J|A|C|S
COMMUNICATIONS

Published on Web 02/14/2006

Regiospecificity of the Peptidyl tRNA Ester within the Ribosomal P Site

Kevin S. Huang, Joshua S. Weinger, Ethan B. Butler, and Scott A. Strobel*

Department of Molecular Biophysics and Biochemistry, Yale University, 260 Whitney Avenue, New Haven, Connecticut 06520-8114

Received August 8, 2005; E-mail: scott.strobel@yale.edu

The ribosome utilizes two substrates during polypeptide synthesis: an aminoacyl tRNA in the A site and a peptidyl tRNA in the P site. Peptide bond formation involves aminolysis of the P site ester by the A-site amino acid. Both the peptide and amino acid are linked to their tRNAs through an ester bond to A76, the 3′-terminal residue of each tRNA. The reaction is expected to be regiospecific with regard to its tRNA substrates, yet the ester linkages between the tRNA and the amino acid or peptide are susceptible to isomerization between the O2′ and O3′ hydroxyls of the terminal A76 ribose sugar. The uncatalyzed rate of amino acid isomerization in aqueous solution is approximately 5 s^{-1}, and the thermodynamic equilibrium between the two isomers is approximately 1.[1] The reaction products are the same beginning with either an O2′- or O3′-linked mechanism, but the geometry of the nucleophile, the leaving group, and the oxyanion are substantially different for reaction of the two regioisomers.

The regiospecificity for the A-site substrate has been established as the O3′ isomer.[2−4] tRNA analogues that force the amino acid onto the O3′ position, such as puromycin (Pm) and 2′-deoxyadenosine (2′-dA), are active as peptide acceptors. Similar analogues that force the amino acid to the O2′ are inactive. The A-site product following peptidyl transfer is also O3′-linked, consistent with this regiospecificity.[5]

In contrast to the A site, the regiospecificity of the peptidyl tRNA in the P site has not been determined (Figure 1a).[2] An early report suggested that a tRNA, which had a 2′-dA substitution at position 76, was active as a peptide donor, while the O2′-linked 3′-dA76 substitution was inactive.[6] This led to the widely held conclusion that the P-site ester is O3′-linked (Figure 1a, left); however, all other studies on P-site substrates lacking either the 2′- or 3′-OH demonstrated that both are inactive as peptide donors.[7−9] Consequently, the functional regioisomer cannot be assigned from such studies. A cocrystal structure of a peptidyl tRNA fragment in the P-site and sparsomycin in the A site suggests that the ribosome binds preferentially to the O3′-linked regioisomer.[10] While this is consistent with reaction via the O3′-linked intermediate, it remains possible that the ribosome catalyzes peptide migration to the O2′ position in the course of the reaction, resulting in an O2′-linked transition state (Figure 1a, right). Potentially consistent with this possibility is the structure of the 50S ribosome in complex with the transition state inhibitor CCdApPm.[11] This compound includes a phosphoramidate to mimic the putative tetrahedral intermediate, but lacked a 2′-OH at the P-site A76.[12] In the cocrystal structure, the nonbridging phosphoramidate oxygen was closer to the C2′ carbon than would be possible if the 2′-OH were present. Such a conformation raises the possibility that the ribosome may prefer to bind a transition state with an O2′ linkage to the P-site tRNA. The ambiguous biochemical and structural data prompted us to explore an alternative approach to establish the regiospecificity of the reaction.

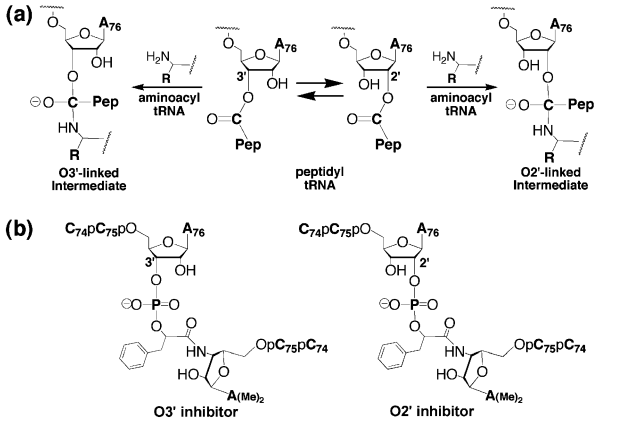

Figure 1. Two distinct pathways for the ribosome-catalyzed peptide bond formation. (a) Isomerization of the peptidyl ester on the terminal A76 of the P-site tRNA leads to two different intermediates linked via the O2′ (right) or the O3′ (left) oxygen. (b) Nonisomerizable mimics of these intermediates feature C74, C75, and A76 of the peptidyl and aminoacyl tRNA acceptor ends. These inhibitors also contain a phosphorus atom to mimic the tetrahedral carbon formed in the intermediate.

We prepared two regioisomers of the peptidyl transferase inhibitor, CCApPmCC, where the phosphodiester is either O3′- or O2′-linked to the P-site A76 (Figure 1b). These analogues were prepared by solid phase chemical synthesis based upon the method described previously.[13] They include the critical A76 hydroxyl vicinal to the phosphodiester linkage.[9] Recent structural studies on A-site substrates indicated that the peptidyl transferase center (PTC) is induced into an active conformation by the stacking of the A-site nucleotide C74.[14] To ensure that the ribosome is in the induced state, both isomers include C74 and C75 on both the P-site and A-site segment. Most importantly, and unlike substrates previously used to explore this question, these compounds cannot undergo regioisomerization.

Transition state theory predicts that the ribosomal PTC will bind tighter to the transition state than to the substrate or product states of the reaction. Consequently, the compound with greatest affinity for the PTC is indicative of the tetrahedral intermediate's regiospecificity. The affinities of the O2′ and O3′ analogues were determined by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) chemical footprinting of active site residue U2585, as previously described.[12,13,15] The extent of CMCT modification as a function of analogue concentration was determined by reverse transcription, and the resulting data were used to calculate the binding constant (Figure 2). The O3′-linked inhibitor bound the ribosome with an affinity of 280 ± 40 nM (Figure 2a). This is 7-fold tighter than that observed for the original CCdApPmn inhibitor under the same conditions (data not shown), consistent with the additional stacking and base pairing interactions by C74 and C75 in the A site. The O2′ analogue showed only slight binding at concentrations greater than 10 μM (Figure 2b). This is

10.1021/ja0554099 CCC: $33.50 © 2006 American Chemical Society

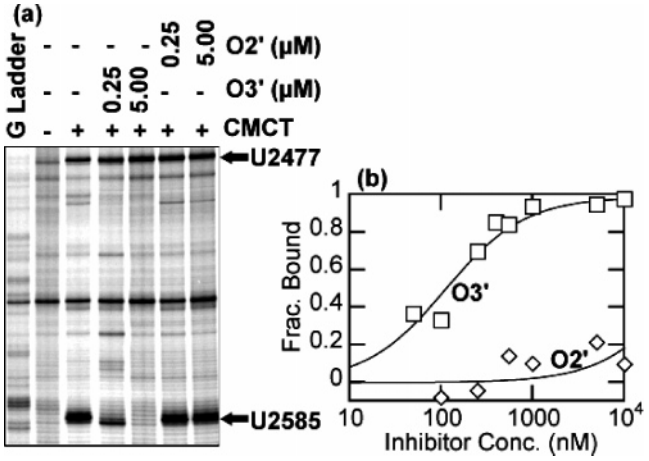

Figure 2. Regiospecificity of analogue binding by chemical footprinting and peptidyl transferase inhibition assays. (a) CMCT footprinting of U2585 in the PTC at 37 °C. Shown is an autoradiograph of primer extension reaction from CMCT modification of U2585 in the presence of the O3′ or the O2′ analogue. Concentrations and presence of CMCT are indicated above each lane. Bands for U2585 and U2477, which is used to normalize the footprinting data, are marked with an arrow. (b) Plot of footprinting data used to estimate the relative dissociation constants (K_d).

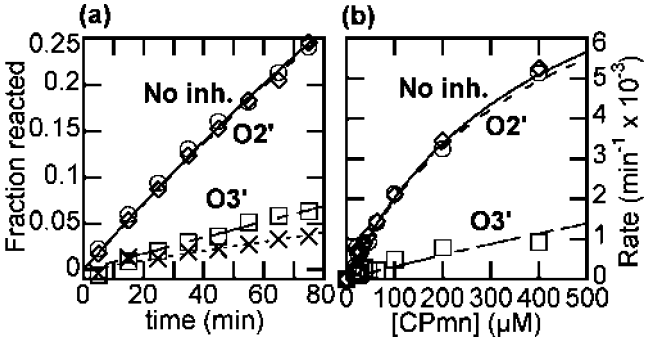

Figure 3. Inhibition of the modified fragment reaction. (a) Sample reaction time courses performed at pH 7.0 with 100 nM *E. coli* 50S ribosomes and 400 nM CCApcb. The reactions shown had 200 μM CPuromycin and no inhibitor (○), 300 nM O3′ inhibitor (□), or 300 nM O2′ inhibitor (◇). Background hydrolysis in the absence of CPuromycin is also shown (×). (B) K_i values for the inhibitors were determined by varying the CPuromycin concentration from 20 to 400 μM.

in the same range as CCA binding alone[12] and suggests that the O2′ regioisomer is unable to bridge between the A site and P site of the PTC due to significant steric hindrance in the active site. A similar preference for the O3′ inhibitor was observed in enzyme inhibition studies,[12,16] where the O3′, but not the O2′ inhibitor, significantly reduced the reaction rate (Figure 3). The inhibition constant (K_i) of the O3′ inhibitor was 45 ± 5 nM, while the O2′-linked inhibitor was greater than 7 μM, which is above the measurable limit of the assay.

The interactions between the O3′ regioisomer and the PTC are likely to provide a reasonable estimate of the reaction intermediate. A crystal structure of the inhibitor in complex with the 50S ribosomal subunit reveals that the CCA portion of both the A-site and P-site segments makes the predicted base pairing interactions in the A loop and P loop, respectively.[14] The structure also shows that the ribosome adopts the active induced conformation upon inhibitor binding.

The marked preference for the O3′ isomer suggests that the ribosome uses the O3′-linked tRNA as the P-site substrate. Because this is the same regioisomer used in the A-site, it indicates that the aminoacyl tRNA ester is not required to undergo regioisomerization during its course through the PTC.

Preferential binding and inhibition by the O3′-linked isomer clarifies an important ambiguity in the previous biochemical data regarding the inactivity of 2′- or 3′-dA76 P-site substrates.[2,6−9] The current data argue that substrates lacking a 3′-OH are inactive as donors because they are constrained into the wrong O2′-linked regioisomer. In contrast, P-site substrates lacking the 2′-OH adopt the correct O3′-linked regioisomer, but are inactive as P-site donors because the 2′-OH plays a critical role in substrate assisted catalysis of the peptidyl transferase reaction.[9] The current data exclude a mechanistic model in which the A76 2′-OH participates as a covalent intermediate in the reaction.

In conclusion, we have established the regiospecificity of the ribosomal peptidyl transferase reaction. We are now in the process of utilizing these inhibitors to understand the role of the critical A76 2′-OH and to probe the stereospecificity and potential metal ion dependence of peptide bond formation.

Acknowledgment. We thank Olke Uhlenbeck and Jesse Cochrane for helpful discussions, and David E. Kitchen (Dharmacon Research Inc.) and Olga Fedorova for technical assistance in solid phase synthesis. This work was supported by a an American Cancer Society Beginning Investigator award to S.A.S. and a National Institutes of Health Postdoctoral Fellowship to K.S.H.

Supporting Information Available: Synthetic, CMCT modification, and kinetic procedures are included. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Taiji, M.; Yokoyama, S.; Miyazawa, T. *Biochemistry* **1983**, *22*, 3220−3225.
(2) Chladek, S.; Sprinzl, M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 371−391.
(3) Chinali, G.; Sprinzl, M.; Parmeggiani, A.; Cramer, F. *Biochemistry* **1974**, *13*, 3001−3010.
(4) Ringer, D.; Chladek, S. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2950−2954.
(5) Taiji, M.; Yokoyama, S.; Miyazawa, T. *Biochemistry* **1985**, *24*, 5776−5780.
(6) Wagner, T.; Cramer, F.; Sprinzl, M. *Biochemistry* **1982**, *21*, 1521−1529.
(7) Dorner, S.; Panuschka, C.; Schmid, W.; Barta, A. *Nucleic Acids Res.* **2003**, *31*, 6536−6542.
(8) Hecht, S. M.; Kozarich, J. W.; Schmidt, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4317−4321.
(9) Weinger, J. S.; Parnell, K. M.; Dorner, S.; Green, R.; Strobel, S. A. *Nat. Struct. Mol. Biol.* **2004**, *11*, 1101−1106.
(10) Hansen, J. L.; Schmeing, T. M.; Moore, P. B.; Steitz, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11670−11675.
(11) Nissen, P.; Hansen, J.; Ban, N.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 920−930.
(12) Welch, M.; Chastang, J.; Yarus, M. *Biochemistry* **1995**, *34*, 385−390.
(13) Weinger, J. S.; Kitchen, D.; Scaringe, S. A.; Strobel, S. A.; Muth, G. W. *Nucleic Acids Res.* **2004**, *32*, 1502−1511.
(14) Schmeing, T. M.; Huang, K. S.; Strobel, S. A.; Steitz, T. A. *Nature* **2005**, *438*, 520−524.
(15) Moazed, D.; Noller, H. F. *Cell* **1989**, *57*, 585−597.
(16) Parnell, M. K.; Seila, A. C.; Strobel, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11658−11663.

JA0554099