRNA to a 1 × 3 cm column of DEAE-cellulose equilibrated with 0.05 M sodium acetate, pH 5, and 0.1 M NaCl. The column was washed with 30 mL of the same buffer and the tRNA eluted with a solution of 0.1 M sodium acetate, pH 5, 2 M NaCl, and 10% ethanol. The eluted tRNA was dialyzed vs. 10 mM Tris, pH 7, and 5 mM $MgCl_2$, concentrated by evaporation to 20 A_{260} units/mL, and precipitated by addition of 2 volumes of ethanol.

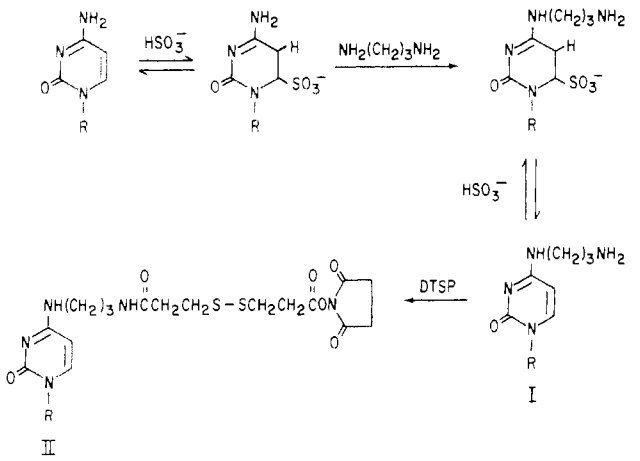
Reaction mixtures for phosphorylation of the 5′-hydroxy-terminated tRNA contained 1.55 A_{260} units/mL tRNA and 420 units/mL polynucleotide kinase in 0.07 M Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 50 μg/mL BSA, and 0.1 mM [γ-^{32}P]ATP (10^4 cpm/pmol). Solutions were incubated at 37 °C for 30 min, resulting in phosphorylation of approximately 90% of the 5′-OH termini. Samples were concentrated 10-fold by evaporation, mixed with an equal volume of 40% sucrose containing 0.1% bromophenol blue dye, and electrophoresed at 100 V on a 3-mm slab gel of 16% polyacrylamide containing 7 M urea, using a running buffer of 0.04 M Tris–borate, pH 8, and 1 mM EDTA. After the dye marker had traveled 24 cm, the tRNA band was located by autoradiography, excised, and electrophoretically eluted (Silberklang et al., 1979). The sample was dialyzed vs. 10 mM Tris-HCl, pH 7, and 5 mM $MgCl_2$ and then adsorbed to a 1 × 2 cm column of DEAE-cellulose. The column was washed and the tRNA eluted and concentrated as described above. Samples isolated from polyacrylamide gels which were not passed through the DEAE-cellulose column were observed to have low methionine acceptor activity. [5′-^{32}P]tRNA^{fMet} was mixed with unlabeled tRNA^{fMet} to the desired specific radioactivity prior to modification with amine/bisulfite.

Cross-Linking of DTSP/PDA-Modified tRNA^{fMet} to MetRS. All procedures were carried out in siliconized culture tubes. Reaction mixtures contained 1.22 μM MetRS and various concentrations of DTSP/PDA-modified tRNA^{fMet} in 20 mM Hepes, pH 7 or 8, 10 mM $MgCl_2$, and 4% glycerol. Solutions were incubated at 25 °C, and aliquots were removed at various times for measurement of residual enzyme activity and/or binding of [5′-^{32}P]tRNA to Millipore filters.

(*A*) *Enzyme Activity.* The cross-linking reaction was quenched by addition of aliquots removed from the reaction to 10 volumes of 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.11 mg of BSA/mL containing 50 mM lysine. The enzyme was diluted with the above buffer minus lysine immediately prior to assaying for residual enzyme activity. Reaction mixtures for assay of methionine acceptance (0.15 mL) contained 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 3 A_{260} units/mL unmodified tRNA^{fMet}, 2 mM ATP, and 17 μM [^{14}C]methionine (360 cpm/pmol). Samples were equilibrated at 25 °C, and reactions were initiated by addition of enzyme to a final concentration of 160 pM. After incubation at 25 °C for 10 min, 100-μL aliquots were pipetted onto 2.5-cm Whatman 3 MM filter disks, added to cold 10% trichloroacetic acid, and washed and counted as previously described (Schulman, 1970). Under these conditions, methionine incorporation was proportional to enzyme concentration and was linear with time for at least 10 min.

Control experiments were carried out with enzyme incubated in the absence of DTSP/PDA-tRNA^{fMet} and in the presence of an equivalent amount of unmodified tRNA^{fMet}. No change

Scheme I



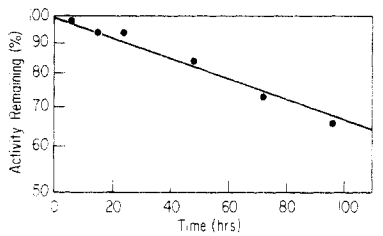


FIGURE 1: Modification of $tRNA^{fMet}$ with PDA/bisulfite. Reaction mixtures contained 16 A_{260} units/mL $tRNA^{fMet}$ in 1 M PDA, 2 M sodium bisulfite, pH 7.0, and 10 mM $MgCl_2$. Incubation was at 25 °C. Aliquots were removed at various times and treated as described under Materials and Methods. Methionine acceptor activity was measured under standard assay conditions (Schulman & Pelka, 1977b).

in enzyme activity was observed in control reaction mixtures incubated for up to an hour at 25 °C.

(B) Millipore Binding. Aliquots from the cross-linking reaction were added to 100 volumes of 10 mM Tris-HCl, pH 8, 2 M KCl, and 50 mM lysine, mixed, and allowed to stand at room temperature for 30 min before filtering through Millipore type HA filters. The filters were washed with ten 5-mL portions of 10 mM Tris-HCl, pH 8, and 2 M KCl followed by ten 5-mL portions of 10 mM Tris-HCl, pH 8, dried, and counted in Aquasol. Blank reactions contained equivalent amounts of 5′-^{32}P-labeled DTSP/PDA-$tRNA^{fMet}$ but no enzyme.

Results

Attachment of Protein Affinity Labeling Groups to E. coli $tRNA^{fMet}$. Attachment of protein affinity labeling groups to single-stranded cytidine residues in *E. coli* $tRNA^{fMet}$ was carried out by using the two-step procedure illustrated in Scheme I. In the first step, the tRNA was incubated with 1 M propane-1,3-diamine (PDA) in the presence of 2 M sodium bisulfite, pH 7.0, at 25 °C, yielding N^4-substituted cytidine residues (I). Pseudo-first-order kinetics were observed for the partial loss of methionine acceptor activity accompanying this modification (Figure 1). A linear relationship was observed between the number of modified residues per molecule of tRNA and the amino acid acceptor activity (Figure 2). The tRNA used in the present cross-linking experiments was modified with PDA/bisulfite for 66 h at 25 °C, resulting in incorporation of an average of 1.0 modified cytidine per tRNA, with retention of 75% of the original methionine acceptor activity.

Modified cytidine residues (I) were coupled to dithiobis(succinimidylpropionate) (DTSP) by incubation in 0.11 M Hepes, pH 7.8, and 60% Me_2SO at 25 °C for 15 min to yield

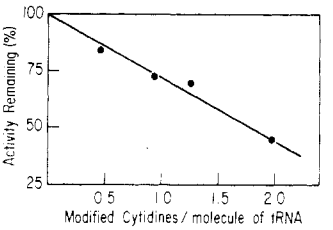
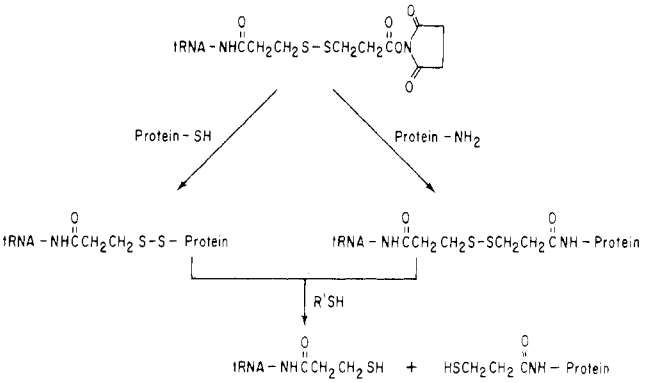


FIGURE 2: Effect of PDA/bisulfite modification on the methionine acceptor activity of $tRNA^{fMet}$. Reactions were carried out as described in the legend to Figure 1. The number of modified cytidine residues per molecule of tRNA was determined as described elsewhere (Schulman et al., 1981).

Scheme II



cytidine derivatives II. Some hydrolysis of the reactive ester groups of the cross-linker occurred during the coupling reaction, and the tRNA was found to contain an average of 0.75 mol of reactive ester per mol of tRNA after isolation. Due to the instability of the *N*-hydroxysuccinimide ester group of II, the modified tRNA was prepared immediately prior to its use in cross-linking reactions.

The sites of the modified cytidine residues carrying DTSP/PDA cross-linking groups were the same as those previously shown to be transaminated with amines in the presence of bisulfite (Schulman et al., 1974b). These include the unpaired cytidine at the 5′ terminus, two cytidine residues in the dihydrouridine loop, the cytidine in the wobble position of the anticodon, and the two cytidine residues in the 3′-terminal CCA sequence.

Cross-Linking of DTSP/PDA-$tRNA^{fMet}$ to E. coli Methionyl-tRNA Synthetase. DTSP/PDA-modified $tRNA^{fMet}$ is capable of cross-linking to protein by reaction of appropriately oriented lysine ε-amino groups with the *N*-hydroxysuccinimide ester moiety or by reaction of cysteine sulfhydryl groups with the disulfide bond of the cross-linker (Scheme II). The rate of cross-linking with purified *E. coli* MetRS (1.22 μM) was followed by carrying out the reaction using 5′-^{32}P-labeled tRNA. Aliquots were withdrawn at various times and quenched by addition to 100 volumes of 50 mM lysine, pH 8, in 2 M KCl, and the amount of covalently bound tRNA was measured by retention of ^{32}P on Millipore filters. In the presence of excess enzyme, reactions were complete within 1 min in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, and 4% glycerol at 25 °C (not shown). At enzyme:tRNA ratios of 20–40:1, 25–30% of the input tRNA was covalently bound to the protein. In the presence of excess tRNA, reactions were complete within about 10 min under the same conditions (Figure 3). Incubations carried out for up to 60 min showed no further change in the amount of ^{32}P retained on the filter. Treatment of the cross-linked complex with 25 mM DTT resulted in rapid release of covalently bound ^{32}P-labeled tRNA by cleavage of the disulfide bond of the cross-linking group

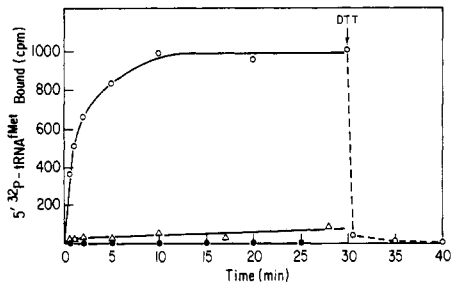


FIGURE 3: Rate of cross-linking of modified [5'-^{32}P]tRNA^{fMet} to proteins. Reactions were carried out in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, and 4% glycerol at 25 °C. Aliquots were removed at various times, quenched by addition to 100 volumes of 10 mM Tris-HCl, pH 8, 50 mM lysine, and 2 M KCl, and filtered through Millipore filters as described under Materials and Methods. (○) 7.4 μM 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet}, 1.22 μM MetRS; (△) 7.4 μM 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet}, 3.1 μM bovine serum albumin; (●) 7.4 μM hydrolyzed 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet}, 1.22 μM MetRS. At the position marked by an arrow, reactions were quenched by addition of 0.1 volume of 0.5 M lysine, pH 8. After incubation at 25 °C for 30 min, 1 M DTT was added to a final concentration of 25 mM and the incubation continued. Aliquots were removed at various times, diluted as described above, and immediately filtered. Reactions carried out in the absence of protein gave a Millipore blank of 75 cpm, which has been subtracted.

Table I: Stoichiometry of Cross-Linking DTSP/PDA-tRNA^{fMet} to MetRS[a]

DTSP/PDA-tRNA^{fMet} added (μM)	[^{32}P]tRNA bound (μM)	enzyme inactivated (μM)	tRNA bound/ mole of enzyme added
1.2	0.20	0.21	0.16
2.4	0.42	0.44	0.34
4.8	0.70	0.71	0.57
29.8	1.22	1.14	1.00
43.5	1.15	1.20	0.96
59.5	1.27	1.18	1.04

[a] Reactions were carrried out with 1.22 μM MetRS and various concentrations of 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet} in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, and 4% glycerol at 25 °C for 30 min. Aliquots were withdrawn and assayed for enzyme-bound ^{32}P and residual enzyme activity as described in the legends to Figures 3 and 4.

(Scheme II). A very low level of cross-linking was observed with a nonspecific protein, bovine serum albumin (Figure 3). All of the coupling of tRNA^{fMet} to MetRS appeared to involve reaction of the *N*-hydroxysuccinimide ester groups of the cross-linker with lysine residues in the protein, since hydrolysis of the ester groups prior to incubation of the modified tRNA with MetRS completely eliminated retention of ^{32}P on the filters (Figure 3). Further evidence for this reaction pathway was obtained by using DTSP/PDA-tRNA^{fMet} radioactively labeled with ^{35}S at the two sulfur atoms of the cross-linker. Incubation of 3.3 μM [^{35}S]DTSP/PDA-5'-^{32}P-tRNA^{fMet} with 1.22 μM MetRS in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, and 4% glycerol at 25 °C for 60 min resulted in covalent attachment of 0.54 μM [^{32}P]tRNA to the enzyme as measured by Millipore filtration. Treatment of the covalent complex with 25 mM DTT resulted in release of protein-bound radioactivity, giving a final value of 0.54 μM ^{35}S-labeled enzyme (based on 1 mol of ^{35}S/mol of coupled cross-linker). Since only the reaction of lysine residues with *N*-hydroxysuccinimide groups on the cross-linker can lead to DTT-stable ^{35}S-labeled protein (Scheme II), the 1:1 ratio of ^{32}P to stable ^{35}S indicates that all of the coupling of tRNA^{fMet} to MetRS occurred by this pathway.

Cross-linking of tRNA^{fMet} to MetRS was accompanied by loss of enzyme activity (Figure 4). Table I compares the

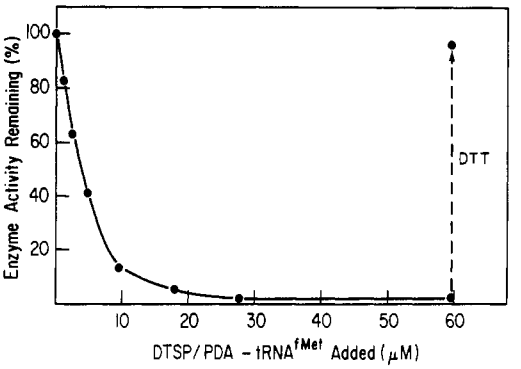


FIGURE 4: Effect of cross-linking on MetRS activity. Reactions were carried out with 1.22 μM MetRS and various concentrations of DTSP/PDA-tRNA^{fMet} in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, and 4% glycerol. Incubations were for 30 min at 25 °C. Aliquots were withdrawn and assayed for residual enzyme activity as described under Materials and Methods. Reactions treated with DTT were first incubated with DTSP/PDA-tRNA^{fMet} for 30 min and quenched by addition of 0.1 volume of 0.5 M lysine, pH 8, adjusted to 25 mM DTT, and the incubation at 25 °C was continued for 90 min. Aliquots were then assayed for enzyme activity as described above.

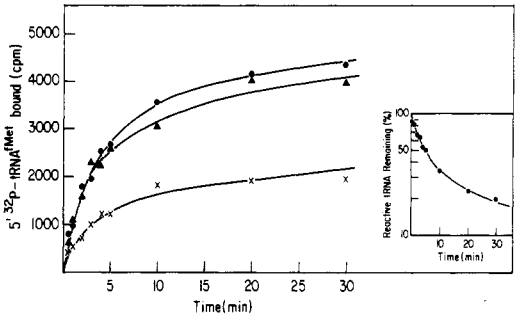


FIGURE 5: Effect of cognate and noncognate tRNAs on the rate of cross-linking of DTSP/PDA-tRNA^{fMet} to MetRS. Reactions were carried out with 1.22 μM MetRS and 7.5 μM 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet} in 20 mM Hepes, pH 7, 10 mM $MgCl_2$, and 4% glycerol at 25 °C. Aliquots were removed at various times and treated as described in the legend to Figure 3. (●) No additions; (▲) plus 33 μM *E. coli* tRNA^{Phe}; (×) plus 33 μM unmodified *E. coli* tRNA^{fMet}. The insert shows a semilogarithmic plot of the rate of cross-linking of the fraction of added DTSP/PDA-tRNA^{fMet} which is capable of covalent reaction with MetRS, based on the end point of the reaction.

amount of ^{32}P-labeled tRNA cross-linked with the amount of enzyme inactivated in the same reaction, using different tRNA/MetRS ratios. It can be seen that there is a 1:1 correspondence between covalent reaction and loss of activity for a wide range of tRNA concentrations and that even in the presence of a large excess of tRNA only 1 mol of tRNA is cross-linked per mol of native enzyme. Treatment of the covalent complex with DTT was found to result in recovery of enzyme activity (Figure 4).

The rate of coupling of primary amino groups to *N*-hydroxysuccinimide esters is sensitive to pH, since the unprotonated form of the amine is the reactive species. As expected, the rate of cross-linking of DTSP/PDA-tRNA^{fMet} to MetRS was significantly slower at pH 7, allowing more accurate measurement of the reaction kinetics (Figure 5). Under these conditions, cross-linking did not occur with a uniform rate but rather exhibited an initial fast reaction followed by a slower reaction. Addition of excess unmodified *E. coli* tRNA^{fMet} to reaction mixtures containing 5'-^{32}P-labeled DTSP/PDA-tRNA^{fMet} and MetRS greatly reduced the rate of covalent attachment of ^{32}P-labeled tRNA to the protein (Figure 5) and the rate of loss of enzyme activity (not shown), indicating that binding of the normal tRNA substrate com-

petes with that of $tRNA^{fMet}$ carrying the chemically reactive cross-linker. Addition of excess *E. coli* $tRNA^{Phe}$ had no effect on the fast cross-linking reaction but partially inhibited the slower reaction (Figure 5). Similar results were obtained with other noncognate tRNAs, including *E. coli* $tRNA_1^{Val}$, *E. coli* $tRNA^{Tyr}$, and yeast $tRNA^{Phe}$.

Discussion

Considerable information is already available on the structure and function of *E. coli* $tRNA^{fMet}$ and *E. coli* methionyl-tRNA synthetase. The three-dimensional structure of the tRNA has recently been elucidated by X-ray crystallographic analysis (Woo et al., 1980), and significant progress has been made in characterizing the enzyme. Native MetRS is a symmetrical dimer of molecular weight 172000 (Lemoine et al., 1968; Koch & Bruton, 1974). A biologically active tryptic fragment of molecular weight 64000 has been crystallized in a form suitable for high-resolution X-ray analysis (Waller et al., 1971), and a 4-Å map has been calculated (Zelwer et al., 1976). Primary sequence studies on the enzyme are also well advanced (Bruton et al., 1974), and detailed mechanistic studies on both the native enzyme and the tryptic fragment have been carried out [Blanquet et al. (1979) and references cited therein]. Some progress has been made in identifying the amino acid sequences at or near the catalytic site of the enzyme. Bruton & Hartley (1970) have isolated and sequenced an octapeptide obtained by cross-linking the amino acid moiety of a chemically reactive derivative of Met-tRNA to the protein. Cross-linking of periodate-oxidized $tRNA^{Met}$ and ATP to MetRS is also expected to yield unique peptides suitable for sequencing, since quantitative reaction of these modified substrates with the enzyme has been achieved (Fayat et al., 1978, 1979; Hountondji et al., 1979).

Complete understanding of the molecular mechanism of the $tRNA^{Met}$–MetRS interaction will require identification of the specific functional groups on both the nucleic acid and protein which are required for binding and catalytic function. An essential step toward this end is to determine the relative orientation of specific regions of each macromolecule within the protein–nucleic acid complex. Nucleic acid bases which are required for recognition of $tRNA^{fMet}$ by MetRS have been found to be widely separated in the three-dimensional structure of the tRNA [Schulman & Pelka, 1977a; Schulman (1979) and references cited therein]. Direct UV irradiation of the enzyme complexed with a number of $tRNA^{Met}$ substrates has also shown that the contact points between the nucleic acid and protein include regions of tRNA structure extending from the anticodon loop to the 3′ terminus (Rosa et al., 1979). Thus, efforts to determine the orientation of these two macromolecules will require identification of peptide sequences located at sites which span essentially the entire length of the tRNA.

We have developed procedures for attachment of reactive side chains to cytidine residues in four structural regions in $tRNA^{fMet}$: the 5′ terminus, the dihydrouridine loop, the anticodon, and the 3′ CCA terminus (Schulman et al., 1981). Our aim is to vary the length and chemical reactivity of these side chains until quantitative cross-linking of MetRS to each of these regions is achieved, allowing isolation of specific peptides in high yield for sequencing studies. The data reported here describe the use of a cross-linker which is potentially capable of reacting with either lysine or cysteine residues in proteins. All of the observed cross-linking of DTSP/PDA-$tRNA^{fMet}$ to MetRS occurs by coupling to lysine groups, however, indicating that none of the 24 SH groups in the native enzyme are properly oriented for reaction with the modified tRNA.

The lysine-reactive *N*-hydroxysuccinimide ester groups of the cross-linker are unstable, undergoing hydrolysis with a half-life of approximately 10 min when incubated at pH 8, 25 °C, in the absence of enzyme. In the presence of excess tRNA, cross-linking to MetRS is complete in 10–15 min under the same conditions; thus, some hydrolysis of the ester groups occurs during the incubation, reducing the concentration of the reactive tRNA species. In the presence of a large excess of enzyme, cross-linking is complete within 1 min, and hydrolysis is minimized. Under these conditions, approximately 30% of the input tRNA (0.75 reactive ester group/molecule) can be covalently bound to protein, indicating that lysine residues react with cross-linking groups attached to certain sites in the tRNA but not to others. Cross-linking occurs at more than one site, since the extent of reaction exceeds the amount of cross-linker present in any one structural region. Further evidence for several reactive sites is obtained from the multiphasic kinetics of cross-linking.

The data indicate that the tRNA is covalently linked to the enzyme at the tRNA binding site. Loss of MetRS activity equal to the amount of covalent reaction is observed over the entire range of DTSP/PDA-$tRNA^{fMet}$ concentrations used. In the presence of excess tRNA, only one molecule of tRNA cross-links to each molecule of native dimeric synthetase. This stoichiometry is consistent with the known anticooperative tRNA binding properties of the enzyme (Blanquet et al., 1973a). Cross-linking to a nonspecific protein, bovine serum albumin, occurs only very slowly and in low yield, further indicating that reaction of the tRNA with MetRS is facilitated by its specific association with the enzyme. Both kinetic phases of the cross-linking reaction with MetRS are effectively competed out by unmodified $tRNA^{fMet}$. The fast coupling reaction is unaffected by noncognate tRNAs, while the slower reaction is partially inhibited. MetRS, like other aminoacyl-tRNA synthetases, is known to bind noncognate tRNA species (Blanquet et al., 1973b). The present data suggest that the binding of such tRNAs occurs at a site which at least partially overlaps the binding site of the cognate species.

Treatment of the covalent $tRNA^{fMet}$–MetRS complex with DTT releases the tRNA and leads to recovery of enzyme activity, indicating that the loss of catalytic function is due only to the presence of the bound tRNA in the complex. Release of the cross-linked tRNA leaves a modified lysine residue on one of the two subunits of the enzyme (Scheme II). Since native MetRS contains identical tRNA binding sites on each subunit, the modified enzyme could function by binding tRNA exclusively to the unmodified subunit. The full recovery of catalytic activity following DTT treatment suggests, however, that both subunits retain the ability to bind tRNA and that the lysine residues modified by the cross-linking reaction may be nonessential for enzyme function. Experiments are in progress to identify the sites in $tRNA^{fMet}$ which carry the reactive cross-linking groups and to isolate corresponding peptide fragments attached to each site.

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