Escherichia coli Methionyl-tRNA Formyltransferase: Role of Amino Acids Conserved in the Linker Region and in the C-Terminal Domain on the Specific Recognition of the Initiator tRNA†

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ABSTRACT: The formylation of initiator methionyl-tRNA by methionyl-tRNA formyltransferase (MTF) is important for the initiation of protein synthesis in eubacteria. We are studying the molecular mechanisms of recognition of the initiator tRNA by *Escherichia coli* MTF. MTF from eubacteria contains an approximately 100-amino acid C-terminal extension that is not found in the *E. coli* glycinamide ribonucleotide formyltransferase, which, like MTF, use N^{10}-formyltetrahydrofolate as a formyl group donor. This C-terminal extension, which forms a distinct structural domain, is attached to the N-terminal domain through a linker region. Here, we describe the effect of (*i*) substitution mutations on some nineteen basic, aromatic and other conserved amino acids in the linker region and in the C-terminal domain of MTF and (*ii*) deletion mutations from the C-terminus on enzyme activity. We show that the positive charge on two of the lysine residues in the linker region leading to the C-terminal domain are important for enzyme activity. Mutation of some of the basic amino acids in the C-terminal domain to alanine has mostly small effects on the kinetic parameters, whereas mutation to glutamic acid has large effects. However, the deletion of 18, 20, or 80 amino acids from the C-terminus has very large effects on enzyme activity. Overall, our results support the notion that the basic amino acid residues in the C-terminal domain provide a positively charged channel that is used for the nonspecific binding of tRNA, whereas some of the amino acids in the linker region play an important role in activity of MTF.

The formylation of initiator methionyl-tRNA (Met-tRNA)[1] by methionyl-tRNA formyltransferase is important for the initiation of protein synthesis in *Escherichia coli* (*1*−*3*). The formylation reaction is highly specific. The enzyme formylates the initiator Met-tRNA, but no other aminoacyl-tRNA including the elongator species of Met-tRNA (*4*, *5*). In addition, the enzyme has a preference for the amino acid methionine (*6*−*8*). The major determinants for formylation are clustered in the acceptor stem of the initiator tRNA (*9*−*11*). A minor determinant is the A11:U24 base pair in the dihydrouridine stem (*9*, *12*). We are interested in the molecular mechanisms of recognition of these determinants by the *E. coli* MTF.

The genes for the *E. coli* and several other eubacterial MTFs have been cloned and sequenced, and the crystal structure of *E. coli* MTF has been established (*13*, *14*). The crystal structure of *E. coli* MTF shows that the enzyme consists of two domains connected by a linker region (amino acids 189−209). The N-terminal domain consists of a Rossmann fold and is strikingly homologous in secondary and tertiary structure to *E. coli* glycinamide ribonucleotide formyltransferase (GARF), another enzyme that, like MTF, uses N^{10}-formyltetrahydrofolate as a formyl group donor in formylation reactions (Figure 1). The N-terminal domain of MTF also contains the same amino acid residues thought to be important for catalysis in GARF (*15*−*17*). Two main differences between MTF and GARF sequences are: (*i*) the insertion in MTF of a 16 amino acid sequence in the loop region between the second β-strand and the second α-helix from the N-terminus of the protein and (*ii*) the presence in

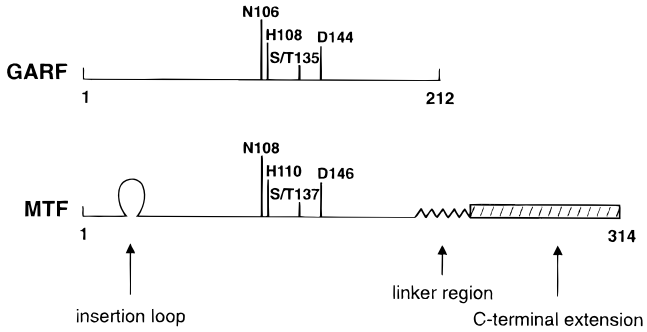

FIGURE 1: Schematic alignment of GARF and MTF sequences from various sources. The amino acid numbering of MTF begins with the serine found at the N-terminus of the *E. coli* protein. The amino acids Asn, His, Ser/Thr, and Asp, thought to be involved in catalysis in GARF and also found in MTF, are indicated. Arrows indicate sites of insertion in MTF compared to GARF.

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[1] Abbreviations: MTF, methionyl-tRNA formyltransferase; GARF, glycinamide ribonucleotide formyltransferase; MetRS, methionyl-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase; VMA, vacoular membrane ATPase; Met-tRNA, methionyl-tRNA; fMet-tRNA, formylmethionyl-tRNA; $tRNA_N$, *E. coli* fMet-$tRNA_2$ species carrying 3′-amino 3′-deoxyA in place of the 3′-terminal A residue.

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MTF of an approximately 100 amino acid extension (amino acids 210−314 in the *E. coli* enzyme) at the C-terminus. The C-terminal extension, which consists of a β-barrel with five antiparallel β-strands, is structurally homologous to the anticodon-binding domains of the *E. coli* lysyl-tRNA synthetase and the yeast aspartyl-tRNA synthetase (*18*−*20*). The C-terminal extension has a positive electrostatic potential on the surface pointing toward the catalytic center of MTF and binds tRNA on its own, although nonspecifically (*14*). These findings have led to the suggestion that the insertion loop in the Rossmann fold and the C-terminal extension together are important in recognition of the initiator tRNA by MTF, with the C-terminal extension being used for nonspecific binding of tRNA and for orientation of the 3′-end of the initiator Met-tRNA toward the catalytic center of the enzyme (*14*, *21*).

Analysis of suppressor mutations in MTF, which compensate for the formylation defect of mutant initiator tRNAs, has provided support to the notion of the importance of the 16 amino acid insertion sequence in the specific recognition of the initiator tRNA (*21*). Further support has come from the results of site-specific mutagenesis within this insertion sequence (*22*), and the findings that amino acids in this region of MTF are important for recognition of some of the major determinants in the initiator tRNA. In this paper, we describe the effect of (*i*) substitution mutations of basic, aromatic and other highly conserved amino acids in the linker region and in the C-terminal domain, and (*ii*) deletion mutations from the C-terminus of MTF on its activity. Our results are consistent with the notion that the positively charged amino acids in the C-terminal domain mostly contribute nonspecific binding energy, whereas the amino acids in the linker region, which come close to the 3′-terminal A of the tRNA (*23*), play important roles in discrimination of the initiator tRNA from elongator tRNA. Results of deletion mutagenesis show, however, that the C-terminal domain is important for the function of the enzyme. We discuss the results of these biochemical studies in the framework of the crystal structure of the *E. coli* MTF−fMet-tRNA complex published recently (*24*). Our results provide additional support to the molecular mechanisms of specificity in recognition of the initiator tRNA by MTF based on the crystal structure. In addition, they help determine which of the contacts seen in the crystal structure play important roles and which ones play marginal roles.

MATERIALS AND METHODS

Radioisotopes, Enzymes, and Chemicals. Radioactive isotopes were purchased from NEN-DuPont. Restriction enzymes and DNA-modifying enzymes were obtained either from New England Biolabs or Boehringer Mannheim. *Pyrococcus furiosus* DNA polymerase was from Stratagene, and Talon-Sepharose was from Clontech. Oligonucleotide primers were custom synthesized by Genosys Biotechnologies. DNA sequencing kit, Sequenase (Version 2.0), was from Amersham. IMPACT I, the one step protein purification system was from New England Biolabs. All other routinely used chemicals were of the highest purity grade available.

Bacterial Strains and Plasmids. E. coli JM109 and XL1-Blue were used as hosts in this study. The expression vectors pQE16 and pCYB3 were from Qiagen and New England Biolabs, respectively. The pCYB3 vector, which is part of the IMPACT I system, is used to express MTF C-terminal deletion mutant as a fusion protein with *Saccharomyces cerevisiae* vacoular membrane ATPase (VMA) intein and a chitin-binding domain, CBD, from *Bacillus circulans* (25). The construct pQE16FMTp, expressing the *E. coli* MTF as a fusion protein with 6XHis tag at the C-terminus, has been described before (*21*). The plasmid pQE16FMTp was used as a template for mutagenesis by the Quik-Change procedure (Stratagene) to obtain the site-specific mutants.

Generation of Deletion Mutants of MTF. Plasmid pQE16-FMTp was digested with *Bss*HII and *Bgl*II to completion, the single-stranded ends generated were removed using mung bean nuclease, and the large fragment was purified and circularized to obtain pQE16FMTpΔC20. This plasmid was used to express MTFΔC20, which lacks the C-terminal 20 amino acids, but has six histidine residues at the very C-terminus. For the construction of pQE16FMTpΔC80, expressing MTFΔC80 lacking the C-terminal 80 amino acids, the plasmid pQE16FMTp was digested with *Bst*XI and *Bgl*II and the ends were filled in with T4 DNA polymerase in the presence of all four dNTPs. The resulting large fragment was purified and circularized with T4 DNA ligase. The overexpression of the deletion mutants was followed by immunoblotting using anti-His tag antibodies (Clontech).

The construct pCYB3FMTΔC18, expressing MTFΔC18 lacking the C-terminal 18 amino acids and the six histidines present in the other mutants, was generated as follows. The *fmt* gene was amplified by PCR using the following two primers: 5′CTAACATGTCAGAATCACTAC3′ (forward primer carrying the site for the *Afl*III restriction enzyme) and 5′ACCATTCCCGAGCAGAGTTCAGGAGG3′ (reverse primer). The PCR product was digested with *Bss*HII, and the *Bss*HII end was filled in using T4 DNA polymerase in the presence of all four dNTPs. Following enzyme inactivation, the product was then digested with *Afl*III and purified on low-melting agarose gel. Similarly, the pCYB3 vector was digested with *Sap*I, the *Sap*I ends filled in using T4 DNA polymerase, and the resulting DNA was digested with *Nco*I. After purification on low-melting agarose gel, the pCYB3 vector DNA fragment was ligated to the *fmt* gene fragment. The ligation mix was used to transform *E. coli* XL 1 Blue, and the recombinant clones were identified using restriction digestion. Expression of the ∼85 kDa MTFΔC18-Intein-CBD fusion protein was detected using both anti-Intein antiserum and anti-MTF antiserum.

Protein Purification. The His-tagged wild-type and deletion mutants of MTF were purified using Talon affinity resin as described before (*21*). Expression and purification of native *E. coli* MTF lacking the His-tag has also been described before (*23*). Construction of plasmids for the expression and purification of His-tagged *E. coli* methionyl-tRNA synthetase (MetRS-6xHis) has been described (*22*). The purified enzymes showed no detectable loss in activity on storage at −20 °C for 2−4 months in 20 mM imidazole, pH 7.5, 150 mM KCl, 10 mM β-mercaptoethanol, and 50% glycerol.

For purification of MTFΔC18, *E. coli* XL1-Blue, harboring the pCYB3FMTΔC18 plasmid, was grown in 1 L 2xYT media containing 100 μg/mL Amp and 10 μg/mL Tet for 4 h at 37 °C. The culture was induced with IPTG (1 mM) for 10 h at 25 °C. Cells were recovered and resuspended in buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM

EDTA, and 0.1% Triton X-100) and sonicated for 2 min (40 pulse X 4) using Vibracell sonicator (Sonic and Materials Inc., Danbury, CT). The extract was clarified by ultracentrifugation (100000*g*; 60 min) and loaded onto a Chitin-agarose column (2.5 mL bed volume) preequilibrated with buffer I. The column was washed extensively with buffer I (100 mL) followed by buffer I + 500 mM NaCl (100 mL). Intein-mediated cleavage of MTFΔC18 from the MTFΔC18-Intein-CBD fusion protein was initiated by rapidly flushing the column with 6.0 mL of freshly prepared cleavage buffer containing DTT (20 mM Tris-HCl buffer, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, and 100 mM DTT). After the column was drained by gravity flow, 6.0 mL of cleavage buffer was added again to the column and the mixture was incubated for 12 h at 4 °C. The eluted fractions containing MTFΔC18 were pooled and dialyzed against 20 mM imidazole, pH 7.5, containing 150 mM KCl, 10 mM β-mercaptoethanol, and 50% glycerol. Approximately 1 mg of purified native MTFΔC18 protein was obtained from 1 L of culture.

Protein concentration was determined by Bradford dye-binding assay (*26*) using Bio-Rad dye binding assay kit employing IgG as a standard. SDS-polyacrylamide gel electrophoresis was carried as described by Laemmli (*27*). Gels were stained with Coomassie Brilliant Blue R-250.

fMet-tRNA$_2$ and the 3′-Amino-3′-deoxy Analogue of fMet-tRNA$_2$ (tRNA$_N$). fMet-tRNA$_2$ was expressed and purified as described (*28*, *29*). The purity of tRNA, assessed by aminoacylation was >95%. The 3′-terminal A of fMet-tRNA was exchanged for 3′-amino-3′-deoxy A as described previously to yield tRNA$_N$ (*30*). In tRNA$_N$, the 3′-hydroxyl group of the terminal A is replaced by an amino group. The purified tRNA$_N$ was aminoacylated with *E. coli* MetRS to yield Met-tRNA$_N$ in which the methionine is linked to the tRNA by a base stable peptide linkage as described before (*31*, *32*).

Gel Mobility Shift Analysis of MTF−Met-tRNA$_N$ Complexes. Binding reactions (10 μL) were performed in 20 mM imidazole·HCl (pH 7.6), 5 mM $MgCl_2$, 150 mM NaCl, and 5% glycerol. [^{35}S]Met-tRNA$_N$ (0.1 μM) and MTF (0.04−10.24 μM for wild-type and 1.1−18.8 μM for MTFΔC20) were mixed and incubated at room temperature for 15 min, and the complexes were resolved on a 6% native polyacrylamide gel at 50 V for 1 h. The gels were fixed, dried, and the radioactivity was quantitated by phosphorimaging (Molecular Dynamics). The apparent K_d values were obtained from the amount of enzyme required to convert 50% of the Met-tRNA$_N$ to the MTF−Met-tRNA$_N$ complex.

Assays for MTF and Measurement of Kinetic Parameters in Formylation of fMet-tRNA$_2$. These were as described elsewhere (*21*).

RESULTS

Amino Acid Residues in MTF Selected for Mutagenesis. Previous work (*22*) has focused on the effects of mutagenesis of amino acid residues in and around the 16-amino acid insertion loop of *E. coli* MTF (Figure 1). Here, we have focused on effects of mutagenesis of residues in the linker region and the C-terminal domain. Figure 2 shows a sequence alignment of these regions in MTFs from 17 eubacterial sources. Within the approximately 135 amino acid stretch of *E. coli* MTF, there are 29 sites that contain very similar amino acids in all 17 sequences. There are some that are highly conserved such as Gln196, Phe or Tyr203, Arg or Lys209, Trp or Tyr216, Gln287, Pro232, Pro234, Arg or Lys291, Arg or Lys292, etc. All in all, we have mutated 19 amino acids, 18 to Ala, and some to several different amino acids. The sites that have been mutagenized are indicated by closed circles in Figure 2. The rationale for selection of sites for mutagenesis was as follows: (1) Using chemical cross-linking, we had shown previously that Lys206 of *E. coli* MTF comes close to the 3′-end of the tRNA (*23*). The sequence following this Lys206 in *E. coli* MTF is Lys-Leu-Ser-Lys-Glu, which is similar to the Lys-Met-Ser-Lys-Ser or Lys-Leu-Ser-Lys-Ser sequences often found in class I aminoacyl-tRNA synthetases (*33*). These sequences in class I aminoacyl-tRNA synthetases are important for aminoacyl-adenylate formation and for transfer of the amino acid to the tRNA. Therefore, to investigate whether amino acids around the Lys206 of *E. coli* MTF are important for function, we mutagenized several of the amino acids in and around Lys206, including the Gln196 conserved in all MTFs. Lys206 is within the linker region of *E. coli* MTF, which includes amino acids 190−208, and Lys209 is assigned to be the first amino acid of the C-terminal domain (*14*). Because both Lys206 and 209 are part of the Lys-Leu-Ser-Lys-Glu sequence, for the sake of convenience in discussion, we have extended the linker region to include Lys209 in this paper. (2) The crystal structure of *E. coli* MTF shows that the C-terminal domain has a positive electrostatic potential on the surface pointing toward the catalytic center of the enzyme (*14*). This surface is lined up with basic and aromatic amino acid residues that are conserved. It has been proposed that the positively charged channel formed by the basic amino acids bind to tRNA and help orient the tRNA acceptor stem and the 3′-end toward the catalytic center of the enzyme. Our mutagenesis of this region has, therefore, focused on those basic, aromatic and other amino acids that are conserved in the C-terminal domain. The mutant enzymes were all purified as fusion proteins with six histidine residues at the C-terminus, except for the ΔC18 mutant and are essentially homogeneous (Figure 3).

Activity of the Mutant Proteins. As a quick screen for the effect of mutations to alanine on enzyme activity, the specific activities of the mutant proteins were measured at high- and low-initiator tRNA concentrations as described before (*22*). The results are presented in the form of a bar diagram (Figure 4), with the specific activity of the wild-type enzyme being set at 100%. It was found that within the linker region, mutation of the invariant Q196 or the highly conserved Y203 to A had minimal effects on enzyme activity, whereas mutation of amino acids 206, 207, and 209 had significant effects. In the C-terminal domain, most of the mutations studied had only small effects (activity reduced by a factor of 2−5 at the most) even at low-initiator tRNA concentrations (Figure 4B).

Steady-state Kinetic Parameters of MTF Mutants in the Linker Region. After the amino acid residues were identified, mutations of which are detrimental to enzyme activity, we mutated the amino acids in this region to several other amino acids and measured the steady-state kinetic parameters in formylation of the initiator tRNA (Table 1). It can be seen that, while the K206A mutant has a k_{cat}/K_m that is about 17-fold lower than that of the wild-type enzyme, the K206R mutant has a k_{cat}/K_m that is only 3-fold lower. Thus, a positive

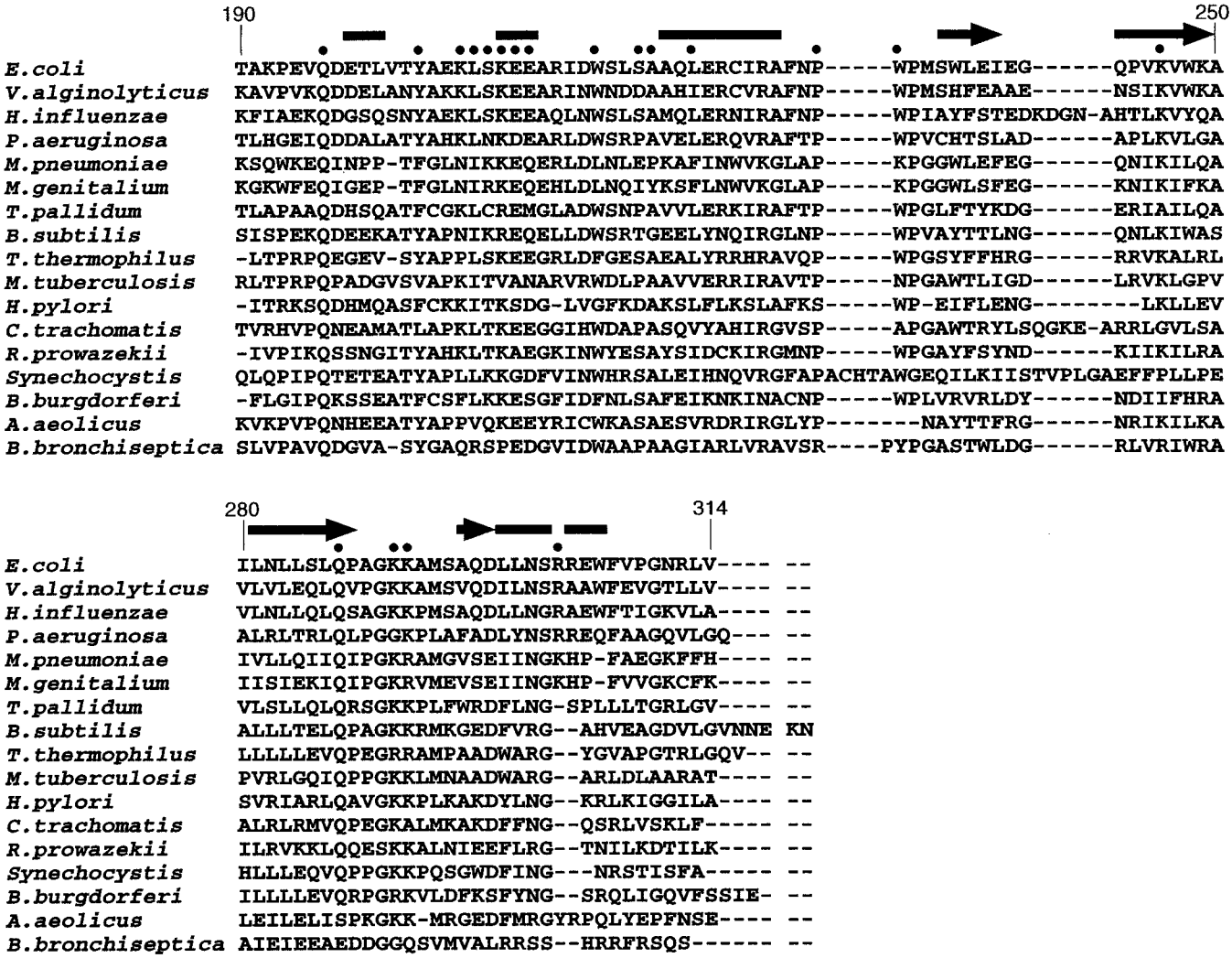

FIGURE 2: Alignment of MTF sequence from eubacteria in the linker region (amino acids, 190−208) and the C-terminal domain (209−314). α-Helices (rods) and β-strands (arrows) as seen in the crystal structure of the *E. coli* MTF. Closed circles indicate some of the basic, aromatic or highly conserved amino acids mutagenized in this study. The database accession numbers for the various MTF sequences are: *Escherichia coli* (P23882), *Vibrio alginolyticus* (O87726), *Haemophilus influenzae* (P44787), *Pseudomonas aeruginosa* (O85732), *Mycoplasma pneumoniae* (P75235), *Mycoplasma genitalium* (P74605), *Treponema pallidum* (O83737), *Bacillus subtilis* (P94463), *Thermus thermophilus* (P43523), *Mycobacterium tuberculosis* (Z80108), *Helicobacter pylori* (AE001534), *Chlamydia trachomatis* (AE001324), *Rickettsia prowazekii* (P50932), *Synechocystis sp.* (D64001), *Borrelia burgdorferi* (AE001119), *Aquifex aeolicus* (O67890), and *Bordetella bronchiseptica* (AJ007747).

charge at this position contributes to *E. coli* MTF function. The K206 to L mutation has basically the same effect as the K206 to A mutation, suggesting that the hydrophobic part of the side chain of Lys206 is probably not very important for enzyme function. In striking contrast to the effect of the K206 to R mutation, K206 to E mutation results in a large decrease of 124-fold in k_{cat}/K_m. This result suggests that a negative charge at this position is highly detrimental to enzyme activity. Much of this is due to a decrease in k_{cat}.

Mutation of L207 to A, V, or Q lowers k_{cat}/K_m by a factor of about 17, 9, and 12, respectively, suggesting that L207 also contributes to enzyme function. In contrast, mutation of S208 to A has only a very small effect (about 3-fold) on k_{cat}/K_m.

Mutation of K209 to A, L, and E has very strong effects on k_{cat}/K_m (down by a factor of 124), whereas mutation to R has only a very small (3-fold) effect. These results suggest that a positive charge at this position is very important.

Steady-state Kinetic Parameters of C-Terminal Substitution Mutants. Mutation of K292 to A and of R303 to A appeared to have very little effect on enzyme activity (Figure 4), whereas mutation of K246, Q287, and K291 appeared to have some small effect. Therefore, we have changed these three latter amino acids also to Glu to study in detail the effect of such mutations on the kinetic parameters. The results are shown in Table 2. While mutation of K246 to A had only a minor effect on k_{cat}/K_m, mutation to E had a major effect of lowering k_{cat}/K_m by a factor of 149-fold. Much of this effect is due to an increase in K_m from 0.5 to 15.4 μM. Similarly, while the Q287 to A mutation has only a small effect, lowering k_{cat}/K_m by about 10-fold, the Q287 to E mutation has a large effect, lowering k_{cat}/K_m by about 187-fold; the effect being due to both an increase in K_m and a decrease in k_{cat}. The results with K291 mutations are also basically the same in that the mutation to A has less of an effect than a mutation to E, although the K291 to A mutation has a substantial effect on kinetic parameters. These results suggest that, in the C-terminal domain, perhaps with the exception of K291, the basic amino acid residues that form part of the positively charged channel on the surface of the

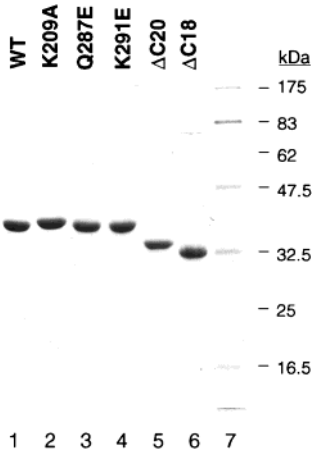

FIGURE 3: SDS/15% polyacrylamide gel electrophoresis of wild-type MTF and some of the least active of the mutant MTFs used in this study. Proteins were detected by staining with Coomassie blue dye. The wild-type MTFs and all of the mutant MTF except for the ΔC18 mutant contained a sequence of six consecutive histidine residues at the C-terminus.

enzyme make relatively small contributions, individually, to its overall activity. However, introduction of a negative charge is highly detrimental. The overproduction and stability of these mutant enzymes are not affected in any way by these mutations. Therefore, it is unlikely that the detrimental effect of the mutation to Glu is due to a major structural alteration of the enzyme.

Figure 5 indicates the relative locations of the positively charged lysine and arginine residues, and the glutamine residue mutated above in the framework of a drawing of the crystal structure of the *E. coli* MTF−fMet-tRNA complex (*24*). K206, K246, K291, and Q287, which when mutated to A, had relatively minor effects, but when mutated to E, had strong effects on enzyme activity are all close to either the minor groove of the tRNA acceptor stem or to the CCA end. K209, which when mutated to A, L, or E, had equally strong effects is highlighted with an asterisk.

Steady-state Kinetic Parameters of the C-Terminal Deletion Mutants. The deletion mutants MTFΔC20 and MTFΔC80 are both essentially inactive in formylation (Table 3 and data not shown), suggesting that the C-terminal region of MTF is very important for enzyme activity. The k_{cat}/K_m is down by factors of 1428−1492-fold. This is due both to an increase in K_m of about 7-fold and a decrease in k_{cat} of about 200-fold for the MTFΔC20 enzyme.

Both deletion mutants were stably overproduced in *E. coli*, were soluble, and could be purified easily. Also, the MTFΔC20 mutant binds to the substrate Met-tRNA, although somewhat weakly (see below). Therefore, while the possibility that there is a major change in the structure of MTF in the deletion mutants cannot be ruled out, this is unlikely at least for the MTFΔC20 mutant. Also, the crystal structure of *E. coli* MTF and the MTF−fMet-tRNA ternary complex show no extensive contacts between the C-terminal 20 amino acids and the rest of the protein. Like all the other mutants of MTF, however, the deletion mutants also contain six histidines at the very C-terminus. In the crystal structure of MTF, the C-terminus is almost at one end of the molecule (*14*) (Figure 5). Therefore, one possible explanation for the strong effect of the C-terminal deletions on MTF activity is that the deletion brings the six histidine residues closer to the rest of the protein and this proximity of the histidines interferes with the folding of the C-terminal domain. To rule

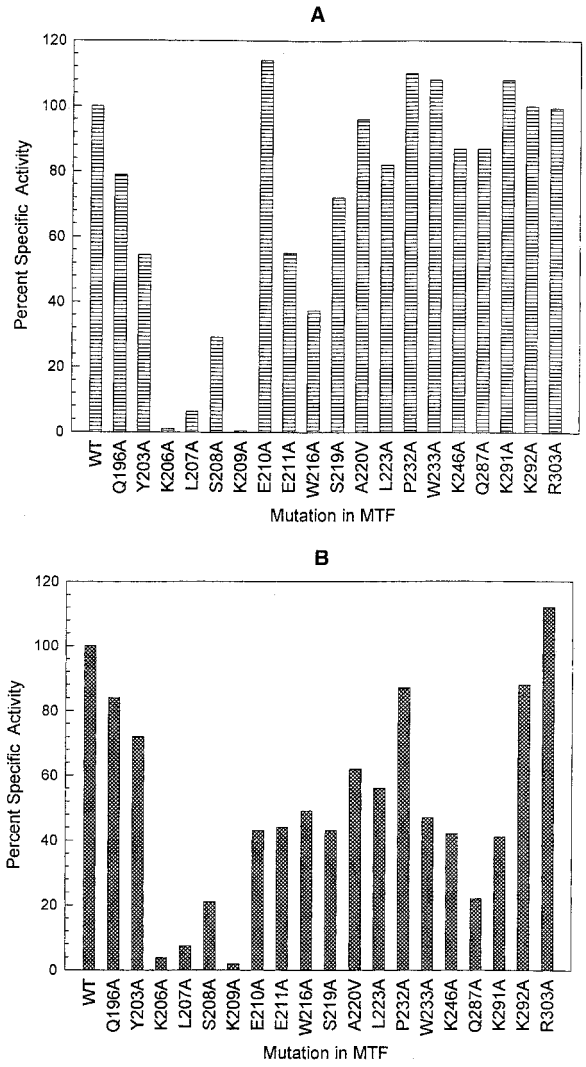

FIGURE 4: Specific activities of mutant MTFs measured at (A) high concentration ($10 \times K_m$) and (B) low concentration ($1 \times K_m$) of the initiator tRNA substrate presented in the form of a bar diagram with the specific activity of the wild-type enzyme fixed at 100%. The specific activities correspond to the initial rates observed per unit amount of the enzyme.

Table 1: Kinetic Parameters of Wild-type MTF and Mutants in the Linker Region[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	(k_{cat}/K_m) WT vs (k_{cat}/K_m) mutant
WT MTF	0.5 ± 0.2	37.3 ± 3.7	74.6	1.0
K206A	2.4 ± 0.4	10.7 ± 3.7	4.5	16.6
K206R	1. 7 ± 0.3	37.3 ± 3.7	21.9	3.4
K206E	2.7 ± 0.6	1.7 ± 0.5	0.6	124.3
K206L	2.8 ± 0.7	18.7 ± 0.9	6.7	11.1
L207A	4.3 ± 1.0	19.0 ± 4.3	4.4	16.9
L207V	4.1 ± 1.6	35.7 ± 11.2	8.7	8.6
L207Q	1.9 ± 0.6	12.3 ± 4.8	6.4	11.6
S208A	0.5 ± 0.1	13.6 ± 0.3	27.2	2.7
K209A	4.6 ± 1.1	2.6 ± 0.5	0.6	124.0
K209R	1.5 ± 0.4	32.5 ± 9.0	21.7	3.4
K209L	3.5 ± 1.3	2.1 ± 0.8	0.6	124.3
K209E	3.1 ± 1.3	1.9 ± 0.7	0.6	124.3

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA concentrations used varied from 0.2 to 2.0 μM, and the enzyme concentration, depending on the mutant, was 0.025 to 1.31 nM.

Table 2: Kinetic Parameters of Wild-type MTF and Mutants in the C-Terminal Domain[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	(k_{cat}/K_m) WT vs (k_{cat}/K_m) mutant
WT MTF	0.5 ± 0.2	37.3 ± 3.7	74.6	1.0
K246A	0.7 ± 0.3	12.0 ± 1.9	17.1	4.4
K246E	15.4 ± 0.5	7.8 ± 0.4	0.5	149.2
Q287A	1.8 ± 0.6	13.7 ± 3.4	7.6	9.8
Q287E	6.0 ± 0.6	2.5 ± 0.4	0.4	186.5
K291A	6.4	16.3 ± 5.6	2.5	29.8
K291E	8.4 ± 0.2	2.6 ± 0.5	0.3	248.6

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA concentrations used varied from 0.2 to 4.0 μM, and the enzyme concentration, depending on the mutant, was 0.025 to 0.293 nM.

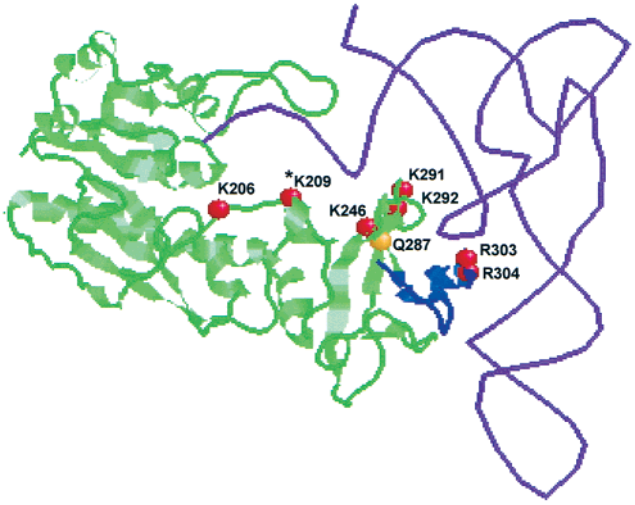

FIGURE 5: X-ray structure of *E. coli* MTF−fMet-tRNA complex (*24*) with the MTF displayed in cartoon format and the tRNA in background format. The figure was drawn using Ras Mol Version 2.6 with coordinates obtained from the Protein Data Bank (PDB code 2FMT). The MTF is colored green except for the C-terminal 20 amino acids, which are deleted in the MTFΔC20 mutant described in the paper, colored blue. The tRNA is colored violet. The backbone nitrogen atoms of the basic amino acids which line the surface of MTF leading to the catalytic center are highlighted as red spheres, that of glutamine 287, which is highly conserved, is highlighted as orange sphere. The single lysine residue, Lys209, which when mutated to alanine, leucine, or glutamic acid, had equally strong effects on activity of MTF is indicated with an asterisk.

Table 3: Kinetic Parameters of Wild-type MTF and C-Terminal Deletion Mutants

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}/\mu M^{-1}$)	(k_{cat}/K_m) WT vs (k_{cat}/K_m) mutant
WT MTF	0.5 ± 0.2	37.3 ± 3.7	74.6	1.0
MTFΔC20	3.7 ± 0.3	0.2	0.05	1492

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA concentrations used varied from 0.2 to 2 μM for the wild-type enzyme and 0.62 to 10 μM for the MTFΔ20 mutant. The enzyme concentration used was 0.025 nM for the wild-type enzyme and 21 nM for the mutant enzyme.

out this possibility, we generated another deletion mutant, MTFΔC18, which has 18 C-terminal amino acids of MTF deleted, but without the addition of any histidine residues. For this, the MTFΔC18 mutant was expressed as a fusion protein with the yeast VMA intein and a bacterial chitin-binding domain (Materials and Methods). The fusion protein

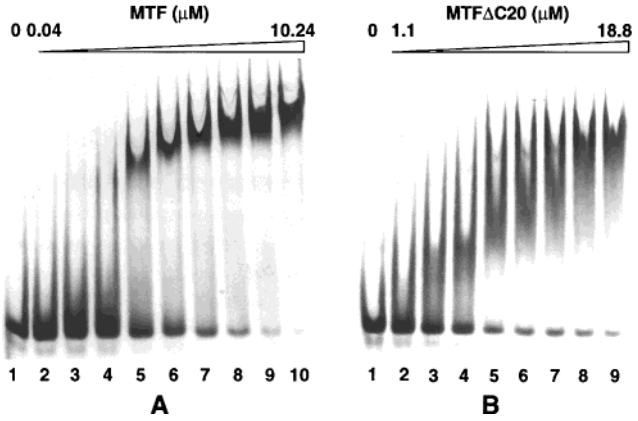

FIGURE 6: Gel mobility shift analysis, using 6% native polyacrylamide gel, of the binding of wild-type MTF (A) and the deletion mutant MTFΔC20 (B) to the *E. coli* [^{35}S]Met-tRNA$_N$. The concentration of MTF varied from 0.04 to 10.24 μM for wild-type MTF and 1.1 to 18.8 μM for the mutant MTF. Other details are provided in Materials and Methods.

was purified by binding it to a chitin-affinity column and the MTFΔC18 portion cleaved off the fusion protein with dithiothreitol (*25*). Activity analysis showed that the MTFΔC18 mutant was essentially as inactive as the MTFΔC20 mutant (data not shown).

Binding Affinity of MTF and MTFΔC20 to Met-tRNA$_N$. The formation of a complex between the MTFΔC20 mutant and the tRNA was analyzed by gel retardation (*34*, *35*). For this purpose, the substrate tRNA, [^{35}S]Met-tRNA$_N$, in which the amino acid is linked to the tRNA by a base-stable peptide linkage was used. The Met-tRNA$_N$ was incubated with increasing amounts of purified enzymes, and the complexes were resolved by electrophoresis (Figure 6). Under the conditions of gel electrophoresis, stable complexes between MTF and Met-tRNA$_N$ were detected at ratios of >1.6:1 (Figure 6A, lanes 5−10). In contrast, the complexes between the MTFΔC20 and Met-tRNA$_N$ were not detected at similar concentrations of the enzyme. Relatively stable complexes could only be detected at enzyme/tRNA ratios of >11:1 (Figure 6B, lanes 3−9). The regions of radioactivity corresponding to the bound and unbound [^{35}S]Met-tRNA$_N$ fractions from this gel were quantified using Phosphorimager and used for determining the binding affinity. Apparent K_d values for complex formation were estimated by the amount of enzyme required for binding 50% of the Met-tRNA$_N$ present. The K_d values for MTF and MTFΔC20 were 0.3 and 3.6 μM, respectively. The 12-fold difference in K_d values is about the same as the difference in K_m values for tRNA in the formylation reaction (Table 3).

DISCUSSION

Previous work has shown that a 16 amino acid insertion loop in the N-terminal domain of *E. coli* MTF (Figure 1) plays an important role in the specific recognition of the determinants for formylation in the acceptor stem of the initiator tRNA (*14*, *21*, *22*, *30*). Here, we have studied the effect of mutations in the C-terminal domain and in the linker region between the N- and C-terminal domains on activity of the *E. coli* MTF. Our results show that some of the amino acids in the linker region play important roles, whereas the basic amino acids in the C-terminal domain contribute mostly toward the nonspecific binding and orientation of the tRNA

3′-end toward the catalytic site of MTF (*14*, *21*). The C-terminal domain of MTF is, however, clearly important, since deletion of 18 or 20 amino acids from the C-terminus leads to a precipitous drop in activity of the enzyme.

Mutations in the Linker Region. Mutations of amino acids Lys206, Leu207, and Lys209 have significant effects on activity of the enzyme. Lys206 was previously shown (*23*) to chemically cross-link to the 3′-end of periodate-oxidated tRNA; therefore, this region of MTF comes close to the acceptor stem of the tRNA where the main determinants for formylation are located. In particular, the positive charge in Lys209 appears to be very important for MTF activity. Mutation of Lys209 to Ala, Leu, or Glu lowers k_{cat}/K_m by a factor of 124-fold, whereas mutation to Arg has only a small (3-fold) effect. The effect of mutations to Ala, Leu, or Glu is on both K_m and k_{cat}, suggesting that the positive charge on Lys209 contributes both toward binding and toward formation of the transition state, perhaps by stabilizing a form of the tRNA acceptor stem structure that mimics the transition state. The importance of a positively charged amino acid at this position is also underscored by the fact that 15 out of 17 known MTF sequences contain a Lys or Arg at this position.

The observed effects of the mutations of Lys209 to Ala on the kinetic parameters differ somewhat in nature and magnitude from those reported by Schmitt and co-workers (*24*). These workers have also shown that Lys206 and Lys209 are important for enzyme activity. While the relative k_{cat}/K_m for the Lys206 to Ala mutant is similar to that reported here, the k_{cat}/K_m for the Lys209 to Ala mutant is lower, by a factor of approximately 22 compared to 124 found by us. Furthermore, the effects of the Lys209 to Ala mutation is reported to be mostly due to an increase in K_m rather than an increase in K_m and a decrease in k_{cat} as noted by us. It is not known whether the reason for this difference is because the mutant enzymes used by us have a His-6 tag at the C-terminus (*21*), whereas those used by Schmitt et al. do not.

The importance of amino acids 206, 207, and 209 in the linker region for MTF activity is also consistent with the finding that amino acids in the linker region interact with the loop containing the SLLP sequence motif, which is highly conserved in many tetrahydrofolate utilizing enzymes. In particular, Schmitt et al. (*14*) have noted that the loop containing the SLLP sequence is disordered in glycinamide ribonucleotide formyltransferase (GARF) and adopts an ordered structure only in the GARF-formyltetrahydrofolate substrate analogue complex (*15*, *16*). In contrast, in the *E. coli* MTF crystal structure, the loop containing the SLLP sequence is already well-ordered, presumably due to the tight electrostatic interactions between some of the amino acids in the SLLP loop and the amino acids in the linker region of MTF.

The Lys206-Leu207-Ser208-Lys209-Glu210 sequence of MTF is related to a similar sequence KLSKS or KMSKS, which is part of a "signature sequence" found in Class I aminoacyl-tRNA synthetases (*33*). As in the case of *E. coli* MTF, where Lys206 can be cross-linked to the 3′-end of periodate-oxidized *E. coli* $tRNA^{fMet}$, one or the other of the lysine residues of *E. coli* MetRS or TyrRS can be cross-linked to the 3′-end of the corresponding periodate-oxidized tRNA (*36*, *37*). In the *E. coli* MetRS or TyrRS, these lysine residues are also important for stabilization of the ground state and/or the transition state during the formation of the aminoacyl-adenylate (*38*). The significance of the conservation of the KLSKS/KMSKS sequence in Class I aminoacyl-tRNA synthetases and of the KLSKE sequence in *E. coli* MTF, which catalyze very different kind of enzyme reactions, is not known. It is interesting to note that in a Class II aminoacyl-tRNA synthetase also, a lysine residue that gets cross-linked 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 (*39*, *40*). It should also be noted that, while Lys209 or Arg209 is conserved in 15 out of 17 known sequences of MTF, Lys206 is found in only 9 out of 17 sequences.

Mutations in the C-Terminal Domain. Our results on mutations of the conserved basic and hydrophilic amino acids in this domain provide support to the idea that the C-terminal domain of MTF is involved in the initial anchoring of the tRNA and in the orientation of the 3′-end of aminoacyl-tRNA toward the catalytic center of the enzyme (*14*). Mutations of amino acids such as Lys246, Gln287, and Lys291 to alanine have smaller effects than mutations to glutamic acid (Table 2). Also mutations of Lys292 and Arg303 to alanine have virtually no effects (Figure 4). Thus, of the amino acids that comprise the positive electrostatic channel on the surface of the enzyme, Lys206, Lys209, Lys246, Lys291, Lys292, Arg303, and Arg304 (Figure 5), the amino acids that stand out as playing a clearly important role are Lys209 and to a smaller extent, Lys206 and Lys291. The other basic amino acids have relatively smaller effects individually, however, because Arg304 is not among the highly conserved amino acids (Figure 1); we did not mutagenize Arg304 and cannot comment on its contribution. Schmitt et al. (*24*) have mutated Arg304 to Ala and noted only a 3-fold drop in k_{cat}/K_m.

Crystal Structure of E. coli MTF Complexed With fMet-tRNA: Comparison of Conclusions Derived from the Crystal Structure to Conclusions from Solution Studies. The crystal structure of the *E. coli* MTF−fMet-tRNA complex was published recently (*24*). Although the structure of the MTF−tRNA complex involves the product fMet-tRNA rather than the substrate Met-tRNA, the crystal structure data are in excellent agreement with much of the biochemical and genetic data, published previously, on the important role of the 16 amino acid insertion loop I (Loop I) in the N-terminal domain of MTF on recognition of the determinants for formylation in the acceptor stem of the initiator tRNA substrate (*21*, *22*, *30*) and with the results described here. The crystal structure of the MTF−fMet-tRNA complex (Figure 5) also supports the previous conclusions: (*i*) that the determinants for formylation of the tRNA are located mostly in the acceptor stem and partly in the D stem (*7*−*12*) and (*ii*) that the enzyme makes no contacts with the D loop, the anticodon stem and loop, and the TΨC stem and loop regions (*30*).

The crystal structure of the complex (*24*) also supports the conclusion that there is an induced fit upon binding of the Met-tRNA substrate to the protein (*14*, *22*, *30*). Two regions of the protein are found to undergo conformational changes in the complex. Loop I, which is "unstructured" in the free enzyme, adopts a defined structure in the MTF−fMet-tRNA complex, allowing several of the amino acids in the loop, including the critical Arg42, to interact with the determinants for formylation in the acceptor stem of the

tRNA. The C-terminal domain of MTF undergoes a locking movement using Lys206 in the linker region as a hinge. This allows amino acids in the C-terminal domain to come close to the D stem and the minor groove of the acceptor stem. The acceptor stem of the tRNA is thus clamped between the N-terminal and the C-terminal regions of MTF (Figure 5).

The crystal structure of the complex shows that Lys209 interacts with phosphates 71 and 72 on the 3′-side of the acceptor stem of the initiator tRNA (*24*). This is the region of the tRNA that contains the major determinants for formylation (*7*−*11*). As shown above (Table 1), the positive charge on Lys209 is very important for activity of MTF. Thus, the electrostatic interactions of Lys209 with the tRNA, as seen in the crystal structure of the complex, contribute significantly toward activity of MTF (Figure 5). With respect to the other amino acids in the linker region, while our results show an approximately 12−17-fold lowering of k_{cat}/K_m in the Lys206 to Ala and the Leu207 to Ala mutants, the crystal structure of the MTF−fMet-tRNA complex shows no contacts between MTF and Lys206 or Leu207. It is possible that these amino acids contribute toward the formation of the ground state or the transition state complex between MTF and the initiator Met-tRNA. Alternatively, the effect of mutations of these amino acids is indirect. One possibility is that these mutations alter the structure of the linker region in such a way that it affects the interaction of Lys209 with phosphates 71 and 72 in the acceptor stem. Another possibility is that mutations here affect the locking movement of the C-terminal domain necessary for MTF to interact with the D stem and the minor groove of the acceptor stem of the tRNA.

The crystal structure of the complex shows that the basic amino acids, Lys246, Lys291, Arg303, and Arg304, in the C-terminal domain all contact either the acceptor stem or the D stem of the tRNA. Most of these contacts are with the tRNA backbone. This is in agreement with the results of this study and with previous suggestions (*14*) that the C-terminal domain is involved mostly in nonspecific binding of the tRNA.

Finally, the crystal structure of the MTF−fMet-tRNA complex shows a cluster of amino acids of MTF, Asp298, Asn301, Ser302, Arg303, and Arg304, near the C-terminus, in contact with the sugar-phosphate backbone in the D stem and loop of the initiator tRNA (*24*). The only base specific contact here is between O2 of U24 and the amide side chain of Asn301. These results support our previous findings that the purine 11:pyrimidine 24 base pair conserved in all eubacterial initiator tRNAs contributes toward formylation of the initiator tRNA in vitro (*9*) and in vivo (*12*). The involvement of many amino acids from the very C-terminal region of MTF in these interactions explains the severe detrimental effect of the C-terminal deletion mutations described here.

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