Mapping the active site of the *Haemophilus influenzae* methionyl-tRNA formyltransferase: residues important for catalysis and tRNA binding

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Formylation of the initiator methionyl-tRNA by methionyl-tRNA formyltransferase (MTF) is an essential step in initiation of protein synthesis in eubacteria. Here, site-directed mutagenesis was used to identify active site residues of the *Haemophilus influenzae* MTF. Of the nine residues investigated, only Arg-41, Asn-107, His-109 and Asp-145 were important for the function of the *H. influenzae* MTF. Replacement of these residues with Ala resulted in a significant reduction in the efficiency of catalysis. Intrinsic fluorescence analysis indicated that this was not due to a defect in *N*¹⁰-formyltetrahydrofolate (fTHF) binding. The Asp-145 and Arg-41 mutations reduced the affinity of the enzyme for the initiator tRNA, whereas the Asn-107 and His-109 mutations affected catalysis but not tRNA binding. Replacement of Arg-

41, His-109 and Asp-145 with functionally similar residues also affected the activity of the enzyme. The data suggest that Asn-107, His-109 and Asp-145 are catalytic residues, whereas Arg-41 is involved in tRNA recognition. In the *Escherichia coli* glycylamide ribonucleotide formyltransferase, which also uses fTHF as the formyl donor, Asn-106, His-108 and Asp-144 participate in the catalytic step. Together, these observations imply that this group of enzymes uses the same basic mechanism in formylating their substrates.

Key words: enzymology, formylation, initiation of protein synthesis, mutagenesis, protein expression.

INTRODUCTION

Formylation of the initiator methionyl-tRNA is an essential step in initiation of protein synthesis in eubacteria, and in the mitochondria and chloroplasts of eukaryotes [1]. Analyses *in vivo* and *in vitro* suggest that the formyl-aminoacyl moiety allows initiation factor IF2 to recognize the initiator tRNA over all other tRNAs [2–5]. The formyl group was also shown to prevent the tRNA from participating in elongation by blocking binding of elongation factor EF-Tu to the initiator tRNA [2,6,7]. The features in the *Escherichia coli* initiator tRNA that are required for formylation are the base–base mismatch between nucleotides 1 and 72, and the second and third bp of the acceptor stem [7,8]. The most critical of the three formylation determinants is the lack of base pairing between nucleotides 1 and 72.

Formylation of the initiator methionyl-tRNA is catalysed by methionyl-tRNA formyltransferase (MTF), which is encoded by the *fmt* gene [9]. The *fmt* gene has been cloned from *E. coli* [9], *Thermus thermophilus* [10], *Rickettsia prowazekii* [11] and *Pseudomonas aeruginosa* [12]. Sequencing of the genomes of *Haemophilus influenzae* [13], *Bacillus subtilis* and *Mycoplasma genitalium* [14] resulted in the identification of a putative *fmt* gene in these organisms. The crystal structure of the *E. coli* MTF showed that the enzyme is composed of two domains connected by a linker [15]. The N-terminal domain consists of a Rossmann fold, involved in nucleotide binding, and is highly similar in structure to *E. coli* glycylamide ribonucleotide formyltransferase (GARF), an enzyme that uses *N*¹⁰-formyltetrahydrofolate (fTHF) as the formyl donor [15,16]. Furthermore, the sequences of the MTF N-terminal domain and GARF are similar [15]. These observations led to the proposal that the N-terminal domain contains the active site.

The N-terminal domains of *E. coli* and other MTFs contain a loop (loop 1) that connects β -strand 2 and α -helix 2 [15]. The loop contains several basic amino acid residues, and is located above the putative active site [15]. In the absence of the initiator tRNA, loop 1 is cleaved by trypsin at Arg-42 and the enzyme is inactivated [15]. This loop is protected in the presence of the initiator tRNA but not by the elongator methionyl-tRNA. In addition, an MTF mutant containing a Gly-41 $\rightarrow$ Arg change was shown to rescue an initiator tRNA mutant that is defective in formylation because of a mutation in the acceptor stem [17]. Taken together, these results suggest that loop 1 in the N-terminal domain of MTF is involved in the specific recognition of the initiator tRNA.

The C-terminal domain of the *E. coli* MTF is similar in structure to an oligonucleotide-binding fold found in the lysyl- and aspartyl-tRNA synthetases [5]. Moreover, this domain was cross-linked with periodate-oxidized initiator tRNA, and a polypeptide corresponding to the C-terminal domain was shown to bind the initiator tRNA. These findings imply that this part of MTF is involved in tRNA binding [15,18]. This binding, however, is not specific, since the C-terminal domain polypeptide binds the initiator and methionine elongator tRNAs with comparable affinities [15].

In this work we show that Arg-41, Asn-107, His-109 and Asp-145 in the N-terminal domain of the *H. influenzae* MTF are important for the function of the enzyme in formylation of the initiator methionyl-tRNA. The data suggest that Asn-107, His-109 and Asp-145 are catalytic residues, whereas Arg-41 is involved in tRNA recognition. The fact that His-108, Asn-106 and Asp-144 in GARF also participate in the catalytic step indicates that this group of enzymes uses the same basic mechanism in formylating their substrates.

Abbreviations used: MTF, methionyl-tRNA formyltransferase; fTHF, *N*¹⁰-formyltetrahydrofolate; GARF, glycylamide ribonucleotide formyltransferase; MetRS, methionyl-tRNA synthetase.

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MATERIALS AND METHODS

Strains and plasmids

E. coli BL21(DE3)pLysS was obtained from Novagen. The plasmids pACYC177 and pCYB3 were purchased from New England Biolabs, and pET23a(+) was purchased from Novagen. The pBlueXET plasmid was described previously [3].

Cloning of the *H. influenzae* *fnt* gene and site-directed mutagenesis

A DNA fragment containing the *H. influenzae* *fnt* open reading frame was obtained by PCR amplification of *H. influenzae* chromosomal DNA using the primers 5'-GGTTCCATGGCG-ATGAAATCACTTAAT-3' and 5'-TTTTGGATCCTTATGCAAGCACCTT-3'. The *H. influenzae* *fnt* gene was cloned into the *Nco*I and *Bam*HI sites in the pACTN vector for site-directed mutagenesis. This vector was constructed by replacing a 1.5-kb *Bam*HI–*Dra*III fragment of pACYC177 with an 860-bp *Pvu*II–*Sal*I fragment from pBlueXET. The latter fragment carries the *Lac* promoter, and the Shine–Dalgarno and transcription-termination sequences of *gene 10* of bacteriophage T7. Incompatible cohesive ends were filled in using the Klenow fragment of DNA polymerase I in the presence of dNTPs. Site-specific mutations were introduced into the *fnt* gene on the pACTN plasmid using *Pfu* DNA polymerase and the Stratagene QuikChange mutagenesis protocol.

Overproduction and purification of mutant MTFs containing an N-terminal His tag

An N-terminal (His)₆ tag connected to a linker consisting of Gly-Ser-Met-Ala was introduced into the wild-type MTF by PCR amplification of the *fnt* gene in pACTN. The fusion gene was cloned into the *Nde*I–*Sal*I site of pET23a(+). The resulting pET(His)₆MTF plasmid was then used as the vector to clone the mutant *fnt* genes. The wild-type *fnt* gene in pET(His)₆MTF was excised with *Nco*I and *Bam*HI. The mutant *fnt* genes were obtained as *Nco*I–*Bam*HI fragments from pACTN and cloned into the pET(His)₆ vector. *E. coli* BL21(DE3) transformants harbouring the pET vector carrying the wild-type and mutant *fnt* genes were grown overnight at 37 °C in Luria–Bertani medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. The cultures were diluted 50-fold into 100 ml of fresh medium containing antibiotics and grown at 37 °C. When the *D*₆₀₀ was 0.5, protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 90 min at 37 °C. The cells were pelleted by centrifugation, and resuspended in 10 ml of 20 mM Tris/HCl (pH 7.9) buffer containing 5 mM imidazole and 500 mM NaCl (binding buffer). The cells were lysed at 70000 kPa using a French press, and unlysed cells were removed by centrifugation at 10000 *g* for 10 min at 4 °C. The supernatant was applied to a Pharmacia Fast Flow Sepharose Ni²⁺ affinity column (2.5-ml bed volume) pre-equilibrated with binding buffer. The column was washed with 25 ml of binding buffer, followed by 15 ml of binding buffer containing 100 mM imidazole. The bound protein was eluted from the column using binding buffer containing 200 mM imidazole. The fractions containing MTF were pooled and dialysed against 20 mM imidazole/HCl (pH 7.5) buffer containing 10 mM 2-mercaptoethanol, 150 mM KCl and 50 % glycerol at 4 °C.

Overproduction and purification of methionyl-tRNA synthetase (MetRS) as an intein–chitin-binding domain-fusion protein

A fragment containing the coding region of the MetRS gene was obtained by PCR amplification of *E. coli* chromosomal DNA,

and cloned between the *Nco*I and *Sap*I sites in pCYB3. An overnight culture of *E. coli* JM109 harbouring the pCYB3/MetRS vector was diluted 50-fold into 500 ml of Luria–Bertani medium supplemented with 100 µg/ml ampicillin, and grown at 37 °C to a *D*₆₀₀ of 0.5. After induction of protein expression with 1 mM isopropyl β-D-thiogalactopyranoside for 90 min at 37 °C, the cells were pelleted by centrifugation. The cell pellet was resuspended in column buffer (20 mM Tris/HCl, pH 8.0/500 mM NaCl/0.1 mM EDTA/0.1 % Triton X-100), and lysed at 70000 kPa using a French press. The lysate was centrifuged at 10000 *g* for 10 min at 4 °C, and the supernatant was applied to a chitin-affinity column (3-ml bed volume; New England Biolabs). The column was washed with column buffer to remove unbound protein. The proteins bound to the column were incubated overnight at 4 °C with cleavage buffer (30 mM dithiothreitol/20 mM Tris/HCl, pH 8.0/50 mM NaCl/0.1 mM EDTA). This treatment releases MetRS from the intein–chitin-binding domain-fusion protein, which remains bound to the column. MetRS was eluted with cleavage buffer lacking dithiothreitol, and dialysed against 10 mM Tris/HCl (pH 7.6) buffer containing 50 % glycerol at 4 °C.

Purification of the *E. coli* initiator tRNA

A fragment containing the gene of the *E. coli* initiator tRNA_{2^{fmet}} was obtained by PCR amplification of chromosomal DNA, and cloned into the *Pst*I and *Bam*HI sites of pUC57 [19]. A transformant of *E. coli* B105 containing the pUCtRNA_{2^{fmet}} vector was grown overnight at 37 °C in 2 × YT medium containing 100 µg/ml ampicillin. An aliquot of the overnight culture was diluted 50-fold into 21 of 2 × YT medium containing 100 µg/ml ampicillin and grown at 37 °C for 6 h. The cells were pelleted by centrifugation and resuspended in 200 ml of 10 mM Tris/HCl, pH 8.0. Total RNA was isolated from the cells by the addition of an equal volume of phenol followed by vigorous mixing at room temperature for 15 min [20]. After centrifugation the aqueous phase was extracted with an equal volume of chloroform, and the RNAs were precipitated at 4 °C by the addition of a 1/10 volume of 3 M sodium acetate, pH 6.0, and 2.5 volumes of ethanol. The RNA pellet was dissolved in 120 ml of TE buffer (10 mM Tris/HCl, pH 8.0/1 mM EDTA), and NaCl was added to a final concentration of 1 M to precipitate the large RNAs. After 3 h at 4 °C the RNA precipitate was pelleted by centrifugation, and the small RNAs in the supernatant were precipitated as described above. The RNA pellet was dissolved in 20 mM Tris/HCl, pH 7.5/375 mM NaCl/8 mM MgCl₂ (column buffer) and applied to a DEAE-Sepharose column (Pharmacia) pre-equilibrated with column buffer. The column was eluted with a 1 litre linear gradient of column buffer containing 375 mM NaCl and 8 mM MgCl₂ to 500 mM NaCl and 16 mM MgCl₂. Fractions containing the initiator tRNA were identified by testing for formyl acceptance [21].

Measurement of kinetic parameters of formylation

Aminoacylation of the initiator tRNA substrate was performed at 37 °C for 30 min [21]. The incubation mixture (20 µl) consisted of 20 mM imidazole/HCl, pH 7.5, 150 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA, 10 µg/ml BSA, 0.1 mM [³⁵S]methionine (specific radioactivity 13000–15000 c.p.m./pmol), 2 mM ATP, various concentrations of tRNA (0.2–10.0 µM) and a large excess of purified MetRS (1 µg). To measure the initial rate of formylation, 5 µl of 1.8 mM fTHF in 20 mM imidazole/HCl, pH 7.5, and 5 µl of appropriately diluted MTF were added and the mixture was incubated for 10 min at 37 °C. The reaction was

Figure 1 Comparison of the deduced amino acid sequences of MTF from various species

The amino acids conserved in 5, 6 and 7 of the sequences are in shaded boxes. Residues that were mutated are indicated with an asterisk.

terminated by the addition of 30 μ l of 360 mM CuSO_4 in 1.1 M Tris, pH 7.3 [21]. After 5 min, an aliquot was spotted on to 3 MM Whatman filter paper, and washed sequentially with 10% trichloroacetic acid containing 0.2% casamino acids, 5% trichloroacetic acid containing 0.2% casamino acids and cold 95% ethanol. The retained radioactivity was determined by liquid-scintillation counting. Lineweaver–Burk and Hanes–Wolf plots were used to calculate K_m and $V_{\max}$.

Substrate-induced intrinsic fluorescence quenching

The affinity of MTF for fTHF was determined by substrate-induced intrinsic fluorescence quenching [21]. Trp fluorescence was measured using a Spex Model DM2000 spectrofluorimeter (Spex Industries Inc., Edison, NJ, U.S.A.), with excitation and emission slits set to 4 nm, and excitation and emission wavelengths of 295 and 332 nm, respectively [22,23]. Before fluorescence measurements were taken, MTF (50 μ g/ml) was incubated with various concentrations of fTHF (0–15 μ M) at 4 °C for 1 h in 20 mM Tris/HCl, pH 7.5, containing 150 mM KCl, 7 mM MgCl_2 and 0.1% Tween 80 to ensure that equilibrium had been achieved.

General methods

Protein concentration was determined using the modified Lowry DC method as specified by the supplier (BioRad), using IgG as the standard. Samples for SDS/PAGE were solubilized by boiling for 5 min in 62.5 mM Tris/HCl, pH 6.8, containing 5% SDS (w/v), 10% glycerol (v/v), 10% 2-mercaptoethanol (v/v) and 0.02% Bromophenol Blue (v/v) [3]. DNA sequencing and oligonucleotide primer synthesis were carried out at the Guelph Molecular Supercentre (Guelph, Ontario, Canada).

RESULTS

MTF mutants constructed

The importance of Phe-7, Gly-9, Leu-18, Val-31, Lys-37, Arg-41, Asn-107, His-109 and Asp-145 for the function of the *H. influenzae* MTF in formylation of the initiator methionyl-tRNA was investigated by site-specific mutagenesis. These residues were chosen because they are conserved in various eubacterial MTFs, and are found in regions proposed to be part of the active site of the *E. coli* enzyme (Figure 1). To obtain the mutant enzymes free of the endogenous wild-type MTF, they were expressed in *E. coli*

Figure 2 Analysis of the purity of the wild-type and mutant MTFs

Aliquots containing 1.5 μ g of purified protein were used to assess the purity of the wild-type (lane 1) and mutant MTFs (lanes 2–12). MTF is indicated with an arrow.

as fusion proteins with an N-terminal (His)₆ tag, and purified by chromatography using Sepharose Ni²⁺ affinity resin. SDS/PAGE analysis showed that the mutant enzymes were essentially pure (Figure 2). The Ala mutants of Phe-7, Gly-9 and Leu-18 were expressed in an insoluble form (results not shown); therefore, these mutants were not characterized further.

Functional characterization of the mutant MTF enzymes

The sequence of the initiator tRNA from *H. influenzae* is nearly identical to that of the *E. coli* tRNA (Figure 3). The formylation

determinants in the *E. coli* initiator tRNA are also found in the *H. influenzae* tRNA. Overproduction of the *H. influenzae* MTF rescued the initiator activity of a formylation-defective *E. coli* initiator tRNA mutant (G72) with a base pair between nucleotides 1 and 72, as well as the slow-growth phenotype of an *E. coli* MTF mutant strain (results not shown). The affinity of the wild-type *H. influenzae* MTF for the *E. coli* tRNA was $1.12 \pm 0.60 \mu\text{M}$ and that of the *E. coli* enzyme was $1.19 \pm 0.34 \mu\text{M}$. In addition, the turnover numbers of both enzymes are comparable (results not shown). Together, these results indicate that the *H. influenzae* MTF recognizes and formylates the *E. coli* initiator tRNA as equally well as the *E. coli* enzyme. Therefore, this tRNA was used as the substrate to functionally characterize the *H. influenzae* MTF mutants (Table 1). Mutation of Val-31, Lys-37, Arg-41, Asn-107 and His-109 to Ala reduced the rate of formylation by 1.7-, 3.5-, 470-, 48- and 4677-fold, respectively. The Asp-145 $\rightarrow$ Ala mutation drastically reduced the activity of the enzyme. The importance of Arg-41, His-109 and Asp-145 was investigated further by replacing these residues individually with functionally similar ones. Replacement of His-109 with Arg, Asp-145 with Glu and Arg-41 with Lys decreased the rate of formylation by 7172-, 4031- and 140-fold, respectively. Changing His-109 to Lys resulted in an inactive enzyme, and mutation of Asp-145 to Asn reduced the rate by 881-fold. These results show that Arg-41, Asn-107, His-109 and Asp-145 were important for MTF activity.

Kinetic parameters of MTF mutants in formylation

To ascertain the role of Arg-41, Asn-107, His-109 and Asp-145, the formylation kinetics of MTF enzymes carrying mutations at these residues were analysed. The K_m values obtained for the K37A, N107A and H109A mutant enzymes were comparable

Figure 3 Nucleotide sequences of the *H. influenzae* and *E. coli* initiator tRNAs

The *H. influenzae* initiator tRNA sequence was deduced from the gene sequence obtained from the *H. influenzae* Rd Genome Database (or HIGB). The boxes indicate the determinants that are important for MTF recognition of the initiator tRNA. The arrows indicate the most crucial of the three formylation determinants. The asterisks indicate the nucleotides that are different between the *H. influenzae* and *E. coli* tRNAs.

Table 1 Activities of wild-type and mutant *H. influenzae* MTFs in formylation of the initiator tRNA

The aminoacylation reaction mixture consisted of 20 mM imidazole/HCl (pH 7.5)/150 mM NH_4Cl /10 mM MgCl_2 /0.1 mM Na_2EDTA /10 $\mu\text{g/ml}$ BSA/2 mM ATP/0.1 mM [^{35}S]methionine (specific radioactivity 15000 c.p.m./pmol)/excess MetRS/0.25 μM tRNA. The formylation reaction was allowed to proceed for 10 min as described in the Materials and methods section. Relative rates were calculated by dividing by the wild-type value and multiplying by 1000. NA, not active.

MTF	Rate (nmol/mg per min)	Relative rate
Wild-type	1230.0	1000.0
V31A	726.0	588.0
K37A	351.0	285.0
R41A	2.6	2.11
N107A	25.5	20.7
H109A	0.3	0.21
D145A	NA	—
R41K	8.8	7.11
H109R	0.2	0.14
H109K	NA	—
D145E	0.3	0.25
D145N	1.4	1.14

with that obtained for the wild-type enzyme (Table 2). The tRNA affinity of the enzyme was reduced by about 3-fold when Arg-41 was changed to Ala. Replacement of Arg-41, Asn-107 and His-109 with Ala reduced the efficiency of catalysis by 250-, 8.5- and 19600-fold, respectively. This was primarily due to a reduction in the catalytic constants of the mutant enzymes. For the R41A mutant, the small increase in K_m also contributed to the marked reduction in the k_{cat}/K_m . The kinetic parameters for the D145A mutant could not be determined since it was essentially inactive in formylation. However, replacement of Asp-145 with Glu or Asn increased the K_m by about 3–4-fold. In comparison with the wild-type enzyme, the k_{cat} values for the D145E and D145N mutants were reduced by about 11300- and 1826-fold, respectively. The efficiency of catalysis of the H109R mutant was reduced by about 17027-fold compared with that of the wild-type enzyme. This was caused by a dramatic reduction in the catalytic constant. Mutation of Arg-41 to Lys increased the K_m by about 5-fold, and decreased the catalytic efficiency about 78-fold. The catalytic efficiency of the R41K mutant enzyme was about 3-fold higher than that of the R41A mutant.

Table 3 Measurement of the affinity of wild-type and mutant MTFs for fTHF using substrate-induced intrinsic fluorescence quenching

The analysis was performed in 20 mM Tris/HCl (pH 7.5), containing 150 mM KCl/7 mM MgCl_2 /0.1% Tween 80/50 $\mu\text{g/ml}$ MTF/0–15 μM fTHF. Lineweaver–Burk and Hanes–Wolf plots were used to calculate K_d . Relative ratios were calculated by dividing K_d values by the wild-type value and multiplying by 100.

MTF	K_d (μM)	Relative ratio
Wild-type	4.25 ± 0.33	100.0
K37A	3.08 ± 0.17	72.5
R41A	8.64 ± 2.1	203.0
R41K	3.13 ± 0.93	73.6
N107A	8.61 ± 1.11	203.0
H109A	10.30 ± 4.95	243.0
H109R	5.09 ± 3.52	120.0
H109K	4.45 ± 1.36	105.0
D145A	3.18 ± 0.48	74.8
D145E	3.34 ± 1.28	78.6
D145N	6.45 ± 1.17	152.0

Analysis of fTHF binding by intrinsic fluorescence quenching

The intrinsic fluorescence of the *E. coli* MTF has been shown to be quenched by fTHF binding [21]. This quenching was used to show that the dissociation constant, K_d , of the *E. coli* MTF for fTHF was 1 μM [21]. Thus, this method was used to determine whether the reduction in catalysis of the mutant enzymes was due to an effect on fTHF binding (Table 3). The K_d for the *H. influenzae* MTF for binding fTHF was 4.25 ± 0.33 μM , which is comparable with that obtained for the *E. coli* MTF (K_d 4.45 μM). The K_d values obtained for the mutants are shown in Table 3. None of the mutations had large effects on the affinity of MTF for fTHF. However, a 2-fold decrease in affinity was observed for the R41A, N107A and H109A mutant enzymes.

DISCUSSION

MTF belongs to the group-transfer class of enzymes. The basic reaction that this enzyme catalyses is transfer of the formyl group of fTHF to the amino moiety of methionine attached to the tRNA, removal of an amino proton of methionine and protonation of N^{10} of the tetrahydrofolate product. However, it is not

Table 2 Steady-state kinetic parameters of wild-type and mutant MTFs for formylation of the initiator tRNA

Lineweaver–Burk and Hanes–Wolf plots were used to calculate K_m and V_{max} values. The aminoacylation reaction mixture was as in Table 1, except with 0.25–2.0 μM tRNA. The formylation reaction was allowed to proceed for 10 min, as described in the Materials and methods section. Relative k_{cat}/K_m values were calculated by dividing by the wild-type k_{cat}/K_m and multiplying by 1000.

MTF	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	Relative k_{cat}/K_m
Wild-type	1.12 ± 0.6	11.29 ± 4.0	10.08	1000.0
K37A	1.14 ± 0.13	5.62 ± 1.13	4.93	490.0
R41A	3.36 ± 0.73	0.14 ± 0.01	0.04	4.13
R41K	5.20 ± 1.2	0.67 ± 0.14	0.13	12.7
N107A	1.28 ± 0.89	1.51 ± 0.59	1.18	120.0
H109A	1.81 ± 0.4	$(9.29 \pm 2.0) \times 10^{-4}$	5.13×10^{-4}	0.05
H109R	1.40 ± 0.69	$(8.29 \pm 1.36) \times 10^{-4}$	5.92×10^{-4}	0.06
D145N	4.22 ± 1.12	$(2.33 \pm 0.65) \times 10^{-2}$	5.52×10^{-3}	0.55
D145E	3.26 ± 1.61	$(1.03 \pm 0.43) \times 10^{-3}$	3.16×10^{-4}	0.03

known whether this enzyme uses a ping-pong or a sequential mechanism. GARF, an enzyme that catalyses formylation of glycylamide ribonucleotide using the same reactions as MTF, employs a sequential random mechanism [24]. Furthermore, mutational analysis and elucidation of the three-dimensional structure of GARF complexed with an analogue of fTHF (5-deaza-5,6,7,8-tetrahydrofolate) and glycylamide ribonucleotide showed that Asp-144, His-108 and Asn-106 participate in the catalytic step [16,25]. These residues are also found in various eubacterial and mitochondrial MTFs. Like GARF, mutation of Asn-107, His-109 or Asp-145 in the *H. influenzae* enzyme affected catalysis. Thus it is possible that MTF uses a catalytic mechanism that is similar to GARF. However, there appears to be some mechanistic differences between MTF and GARF. For instance, mutation of Asn-107 in MTF to Ala reduced the k_{cat} by about 8-fold, whereas in GARF, changing Asn-106 to a variety of residues including Ala dramatically affected the activity of the enzyme [25].

Mutations of Asn-107 and His-109 in the *H. influenzae* MTF affected k_{cat} but not fTHF or tRNA binding (Tables 2 and 3). Although mutants of the Asp-145 residue showed a modest decrease in the affinity for the tRNA substrate, the primary defect was in catalysis. The reduction in k_{cat} observed for the Asn-107 → Ala mutant was about 8-fold. In contrast, k_{cat} was profoundly reduced when His-109 was changed to Ala or Lys, and Asp-145 was mutated to Asn or Glu. Computer modelling studies using the three-dimensional co-ordinates of the *E. coli* enzyme showed that an fTHF analogue can be stably accommodated in the crevice formed by β -strand 5 containing Asn-107 and His-109, and loop 2 containing Asp-145 [15]. In addition, the side chains of the residues adopted a conformation similar to those observed in the co-crystal structure of GARF and 5-deaza-5,6,7,8-tetrahydrofolate. Taken together, these findings suggest that Asn-107, His-109 and Asp-145 in MTF may be catalytic residues. However, His-109 and Asp-145 play a more critical role than Asn-107.

The functions of Asn-107, His-109 and Asp-145 of MTF in catalysis are not understood. Studies of GARF have suggested that Asp-144 facilitates removal of the amino proton of glycylamide ribonucleotide, and protonation of N^{10} of the tetrahydrofolate substrate [25]. This is accomplished via a water molecule, which is H-bonded to the carboxylate of Asp, N^{10} of fTHF and the amino group of glycylamide ribonucleotide. The His-108 residue is thought to stabilize the oxyanion of the tetrahedral transition-state intermediate. Based on these studies, it is possible that the roles of Asp-145 and His-109 of MTF are similar to those of His-108 and Asp-144 of GARF.

Mutational analyses of the *E. coli* initiator tRNA *in vitro* and *in vivo* indicated that the presence of unpaired bases between nucleotides 1 and 72 of the acceptor stem is crucial for MTF recognition of the initiator tRNA [6–8]. NMR analysis of microhelices of the acceptor stem of the wild-type tRNA, and of a mutant tRNA that is a poor substrate for MTF because of the presence of a base pair between nucleotides 1 and 72, showed that the enzyme also prefers a tRNA structure in which the 3' end of the acceptor arm is folded back towards the 5' end of the RNA [26]. However, the significance of the two unpaired bases and the unique structure of the acceptor arm of the tRNA in formylation is not known. NMR analysis of the initiator tRNA indicated that bases of the acceptor stem are more accessible to the solvent when complexed to MTF [27]. This observation led to the suggestion that MTF alters the structure of the acceptor stem in order to position the methionyl moiety in the catalytic pocket. This is facilitated by the presence of the two unpaired bases between nucleotides 1 and 72 at the end of the acceptor stem.

Loop 1, located above the active site of MTF, is thought to aid in altering the structure of the acceptor stem by interacting with the formylation determinant. This notion is based on studies showing that trypsin cleavage of loop 1 is protected by the initiator tRNA but not the elongator tRNA [15]. This possibility was further supported by the finding that changing Gly-41 of loop 1 in the *E. coli* enzyme to Lys or Gln rescued a formylation-defective mutant initiator tRNA with a base pair between nucleotides 1 and 72 [17]. Loop 1 contains several basic residues that are found in various eubacterial MTFs (Figure 1). Therefore, we investigated the effect of Ala replacement of two residues in this loop, Lys-37 and Arg-41, on the activity of the *H. influenzae* MTF. The mutational analysis showed that alterations in Arg-41 and Lys-37 significantly affected MTF activity (Table 1). Determination of the formylation kinetic parameters indicated that the k_{cat} of the R41A mutant was reduced by 80-fold, whereas that of the K37A mutant was only modestly affected (Table 2). These results suggest that Arg-41, but not Lys-37, is playing an essential role. The tRNA affinity of the R41A mutant was also reduced by about 3-fold (Table 2). Furthermore, changing Arg-41 to Lys resulted in a 17-fold reduction in k_{cat} and a 5-fold increase in K_m . These findings imply that Arg-41 is most probably participating in tRNA binding rather than catalysis. The location of loop 1 outside of the putative catalytic pocket is consistent with this interpretation. It is possible that Arg-41 interacts with the formylation determinants of the initiator tRNA to position the end of the acceptor arm into the catalytic pocket. The fact that mutation of Arg-41 did not abolish tRNA binding completely may indicate that it is one of several residues required for interaction with the acceptor stem. Alternatively, the observed K_m values of the mutant enzymes could be an overestimation of the true value due to non-specific binding of the C-terminal domain of MTF [15].

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