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Covalent Coupling of 4-Thiouridine in the Initiator Methionine tRNA to Specific Lysine Residues in *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: A new method has been developed to couple a lysine-reactive cross-linker to the 4-thiouridine residue at position 8 in the primary structure of the *Escherichia coli* initiator methionine tRNA ($tRNA^{fMet}$). Incubation of the affinity-labeling $tRNA^{fMet}$ derivative with *E. coli* methionyl-tRNA synthetase (MetRS) yielded a covalent complex of the protein and nucleic acid and resulted in loss of amino acid acceptor activity of the enzyme. A stoichiometric relationship (1:1) was observed between the amount of cross-linked tRNA and the amount of enzyme inactivated. Cross-linking was effectively inhibited by unmodified $tRNA^{fMet}$, but not by noncognate $tRNA^{Phe}$. The covalent complex was digested with trypsin, and the resulting tRNA-bound peptides were purified from excess free peptides by anion-exchange chromatography. The tRNA was then degraded with T1 ribonuclease, and the peptides bound to the 4-thiouridine-containing dinucleotide were purified by high-pressure liquid chromatography. Two major peptide products were isolated plus several minor peptides. N-Terminal sequencing of the peptides obtained in highest yield revealed that the 4-thiouridine was cross-linked to lysine residues 402 and 439 in the primary sequence of MetRS. Since many prokaryotic tRNAs contain 4-thiouridine, the procedures described here should prove useful for identification of peptide sequences near this modified base when a variety of tRNAs are bound to specific proteins.

Recent studies from this laboratory have involved development of new methods for the covalent coupling of specific protein–nucleic acid complexes, with a view to isolation and sequencing of peptides at nucleic acid binding sites. To date, we have focused our attention on the interaction of *Escherichia coli* methionine tRNAs with *E. coli* methionyl-tRNA synthetase (MetRS).[1] High-resolution X-ray crystallographic data is available for a biologically active monomeric form of MetRS (Zelwer et al., 1982), providing an opportunity to interpret the results of cross-linking studies in terms of the known three-dimensional structure of the enzyme. Affinity-labeling derivatives of $tRNA^{fMet}$ carrying cross-linkers at the 5′ terminus, the dihydrouridine loop, and the anticodon have been coupled to MetRS in high yield, and specific peptides attached to each site have been identified (Schulman et al., 1981a,b; Valenzuela et al., 1984; Valenzuela & Schulman, 1986; Leon & Schulman, 1987a). In addition, we have made use of the presence of the minor base 3-(3-amino-3-carboxypropyl)uridine in the elongator methionine tRNA to attach a site-specific cross-linker to the variable loop of this tRNA and have determined the sequence of the major MetRS peptide coupled to this site (Leon & Schulman, 1987b). The initiator tRNA also contains a unique minor base, 4-thiouridine, which can be used to prepare a site-specific affinity-labeling tRNA derivative carrying a cross-linker on the inside of the L-shaped three-dimensional structure of the tRNA. This region of tRNAs has been suggested to form part of a general binding domain for aminoacyl-tRNA synthetases (Rich & Schimmel, 1977) and has been shown to directly interact with cognate

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[1] Abbreviations: $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; $tRNA_m^{Met}$, *E. coli* elongator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; SBrAB, *N*-succinimidyl [(bromoacetyl)amino]-benzoate; SBrAB-$tRNA^{fMet}$, $tRNA^{fMet}$ modified by coupling SBrAB to the 4-thiouridine residue at position 8 from the 5′ terminus; [$^{35}S_8$]-$tRNA^{fMet}$, $tRNA^{fMet}$ labeled with ^{35}S in the sulfur atom of the 4-thiouridine residue at position 8; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DEAE, *N*,*N*-diethylaminoethyl; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; NHS, *N*-hydroxysuccinimide; ODS, octadecylsilane; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; RNase, ribonuclease.

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synthetases in certain tRNA–enzyme complexes (Schimmel, 1979). It is therefore of particular interest to examine the protein structure near this region of tRNAs bound to their specific synthetases.

Photoaffinity probes have previously been coupled to 4-thiouridine residues in several *E. coli* tRNAs, and covalent cross-linking to cognate synthetases has been observed (Budker et al., 1974; Gorshkova et al., 1976; Wetzel & Söll, 1977); however, no peptide sequences have emerged from these studies. In our experience, higher yields of specific peptides are obtained by using affinity-labeling derivatives directed to react with specific amino acid residues in the protein. We have therefore developed a procedure for coupling a lysine-reactive cross-linker to the 4-thiouridine residue at position 8 in $tRNA^{fMet}$. In the present paper, we describe this procedure and demonstrate the use of the site-specific tRNA affinity-labeling derivative to identify peptide sequences at the tRNA binding site of MetRS which are close to this modified base in the tRNA.

Materials and Methods

Materials

Sequenal grade trifluoroacetic acid was obtained from Pierce Chemical Co. *N,N'*-Dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, bromoacetic acid, dioxane, 2-propanol, *p*-aminobenzoic acid, cyanogen bromide, and spectrophotometric grade dimethyl sulfoxide were purchased from Aldrich Chemical Co. HPLC grade acetonitrile was from Fisher, and water was purified in a MilliQ reagent grade water system from Millipore Corp. [^{14}C]Methylamine was from New England Nuclear, and [γ-^{32}P]ATP, [α-^{32}P]ATP, and [^{35}S]-sodium sulfide were obtained from Amersham. TPCK-treated trypsin was obtained from Worthington Biochemical Corp. and further purified as described before (Leon & Schulman, 1987a). Calf intestinal alkaline phosphatase was from Boehringer-Mannheim, and T_1 and T_2 RNases were from Calbiochem. *E. coli* $tRNA^{fMet}$ and *E. coli* $tRNA^{Phe}$ having specific amino acid acceptor activities of 1650 and 1750 pmol/A_{260} unit, respectively, were purchased from Subriden. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* CSR 603/pLC 20-25 as described before (Leon & Schulman, 1987b).

Methods

Synthesis of N-Succinimidyl [(Bromoacetyl)amino]-benzoate (SBrAB). *N*-(Bromoacetoxy)succinimide was synthesized by the method of Santi and Cunnion (1974) and recrystallized from 2-propanol. (Bromoacetamido)benzoic acid was synthesized by addition of 1 mmol (232 mg) of *N*-(bromoacetoxy)succinimide to 1 mmol (137 mg) of *p*-aminobenzoic acid in 10 mL of dry dioxane and stirring at 25 °C for 16 h in the dark. Fifteen milliliters of 0.5 M HCl and 40 mL of ether were added, and the solution was mixed vigorously. The aqueous phase was discarded, and the organic phase was evaporated to dryness at 37 °C under vacuum. The residue was dried over P_2O_5 (mp 234 °C). *N*-Succinimidyl [(bromoacetyl)amino]benzoate (SBrAB) was synthesized by addition of dicyclohexylcarbodiimide (164 mg, 0.8 mmol) to *p*-(bromoacetamido)benzoic acid (208 mg, 0.8 mmol) and *N*-hydroxysuccinimide (92 mg, 0.8 mmol) in 15 mL of dry dioxane and stirring at 25 °C for 6 h. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated at 37 °C under vacuum, yielding an oily residue. The product was crystallized from 2 mL of methanol, recrystallized from petroleum ether, and stored over $CaCl_2$ at −20 °C in a vacuum desiccator; mp 195–197 °C with decomposition. Anal. Calcd: C, 44.50; H, 3.16; N, 8.00; O, 22.80; Br, 21.42. Found: C, 44.50; H, 3.63; N, 7.87; O, 22.29; Br, 20.76. The proton NMR spectrum of SBrAB was taken on a Varian XL-200 with $DCCL_3$ as solvent: δ 2.85 (s, 4, NHS), 3.98 (s, 2, CH_2Br), 7.19–8.07 (m, 4, aromatic), 8.3 (s, 1, NH) (standard = hexamethyldisilane).

Preparation of ^{32}P-Labeled $tRNA^{fMet}$. All steps were carried out in the dark in order to avoid light-catalyzed modification of 4-thiouridine. The 3'-terminal adenosine residue of $tRNA^{fMet}$ was labeled with ^{32}P by using an exchange reaction catalyzed by *E. coli* tRNA nucleotidyltransferase in the presence of [α-^{32}P]ATP and sodium pyrophosphate, as described by Francis et al. (1983).

Preparation of ^{35}S-Labeled 4-Thiouridine in $tRNA^{fMet}$. All steps were carried out under a hood. The procedure used was based on the method of Pal and Schmidt (1974). $tRNA^{fMet}$ (160 A_{260} units) was dissolved in 3.4 mL of 20 mM sodium phosphate, pH 8.0. Two-tenths milliliter of 0.5 M cyanogen bromide in ethanol was added and the mixture incubated at 25 °C for 15 min. Sodium chloride was added to a final concentration of 0.2 M, and the tRNA was precipitated with 2 volumes of ethanol. The tRNA (40 A_{260} units/mL) was precipitated once from 0.2 M NaCl and then dissolved in 1.7 mL of 20 mM sodium phosphate, pH 8.0. Nine-tenths milliliter of 40 mM sodium phosphate, pH 7.0, containing 10 μmol of [^{35}S]sodium sulfide was added, yielding a solution of pH 8.5. The reaction vessel was flushed with N_2 and the solution incubated for 1 h at 25 °C and then overnight at 4 °C. Excess [^{35}S]sodium sulfide was removed by three ethanol precipitations. The tRNA was dissolved in 0.2 M NaCl and dialyzed in the dark vs. 200 volumes of 0.2 M NaCl and 25 mM NaOAc, pH 6.5, with two changes of buffer. The tRNA was precipitated with 2 volumes of ethanol, dissolved in 0.1 M sodium acetate, pH 6.5, and stored at −20 °C.

One A_{260} unit of carrier $tRNA^{fMet}$ was mixed with 0.5 A_{260} unit (20000 cpm) of the ^{35}S-labeled tRNA in 50 μL of 40 mM ammonium formate, pH 4.5. T_2 RNase (15 units) and T_1 RNase (40 units) were added, and the mixture was incubated at 37 °C for 16 h. The solvent was evaporated and the residue taken up in 2 μL of water, and the nucleotides were separated by chromatography on a plastic-backed cellulose thin-layer plate with isobutyric acid/0.5 N NH_4OH (5/3 v/v) as solvent. The nucleotides were visualized by their UV absorbance. The chromatogram was cut into 1-cm strips, and these were added to 5 mL of Econofluor for measurement of radioactivity.

Attachment of SBrAB to $tRNA^{fMet}$. Due to the instability of the lysine-reactive cross-linking group, SBrAB was coupled to $tRNA^{fMet}$ immediately prior to use in cross-linking reactions. $tRNA^{fMet}$ was dissolved in 5 mM potassium phosphate, pH 7.4, at a concentration of 100 A_{260} unit/mL, and 5 volumes of dimethyl sulfoxide containing 11 mg/mL SBrAB was added. The reaction mixture was incubated at 25 °C for 7 min in the dark. Sodium chloride was added to a final concentration of 0.2 M, and the tRNA was precipitated with 2 volumes of ethanol. Excess SBrAB was removed by reprecipitation 4 times from 0.1 M sodium acetate, pH 6.0. The tRNA was dissolved in 10 mM $MgCl_2$ just before use.

The amount of reactive ester remaining attached to the tRNA following the coupling reaction was determined by quenching samples of SBrAB-$tRNA^{fMet}$ with [^{14}C]methylamine and measuring the incorporation of ^{14}C into a TCA-insoluble form. The modified tRNA was incubated in 0.2 M Hepes, pH 7.8, and 5 mM [^{14}C]methylamine at 25 °C for 4 h in the dark. Aliquots were removed, added to 1 mL of 10% TCA, mixed, filtered through nitrocellulose disks, and washed

with 15 × 5 mL portions of 5% TCA, followed by 5 mL of ethanol. The filters were dried and counted in Econofluor (New England Nuclear). Blanks were determined with samples of unmodified tRNA^{fMet} treated in the same way.

Complete hydrolysis of the reactive *N*-hydroxysuccinimide ester group of SBrAB-tRNA^{fMet} was accomplished by incubation of the derivatized tRNA in 0.1 M Hepes, pH 9.0, at 37 °C for 4 h. The tRNA was precipitated with ethanol, reprecipitated once from 0.1 M NaCl, and stored frozen in 10 mM $MgCl_2$.

The kinetic parameters for aminoacylation of ester-hydrolyzed SBrAB-tRNA^{fMet} were determined as described elsewhere (Schulman & Pelka, 1983).

Site of Attachment of SBrAB to tRNA^{fMet}. SBrAB-tRNA^{fMet} was quenched with [^{14}C]methylamine was described above. The ^{14}C-labeled derivative was digested with T_1 RNase, and the resulting oligonucleotides were separated by chromatography on a column of DEAE-cellulose in the presence of 7 M urea as described by Valenzuela et al. (1984). Fractions (2 mL) were collected and mixed with 20 mL of ACSII (Amersham) for measurement of radioactivity. The absorbance at 260 nm was monitored with a Gilford 2400 spectrophotometer. The site of attachment of SBrAB to tRNA^{fMet} was determined by comparison of the elution position of the ^{14}C-labeled oligonucleotide with the elution positions of the A_{260} T_1 RNase oligonucleotide markers of known sequence.

[$^{35}S_8$]SBrAB-tRNA^{fMet} was quenched by incubation with glycine. The tRNA was then digested with T_2 RNase and T_1 RNase, and the resulting nucleotides were separated by thin-layer chromatography as described above. The mobility of the radioactive nucleotide was compared with that of nucleotide A_{260} markers.

Cross-Linking of SBrAB-tRNA^{fMet} to MetRS. Reaction mixtures contained 1–4 μM MetRS and 4–12 μM SBrAB-tRNA^{fMet} in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$. Solutions were incubated in siliconized culture tubes at 25 °C. Aliquots were removed at various times, quenched by incubation with 50 mM glycine for 30 min at 25 °C, and assayed for residual enzyme activity and/or binding of ^{32}P-labeled SBrAB-tRNA^{fMet} to nitrocellulose filters (Millipore, type HA) in the presence of high salt as described before (Schulman et al., 1981a). Control experiments were carried out with enzyme incubated in the absence of SBrAB-tRNA^{fMet} in the presence of an equivalent amount of unmodified tRNA.

Competition experiments were carried out at 25 °C with 1.2 μM MetRS in 20 mM Hepes, pH 8, 10 mM $MgCl_2$, 4.4 μM [^{32}P]SBrAB-tRNA^{fMet}, and 16 μM unmodified tRNA^{fMet} or tRNA^{Phe}. The unmodified tRNAs were added to the enzyme solution immediately prior to addition of SBrAB-tRNA^{fMet}.

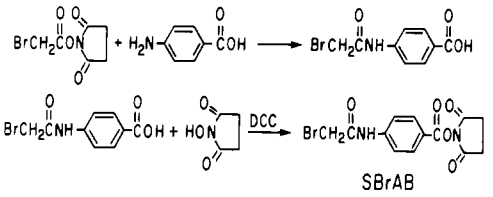
Isolation of Peptides Cross-Linked to SBrAB-tRNA^{fMet}. Cross-linking was carried out in 4 mL of 15 mM Hepes, pH 8, and 10 mM $MgCl_2$ containing 4.3 μM MetRS and 12 μM [$^{35}S_8$]SBrAB-tRNA^{fMet} (15 cpm/pmol). The solution was incubated in the dark for 60 min at 25 °C. Glycine, pH 7, was added to a final concentration of 50 mM and the incubation at 25 °C continued for another 30 min. Aliquots were withdrawn for measurement of residual enzyme activity and determination of the amount of cross-linked complex by nitrocellulose filtration. The remaining reaction mixture was concentrated in a collodion bag to 2.5 mg/mL protein and dialyzed against 0.1 M ammonium acetate, pH 6.5, and 0.1 mM $CaCl_2$ for 2 h. The protein was digested with 3% RNase-free trypsin (w/w) for 7 hrs at 25°. The tryptic digest was adjusted to 0.1 M NaCl by addition of 4 M NaCl and precipitated by addition of 3 volumes of ethanol. The pellet 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 equilibrated with the same buffer. The column was washed with a gradient from 0% to 20% buffer B (buffer B = 7 M urea, 1 M NaCl, 0.05 M ammonium acetate, pH 6.5) over 5 min followed by a 25-min wash with 20% buffer B. This was followed by a second gradient from 20% to 100% buffer B over 30 min. One-minute fractions were collected at a flow rate of 0.5 mL/min, and aliquots were taken for measurement of radioactivity. The radioactive fractions were pooled (6 mL), dialyzed twice for 3 h against 2 L of 0.1 M sodium acetate, pH 6.5, and once against 2 L of 25 mM sodium acetate, pH 6.5, overnight. The dialysate was concentrated by evaporation and precipitated from 0.3 M sodium acetate. The pellet was reprecipitated once and then dissolved in 0.1 M NaOAc, pH 6.5, at a concentration of 44 A_{260} units/mL and digested with 1430 units/mL T_1 RNase at 37 °C for 1 h. Calf intestinal alkaline phosphatase was added to a final concentration of 15 units/mL and the incubation at 37 °C was continued for another 2 h. Two volumes of 7 M urea, 20 mM sodium acetate, pH 6.5, and 10% acetonitrile was added, and the sample was injected onto a 7.5 × 75 mm column of Bio-Gel TSK-DEAE 5-PW equilibrated with the same buffer. Oligonucleotides were eluted with a linear salt gradient from 0 to 0.3 M NaCl in the same buffer over 3 h at a flow rate of 0.3 mL/min. Two-minute fractions were collected in siliconized culture tubes, and 10-μL 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. Radioactive peaks were pooled, diluted with an equal volume of 7 M urea and 20 mM sodium acetate, pH 6.5, and injected onto a 250 × 4.6 mm Altex PTH-amino acids ODS reverse-phase HPLC column equipped with a 45 × 4.6 mm precolumn (Rainin) and preequilibrated with 0.1% TFA in water (v/v) (solvent A). The column was washed with solvent A at a flow rate of 0.65 mL/min until the absorbance of the effluent returned to base line. The oligonucleotide-bound peptides were then eluted by using a Rainin gradient HPLC system and the following gradient profile: solvent A from 0 to 10 min, followed by a linear gradient from 0% to 42% solvent B in 120 min (solvent B = 0.1% TFA in CH_3CN v/v). A second linear gradient from 42% to 70% solvent B was run from 130 to 150 min, followed by a 10-min wash with 70% solvent B. The absorbance of the effluent at 210 and 260 nm was monitored with a Gilson Model 116 detector. One-minute fractions were collected in siliconized, heat-treated (300 °C, 24 h) culture tubes and, 50-μL aliquots were taken for measurement of radioactivity.

Peptide Sequencing. Peptide sequencing was carried out by automated N-terminal degradation with PITC on an Applied Biosystems gas-phase sequenator at Yale University (Stone & Williams, 1986). Amino acids were determined by the high-sensitivity PTH-amino acid detection method of Merrill et al. (1984).

RESULTS

Synthesis of SBrAB. *N*-Succinimidyl [(bromoacetyl)-amino]benzoate (SBrAB) was prepared as outlined in Scheme I. In the first step, *p*-aminobenzoic acid was reacted with the *N*-hydroxysuccinimide ester of bromoacetic acid. The resulting (bromoacetamido)benzoic acid was then coupled to *N*-

Scheme I: Procedure for the Synthesis of SBrAB



hydroxysuccinimide in the presence of DCC. The final recrystallized product had a melting point of 195–197 °C with decomposition. Elemental analysis and NMR spectroscopy confirmed the predicted structure (see Materials and Methods).

Attachment of a Lysine-Reactive Cross-Linker to 4-Thiouridine in tRNA^{fMet}. *E. coli* tRNA^{fMet} contains the minor base 4-thiouridine at position 8 in the primary sequence (Dube et al., 1969). The sulfur atom of this minor base was alkylated by incubation of the tRNA (17 A_{260} units/mL) with SBrAB in 0.83 mM potassium phosphate, pH 7.4, and 83% DMSO at 25 °C for 7 min in the dark (Scheme II). The amount of reactive ester remaining intact following the coupling reaction was determined by quenching with [^{14}C]methylamine and measurement of cold TCA insoluble radioactivity. By this criterion, 0.6 mol of unhydrolyzed ester remained per mole of tRNA after isolation of SBrAB-tRNA^{fMet}. The coupling reaction was complete with SBrAB concentrations between 2.5 and 25 mM. Direct reaction of the starting tRNA^{fMet} with [^{14}C]iodoacetate resulted in ^{14}C labeling of 0.75 mol of 4-thiouridine per mole of tRNA, suggesting that a portion of the tRNA lacked the minor base and that about 80% of the S_8-containing tRNA was converted to the lysine-reactive SBrAB-tRNA^{fMet} derivative.

Complete reaction of SBrAB with the available 4-thiouridine in tRNA^{fMet} was indicated by loss of the characteristic absorbance of the minor base at 335 nm following the coupling reaction. In addition, tRNA^{fMet} labeled with ^{35}S in the sulfur atom of the 4-thiouridine residue was modified with SBrAB. Complete reaction of the minor base was indicated by a shift in the mobility of the ^{35}S-labeled nucleotide following ribonuclease digestion of the modified tRNA to nucleotides and chromatography on thin-layer plates (data not shown).

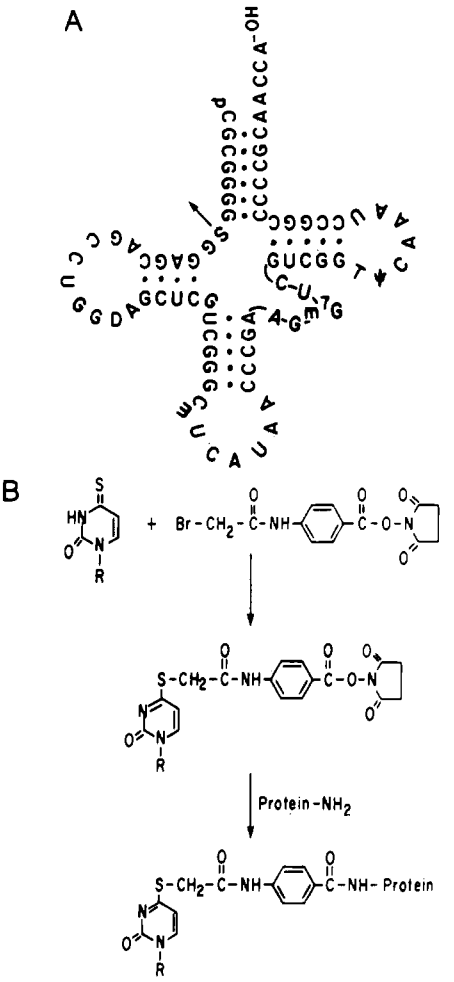
In order to access the specificity of the alkylation reaction, [^{14}C]methylamine-quenched SBrAB-tRNA^{fMet} was digested with T_1 RNase and the resulting oligonucleotides were chromatographed on a column of DEAE-cellulose under denaturing conditions (data not shown). A single ^{14}C-labeled peak was obtained that eluted in the position expected for the modified dinucleotide SpGp. These results confirm the specific coupling of SBrAB to 4-thiouridine in tRNA^{fMet}.

The half-life of the reactive ester of SBrAB-tRNA^{fMet} in the buffer used for the cross-linking experiments (20 mM Hepes, pH 8.0, 10 mM $MgCl_2$) was 1 h at 25 °C in the absence of MetRS. In 0.1 M sodium acetate, pH 6.0, and at 4 °C, no hydrolysis was observed in 2 h.

Attempts were also made to directly couple the *N*-hydroxysuccinimide ester of bromoacetic acid to 4-thiouridine in tRNA^{fMet} by using conditions similar to those described above. Complete alkylation of the minor base was achieved; however, the NHS ester group was hydrolyzed to the corresponding carboxylic acid during the coupling reaction. The speed of coupling and relative stability of the ester group of SBrAB made this reagent particularly suitable for preparation of the desired affinity-labeling derivative of tRNA^{fMet}.

Aminoacylation of 4-Thiouridine-Modified tRNA^{fMet}. In order to determine the effect of SBrAB coupling on the ability

Scheme II: Labeling of 4-Thiouridine in tRNA^{fMet} with SBrAB and Coupling of Reactive NHS Ester to Protein–NH_2 Groups[a]



[a] (A) Cloverleaf structure of *E. coli* tRNA^{fMet} (Dube et al., 1969) showing the site of attachment of SBrAB (arrow). (B) Coupling of SBrAB to 4-thiouridine followed by reaction of the *N*-hydroxysuccinimide ester with protein amino groups.

of modified tRNA^{fMet} to interact with MetRS, the ester group of SBrAB-tRNA^{fMet} was hydrolyzed and the amino acid acceptor activity of the resulting carboxylic acid derivative was measured. In the presence of excess MetRS, 73% of ester-hydrolyzed SBrAB-tRNA^{fMet} could be aminoacylated. Measurement of the kinetic parameters for aminoacylation showed that V_{max} was unchanged; however, the modified tRNA had a K_m that was 6-fold higher than that observed for unmodified tRNA^{fMet}.

Cross-Linking of SBrAB-tRNA^{fMet} to MetRS. The *N*-hydroxysuccinimide ester group of SBrAB-tRNA^{fMet} is capable of cross-linking to proteins by reaction with appropriately oriented lysine ϵ-amino groups (Scheme II). Incubation of SBrAB-tRNA^{fMet} (6.4 μM) with MetRS (1.2 μM) in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$ at 25 °C resulted in rapid formation of a covalent complex of protein and nucleic acid, as measured by retention of ^{32}P-labeled tRNA on nitrocellulose filters. Enzyme activity was also determined by assaying diluted aliquots of the cross-linking reaction mixture. The kinetics of cross-linking and MetRS inactivation were found to closely parallel each other (Figure 1). Comparison of the amount of [^{32}P]SBrAB-tRNA^{fMet} cross-linked with the amount of enzyme inactivated showed a 1:1 correspondence between covalent reaction and loss of biological activity.

The specificity of the cross-linking reaction was examined by determining the effect of cognate and noncognate tRNAs

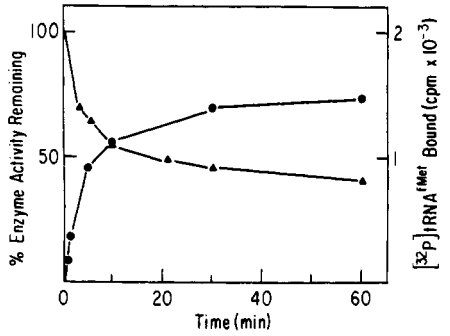


FIGURE 1: Cross-linking of SBrAB-tRNA^{fMet} to MetRS. MetRS (1.2 μM) was incubated with [^{35}P]SBrAB-tRNA^{fMet} (3.8 μM) in 20 mM Hepes, pH 8.0, and 10 mM $MgCl_2$ at 25 °C. Aliquots were removed at various times for measurement of residual enzyme activity (▲) and retention of ^{32}P on nitrocellulose filters (●), as described under Materials and Methods.

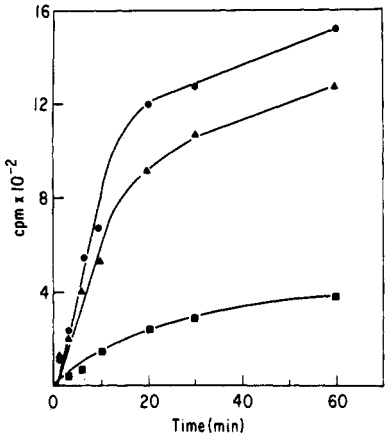


FIGURE 2: Effect of cognate and noncognate tRNAs on the rate of cross-linking of SBrAB-tRNA^{fMet} to MetRS. Reactions were carried out with 1.1 μM MetRS and 4.4 μM [^{32}P]SBrAB-tRNA^{fMet} in 20 mM Hepes, pH 8.0, and 10 mM $MgCl_2$ at 25 °C. Aliquots were removed at various times for measurement of ^{32}P retained on nitrocellulose filters as described under Materials and Methods. Symbols: ●, no additions; ▲, plus 16 μM *E. coli* tRNA^{Phe}; ■, plus 16 μM unmodified *E. coli* tRNA^{fMet}.

on the rate of MetRS inactivation. Figure 2 shows the kinetics of cross-linking of 4.4 μM [^{32}P]SBrAB-tRNA^{fMet} to 1.2 μM MetRS in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$ at 25 °C. Addition of excess unmodified *E. coli* tRNA^{fMet} (16 μM) to the cross-linking reaction mixture reduced the initial rate of the reaction approximately 4-fold. In contrast, addition of excess *E. coli* tRNA^{Phe} (16 μM) had little effect on the initial rate of inactivation but partially decreased its final extent (Figure 2).

Isolation and Sequencing of Peptides Cross-Linked to [$^{35}S_8$]SBrAB-tRNA^{fMet}. 4-Thiouridine in tRNA^{fMet} was labeled in the sulfur atom with ^{35}S according to the method of Pal and Schmidt (1974). The tRNA was first treated with cyanogen bromide to convert the sulfhydryl group of 4-thiouridine to a thiocyanate group. This derivative was then displaced by NaH^{35}S, generating [^{35}S]-4-thiouridine. Thin-layer chromatography of nucleotides obtained by T_2 RNase digestion of the ^{35}S-labeled tRNA confirmed that the label was attached exclusively to the 4-thiouridine residue. The extent of labeling was 0.6 mol of ^{35}S/mol of tRNA^{fMet}. Cross-linking was carried out with 4.3 μM MetRS and 12 μM [$^{35}S_8$]SBrAB-tRNA^{fMet} in 20 mM Hepes, pH 8, and 10 mM $MgCl_2$ at 25 °C for 30 min. Glycine was added to a final concentration of 50 mM, and the incubation was continued for 30 min. Due to the incomplete regeneration of [^{35}S]-4-thiouridine, the reaction also contained 8 μM [U_8]tRNA^{fMet}, carrying no cross-linker. Under these conditions, 43% of the input enzyme was cross-linked, as measured by retention of ^{35}S on nitrocellulose filters and loss of enzyme activity. The cross-linked tRNA–enzyme complex was digested with 3% trypsin (w/w) for 7 h, at 25 °C. Unreacted tRNA and tRNA-bound peptides were precipitated with ethanol, and the supernatant containing free 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 the remaining free peptides by chromatography on a Bio-Gel TSK-DEAE 5-PW ion-exchange HPLC column under denaturing conditions (data not shown). Free, unlabeled peptides eluted early from the column, and a single radioactive peak was obtained at the elution position of unmodified tRNA^{fMet}. The recovery of radioactivity applied to the column was 79%. The fractions containing ^{35}S were pooled, dialyzed, and concentrated, and the tRNA was digested with T_1 RNase and alkaline phosphatase. The resulting [^{35}S]SpG-bound peptides, unreacted [^{35}S]SpG derivatives, and unlabeled oligonucleotides were chromatographed on a TSK-DEAE column in 7 M urea, 20 mM sodium acetate, pH 6.5, and 10% acetonitrile with a gradient of NaCl (Figure 3). A small radioactive peak (A) eluted at the front of the column, and a large peak (B) eluted near the trinucleotide region. The second peak corresponded to the elution position of the free ester hydrolyzed and glycine-quenched SBrAB-[^{35}S]SpG derivatives, which have a net charge of −2 at pH 6.5. A third small peak (C) was obtained following a high salt (0.45 M) wash of the column. The recovery of radioactivity applied to the column was 94%. Peaks B and C were also present in a control sample of [^{35}S]SBrAB-tRNA^{fMet} that had been carried through a mock cross-linking reaction in the absence of MetRS and then digested and chromatographed as described above. Radioactive peak A (Figure 3) was not seen in the control sample (data not shown).

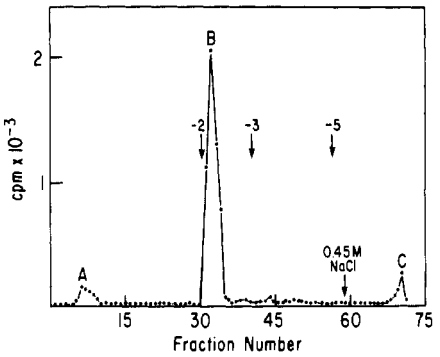


FIGURE 3: Separation of oligonucleotide-bound peptides by ion-exchange HPLC. The ^{35}S-labeled tRNA–peptide/free tRNA fraction was digested with T_1 RNase and phosphomonoesterase as described under Materials and Methods. The sample was dissolved in 7 M urea, 20 mM sodium acetate, pH 6.5, and 10% acetonitrile and injected onto a TSK-DEAE 5-PW column equilibrated with the same buffer. Oligonucleotides were eluted with a linear salt gradient from 0 to 0.2 M NaCl over 2 h at a flow rate of 0.3 mL/min. At the position marked by an arrow, the column was washed with 0.45 M NaCl. Two-minute fractions were collected, and 10-μL aliquots were taken for measurement of radioactivity. The charges and elution positions of A_{260} oligonucleotide markers are indicated on the figure.

Each of the radioactive peaks (Figure 3) was pooled and separately analyzed for [^{35}S]SpG-bound peptides by chromatography on a reverse-phase HPLC column. After the injection of each sample, the column was washed extensively with 0.1% TFA in water until the UV absorbance returned to base line, and then the column was developed with a gradient of acetonitrile. The absorbance at 210 and 260 nm was

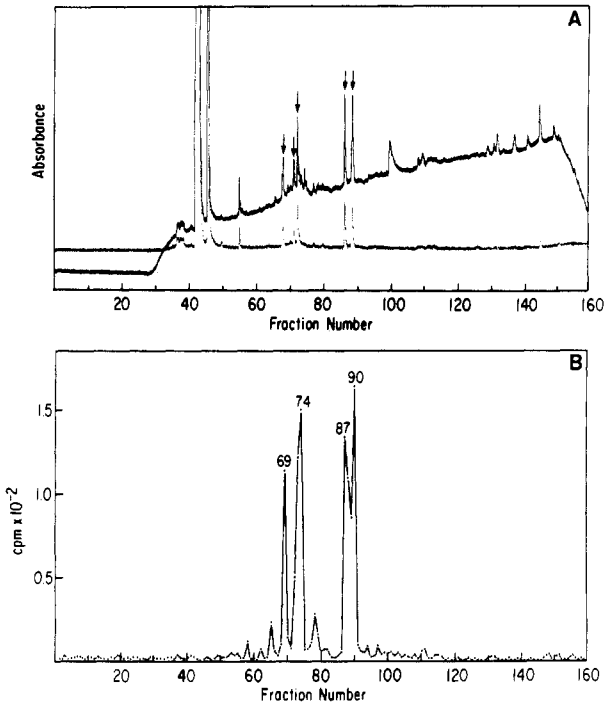


FIGURE 4: Reverse-phase chromatography of [^{35}S]SpG-bound peptides from peak A. Peak A (Figure 3) was injected onto a reverse-phase HPLC column equilibrated with 0.1% TFA in water (v/v), and the column was washed with the same buffer until the absorbance returned to base line (not shown). The oligonucleotide-bound peptides were eluted with a gradient of CH_3CN as described under Materials and Methods. Panel A: Absorbance at 210 (upper) and 260 (lower) nm was continuously monitored by using a Gilson 116 detector and a full scale of 0.2 OD. Arrows on the absorbance profile indicate the elution positions of the radioactive peaks. Panel B: Fractions (0.65 mL) were collected and aliquots (50 μL) withdrawn for measurement of radioactivity.

monitored, and aliquots were taken for measurement of radioactivity. No radioactivity was found in the flow-through fractions.

Figures 4 and 5 show the gradient elution profiles of peaks A and B (Figure 3). Peak C was found to contain only nucleotide material, and no ^{35}S-labeled peaks were observed in the HPLC profile (data not shown).

Chromatography of peak A yielded four main radioactive peaks which absorbed at both 210 and 260 nm (Figure 4). The peaks centered at fractions 69 and 74 were also found in the elution profile of a control sample that was not subjected to cross-linking, indicating that these peaks do not correspond to peptide products. The radioactive peaks eluting at fractions 87 and 90 were found only after cross-linking [^{35}S]SBrAB-tRNA^{fMet} to MetRS. Peak B contained mainly unlabeled nucleotide material plus two large radioactive peaks derived from the unreacted [^{35}S]SpG-bound cross-linker (fractions 68 and 73, Figure 5). In addition, two small ^{35}S-labeled peaks absorbing at 260 and 210 nm eluted at fractions 104 and 107 (Figure 5). These fractions are expected to contain peptides on the basis of their retention times on reverse-phase HPLC; however, insufficient material was available for sequence analysis.

Fractions 87 and 90, containing the [^{35}S]SpG-bound peptides isolated in highest yield, were subjected to automated degradation on an Applied Biosystems gas-phase sequencer (Stone & Williams, 1986). The results of the sequence analysis (Table I) indicate that the cross-linker attached to the 4-thiouridine residue in tRNA^{fMet} is coupled to lysine residues at positions 402 and 439 in the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984).

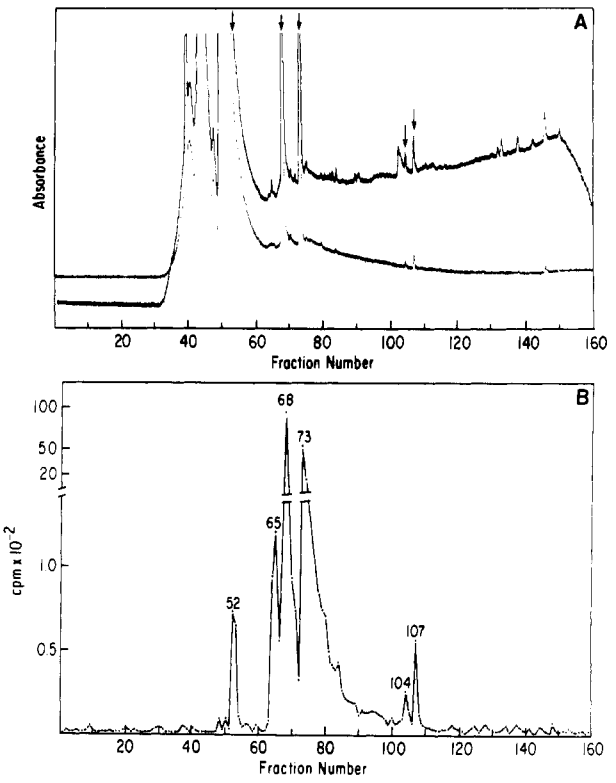


FIGURE 5: Reverse-phase chromatography of [^{35}S]SpG-bound peptides from peak B. Peak B (Figure 3) was chromatographed on a reverse-phase HPLC column as described in the legend to Figure 4. Panel A: Absorbance at 210 (upper) and 260 (lower) nm was recorded at a full scale of 0.2 OD. Arrows on the absorbance profile indicate the elution positions of the radioactive peaks. Panel B: Fractions (0.65 mL) were collected and aliquots (50 μL) withdrawn for measurement of radioactivity.

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

cycle	residue[b]	amino acid identified	amount (pmol)
	Peptide Fraction 87 (113 pmol)[c]		
1	Glu-436	Glu	80
2	Phe-437	Phe	60
3	Gly-438	Gly	74
4	Lys-439	Lys*[d]	38
5	Ala-440	Ala	83
6	Val-441	Val	46
7	Arg-442	Xxx[e]	
	Peptide Fraction 90 (140 pmol)[c]		
1	Asn-396	Asn	51
2	Ala-397	Ala	49
3	Gly-398	Gly	40
4	Phe-399	Phe	41
5	Ile-400	Ile	37
6	Asn-401	Asn	39
7	Lys-402	Xxx[e]	
8	Arg-403	Xxx[e]	

[a] The peptides were subjected to automated degradation on an Applied Biosystems gas-phase sequencer (Stone & Williams, 1986). Phenylthiohydantoin amino acids were determined as described by Merrill et al. (1984). [b] Residue number corresponding to the primary sequence of MetRS (Barker et al., 1982; Dardel et al., 1984). [c] The amount of peptide was determined from the radioactivity of the sample. [d] Lys* represents the chemically modified lysine. The amount released was determined by radioactive counting of ^{35}S in an aliquot of the phenylthiohydantoin fraction. [e] Xxx represents a cycle in which no identifiable amino acid derivative was detected.

DISCUSSION

The unique chemical reactivity of the 4-thiouridine residue present at position 8 in many *E. coli* tRNAs has previously allowed preparation of a variety of site-specific fluorescent, spin-labeled, and photoreactive tRNA derivatives (Hara &

Horiuchi, 1970; Yang & Söll, 1973; Budker et al., 1974; Caron & Dugas, 1976; Gorshkova et al., 1976; Wetzel & Söll, 1977; Johnson et al., 1977; Ofengand et al., 1977; Pande & Wishnia, 1985). In the present work, we have developed a procedure suitable for coupling a lysine-reactive cross-linker to 4-thiouridine residues. A new heterobifunctional cross-linker, SBrAB, has been synthesized by using a method similar to that previously described for the corresponding iodo compound *N*-succinimidyl [(iodoacetyl)amino]benzoate (SIAB) (Weltman et al., 1983). The bromo derivative was found to react more rapidly with 4-thiouridine than SIAB, and at a lower pH. This allowed coupling of SBrAB to tRNA^{fMet} with retention of about 80% of the lysine-reactive side chains.

The effect of the modification on the ability of tRNA^{fMet} to interact with MetRS was evaluated by measuring the aminoacylation activity of the ester-hydrolyzed derivative of SBrAB-tRNA^{fMet}. This modified tRNA could be aminoacylated to the extent of 73% and showed a 6-fold increase in the K_m for methionine acceptance, suggesting some change in the binding affinity. As has been observed with other chemically modified tRNAs, the incomplete level of aminoacylation is probably due to reduction in the rate of the forward aminoacylation reaction compared to the reverse deacylation reaction, leading to a lower equilibrium level of aminoacyl-tRNA. The alternative explanation that two functionally different populations of the modified tRNA exist has not been ruled out; however, we consider this less likely since the modification occurs at a single site and various attempts to "renature" the modified tRNA did not lead to a change in the level of methionine acceptance. Differences of similar magnitude in K_m's (4-fold) have been reported for the two naturally occurring isomers of tRNA^{fMet} that differ by a single base at position 47 (Daniel & Cohn, 1976). Modification of tRNA^{fMet} with a photoaffinity reagent having a structure very similar to SBrAB was previously shown to reduce the binding affinity of the tRNA for MetRS by 35% (Wetzel & Söll, 1977).

Cross-linking of SBrAB-tRNA^{fMet} to MetRS was accompanied by loss of aminoacylation activity. One mole of enzyme was inactivated per mole of cross-linked tRNA, in keeping with the known anticooperative tRNA binding properties of the native dimeric synthetase. The specificity of the reaction was further indicated by the inhibition of cross-linking on addition of excess unmodified tRNA^{fMet}, but not on addition of a noncognate tRNA.

Isolation of the peptides cross-linked to 4-thiouridine was facilitated by in vitro labeling of the thio base with ^{35}S. The cross-linked complex of [^{35}S]SBrAB-tRNA^{fMet}–MetRS was digested with trypsin, and the tRNA-bound peptides were separated from excess free peptides by ion-exchange chromatography. The tRNA was then digested with T_1 RNase, and the cross-linked peptides bound to [^{35}S]SpG were purified by HPLC chromatography. The positions of the cross-linked residues in the known primary structure of MetRS were determined by N-terminal sequencing of the dinucleotide-bound peptides. Due to the fact that several chromatographic steps were performed prior to analysis of the cross-linked products, preferential losses of specific peptides cannot be ruled out, making the relative amounts of initial reaction at each of the identified sites uncertain. Nevertheless, the small number of peptides found coupled to SBrAB-tRNA^{fMet} provides further evidence for the specificity of the cross-linking reaction, and the sequencing results place the inner portion of the L-shaped tRNA structure within 10 Å of Lys-402 and Lys-439 (Figure 6). In keeping with this result is the finding that Lys-402 is one of two lysine residues protected from reaction with acetic anhydride following noncovalent binding of either tRNA^{fMet} or tRNA$_m^{Met}$ to MetRS (Bruton, 1979).

FIGURE 6: Location of cross-linker attached to 4-thiouridine in the three-dimensional structure of the tRNA. The attachment site for SBrAB at position 8 is indicated by an arrow on a generalized three-dimensional tRNA structure based on the model of yeast tRNA^{Phe} (Kim et al., 1974; Klug et al., 1974). The length of the line extending from the attachment site approximates the length of the cross-linker side chain (10 Å) in its maximally extended form. Residues 16 and 17 in the D loop are numbered.

Lysine residues 402 and 439 have previously been found to cross-link to tRNA^{fMet} affinity-labeling derivatives carrying lysine-reactive groups attached to cytidine residues 16 and 17 in the D loop of the tRNA (Leon & Schulman, 1987a). The length of the cross-linker coupled to the D loop (14 Å) allows a maximum distance of 21 Å between the peptide backbone and the cytidine base when the coupled side chains of the cross-linker and the lysine residue are fully extended. It is therefore possible for the same amino acid residues on the enzyme to cross-link to both sites in tRNA^{fMet}. Free lysine-reactive compounds do not preferentially modify these lysine residues in MetRS (L. Schulman, unpublished results), and lysine-reactive cross-linkers coupled to the anticodon, the 5′ terminus, and the variable loop of tRNA^{fMet} react with other lysine residues in the protein (Leon & Schulman, 1987a,b). Thus, the specificity of the reaction of Lys-402 and Lys-439 with cross-linkers coupled to S_8 and $C_{16,17}$ is due to the proximity of the amino acids to this region of the bound tRNA. It is likely that the 4-thiouridine residue of tRNA^{fMet} is oriented toward the surface of MetRS, since the lysine-reactive side chain of SBrAB-tRNA^{fMet} does not extend a significant distance beyond the surface of the tRNA structure (Figure 6). Rotation of the D loop slightly away from the protein would still allow lysine residues on the surface of MetRS to react with the highly flexible cross-linker attached to this site on the outer perimeter of the tRNA structure (see Figure 6). Earlier biochemical studies have indicated that there is no direct interaction between the D loop and MetRS, since extensive structural modifications within this region have little effect on aminoacylation of tRNA^{fMet} (Schulman & Pelka, 1977).

Native MetRS is composed two identical subunits of 676 amino acids, each containing 40 lysine residues (Barker et al., 1982; Dardel et al., 1984). The enzyme is converted to a fully biologically active monomeric form by removal of approximately 100 amino acids from the carboxy terminus by limited digestion with trypsin (Casio & Waller, 1971). 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). It is an elongated molecule having dimensions of 90 Å × 52 Å × 44 Å, which is organized in a biglobular structure composed of N-terminal and C-terminal domains. The N-terminal domain contains the catalytic site (Risler et al., 1981), while tRNA substrates bind to the C-

terminal domain (Valenzuela & Schulman, 1986). In addition to lysine residues 402 and 439, the truncated enzyme contains Lys-465, which has been coupled to the anticodon of $tRNA^{fMet}$ (Leon & Schulman, 1987a), and lysine residues 61 and 335, previously cross-linked to the periodate-oxidized 3′ terminus of the tRNA (Hountondji et al., 1985). The crystal structure of the enzyme has recently been refined to 1.8-Å resolution (S. Brunie, J. L. Risler, and C. Zelwer, unpublished results), providing the opportunity to model the tRNA–synthetase interaction in detail.

4-Thiouridine is present in approximately 70% of all *E. coli* tRNAs. The cross-linking method described in the present work should prove useful in identification of peptide sequences in close proximity to this site when tRNAs are bound to a variety of specific proteins. Of particular interest is the complex between tyrosine tRNA and its cognate synthetase. Schimmel and co-workers have shown that *E. coli* $tRNA^{Tyr}$ undergoes TyrRS-catalyzed tritium exchange at position 8 in the tRNA (Schoemaker & Schimmel, 1977) and have suggested that a transient covalent bond is formed by addition of a nucleophilic residue in the enzyme to the C_5–C_6 double bond of the pyrimidine base in the tRNA–enzyme complex. Saturation of this double bond has recently been shown to inactivate $tRNA^{Tyr}$, suggesting that an important interaction occurs at this site during the aminoacylation reaction (Starzyk et al., 1985). *Bacillus stearothermophilus* $tRNA^{Tyr}$ also contains a 4-thiouridine at position 8, which could be modified to yield a site-specific affinity-labeling derivative. The availability of a high-resolution crystal structure for *B. stearothermophilus* TyrRS (Blow & Brick, 1985) makes this an attractive system for study, since it may be possible to interpret cross-linking results in light of the three-dimensional structure of the enzyme. The lysine-reactive cross-linker described here may also find application in other systems since several methods are available for in vivo and in vitro incorporation of a limited number of 4-thiouridine residues into other nucleic acids (Favre & Fourrey, 1974; Baltzinger et al., 1979; Eshaghpour et al., 1979; Favre et al., 1986).

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The Active Form of *Escherichia coli* DNA Photolyase Contains a Fully Reduced Flavin and Not a Flavin Radical, both in Vivo and in Vitro†

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ABSTRACT: *Escherichia coli* DNA photolyase is a flavoprotein that when purified is blue in color and contains a stable neutral radical FAD (E-FADH•). In the presence of a suitable electron donor (i.e., thiols, tyrosine, or NADH) the radical FAD adsorbs visible light and undergoes photoreduction to the fully reduced FAD ($E\text{-}FADH_2$). The in vitro quantum yield of dimer repair for E-FADH• is 0.07 while that of $E\text{-}FADH_2$ approaches the in vivo value of 1. Electron paramagnetic resonance studies on whole cells indicate that the in vivo form of photolyase is $E\text{-}FADH_2$ with enzyme containing radical FAD generated predominantly during the ammonium sulfate precipitation step of the purification. Activity measurements of E-FADH• using long-wavelength photoreactivating light indicate that enzyme containing FAD in the radical form is not active in dimer repair. Dimer repair observed with E-FADH• at shorter wavelengths is probably photoreduction of E-FADH• followed by dimer repair by $E\text{-}FADH_2$.

Irradiation of cells with UV light results in the formation of pyrimidine dimers between adjacent pyrimidines in DNA. DNA photolyases catalyze the light-dependent photoreversal of these mutagenic lesions, thus restoring the integrity of the DNA. Enzyme purified from *Escherichia coli* contains a stable neutral blue radical FAD and a second chromophore that has not yet been identified (Sancar & Sancar, 1984; Jorns et al., 1984).

The in vivo action spectrum of *E. coli* DNA photolyase has been determined by Harm (1970) and Jagger (Jagger et al., 1969). Harm reported a photoreactivation cross section ($\epsilon\phi$) of $(1.5\text{–}2.5) \times 10^4$ M^{-1} cm^{-1} at 384 nm. Assuming that it was unlikely that ϵ_{384} would be greater than 10^5 M^{-1} cm^{-1}, it was proposed that the quantum yield of photoreactivation (ϕ) must be between 0.1 and 1. Following purification of photolyase (Sancar et al., 1984) the molar extinction coefficient (ϵ) at 384 nm was determined to be 18.1×10^4 M^{-1} cm^{-1} (Jorns et al., 1987). By use of ϵ_{384} of purified photolyase, the in vivo quantum yield is estimated to be ~1. However, Sancar et al. (1987a) determined the in vitro photoreactivation cross section at 384 nm of purified radical FAD containing photolyase to be 1209 M^{-1} cm^{-1}, which gives an in vitro quantum yield of photoreactivation of 0.07. The disparity between the in vivo and in vitro quantum yields led us to suspect that the in vivo form of the enzyme was in some way different from the purified form. Several possibilities were ruled out (Sancar et al., 1987a). In particular, it was demonstrated that the discrepancy between the ϕ values obtained in vitro and those calculated in vivo was not due to the fact that the enzyme used for the in vitro experiments was purified from a strain that grossly overproduces photolyase. Other causes for the discrepancy were considered and investigated. The results of these investigations suggested that the purified blue radical form of photolyase (E-FADH•) was an artifact and that the in vivo form of photolyase contained a fully reduced FAD ($E\text{-}FADH_2$). In support of this it was found that dithionite-reduced photolyase has a photoreactivation cross section at 384 nm of 16 250 M^{-1} cm^{-1} (Sancar et al., 1987a). With ϵ_{384} = 16 100 M^{-1} cm^{-1} for the reduced enzyme (Jorns et al., 1987), ϕ was calculated to be 1.0 (Sancar et al., 1987a). This strongly suggested that the in vivo form of the enzyme contains a fully reduced FAD.

The observation that E-FADH• can undergo photoreduction (ϕ = 0.1) in the presence of certain appropriate electron donors (Heelis & Sancar, 1986; Heelis et al., 1987) led us to suspect that photolyase in the radical form may not be active and that the apparent photoreactivation activity observed with the blue radical flavin form of the enzyme is in fact photoreduction, followed by several rounds of dimer splitting.

In this paper we demonstrate that photolyase is not present as E-FADH• in vivo and that the radical flavin is generated during purification. Comparison of the photolytic cross section at 366 nm of E-FADH• and photoreduced enzyme, $E\text{-}FADH_2$, demonstrates that $E\text{-}FADH_2$ has a 4–8-fold higher quantum yield depending upon the electron donor present in the buffer

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