Peptidyltransferases

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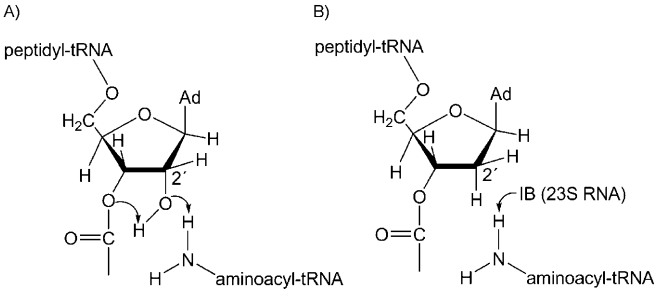
Peptide-Bond Synthesis on the Ribosome: No Free Vicinal Hydroxy Group Required on the Terminal Ribose Residue of Peptidyl-tRNA**

*Miriam Koch, Yiwei Huang, and Mathias Sprinzl**

The synthesis of a peptide bond is a reaction of central importance for biology. Despite remarkable progress in the last decade in the elucidation of ribosome structure,[1] the catalytic mechanism of this reaction, which takes place in the peptidyltransferase center of the ribosome, remains unclear.[2] According to the currently accepted view, peptide transfer is an entropically driven reaction[3] in the protein-free active site formed by 23S RNA and bound 3′ tails of peptidyl- and aminoacyl-tRNA molecules.[4] Peptide-bond formation occurs through nucleophilic attack of the α-amino group of aminoacyl-tRNA at the carbonyl group of peptidyl-tRNA with the formation of a tetragonal transition state. In this process, a proton is transferred from the α-amino group to the 3′-OH group of the peptidyl-tRNA. Free vicinal OH groups at the 3′ end of the aminoacyl- and peptidyl-tRNA are potential proton donors.[5] Therefore, positional isomers of aminoacyl- and peptidyl-tRNA molecules have been studied extensively with respect to the attachment of the acyl residue to the 2′- or 3′-OH group and requirements for the free vicinal OH groups during translation.[6–8]

For peptide transfer by a peptidyltranferase to occur, both the aminoacyl residue in the A site and the peptidyl residue in the P site have to be attached to the 3′-OH group of the terminal adenosine residue. The absence of the vicinal 2′-OH group in the aminoacyl-tRNA does not impair peptide transfer; however, the role of the 2′-OH group in the peptidyl-tRNA remains unclear. Peptidyltransferase activity measured by in vitro assays with tRNA fragments or puromycin as models for aminoacyl- and peptidyl-tRNA, or in assays in which short oligonucleotides were used as mRNA substitutes (again not a complete tRNA system), was inhibited by the absence of the 2′-OH peptidyl analogues bound in the P site.[5,9,10] However, in assays in which complete peptidyl-tRNA-2′dA and long mRNA were used, the peptidyltransferase tolerated the absence of the 2′-OH group.[11]

To resolve this discrepancy, we tested the activity of suppressor $tRNA^{Ser(CUA)}$-2′dA in vitro by translating a complete mRNA of esterase 2 from *Alicyclobacillus acidocaldarius* with a nonsense UAG codon 155 and RF2-dependent termination codons (RF2 = release factor 2). Codon 155 codes for a serine residue, an essential member of the catalytic triad, in esterase 2.[12] Only the suppression of UAG-155 by Ser-$tRNA^{Ser(CUA)}$ enables the synthesis of the active esterase. In the absence of Ser-$tRNA^{Ser(CUA)}$, premature termination, frame shifting, or suppression with endogenous aminoacyl-tRNA occurs.[13] Premature termination can be suppressed almost completely by the removal of release factor 1 (RF1) from the in vitro translation mixture.[13] This assay, previously described for $tRNA^{Ser(CUA)}$-A (aminoacyl-$tRNA^{Ser(CUA)}$), provides a means to test the activity of $tRNA^{Ser(CUA)}$-2′dA in the elongation cycle. If the mechanism for "substrate-assisted catalysis"[10] involving the 2′-OH group during peptide transfer (Scheme 1 A) is correct, the replacement of $tRNA^{Ser(CUA)}$-A with $tRNA^{Ser(CUA)}$-2′dA should prevent the in vitro synthesis of esterase 2.



Scheme 1. A) Suggested mechanism for the participation of the 2′OH group of the 3′-terminal adenosine residue (Ad = adenine residue) of peptidyl-tRNA in peptidyl transfer.[10] B) The activity of peptidyl-tRNA-2′dA in the peptidyltransferase reaction implies an alternative mechanism.

$tRNA^{Ser(CUA)}$-2′dA was prepared by exchanging the 3′-terminal adenosine residue for a 2′-deoxyadenosine residue in the presence of pyrophosphate under the catalysis of ATP-(CTP)tRNA nucleotidyltransferase (NTase; ATP = adenosine-5′-triphosphate, CTP = cytidine triphosphate).[14] To ensure the full occupancy of the 3′ terminus by 2′-deoxyadenosine, the product of the exchange reaction was treated with excess periodate to oxidize any tRNA that terminated with ribose. tRNA-2′dA is resistant to periodate oxidation.[15] The resulting $tRNA^{Ser(CUA)}$-2′dA was analyzed by electrophoresis on a boronate-containing polyacrylamide gel. No residual $tRNA^{Ser(CUA)}$-A was detected in the reaction product after periodate treatment (see the Supporting Information). The ability of $tRNA^{Ser(CUA)}$-A to undergo aminoacylation after treatment with periodate was more than 100 times lower than that of $tRNA^{Ser(CUA)}$-2′dA after treatment with periodate

[*] M. Koch, Dr. Y. Huang, Prof. Dr. M. Sprinzl
Laboratorium für Biochemie
Universität Bayreuth
Universitätsstrasse 30, 95440 Bayreuth (Germany)
Fax: (+49) 921-55-2432
E-mail: mathias.sprinzl@uni-bayreuth.de

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under identical conditions. To eliminate the possibility that $tRNA^{Ser(CUA)}$-A is formed from $tRNA^{Ser(CUA)}$-2′dA during in vitro translation in the presence of soluble *Escherichia coli* enzymes, we performed control experiments in which $tRNA^{Ser(CUA)}$-2′dA was incubated with the translation mixture (S30 extract) or with purified *E. coli* NTase. We did not detect any formation of $tRNA^{Ser(CUA)}$-A from $tRNA^{Ser(CUA)}$-2′dA, either by electrophoresis on boronate gels or in aminoacylation assays.

Seryl-tRNA synthetase aminoacylates $tRNA^{Ser}$ at the 3′-OH group.[15] Thus, the sample of $tRNA^{Ser(CUA)}$-2′dA used in this investigation could be aminoacylated in the translation mixture. Total in vitro polypeptide synthesis was monitored by measuring [^{14}C]leucine incorporation into polypeptides. The degree of [^{14}C]leucine incorporation was determined by scintillation counting of proteins that precipitated in hot trichloroacetic acid, or, alternatively, by autoradiography after SDS-PAGE. In parallel, esterase 2 activity in each sample was determined by activity staining after electrophoretic separation on SDS–polyacrylamide gels[16] or by spectroscopic monitoring of *p*-nitrophenyl acetate hydrolysis.[17]

Proteins were synthesized in the presence of different amounts of $tRNA^{Ser(CUA)}$-A (Figure 1). After activity staining, the gels were stained additionally with Coomassie blue to visualize all proteins, including endogenous *E. coli* proteins. The amount of active esterase (magenta bands) depends on the concentration of added suppressor tRNA. No active esterase is visible in lane 1 (Figure 1 A), although [^{14}C]leucine was incorporated into the protein with the molecular mass of esterase 2 (Figure 1 B). Suppression can evidently be caused by some endogenous tRNA present in the *E. coli* S30 extract; however, in that case the essential serine-155 residue is missing, and the polypeptide is void of esterase 2 activity.[13] The presence of antibodies against RF1 blocks termination at UAG and increases the probability of suppression by natural suppressor tRNAs, such as Tyr-$tRNA^{Tyr}$ (codon UAY). This effect is the most probable explanation for synthesis of the inactive full-length protein. Ser-$tRNA^{Ser(CUA)}$ competes with natural suppressor tRNA for UAG codons. Therefore, an increase in the concentration of suppressor $tRNA^{Ser(CUA)}$-A (Figure 1, lines 2–5) results in increasing amounts of active esterase 2 and decreasing amounts of the inactive polypeptide. However, a large increase in the concentration of $tRNA^{Ser(CUA)}$-A again results in the formation of the inactive protein.[13]

When $tRNA^{Ser(CUA)}$-2′dA was used instead of $tRNA^{Ser(CUA)}$-A in analogous experiments, suppression of the UAG-155 codon took place, and the active esterase was synthesized (Figure 2, lanes 2–5). The amount of active esterase 2 synthesized in vitro is dependent on the concentration of $tRNA^{Ser(CUA)}$-2′dA. Comparison of the results in Figures 1 and 2 shows that $tRNA^{Ser(CUA)}$-2′dA, like $tRNA^{Ser(CUA)}$-A, participates in all reactions in the peptide-elongation cycle on the ribosome. Identical results were observed in the two cases for the synthesis in the absence of suppressor $tRNA^{Ser(CUA)}$ (lane 1, Figures 1 and 2): Only inactive polypeptide of a length corresponding to the esterase 2 was produced (see above). The addition of

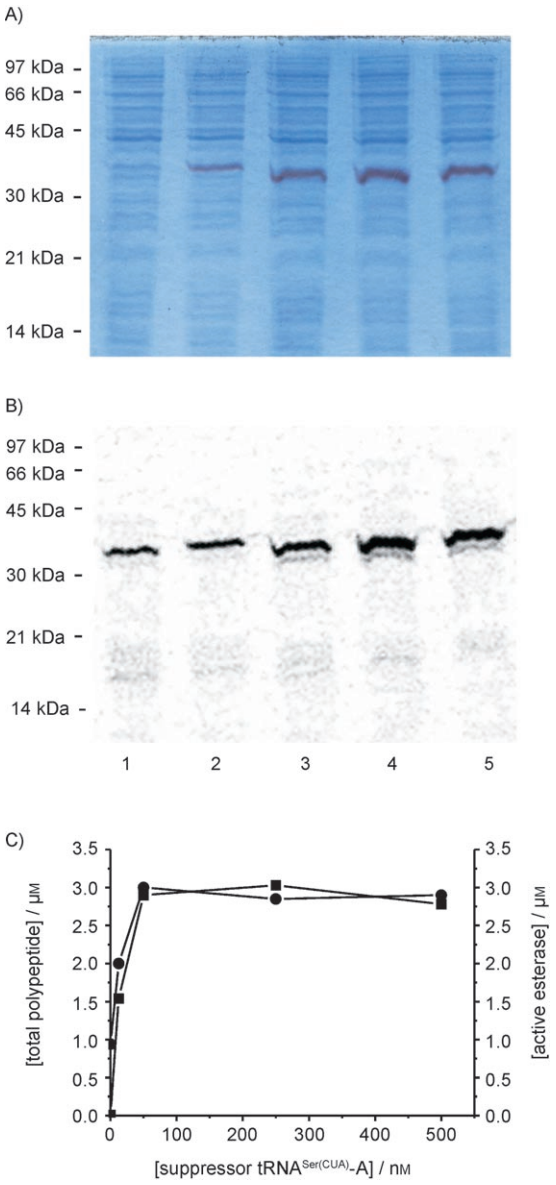

Figure 1. Suppression of the amber stop codon by $tRNA^{Ser(CUA)}$-A in the in vitro translation system programmed by the plasmid pIVEX-Est2-S155X. The translation assay was performed in the presence of RF1 antibodies (0.6 μg) and an increasing amount of suppressor $tRNA^{Ser(CUA)}$-A (lane 1: 0 nM, lane 2: 12.5 nM, lane 3: 50 nM, lane 4: 250 nM, lane 5: 500 nM). For radioactive labeling of the protein synthesized in vitro, [^{14}C]leucine (0.5 mM) was present in the translation assay. Aliquots withdrawn from the reaction mixture after 30 min were analyzed by SDS-PAGE. A) Electropherograms obtained by staining for esterase activity (magenta bands)[21] followed by staining with Coomassie blue (blue bands). B) Radioactive images of the [^{14}C]leucine-labeled total polypeptide. C) Yields for in vitro protein synthesis in the presence of $tRNA^{Ser(CUA)}$-A were determined by measuring [^{14}C]leucine incorporation into polypeptide (●) that could be precipitated from hot 10% aqueous trichloroacetic acid and by measuring the total activity of the active esterase in the samples (■). Further experimental details are provided in the Supporting Information.

$tRNA^{Ser(CUA)}$-2′dA led to the synthesis of active esterase 2 (with serine at position 155). At the same time, there was a

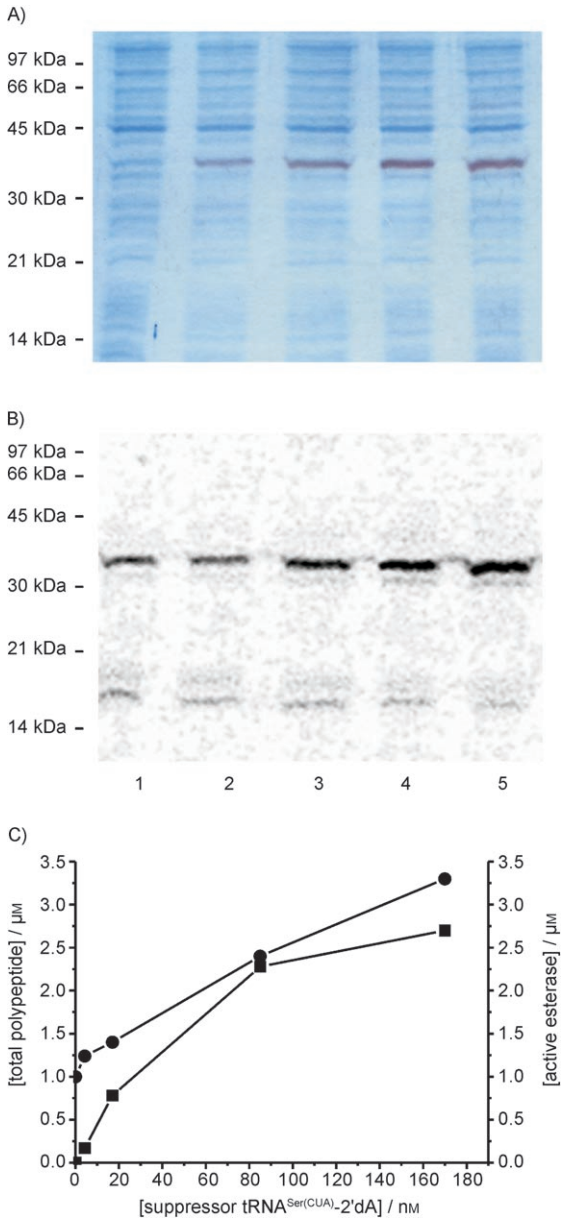

Figure 2. Suppression of the amber stop codon by tRNA$^{Ser(CUA)}$-2′dA in the in vitro translation system (lane 1: 0 nM, lane 2: 4.25 nM, lane 3: 17 nM, lane 4: 85 nM, lane 5: 170 nM). The experiments and presentation of the results are analogous to those for tRNA$^{Ser(CUA)}$-A; see the legend to Figure 1 for details.

decrease in the amount of inactive polypeptide formed (with an amino acid residue other than serine at position 155).[13] Quantitative data for the experiments shown in Figure 2B are presented in Figure 2C. As observed for tRNA$^{Ser(CUA)}$-A (Figure 1C), the ratio of total protein to active esterase 2 changes with the concentration of tRNA$^{Ser(CUA)}$-2′dA.[13] Under the given conditions, suppression of UAG-155 was most efficient at a tRNA$^{Ser(CUA)}$-2′dA concentration of about 85 nM (Figure 2C), whereas 50 nM was the optimal concentration in the case of tRNA$^{Ser(CUA)}$-A (Figure 1C).

The kinetics of polypeptide synthesis with respect to the total protein or to active esterase 2 were similar irrespective of whether tRNA$^{Ser(CUA)}$-A or tRNA$^{Ser(CUA)}$-2′dA was used (Figure 3). The maximal yield of active esterase 2 was reached in both experiments after approximately 30 min.

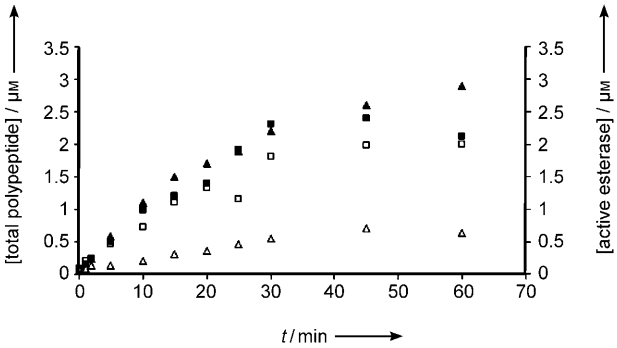

Figure 3. Kinetics of the in vitro synthesis of total polypeptide (squares) and active esterase 2 (triangles) in the presence of tRNA$^{Ser(CUA)}$-A (12.5 nM; filled symbols) or tRNA$^{Ser(CUA)}$-2′dA (17 nM; open symbols), as determined by [^{14}C]leucine incorporation and esterase 2 activity, respectively. The activity of esterase 2 in samples withdrawn at the indicated time intervals was monitored photometrically[22]. The specific activity of the formed esterase 2 was 152 μmol min^{-1} mg^{-1}. The degree of [^{14}C]leucine incorporation was determined for separately withdrawn aliquots by protein precipitation in hot trichloroacetic acid and scintillation counting. Experimental details are provided in the Supporting Information.

The results of the present investigation provide evidence that tRNA$^{Ser(CUA)}$-2′dA, which lacks a 2′-OH group on the 3′-terminal adenosine residue, can be utilized in all steps of the elongation cycle. Previously,[18] we used polyA as mRNA for the synthesis of polylysine in the presence of different tRNA^{Lys} analogues. We did not detect polylysine formation when Lys-tRNA^{Lys}-2′dA was used. However, Lys-tRNA^{Lys}-2′dA and *N*-Ac-Lys-tRNA^{Lys}-2′dA were found to be active in partial reactions as an acceptor and a donor, respectively, when the formation of Ac-Lys-Lys dipeptide was measured on ribosomes whose synthesis was programmed by polyA.[11] More recently, Weinger et al.[10] used a ribosomal fragment reaction[19] in which the release of fMet-Lys from P-site-bound fMet-Lys-tRNA^{Lys}-2′dA by puromycin was determined. They demonstrated clearly that the formation of fMet-Lys–puromycin is at least 10^6 times faster when fMet-Lys-tRNA^{Lys}-A is present in the ribosomal P site than when fMet-Lys-tRNA-2′dA occupies the P site. These results agreed with those of similar experiments performed earlier with a peptidyl-tRNA fragment bound to the P site and an aminoacyl-tRNA molecule bound to the A site.[5] An attractive hypothesis derived from these observations explained the mechanism of peptide transfer in the ribosomal peptidyltransferase in terms of "substrate-assisted catalysis", whereby the 2′-OH group of the adenosine residue in the P site functions as a proton shuttle to accept a proton from the incoming α-amino group and deliver it to the outgoing deacylated tRNA (Scheme 1A). However, this mechanism is not supported by the results of the current study (Figures 2 and 3). We have shown herein that the absence of the 2′-hydroxy group on the adenosine-76 residue of the peptidyl-

transferase does not render the peptidyltransferase inactive. Instead, peptide transfer still takes place at a reasonable rate.

In summary, when peptidyltransferase reactions were carried out with tRNA fragments or puromycin, a very strong (10^6-fold) inhibitory effect was observed in the absence of the 2′-OH group on the peptidyl substrate. We have shown herein that the same modification on the terminal adenosine residue has little effect when the CCA ends of both the donor and the acceptor tRNA molecules are a part of a complete tRNA structure, and the ribosomes translate a complete mRNA molecule. This result indicates the importance of the precise location of the CCA ends of the reacting tRNA molecules for efficient peptide transfer to occur. The whole body of tRNA, including anticodons and the CCA ends, contributes cooperatively to the correct placement of the reacting elements in the peptidyltransferase center.[20]

The present study also suggests that proton shuttling from the incoming α-amino group of the aminoacyl-tRNA to the 3′-OH group of the peptidyl-tRNA during peptide-bond formation does not involve the 2′-hydroxy group of the peptidyl-tRNA. Instead, it is possible that a nucleophile located on 23S RNA in the vicinity of the reacting tRNA partners acts as the proton shuttle (Scheme 1 B). This site of ribosomal RNA remains to be identified.

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[1] M. Selmer, C. M. Dunham, F. V. Murphy, A. Weixlbaumer, S. Petry, A. C. Kelley, J. R. Weir, V. Ramakrishnan, *Science* **2006**, *313*, 1935 – 1942.
[2] M. Beringer, M. V. Rodnina, *Mol. Cell* **2007**, *26*, 311 – 321.
[3] A. Sievers, M. Beringer, M. V. Rodnina, R. Wolfenden, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7897 – 7901.
[4] T. M. Schmeing, K. S. Huang, S. A. Strobel, T. A. Steitz, *Nature* **2005**, *438*, 520 – 524.
[5] K. Quiggle, G. Kumar, T. W. Ott, E. K. Ryu, S. Chladek, *Biochemistry* **1981**, *20*, 3480 – 3485.
[6] T. H. Fraser, A. Rich, *Proc. Natl. Acad. Sci. USA* **1973**, *70*, 2671 – 2675.
[7] S. M. Hecht, *Acc. Chem. Res.* **1977**, *10*, 239 – 245.
[8] S. Chládek, M. Sprinzl, *Angew. Chem.* **1985**, *97*, 1 – 31; *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 371 – 391.
[9] S. Dorner, C. Panuschka, W. Schmid, A. Barta, *Nucleic Acids Res.* **2003**, *31*, 6536 – 6542.
[10] J. S. Weinger, K. M. Parnell, S. Dorner, R. Green, S. A. Strobel, *Nat. Struct. Mol. Biol.* **2004**, *11*, 1101 – 1106.
[11] T. Wagner, F. Cramer, M. Sprinzl, *Biochemistry* **1982**, *21*, 1521 – 1529.
[12] G. De Simone, S. Galdiero, G. Manco, D. Lang, M. Rossi, C. Pedone, *J. Mol. Biol.* **2000**, *303*, 761 – 771.
[13] D. E. Agafonov, Y. Huang, M. Grote, M. Sprinzl, *FEBS Lett.* **2005**, *579*, 2156 – 2160.
[14] B. E. Nordin, P. Schimmel, *J. Biol. Chem.* **2002**, *277*, 20510 – 20517.
[15] M. Sprinzl, F. Cramer, *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 3049 – 3053.
[16] D. E. Agafonov, K. S. Rabe, M. Grote, Y. Huang, M. Sprinzl, *FEBS Lett.* **2005**, *579*, 2082 – 2086.
[17] G. Manco, L. Mandrich, M. Rossi, *J. Biol. Chem.* **2001**, *276*, 37482 – 37490.
[18] T. Wagner, M. Sprinzl, *Biochemistry* **1983**, *22*, 94 – 98.
[19] B. E. Maden, *Trends Biochem. Sci.* **2003**, *28*, 619 – 624.
[20] L. Cochella, R. Green, *Science* **2005**, *308*, 1178 – 1180.
[21] T. B. Higerd, J. Spizizen, *J. Bacteriol.* **1973**, *114*, 1184 – 1192.
[22] G. Manco, E. Giosue, S. D'Auria, P. Herman, G. Carrea, M. Rossi, *Arch. Biochem. Biophys.* **2000**, *373*, 182 – 192.

