Decoding the Logic of the tRNA Regiospecificity of Nonribosomal FemX_{wv} Aminoacyl Transferase**

Matthieu Fonvielle, Maryline Chemama, Maxime Lecerf, Régis Villet, Patricia Busca, Ahmed Bouhss, Mélanie Ethève-Quelquejeu,* and Michel Arthur*

Aminoacyl-tRNAs are key intermediates in protein synthesis. They act as adapters between the codons of mRNA and the growing polypeptide chain in the ribosome.^[1] The vicinal hydroxy groups at the 2'- and 3'-positions of the terminal nucleotide (A⁷⁶) of tRNA have pivotal roles in the function of these molecules. The tRNA molecules are esterified by aminoacyl-tRNA synthetases,^[2] which catalyze the transfer of a specific aminoacyl residue from an adenylate to the 2'- or 3'-hydroxy group of A⁷⁶ (Scheme 1). Transesterification between the 2'- and 3'-positions occurs in the absence of an enzyme with a rate and thermodynamic equilibrium of the order of 5 s⁻¹ and 1, respectively.^[3] The A site of the ribosome is specific for the 3'-O-aminoacyl isomer, and the 3' linkage to the tRNA is conserved in the product of the peptidyl-transfer reaction.^[4] The 2'-hydroxy group of the peptidyl-tRNA is thought to assist catalysis of this reaction.^[5]

Besides their role in protein synthesis, aminoacyl-tRNAs participate in various metabolic pathways,^[6] such as the synthesis of cyclodipeptides^[7] or the aminoacylation of proteins^[8] and membrane phosphatidylglycerol.^[9] Transferases of the Fem family catalyze the incorporation of amino acids into peptidoglycan precursors to form a side chain that contains the amino group used as an acyl acceptor in the final cross-linking step of cell-wall synthesis^[10] (Scheme 1). The specificity of these enzymes is essential for bacteria, since

	[*]	Dr. M. Fonvielle, ^[+] M. Lecerf, Dr. R. Villet, Dr. M. Arthur Laboratoire de Recherche Moléculaire sur les Antibiotiques Centre de Recherche des Cordeliers, Equipe 12, UMR S 872 INSERM, Université Pierre et Marie Curie—Paris 6 and Université Paris Descartes 15, rue de l'Ecole de Médecine, 75006 Paris (France) Fax: (+33) 1-4325-6812 E-mail: michel.arthur@crc.jussieu.fr
		Dr. M. Chemama, ^[+] Dr. P. Busca, Dr. M. Ethève-Quelquejeu Institut Parisien de Chimie Moléculaire, CNRS UMR 7201 Université Pierre et Marie Curie—Paris 6 4 place Jussieu, 75005 Paris (France)
		Fax: (+ 33) 1-4427-5504 E-mail: melanie.etheve@upmc.fr
		Dr. A. Bouhss Laboratoire des Enveloppes Bactériennes et Antibiotiques Institut de Biochimie et de Biophysique Moléculaire et Cellulaire UMR 8619, CNRS, Université Paris-Sud, 91405 Orsay (France)
	[*]	These authors contributed equally to this work.
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misincorporated amino acids can act as chain terminators and block peptidoglycan polymerization.^[10] Because of their key role in peptidoglycan metabolism, Fem transferases are considered attractive targets for the development of novel antibiotics.^[10]

We previously used chemical acylation^[11] of RNA helices with natural and nonproteinogenic amino acids to gain insight into the specificity of FemX_{Wv} of *Weissella viridescens*,^[12,13] a model enzyme of the Fem family.^[14] A combination of modifications in the RNA and aminoacyl moieties of the substrate revealed that unfavorable interactions of FemX_{Wv} with the acceptor arm of tRNA^{Gly} and with L-Ser or larger residues quantitatively account for the preferential transfer of L-Ala observed with complete aminoacyl-tRNAs.^[12,13] The main FemX_{Wv} identity determinant of Ala-tRNA^{Ala} was found to be the penultimate base pair, G²-C⁷¹, which is replaced with C²-G⁷¹ in tRNA^{Gly} isoacceptors.^[12,13]

In this study, we synthesized nonisomerizable mimics of Ala-tRNA^{Ala} that contained 2'-deoxyadenosine or 3'-deoxyadenosine to lock the amino acid in the 3'- and 2'-position, respectively (Scheme 2). We also synthesized nonisomerizable aminoacyl-tRNA analogues by replacing the ester bond connecting the amino acid residue to the terminal nucleotide with a triazole ring^[15] (Scheme 3). We synthesized these molecules to determine the regiospecificity of FemX_{wv} for the 3' and 2' isomers and to evaluate the role of the adjacent hydroxy group in the transfer reaction.

Ala-tRNA^{Ala} analogues containing a terminal 2'- or 3'deoxyadenosine residue and a 24 nucleotide (nt) helix mimicking the acceptor arm of the tRNA (Figure 1) were obtained by semisynthesis (see the Supporting Information and Scheme 2) and assayed as substrates of FemX_{wy}



Figure 1. Analogues of Ala-tRNA^{Ala} contained a 24 nt helix to mimic the acceptor arm of the tRNA (helix^{Ala}).



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Scheme 1. Participation of Ala-tRNA^{Ala} regioisomers in protein and peptidoglycan synthesis. Highlighted zones show reactions catalyzed by the enzymes indicated in blue. FemX_{wv} transfers an alanyl residue from Ala-tRNA^{Ala} to the side chain of L-Lys in the peptidoglycan precursor UDP-*N*-acetylmuramyl pentapeptide. This reaction initiates synthesis of the L-Ala-L-Ser-L-Ala side chain, which is completed by other Fem transferases.^[10] R stands for glycan chains composed of alternating $\beta(1,4)$ -*N*-acetylglucosamine and *N*-acetylmuramic acid. UDP=uridine diphosphate.

(Figure 2). Ala was transferred from the 2'-position of compound **A** with a turnover number of $(0.19 \pm 0.03) \text{ min}^{-1}$. FemX_{wv} catalyzed aminoacyl transfer only from this position, since compound **B** was not a substrate. The 3'-hydroxy group was not essential for catalysis, although a 240-fold decrease in the turnover number was observed in comparison with **C**, which contained a terminal adenosine residue. Since alanyl-tRNA synthetase (AlaRS) aminoacylates the 3'-position of tRNA^{Ala}, transacylation of Ala must occur prior to transfer to the peptidoglycan precursor.

The Ala-helix^{Ala} derivatives containing a terminal 3'- or 2'deoxyadenosine residue were also tested as inhibitors of FemX_{Wv} in the presence of the complete Ala-tRNA^{Ala} (76 nt) as the substrate (Figure 2). Compound **B** (2'-deoxy), which was not a substrate of FemX_{Wv}, inhibited the reaction with an IC₅₀ value of (0.80 ± 0.03) µM. A threefold lower IC₅₀ value [(0.25 ± 0.04) µM] was observed for **A** (3'-deoxy), which was used inefficiently as a substrate by FemX_{wv}. Inhibition of the transferase activity of $Fem X_{Wv}$ by **B** indicates that the presence of Ala at the 3'-position did not prevent binding of the Ala-tRNA^{Ala} analogue to the enzyme, although aminoacyl transfer was not catalyzed from this position. Thus, FemX_{wy} can recruit the 3'-O-aminoacyl regioisomer produced by AlaRS. Transacylation within the active site is predicted to provide efficient access to the 2'-O-aminoacyl regioisomer required for Ala transfer to the peptidoglycan precursor. FemX_{Wy}catalyzed transacylation is also predicted to prevent inhibition by the 3'-O-aminoacyl regioisomer.

To investigate the inhibition of FemX_{wv} by the product of the reaction, the deacylated tRNA^{Ala}, we exploited the capacity of the enzyme to transfer seryl residues from Ser-tRNA^{Ser} (see Figure 2f in the Supporting Information). Compound D, the unacylated helix^{Ala}, inhibited this reaction moderately, with an apparent IC₅₀ value of (89 \pm 4) µM (Figure 3): a 360-fold difference in comparison to inhibition by regioisomer A, which contained a 2'-O-Ala ester [($0.25 \pm$ Figure 2]. 0.04) µм; Compound Е (Scheme 2), which contained a terminal 2'deoxyadenosine residue, inhibited FemX_{Wy} with an IC₅₀ value of (5.5 ± 0.5) µM. Thus, a free 2'-hydroxy group in the terminal nucleotide was critical for release of the tRNA^{Ala} product following catalysis. This feature is also expected to contribute to the in vivo efficiency of the enzyme by preventing inhibition by unacylated tRNA^{Ala}.

To further analyze the regiospecificity of $Fem X_{Wv}$, we synthesized two stable regioisomers of Ala-tRNA^{Ala} containing a triazole ring to mimic the ester linkage (Scheme 3). These analogues, **F** and **G**,

which retained a vicinal hydroxy group but could not undergo regioisomerization, inhibited $Fem X_{Wv}$ with similar IC₅₀ values $[(2.3\pm0.1)$ and $(2.4\pm0.4) \mu M$, respectively; Figure 3]. This result was expected, since regioisomers A and B inhibited $Fem X_{Wv}$ (Figure 2). Together, these results indicate that the 240-fold decrease observed upon removal of the 3'-hydroxy group from the substrate (compare A and C in Figure 2) affects the enzyme-catalyzed chemical reaction rather than substrate binding. The 3'-hydroxy group is therefore likely to play a role in substrate-assisted catalysis. A model proposing its participation in proton shuttling during catalysis is presented in Scheme 1. Asp108 of $Fem X_{Wv}$ has been proposed to act as a general base;^[14] however, this residue is not located within the enzyme active site.^[16] Substrate-assisted catalysis might account for the inability to identify any catalytic residue.^[16]







Scheme 2. a) 1. α-acetoxyisobutyryl bromide, then Dowex OH⁻, 1 h; 2. TBSCl, pyridine, 3 h, RT; 3. LiEt₃BH, THF, RT, 3 h; 4. BzCl, pyridine, RT, 3 h; 5. TBAF, THF, AcOH, RT, 3 h; b) 1. Ac-dC-CE phosphoramidite, tetrazole, CH₂Cl₂, RT, 1 h; 2. l₂, RT, 30 min; 3. TCA, CH₂Cl₂, RT, 30 min; 4. bis (2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH₂Cl₂, RT, 1 h; 5. l₂, RT, 30 min; 6. CH₃NH₂, RT, 24 h; 7. Dowex ionexchange beads in the tetrabutylammonium form; c) *N*-(4-pentenoyl)-*S*-alanine cyanomethyl ester, DMF, CH₃CN, RT, 24 h; d) 1. 22 nt RNA helix^{Ala}, T4 RNA ligase; 2. l₂, THF/H₂O; e) 1. TBSCl, pyridine, RT, 4 h; 2. BzCl, pyridine, RT, 8 h; 3. TBAF, THF, AcOH, RT, 4 h. Ac-dC-CE phosphoramidite = 5'-dimethoxytrityl-*N*-acetyl-2'-deoxycytidine-3'-(2cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, Bz = benzoyl, DMF = *N*,*N*-dimethylformamide, pdC = 5'-monophosphate-2'-deoxycytidine, TBAF = tetrabutylammonium fluoride, TBS = *tert*-butyldimethylsilyl, TCA = trichloroacetic acid.



Scheme 3. a) NaBH₄, EtOH/H₂O, 30 min; b) TfCl, DMAP, CH₂Cl₂, 1 h; c) NaN₃, DMF, 48 h; d) TFA, H₂O/THF, 0°C, 24 h; e) *tert*-butylbut-3-yn-2-ylcarbamate, CuSO₄, sodium ascorbate, THF, H₂O, RT, 24 h; f) 1. Ac-dC-CE phosphoramidite, tetrazole, CH₂Cl₂, RT, 1 h; 2. I₂, 30 min, RT; 3. TCA, CH₂Cl₂, RT, 30 min; 4. bis(2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH₂Cl₂, RT, 1 h; 5. I₂, RT, 30 min; 6. CH₃NH₂, RT, 24 h; 7. HCl (6N)/THF/CH₃OH, 24 h; g) 1. 22 nt RNA helix^{Ala}, T4 RNA ligase; 2. I₂, THF/H₂O. DMAP=4-dimethylaminopyridine, Tf=trifluoromethanesulfonyl, TFA=trifluoroacetic acid.

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Figure 2. Analogues of Ala-tRNA^{Ala} containing a terminal 3'-deoxyadenosine (compound **A**), 2'-deoxyadenosine (compound **B**), or adenosine residue (compound **C**) were assayed as substrates (top left graph) and inhibitors (top right graph) of FemX_{WV}. The magnification of different ranges of FemX_{WV} concentrations (bottom) shows the linear relationship between the concentration of FemX_{WV} and the concentration of the UDP-MurNAc hexapeptide produced by the aminoacyl-transfer reaction. These ranges were used to calculate the turnover numbers for compounds **A** and **C**. Compound **B** was not a substrate. Compound **C** could not be assayed as an inhibitor, since it was used efficiently by FemX_{WV} as a substrate. N.A. = not applicable.

The 2'-hydroxy group of peptidyl-tRNA at the P site of the ribosome was reported to be essential, since the use of a 2'-deoxy analogue resulted in a 10⁶-fold decrease in the rate of peptide-bond formation.^[5,17] The critical role of the 2'-hydroxy group was proposed to reflect substrate-assisted catalysis in the peptidyltransferase center of the ribosome.^[5,17] According to this proposal, the ribosome provides a template for entropic activation rather than groups for acid–base catalysis.^[18] However, this model remains controversial, since engineered suppressor tRNAs containing a 2'-deoxyadeno-sine^[19] or diacylated adenosine^[20] residue were used efficiently in in vitro transcription–translation coupled systems.

In conclusion, analysis of the tRNA regiospecificity of $FemX_{Wv}$ revealed several features that are relevant to the physiological role of the enzyme. FemX_{Wv} and the ribosome compete for the same pool of aminoacyl-tRNAs (Scheme 1). Since AlaRS acylates the 3'-hydroxy group of tRNA^{Ala}, whereas FemX_{Wv} catalyzes transfer from the 2'-position, the ability of FemX_{Wv} to bind both regioisomers of Ala-tRNA^{Ala} may provide adequate access to activated alanine for



Figure 3. Inhibition of FemX_{Wv} by helix^{Ala} containing unacylated adenosine (compound **D**), 2'-deoxyadenosine (compound **E**), or a 1,2,3-triazole ring linked to the 2'- (compound **F**) or 3'-position (compound **G**) of adenosine.

peptidoglycan synthesis. Efficient binding of the two regioisomers to FemX_{wv} implies that transacylation occurs within the active site to prevent inhibition by the 3'-Oaminoacyl regioisomer. We previously established that the specificity of $Fem X_{Wv}$ for Ala-tRNA $^{\rm Ala}$ does not depend upon an interaction with the methyl side chain of Ala.^[13] In agreement with this conclusion, we have shown herein that $Fem X_{Wv}$ is inhibited to a similar extent by the two AlatRNA^{Ala} regioisomers. In spite of the absence of an interaction between the enzyme and the side chain of Ala, Fem X_{W_v} is not strongly inhibited by the deacylated product of the aminoacyl-transfer reaction. The free 2'-hydroxy group generated by this reaction appears to be critical both in promoting the release of the product of the reaction and in preventing inhibition by unacylated tRNA molecules present in the cytoplasm.

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