Lysine 207 as the Site of Cross-linking between the 3'-End of Escherichia coli Initiator tRNA and Methionyl-tRNA Formyltransferase*

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The specific formylation of initiator methionyl-tRNA by methionyl-tRNA formyltransferase (MTF) is important for the formylation of protein synthesis in Escherichia coli. In attempts to identify regions of MTF that come close to the 3'-end of the tRNA, oxidized [35S]3'-end-labeled E. coli initiator methionine tRNA with sodium metaperiodate and cross-linked it to MTF. The cross-linked MTF was separated from un-cross-linked MTF by DEAE-cellulose chromatography, and the tRNA in the cross-linked MTF was hydrolyzed with nuclease P1 and RNase T1, leaving behind an oxidized fragment of [32P]AMP attached to MTF. Trypsin digestion of the cross-linked MTF followed by high pressure liquid chromatography of the digest yielded two peaks of radioactive peptides, I* and II*. These peptides were characterized by N- and C-terminal sequencing and by matrix-assisted laser desorption ionization mass spectroscopy. Peptide I* contained amino acids Gln186–Lys210 with Lys207 as the site of the cross-link. Peptide II*, a partial digestion product, contained amino acids Gln186–Arg214 also with Lys207 as the site of the cross-link. The molecular masses of peptides I* and II* indicate that the final product of the cross-linking reaction between the perio-date-oxidized AMP moiety of the tRNA and Lys207 is most likely a morpholin derivative rather than a reduced Schiff’s base.

From assembly and packaging of RNA viruses (1) to mRNA localization during development (2), the specific recognition of RNAs by proteins plays an important role in many biological processes. Examples of these biological processes include RNA processing, RNA splicing, RNA transport, ribosome assembly, and translation, and translational regulation (3). As molecules that interact with a variety of different proteins, tRNAs provide an excellent system for studying the molecular basis of specificity in recognition of RNAs by different proteins (4).

We are studying the specific recognition of Escherichia coli initiator methionyl-tRNA (Met-tRNA), during its formylation to formylmethionyl-tRNA by the enzyme methionyl-tRNA formyltransferase (MTF, EC 2.1.2.9; 10-formyltetrahydrofolic acid:t-methionyl-tRNA N-formyltransferase). Formylation of initiator Met-tRNA is important for initiation of protein synthesis in eu-bacteria and in eukaryotic organelles such as mitochondria and chloroplasts (5–9). In E. coli, formylation provides a positive determinant for allowing the initiation factor IF2 to select the initiator tRNA from other tRNAs (10, 11) and a second negative determinant for blocking the binding of elongation factor EF-Tu to the initiator tRNA (12–14). The formylation reaction is highly specific; the enzyme formylates the initiator Met-tRNA but not the elongator Met-tRNA or any other aminoacl-tRNA (15). Previous studies have shown that most of the determinants on the initiator tRNA important for its formylation by MTF are clustered in the acceptor stem (16–19) (Fig. 1). However, although the protein sequence of MTF is known (20), little is known about the amino acid residues in MTF that are important for this recognition and the molecular basis of the specificity in recognition.

As a first step in identifying regions of MTF that come close to the acceptor stem of the tRNA, we have cross-linked periodate-oxidized tRNA to MTF and have analyzed the site(s) of cross-linking. We show that Lys207 in the sequence KLSKE tochondria and chloroplasts (5–9). In attempt to identify regions of MTF that come close to the 3'-end of the tRNA, oxidized 32P-3'-end-labeled E. coli initiator methionine tRNA with sodium metaperiodate and cross-linked it to MTF. The cross-linked MTF was separated from un-cross-linked MTF by DEAE-cellulose chromatography, and the tRNA in the cross-linked MTF was hydrolyzed with nuclease P1 and RNase T1, leaving behind an oxidized fragment of 32PAMP attached to MTF. Trypsin digestion of the cross-linked MTF followed by high pressure liquid chromatography of the digest yielded two peaks of radioactive peptides, I* and II*. These peptides were characterized by N- and C-terminal sequencing and by matrix-assisted laser desorption ionization mass spectroscopy. Peptide I* contained amino acids Gln186–Lys210 with Lys207 as the site of the cross-link. Peptide II*, a partial digestion product, contained amino acids Gln186–Arg214 also with Lys207 as the site of the cross-link. The molecular masses of peptides I* and II* indicate that the final product of the cross-linking reaction between the perio-date-oxidized AMP moiety of the tRNA and Lys207 is most likely a morpholin derivative rather than a reduced Schiff’s base.

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As a first step in identifying regions of MTF that come close to the acceptor stem of the tRNA, we have cross-linked periodate-oxidized tRNA to MTF and have analyzed the site(s) of cross-linking. We show that Lys207 in the sequence KLSKE (207–211) of MTF is the site of cross-link to the 3' terminus of the tRNA.2 The cross-linking of periodate-oxidized E. coli tRNA Met to MTF has been studied by Blanquet and co-workers (21). However, the sites of cross-linking were not analyzed in the previous work.

MATERIALS AND METHODS

Chemicals, Enzymes, and Radiolabeled Nucleotides—Sodium metaperiodate, folinic acid, carboxypeptidase Y (sequencing grade), and methionine were obtained from Sigma. Sodium borohydride, sodium cyanoborohydride, and L-cyano-4-hydroxycinnamic acid were purchased from Aldrich. E. coli tRNA nucleotidytransferase and E. coli methionyl-tRNA synthetase were purified in our laboratory by Mike Dyson. Nuclease P1, modified trypsin (sequencing grade), and chymotrypsin (sequencing grade) were obtained from Boehringer Mannheim. RNase T1 was from Sankyo Chemical Company Ltd. (Tokyo, Japan). [35S]Metionine (specific activity = 1175 Ci/mmol) and [α-32P]ATP (specific activity = 3000 Ci/mmol) were purchased from DuPont NEN. All of the solvents used for HPLC were HPLC grade and procured from EM Science. All other routinely used chemicals were of the highest purity grade available.

Assay for Aminocacylation of tRNA—The reaction was carried out at 37 °C. The incubation mixture (20 μl) contained 20 mM imidazole-HCl buffer (pH 7.5), 0.1 mM EDTA, 2 mM ATP, 150 mM NH4Cl, 10 μg/ml bovine serum albumin, 4 mM MgCl2, 25 μM [35S]methionine (specific activity = 5,000–10,000 cpn/pmol), tRNA (approximately 0.1 A260 of total tRNA or 0.01 A260 of pure tRNA(54)) and saturating amounts of purified methionyl-tRNA synthetase. Aliquots (5 μl) were withdrawn at 5-min intervals and spotted onto 3 MM Whatman paper discs (pre-
soaked in 5% trichloroacetic acid and dried), and the discs were washed for 20 min at 0 °C, followed by a wash with 5% trichloroacetic acid (10 min) and finally one wash with ethanol for 10 min (22). Discs were dried in a ventilation oven, and the radioactivity on each filter was determined by scintillation counting.

Assay for Formylation of Met-tRNAfMet—The incubation (20 μl) carried out at 37 °C, contained 10 μl of the above aminoacylation reaction mixture (which had been preincubated for 30 min), 0.3 mM N10-formyllaetrabehydrofolute, and appropriate amounts of MTF (depending on the purity and specific activity of preparation). The reaction was allowed to proceed for 15 min and was terminated by the addition of 20 μl of 0.36 M CuSO4 in 1.1 M Tris-HCl (pH 7.3) and incubated further for 3 min at room temperature (20). Acid-precipitable radioactivity was measured as described for the aminoclayation assay.

Purification of MTF—Purification of MTF was carried out using a procedure developed by Dr. D. Mangroo (3). The specific activity of the purified enzyme was 2.69 × 109 pmol of formyl group incorporated into Met-tRNAfMet/min/μg of protein. The purified enzyme migrated as a single band on SDS-polycarlylamide gels.

Analytical Methods—Protein concentrations were estimated by the modified Lowry DC method as described by the supplier (Bio-Rad) using IgG as a standard. The concentration of purified MTF was also determined using an absorbance of 1.39 at 280 nm for a solution containing 1 mg/ml bovine serum albumin, 50 mM Tris-Cl, pH 6.8, containing 4% (w/v) SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.02% bromophenol blue.

Purification and 3′-End Labeling of tRNAfMet—Total tRNA was isolated by phenol extraction from E. coli cells (17 g, wet weight) overproducing tRNAfMet (which carries the tRNAfMet gene). The cold RNA (100 μg units) was fractionated on a 10% native polycarlylamide gel, and tRNAs were visualized by UV shadowing (25). The gel slice containing tRNAfMet was cut out, and tRNAfMet was eluted from the gel in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE buffer). The purity of gel-eluted tRNAfMet, estimated by polycarlylamide gel electrophoresis and by aminoacylation assay, was >95% (22). 32P-3′-End labeling of tRNAfMet was carried out using tRNA nucleotidyltransferase. First, the 3′-terminal A76 was removed by Whitefield degradation by treatment of the tRNA with sodium periodate and subsequently with aniline (26). The 3′-terminal phosphate was then removed to treat with alkaline phosphatase. The reaction mixture for 3′-labeling contained 50 μM glycine-NaOH (pH 9.2), 10 μM Mg(OAc)2, 10 μM glutathione-SH, 10 μM bovine serum albumin, 50 μM [γ-32P]ATP, 6 μM tRNAfMet (−A76) and 2 units of E. coli tRNA nucleotidyltransferase. Incubation was at 37 °C for 20 min. One unit of tRNA nucleotidyltransferase is the amount of enzyme that catalyzes the incorporation of 1 pmol of AMP/h (27).

Unreacted ATP was removed by spin column chromatography through a 1-ml Sephadex G-25 column. The 32P-3′-end-labeled tRNAfMet was recovered by ethanol precipitation and dissolved in 20 μl of TE buffer (pH 8.0). The labeled tRNAfMet was subsequently purified by denaturing polyacrylamide gel electrophoresis (8% urea-PAGE) from the gel, and precipitated with ethanol. The pellet was dried and dissolved in distilled water and stored at −20 °C.

Preparation of Periodate-oxidized tRNAfMet—The reaction mixture (40 μl) contained 1.0 A260 unit of 3′-end-labeled [32P]tRNAfMet (30,000 cpm), 2.5 mM sodium periodate, and 100 mM sodium acetate, pH 5.2. The mixture was incubated for 30 min at 0 °C in the dark. Excess periodate was destroyed by the addition of ethylene glycol to 5 mM (final concentration). The oxidized tRNAfMet was ethanol-precipitated, the pellet was dissolved in water, 0.1 volume of 3 M sodium acetate buffer, pH 5.0, was added to it, and the oxidized tRNAfMet was repurified with ethanol. The pellet was collected by centrifugation, excess ethanol was removed, and the pellet was dried and dissolved in 50 μl of distilled water and used immediately for the cross-linking reaction. The extent of oxidation of tRNAfMet was checked by determining the residual methionine acceptance activity (22) of oxidized tRNA and was found to be essentially complete (>96%).

Cross-linking of Periodate-oxidized tRNAfMet to MTF—For most experiments, the periodate-oxidized tRNAfMet (10 pmol) was incubated in a total volume of 0.5 μl with MTF (0.066 μg/μl in 0.01 M HEPES pH 7.3, 10 mM MgCl2, 0.1 mM EDTA at 37 °C for 1 h in the presence of 1 mM sodium cyanoborohydride (21). The reaction was quenched by the addition of sodium borohydride (1 mM final concentration), and the extent of cross-linking was determined by SDS-PAGE followed by autoradiography and quantitation by PhosphorImager analysis. The effect of the addition of either excess substrate (tRNAfMet) or cofactor (N10-formyltetrahydrofolute) to the reaction mixture on the cross-linking efficiency was determined by adding appropriate amounts of these ligands to the reaction mixture followed by incubation for 10 min at 37 °C prior to the addition of periodate-oxidized tRNAfMet. The effect of cross-linking of periodate-oxidized tRNAfMet to MTF on the enzymatic activity of MTF was evaluated by withdrawing (5 μl) aliquots of a large scale reaction mixture (60 pmol of MTF and 300 pmol of either 32P-labeled oxidized initiator tRNA or oxidized initiator tRNA) at various times (every 5 min for 30 min) and estimating the residual enzyme activity after diluting the reaction mixture 500-fold with 20 mM imidazole buffer, pH 7.5, containing 10 mM MgCl2 and 0.1 mg/ml bovine serum albumin. For isolation and analysis of cross-linked MTF, a large scale reaction was carried out using 20 μg of MTF (600 pmol) and 1800 pmol of periodate-oxidized tRNAfMet (5-fold molar excess), and the reaction time was increased to 2 h.

Rate of Cross-linking of Various tRNAs to MTF—MTF (90 pmol) in 40 μl of 20 mM imidazole-HCl, pH 7.5, 10 mM MgCl2, and 0.1 mM EDTA was incubated with 450 pmol of oxidized tRNAs in the presence of 1 mM sodium cyanoborohydride at 37 °C. Aliquots (4 μl) were removed at the indicated time intervals, and the reaction was quenched by the addition of 1 M sodium borohydride (final concentration of 1 mM). The extent of cross-linking in each case was determined by SDS-PAGE followed by autoradiography and by PhosphorImager analysis.

Separation of the Cross-linked MTF from MTF and Digestion of the tRNA Moiety in the Cross-linked MTF—MTF cross-linked to tRNA was separated from uncleaved MTF by DEAE-cellulose chromatography. Following the cross-linking reaction, the reaction mixture was diluted to 1 ml with 0.1% Tris-HCl, pH 7.5, containing 0.2 M LiCl and loaded onto a DEAE-cellulose column (1 ml) preequilibrated with the same buffer. After extensive washing of the column to remove MTF, the cross-linked MTF was eluted with 0.1 M Tris-HCl, pH 7.5, containing 1 M LiCl. Fractions (0.3 ml) were collected. The fractions showing high radioactivity were pooled and subjected to Centricon-10 ultrafiltration for desalting. After centrifugation, excess tRNA and tRNA cross-linked to MTF was hydrolyzed to nucleotides (25) by incubating the cross-linked MTF with 0.3 unit of P1 nuclease and 5 units of RNase T1 at 45 °C for 4 h. The extent of hydrolysis of tRNA from cross-linked MTF was analyzed by SDS-PAGE and autoradiography.

To remove free nucleotides from cross-linked MTF, the reaction mixture with nuclease P1 and RNase T1 was subjected to gel filtration on a Sephadex G-25 column (0.8 × 14 cm). Fractions containing MTF (void volume) were concentrated and used for proteolytic digestion. Alternatively, MTF was digested through the Centricon-10 membrane used to remove the released nucleotides from cross-linked MTF.

Trypsin Digestion of Cross-linked MTF and Separation of Peptides by HPLC—The cross-linked MTF (20 μg and ~10,000 cpm of Cerenkov radiation) in 100 μl of 0.5 M Tris-HCl, pH 7.5, was treated with 2 μg

D. Mangroo and U. RajBhandary, unpublished observations.
of sequencing grade modified trypsin (10%, w/w) at 37 °C for 12 h. The 
trypsic peptides were separated by HPLC on a Hewlett-Packard HP-
1090 HPLC system equipped with a reverse phase Vydac C_{18} column 
(0.46 × 25 cm). The trypsin digest was diluted with 0.1% trifluoroacetic 
acid in water to 250 μl, centrifuged at 14,000 rpm, and injected into the 
column preequilibrated with 90% solvent A (0.1% trifluoroacetic acid in 
water) and 10% solvent B (0.1% trifluoroacetic acid in 100% acetonitrile). 
The elution rate was 0.5 ml/min in 90% solvent A and 10% solvent 
B from 0 to 10 min followed by a linear gradient from 10% solvent B to 
20% solvent B between 10 and 60 min (0.2% per min) and then 20–27% 
B between 60 and 160 min (0.07% per min) and then 27–100% solvent 
B in an additional 40 min (1.82% per min). The effluent was continu-
ously monitored at 210 nm, and fractions of 0.5 ml were collected. The 
radioactivity in each fraction was determined by Cerenkov counting for 
32P in an LKB-1217 RACKBETA liquid scintillation counter.

Chymotrypsin Digestion of Peak II* and HPLC—Fractions contain-
ing peak II* (obtained from the HPLC of trypsin digestion of cross-
linked MTF above) were lyophilized and dissolved in 100 μl of 100 mM 
Tris-HCl, pH 7.5. This mixture was then treated with 2 μg of chymo-
trypsin for 5 h at 37 °C and subjected to reverse phase HPLC on a C_{18} column. 
The column was preequilibrated with 100% solvent A. The 
elution rate was 0.5 ml/min in 100% solvent A from 0 to 10 min followed 
by a linear gradient from 0 to 25% Solvent B between 10 and 135 min 
(0.2% per min) and then 25–100% Solvent B for an additional 35 min. 
The effluent was continuously monitored at 210 nm, and fractions of 0.5 
ml were collected. The radioactivity in each fraction was determined by 
Cerenkov counting for 32P as above.

Mass Spectroscopic Analysis and Amino Acid Sequencing of Radio-
active Peptides—The mass of the cross-linked peptides was determined 
on a MALDI mass spectrophotometer (PerSeptive Biosystems Voyager 
mass spectrophotometer) using delayed extraction technology. For the 
mass analysis of the peptides, approximately 1 pmol of peptide (based on 
radioactivity) was mixed with 1 μl of a-cyano-4-hydroxycinnamic 
acid (matrix). For calibration of the mass spectrophotometer, oxidized 
insulin chain B (Sigma) was used as a standard. C-terminal amino acid 
sequencing was also carried out using limited digestion of peptide with 
carboxypeptidase Y followed by mass spectroscopic analysis. The N-
terminal amino acid sequence of isolated peptides was determined 
using an automated gas phase protein/peptide sequence analyzer from 
Applied Biosystems (model 470A) equipped with an on-line phenylthio-
hydantoin analyzer (model 120) and computer (model 900A).

RESULTS

Cross-linking of MTF to Periodate-oxidized tRNA\textsubscript{2Met}—Perio-
date-oxidized nucleotides and tRNAs have often been used as 
affinity reagents for cross-linking to proteins (29). Oxidation of 
tRNA with sodium periodate converts the terminal ribose moi-
ety in the 5′ of the tRNA to a 2′,3′-dialdehyde. The dialde-
hyde then reacts primarily with the ε-amino group of lysine 
residues in a protein that comes close to the 3′-end of tRNA. 

For following the cross-linking of MTF to the tRNA, 32P-3

- end-labeled periodate-oxidized initiator tRNA (0–90 
m) was incubated with MTF in the presence of 
sodium cyanoborohydride, which reduces the intermediate 
Schiff's base and prevents reversal of the reaction (21). The 
reaction was quenched after 1 h by the addition of sodium 
borohydride, and the products were analyzed by SDS-poly-
acrylamide gel electrophoresis followed by autoradiography of 
the gel. Fig. 2, lane 2, shows that in an incubation mixture 
containing periodate-oxidized tRNA, most of the 32P radioac-
tivity migrated more slowly than tRNA at a position expected 
for that of a tRNA cross-linked to a protein.

Formation of the tRNA-protein cross-link requires oxidation of 
the tRNA with periodate. When 32P-3′-end-labeled tRNA\textsubscript{2Met} 
without periodate oxidation was incubated with MTF under 
identical conditions, there was no cross-link between the tRNA 
and the protein (Fig. 2, lane 1). The presence of sodium cya-
oborohydride during the incubation was found to be impor-
tant for maximal cross-linking (data not shown).

Fig. 3 shows the effect of increasing the concentration of the 
oxidized tRNA on the extent of cross-linking. It can be seen that 
with 2 pmol of MTF (0.2 μM), the extent of cross-linking 
is dependent on the concentration of the tRNA and reaches a 
plateau at approximately 10–20 pmol of the tRNA (1–2 μM).

This result indicates that the cross-linking of periodate-oxi-
dized tRNA\textsubscript{2Met} to MTF is saturable and that it is quite specific. 
Further evidence for the specificity of cross-linking was derived 
from an experiment in which increasing amounts of unoxidized 
tRNA\textsubscript{2Met} was added to the cross-linking reaction containing a 
fixed amount of periodate-oxidized tRNA. It was found that 
there was a gradual decrease in the extent of cross-linking 
dependent upon the concentration of unoxidized tRNA\textsubscript{2Met} in 
the reaction (data not shown). This result suggests that unoxi-
dized tRNA and oxidized tRNA are competing for the same site in 
MTF. In contrast, the addition of N\textsuperscript{10}-formyltetrahydrofo-
late, another substrate of MTF, had no significant effect on the 
extent of cross-linking (data not shown).

The cross-linking of MTF to periodate-oxidized tRNA\textsubscript{2Met} 
leads to loss of enzymatic activity of MTF. Incubation of MTF 
with periodate-oxidized tRNA\textsubscript{2Met} leads to a time-dependent 
inactivation of MTF (data not shown). This inactivation re-

FIG. 2. Analysis of cross-linking of 32P-3′-end-labeled oxidized 
initiator tRNA to MTF by SDS-PAGE. MTF (60 pmol) was incubated 
with 180 pmol of 32P-3′-end-labeled unoxidized initiator tRNA (lane 1) 
or 32P-3′-end-labeled oxidized initiator tRNA (lane 2). After 1 h, an 
 aliquot (5 μl) was removed, the reaction was quenched by the addition 
of NaBH\textsubscript{4}, and the cross-linking mixture was subjected to SDS-PAGE. 
The gel was dried, and radioactivity was detected by autoradiography.

FIG. 3. Effect of initiator tRNA concentration on the extent of 
cross-linking to MTF. MTF (2 pmol) was incubated with increasing 
amounts of 32P-3′-end-labeled periodate-oxidized initiator tRNA (0–90 
pmol). The reaction was carried out under standard cross-linking con-
ditions as described under “Materials and Methods.” Following incuba-
tion, the extent of cross-linking was determined by subjecting the cross-
linking mixture to SDS-PAGE. The quantitation of radioactivity in the 
band corresponding to cross-linked MTF was carried out using Phos-
phrImager analysis.
Another peak designated peptide II* also contains $^{32}$P radioactivity. This peak contains a mixture of two peptides, one of which is radioactive. We show below that the radioactive peptide II* is a partial digestion product and is the same as peptide I* but with an extension of four amino acids at the C terminus.

**Characterization of Peptides I and I*—**A combination of MALDI mass spectroscopy (30), N-terminal sequencing using Edman degradation (31), and C-terminal sequencing using partial digestion with carboxypeptidase Y (32) followed by MALDI mass spectrometric analysis was used to establish the sequence of these peptides. The following lines of evidence summarized in Table I show that peptide I* has the sequence $^{186}$QLADGTKAPE, in which $^{32}$PAMP moiety of the tRNA.

(i) MALDI mass spectroscopy of peptide I yielded two peaks with molecular masses of 2406.95 and 2390.59 Da. The only tryptic peptide of MTF that fits this is Gln$^{186}$–Lys$^{207}$. The difference of 16.36 Da in the two molecular masses is most probably due to the well known conversion of glutamine at the N terminus of peptides or proteins to pyroglutamic acid (33). The complete absence of a peak corresponding to peptide I in digests of cross-linked MTF (Fig. 4B) suggests that one of the two lysine residues in the peptide is linked to the AMP moiety of the tRNA. The most likely possibility is that Lys$^{207}$ is cross-linked to the AMP moiety, thereby making the peptide bond involving this lysine residue resistant to cleavage by trypsin in the cross-linked MTF.

(ii) N-terminal sequencing of peptide I* yielded the sequence QLADGTKAPE, which is the same as that of peptide I. This result confirms the suggestion above that peptide I* is derived from peptide I. Furthermore, the clear identification of lysine as amino acid number 8 of peptide I* shows that lysine 193 is not cross-linked in MTF and supports the conclusion above that Lys$^{207}$ is one of the most probably cross-linked. While the N-terminal sequence data were unambiguous, the yields of phenylthiohydantoin-derivatives in the first and successive cycles were much lower than expected (~35%), based on the $^{32}$P radioactivity present in peptide I*. This is due to the fact that most of the glutamine at the N terminus is converted to pyroglutamic acid, which is inert to the reagents used for N-terminal sequencing.

(iii) Partial digestion with carboxypeptidase Y of peptide I* in situ on the sample plate used for MALDI mass spectroscopy, followed by mass spectrometric analysis of the partial digestion products, indicated the loss successively of the amino acids lysine, serine, and leucine from the C terminus. Thus, the sequence at the C terminus of peptide I* is LSK. These are the amino acids that immediately follow Lys$^{207}$ in the cross-linked MTF. This result supports the conclusion above that peptide I* is derived from peptide I and shows that it has an extension of LSK at the C terminus beyond Lys$^{207}$.

(iv) Final evidence for the sequence of peptide I* was derived by MALDI mass spectrometric analysis. Mass spectroscopy yielded four peaks with molecular masses in decreasing order of 3052.23, 3035.31, 2917.37, and 2901.09 daltons (Fig. 5). The molecular mass of 3052.23 Da is very close to that expected for the cross-linked peptide Gln$^{186}$–Lys$^{207}$ in which Lys$^{207}$ is cross-linked to the AMP moiety of the tRNA (Table I). The peptide with mass of 3035.31 Da differs from the previous one by 16.92 Da and most likely has pyroglutamic acid at the N terminus instead of Gln in the former. The peptides with masses of 2917.37 and 2901.09 Da differ from the above two, respectively, by masses of ~134–135 Da and correspond to those peptides in which the adenine base of AMP has fragmented off during mass spectroscopy (34).

Molecular mass measurement of three different isolates of...
peptide I* yielded values of 3052.23, 3049.19, and 3050.66 Da. The average molecular mass of 3050.7 Da thus obtained for peptide I* differs by only about 2.2 Da from the molecular mass expected for a cross-linked product, which contains a morpholin type of linkage (Fig. 6, III) between the peptide and the ribose moiety of AMP rather than a reduced Schiff's base (Fig. 6, I).

Characterization of Peptide II*—MALDI mass spectroscopic analysis of peptide II* showed that it contained a mixture of peptides with molecular masses of 2641.47 and 3536.71 Da. The peptide with a molecular mass of 2641.47 Da was shown to have the sequence HLDALLSSGHNVVGVFTQPDRPAGR43 by N- and C-terminal sequence analysis. This peptide is also present in digests of uncross-linked MTF (Fig. 4A). The radioactive peptide II* with a molecular mass of 3536.71 Da was established by the following three lines of evidence to have the same sequence as peptide I* except that it has an extension at the C terminus of EEAR.

(i) Peptide II* was treated with chymotrypsin, and the digest was subjected to HPLC chromatography (Fig. 7). A predominant radioactive peak that coeluted with a peptide peak was obtained. This peptide was then used for sequence analysis using Edman degradation, the products of each cycle being also monitored for release of 32P radioactivity.

(ii) Fig. 8 shows that the sequence of the chymotryptic peptide is AEKLSKEEAR, in which X in the third cycle (corresponding to Lys207 in uncross-linked MTF) contains most of the 32P radioactivity. This peptide sequence overlaps with the C-terminal sequence of peptide I*. These results establish that Lys207 is the site of cross-linking between the 3' end of tRNA and MTF. Table I shows the results. While all three tRNAs react with MTF, the rate of cross-linking with tRNA2fMet reaches a plateau after 1 h, whereas even after 2 h 3536.71 Da for peptide II* differs by only ~3 Da from the mass of 3533.66 Da expected for a peptide Gln186–Arg214, in which Lys207 is attached through a morpholin type of linkage (Fig. 6, III) to the AMP moiety derived from the 3'9-end of the tRNA.

Cross-linking of Mutant tRNA2fMet and Yeast tRNAPhe to MTF—Although MTF formylates only the initiator methionyl-tRNA species (15), previous studies have shown that it will nevertheless bind to other tRNAs almost as well as to the initiator tRNA (35). Therefore, we have investigated whether the periodate-oxidized G72 mutant of tRNA2fMet, which is a very poor substrate for MTF (16–18), and yeast tRNApho, which is not a substrate for MTF, will cross-link to MTF. Fig. 9 shows the results. While all three tRNAs react with MTF, the rate of cross-linking with tRNA2pho is significantly higher than with the mutant tRNA2Met or yeast tRNApho. The reaction with tRNA2Met reaches a plateau after 1 h, whereas even after 2 h

![Mass spectrum of peptide I*](image)

**FIG. 5.** Mass spectrum of peptide I*. 1 μl of peptide (1–2 pmol; based on the specific radioactivity) was mixed with 1 μl of matrix, loaded on the sample plate, and allowed to dry completely. Prior to peptide mass determination, the mass spectrophotometer was calibrated using insulin chain B as a standard.

**FIG. 6.** Possible products of the reaction of nucleoside 2',3'-dialdehyde with the ε-amino group of the lysine residue in protein enzymes. E, enzyme.

| Peptide | Mass calculated (M + 1) Da | Mass obtained Da | Amino acid sequence determined | Peptide sequence deduced |
|---------|---------------------------|-----------------|-------------------------------|-------------------------|
| Missing peak I | 2406.21 | 2406.95 | ND* | ND |
| Peak I* | 3068.43b | 3052.23 | LS | QLADGTKPEQDQLTVYAKEK207 |
| | 3064.43c | 3050.66 | -LSK | QLADGTKPEQDQLTVYAKEK207LSK |
| | 3048.43d | 3049.19 | e | |

* ND, not determined.

b Molecular mass based on Schiff's base (Fig. 6, structure I).

c Lysine linked to AMP moiety.

d Molecular mass based on hydroxymorpholine derivative (Fig. 6, structure II).

e Molecular mass based on morpholine derivative (Fig. 6, structure III).
the reactions with the other tRNAs have not reached the level attained with tRNA^{fMet}. These results are in agreement with those of Hountondji et al. (21), who showed that the rate of reaction of periodate-oxidized yeast tRNA^{Phe} with MTF, as followed by inactivation of MTF, was about 6-fold lower than that of tRNA^{fMet}.

The site(s) of cross-linking of the G72 mutant of tRNA^{fMet} and yeast tRNA^{Phe} were also analyzed by HPLC of tryptic digests of MTF cross-linked to these tRNAs. The same two radioactive peaks as seen above for cross-linking of wild type tRNA^{fMet} to MTF were found (data not shown). Thus, the mutant tRNA^{G72} and the yeast tRNA^{Phe} also cross-link to Lys^{207} in MTF.

**DISCUSSION**

As a first step in studies on the topology of interaction of *E. coli* initiator tRNA with MTF, we have shown that reaction of periodate-oxidized tRNA with MTF leads to cross-linking of the tRNA specifically to Lys^{207} of the enzyme. This suggests that Lys^{207} comes close to the 3'-end of the tRNA. Since MTF formylates the amino group of methionine attached to the 3'-end of the tRNA, Lys^{207} is likely to be within or near the active site of the enzyme.

The Lys^{207} of MTF is part of the sequence^{207}KLSKE^{211}. This sequence is related to a similar sequence, KMSKS or KLSKS, found in virtually all class I aminoacyl-tRNA synthetases (36). The significance, if any, of this similarity in sequences is not known. In two of the *E. coli* class I aminoacyl-tRNA synthetases, tyrosyl-tRNA synthetase and methionyl-tRNA synthetase, one or the other of the lysine residues in the KMSKS or KLSKS sequence has been shown to cross-link to the 3'-end of the corresponding periodate-oxidized tRNA (37, 38). These lysine residues are also functionally important for stabilization of the ground state and/or the transition state during the formation of aminoacyl-adenylate (36, 39, 40). In a class II aminoacyl-tRNA synthetase also, a lysine residue that cross-links to the 3'-end of periodate-oxidized tRNA is part of a conserved motif (motif 2) important for aminoacyl-adenylate formation and for transfer of the amino acid to the tRNA (41, 42). Whether Lys^{207} or Lys^{210} plays a functional role in MTF is not known. Although these amino acid residues lie within a conserved region in the six MTF protein sequences deduced on the basis of DNA sequences (Fig. 10), Lys^{207} is present in *E. coli*, *Hemophilus influenzae* and *Rickettsia prowazekii* MTF but not in *Thermus thermophilus*, *Mycoplasma genitalium*, or yeast mitochondrial MTF.

Another region of very strong sequence conservation in MTF includes amino acids 83–150. This region contains amino acids Asn^{109}, His^{111}, and Asp^{147}, thought to be involved in catalysis (43, 44) in *E. coli* glycaminide ribonucleotide formyltransferase, another enzyme that, like MTF, transfers a formyl group using N^{10}-formyltetrahydrofolate as a cofactor (45). These three amino acids are conserved in all six of the MTF
sequences known so far. The strict conservation of these amino acid residues in MTF and the very strong homology in this region among MTF, the glycaminide ribonucleotide formyltransferases, and amino imidazole carboxamidine ribonucleotide formyltransferases (46, 47) from a number of sources suggest that these amino acids also play a similar catalytic role in MTF. If, as stated above, Lys207 is at or near the active site of the MTF, it might be close to amino acids 109, 111, and 147 in the three-dimensional structure.

Reaction of periodate-oxidized G72 mutant initiator tRNA, which is a very poor substrate for MTF and yeast tRNA<sup>apo</sup>, also led to cross-linking to MTF, although at a slower rate compared with the wild type initiator tRNA. This result agrees with the previous observation that although MTF formylates only the initiator methionyl-tRNA species, it binds almost as well to other tRNAs with dissociation constants in the micromolar range. The site of cross-linking to the mutant initiator tRNA and to yeast tRNA<sup>apo</sup> is also Lys<sup>207</sup>. It would thus appear that MTF has a binding pocket for the 3′-end of the tRNA and that all of these tRNAs bind initially to MTF in a similar manner. The differences in rates of reaction of MTF with cognate versus noncognate tRNA or the G72 mutant initiator tRNA could be due to a conformational change, subsequent to binding, of the MTF-cognate tRNA complex, which places Lys<sup>207</sup> of MTF in a favorable position for reaction with the 3′-end of the tRNA in the cognate complex. Evidence for a possible conformational change of the cognate tRNA upon binding to MTF has been obtained by NMR analysis of MTF complexed to initiator and elongator species of methionine tRNA (35). NMR analysis showed a general broadening and loss of intensity of resonances assigned to G:C base pairs in the acceptor stem of the initiator tRNA species but not of the elongator tRNA species. The notion of a conformational change in the MTF-initiator tRNA complex subsequent to binding is similar to the situation with aminoaac-tRNA synthetase-tRNA complexes in which a conformational change of the complex is triggered by cognate tRNAs but not by noncognate tRNAs (48).

Another explanation for the cross-linking of all three tRNAs to Lys<sup>207</sup> of MTF is that Lys<sup>207</sup> is just a particularly reactive lysine residue in MTF. We consider this unlikely. First, the periodate-oxidized initiator tRNA does not react with just any protein. For example, it does not react with bovine serum albumin (data not shown). Second, we have also cross-linked periodate-oxidized <i>E. coli</i> 5 S tRNA carrying a 3′-terminal 32P-PC extension to MTF and analyzed the tryptic digest of the cross-linked MTF by HPLC. In contrast to the specific cross-linking of the 3′-ends of tRNAs to Lys<sup>207</sup>, there were several other radioactive peptides in the tryptic digest of MTF cross-linked to 5 S tRNA (data not shown).

Mass spectral analysis of peptides derived from MTF that were cross-linked to the AMP moiety of the tRNA has proven extremely useful in the characterization of the cross-linked peptides and the nature of the cross-link formed. Earlier reports on the reaction of 2′,3′-dialdehydes derived by periodate oxidation of tRNA, nucleosides, or 5′-mononucleotides with primary amines indicated the production of either a Schiff’s base (Fig. 6, I) or a morpholino derivative in which one of the carbon atoms in the morpholine ring carries a hydroxyl group (49) (Fig. 6, II). The molecular masses that we have obtained for the cross-linked peptides are, however, more consistent with a morpholino derivative described by Brown and Read (50) in which none of the carbon atoms in the morpholine ring carries a hydroxyl group (Fig. 6, III). A possible mechanism for the formation of this is that the secondary amine formed by reduction of the Schiff’s base intermediate with sodium cyanoborohydride attacks the neighboring carbonyl group, leading to the formation of a morpholine ring. Loss of water followed by reduction with sodium cyanoborohydride or sodium borohydride would generate the morpholino derivative III shown in Fig. 6.

Finally, the work described here has provided the first indication of the amino acid residue in MTF that comes close to the 3′-end of the tRNA. In parallel work, we are attempting isolation and identification of suppressor mutations in MTF that compensate for formylation defects of mutant initiator tRNA. The information derived from these experiments, along with knowledge of the amino acid residues in MTF that are highly conserved (Fig. 10), can be used for the selection of amino acid residues in MTF for site-specific mutagenesis and structure-function relationship studies.

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**Note Added in Proof—**The crystal structure of <i>E. coli</i> MTF has been published by P. E. Blanquet, S. Mechulam, Y. (1996) EMBO J. (1996) 15, 4749–4758. The crystal structure shows that Lys<sup>207</sup> is on the surface of the protein and is part of a positively charged channel possibly involved in orientation of the acceptor stem of Met-tRNA toward the active site. The specific cross-linking of Lys<sup>207</sup> to the 3′-terminal A of tRNA<sup>apo</sup> described in this paper is consistent with such a possibility.

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