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The Pseudouridine Synthases: Revisiting a Mechanism That Seemed Settled

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The pseudouridine synthases catalyze the conversion of uridine in RNA to its *C*-glycoside isomer pseudouridine (Ψ). The Ψ synthases fall into five families that share no global sequence similarity.[1] Structures of members of all five families have been solved,[2] and all share a core β-sheet fold[2e,3] and several structurally conserved active-site amino acid residues.[2d,f] The Ψ synthases, then, appear to be homologues and presumably operate by the same chemical mechanism. The single universally conserved amino acid residue is a catalytically essential Asp.[1c,4] Two general mechanisms have been proposed for the Ψ synthases,[4a,5] and they differ in the role played by the conserved Asp.[3,6] In the "Michael mechanism",[5,6] the Asp is a nucleophile in a Michael addition to the pyrimidine ring; subsequent glycosidic bond cleavage allows the pyrimidine ring to rotate about the new ester bond with the Asp, which repositions C5 near C1′ to form the *C*-glycosidic bond. Elimination of the Asp and deprotonation of C6 complete the formation of Ψ. In the "acylal mechanism",[3,4a] the conserved Asp follows the precedent of carboxylate groups in the retaining glycosidases[7] and stabilizes the oxocarbenium intermediate, either by ion pair formation or nucleophilic attack at C1′ to form an acylal intermediate. The tightly bound uracilate anion then rotates in the active site to reposition C5 near C1′, and formation of the *C*-glycosidic bond and deprotonation of C5 generate Ψ.

The Michael mechanism appeared to be established by studies using RNA containing 5-fluorouridine, [f^5U]RNA, which strongly or irreversibly inhibits several Ψ synthases.[4a,6,8] This behavior fit expectations based on similar work with thymidylate synthase, which is inhibited by 5-fluorouridylate due to the formation of a stable Michael adduct.[9] In particular, the reaction between the *Eschericia coli* Ψ synthase TruA (which isomerizes U residues to the 3′ side of the anticodon in tRNA) and [f^5U]tRNA was examined in detail by Santi and co-workers.[4a,6] The TruA-[f^5U]RNA adduct survives denaturing gel conditions, but it is disrupted by heating. Radiolabeling and site-directed mutagenesis indicated an attachment between the pyrimidine ring and the conserved Asp, and mass spectrometry revealed that the product of f^5U was hydrated. On the basis of HPLC retention times, the product was deduced to be the hydrate of unrearranged f^5U (with the *N*-glycosidic bond intact), which was reasonably assumed to result from ester hydrolysis of the Michael adduct (Figure 1a).[6]

However, the *E. coli* Ψ synthase that generates the nearly universal Ψ in the TΨC loop of tRNA, TruB, displays markedly different behavior when incubated with [f^5U]RNA. The structure of TruB was solved as a cocrystal with [f^5U]RNA in an attempt to observe the Michael adduct.[2b] In this case, f^5U replaced the substrate U in a stem-loop RNA that is as good a substrate as full-length tRNA.[10] Rather than being part of a covalent adduct, the f^5U was unexpectedly converted to 5-fluoro-6-hydroxypseudouridine. This unexpected product was reasonably ascribed to the progression of f^5U along the Michael mechanism to the rearranged Michael adduct and subsequent ester hydrolysis (Figure 1b) on the crystallization time scale.[2b] Recently, we showed that on a time scale of minutes to hours, the same [f^5U]RNA stem-loop neither forms an adduct with nor significantly inhibits TruB;[11] instead, the f^5U becomes hydrated[11] and likely rearranged (Spedaliere, unpublished data).

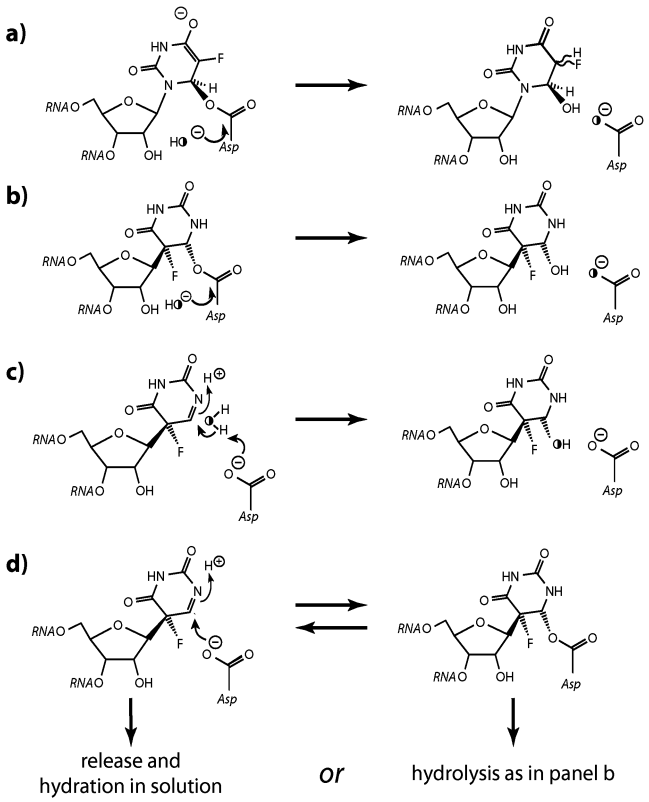

Figure 1. Schemes for the generation of hydrated products from f^5U. The half-filled O denotes 50% ^{18}O content. (a) the Michael adduct between f^5U and the conserved Asp is hydrolyzed. (b) f^5U proceeds along the Michael mechanism with ester hydrolysis after rearrangement. (c) The conserved Asp catalyzes hydration of rearranged f^5U. (d) Rearranged f^5U may be released into solution and undergo direct hydration, or the conserved Asp may add to rearranged f^5U and form an adduct that undergoes ester hydrolysis or reverts to rearranged f^5U.

Our observations could result from an unexpectedly fast ester hydrolysis of the rearranged Michael adduct, which can be detected by running the reaction in buffer containing [^{18}O]water. If ester hydrolysis occurs, the conserved Asp (Asp-48 of TruB) will contain ^{18}O, which can be detected by trypsinolysis and MALDI MS analysis of the resulting peptides. The tryptic peptide containing Asp-48 was observed, and its identity was confirmed by ESI MS MS sequencing. For the labeling experiment to give meaningful results, the oxygen atoms of Asp-48 must not exchange with solvent under reaction conditions or during analysis. Neither incubation of TruB in buffer containing 50% [^{18}O]water nor addition of [^{18}O]-water to the MALDI matrix solution resulted in the labeling of Asp-48, showing that its oxygen atoms do not exchange with solvent.

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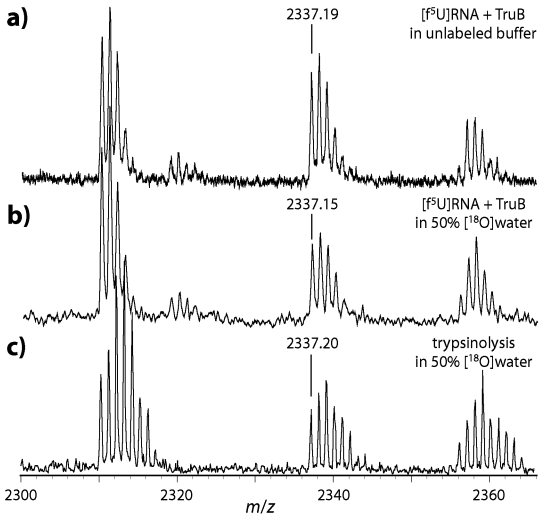

Figure 2. Partial mass spectrum of the tryptic digest of TruB after incubation with 4 equiv of [f^5U]RNA (identical results were obtained with 1 equiv). Asp-48 is in the peptide Ala-41-Lys-64 (m/z_{calc} 2337.19). (a) Incubation in unlabeled buffer, (b) incubation in buffer containing 50% [^{18}O]water, (c) trypsinolysis in buffer containing 50% [^{18}O]water.

TruB was incubated with either 1.0 or 4.0 equiv of [f^5U]RNA in both labeled and unlabeled buffer; in all cases, TruB was preincubated in buffer to allow any water molecules in the active site to equilibrate with bulk solvent. After 3 h, HPLC analysis[11] confirmed the complete reaction of [f^5U]RNA. TruB was heat precipitated, and the pellet was resuspended in 50 mM ammonium bicarbonate buffer, pH 8, and digested with trypsin; the resulting peptides were analyzed by MALDI MS. In no case was ^{18}O observed in Asp-48 (Figure 2a,b). To confirm that ^{18}O would be detected, the trypsinolysis of TruB was carried out in 50% [^{18}O]-water, and all peptides had the ^{18}O content predicted for hydrolytically generated C-termini (Figure 2c). A complementary analysis of the RNA products was achieved by digestion of the 17-mer stem-loop with RNase T_1 and alkaline phosphatase, which leaves the product of f^5U in a tetranucleotide with a 3′-OH end. Controls revealed that the hydroxyl group in the hydrated product of f^5U does not exchange with solvent at any stage of the analysis. As expected in the absence of ester hydrolysis, the ^{18}O content of the hydrated product matched that of the buffer.

These labeling studies definitively show that hydrolysis of an ester adduct with Asp-48 of TruB does not give rise to the observed product of f^5U, and the active site offers no other suitable nucleophile. The hydration of the product of f^5U cannot, therefore, be construed to support the Michael mechanism. At the least, the results with TruA and [f^5U]tRNA and the reasonable assumptions stemming from them are not general to all Ψ synthases. Since TruA and TruB belong to different families, it may seem that the differences fall cleanly along family lines, but they do not. *Thermotoga maritima* TruB forms a covalent adduct with [f^5U]-RNA, but a cocrystal structure shows the same rearranged and hydrated product observed with *E. coli* TruB.[12]

These homologous enzymes might then proceed by different mechanisms, or they may differ in their ability to cleave the glycosidic bond of f^5U. However, we offer another scenario that allows all Ψ synthases to share a common reaction mechanism and to handle f^5U to the same extent. The product of f^5U observed in the TruB cocrystal may be generated when TruA is incubated with [f^5U]tRNA since the conclusion that f^5U was not rearranged rests on an HPLC retention time,[6] and rearranged and hydrated f^5U was not available for direct comparison. In that case, all Ψ synthases generate the rearranged product, which cannot proceed further along the normal reaction coordinate since fluorine replaces the proton that would be abstracted to generate Ψ. Although they are not on the pathway normally followed to convert U into Ψ, three chemically reasonable routes exist for the hydration of rearranged f^5U.[3] The geometry of a particular active site may allow a water molecule to fit between rearranged f^5U and the Asp, which catalyzes hydration as a general base (Figure 1c). Alternatively, rearranged f^5U may be released to solution and spontaneously hydrated, or the Asp may lie near enough to rearranged f^5U for addition to occur. Either ester hydrolysis of the adduct or the reversal of the addition followed by the release of rearranged f^5U and its hydration in solution would generate the product seen in the TruB cocrystal (Figure 1d). Although ester hydrolysis does not occur with TruB, it may occur at the active sites of Ψ synthases that form a covalent adduct with [f^5U]RNA. Work is underway to test this scenario, and our results do not rule out either mechanism proposed for the Ψ synthases. But the mechanism they use is clearly not as settled as it seemed to be.

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Supporting Information Available: Experimental details, figures of the mechanisms, mass spectra of nucleoside products. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Gustafsson, C.; Reid, R.; Greene, P. J.; Santi, D. V. *Nucleic Acids Res.* **1996**, *24*, 3756−3762. (b) Koonin, E. V. *Nucleic Acids Res.* **1996**, *24*, 2411−2415. (c)Kaya, Y.; Ofengand, J. *RNA* **2003**, *9*, 711−721. (d) Ma, X. J.; Zhao, X. L.; Yu, Y. T. *EMBO J.* **2003**, *22*, 1889−1897.
(2) (a) Foster, P. G.; Huang, L.; Santi, D. V.; Stroud, R. M. *Nat. Struct. Biol.* **2000**, *7*, 23−27. (b) Hoang, C.; Ferré-D'Amaré, A. R. *Cell* **2001**, *107*, 929−939. (c) Sivaraman, J.; Sauvé, V.; Larocque, R.; Stura, E. R.; Schrag, J. D.; Cygler, M.; Matte, A. *Nat. Struct. Biol.* **2002**, *9*, 353−358. (d) Sivaraman, J.; Iannuzzi, P.; Cygler, M.; Matte, A. *J. Mol. Biol.* **2004**, *335*, 87−101. (e) Del Campo, M.; Ofengand, J.; Malhotra, A. *RNA* **2004**, *10*, 231−239. (f) Kaya, Y.; Del Campo, M.; Ofengand, J.; Malhotra, A. *J. Biol. Chem* **2004**, *279*, 18107−18110. (g) Ericsson, U. B.; Nordlund, P.; Hallberg, B. M. *FEBS Lett.* **2004**, *565*, 59−64. (h) Hoang, C.; Ferré-D'Amaré, A. R. *RNA* **2004**, *10*, 1026−1033.
(3) Mueller, E. G. *Nat. Struct. Biol.* **2002**, *9*, 320−322.
(4) (a) Huang, L. X.; Pookanjanatavip, M.; Gu, X. G.; Santi, D. V. *Biochemistry* **1998**, *37*, 344−351. (b) Ramamurthy, V.; Swann, S. L.; Paulson, J. L.; Spedaliere, C. J.; Mueller, E. G. *J. Biol. Chem* **1999**, *274*, 22225−22230. (c) Conrad, J.; Niu, L. H.; Rudd, K.; Lane, B. G.; Ofengand, J. *RNA* **1999**, *5*, 751−763. (d) Raychaudhuri, S.; Niu, L. H.; Conrad, J.; Lane, B. G.; Ofengand, J. *J. Biol. Chem* **1999**, *274*, 18880−18886.
(5) Kammen, H. O.; Marvel, C. C.; Hardy, L.; Penhoet, E. E. *J. Biol. Chem* **1988**, *263*, 2255−2263.
(6) Gu, X. R.; Liu, Y. Q.; Santi, D. V. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14270−14275.
(7) Zechel, D. L.; Withers, S. G. *Acc. Chem. Res.* **2000**, *33*, 11−18.
(8) (a) Doong, S. L.; Dolnick, B. J. *J. Biol. Chem* **1988**, *263*, 4467. (b) Samuelsson, T. *Nucleic Acids Res.* **1991**, *19*, 6139−6144. (c) Samuelsson, T.; Olsson, M. *J. Biol. Chem* **1990**, *265*, 8782−8787. (d) Yu, Y. T.; Shu, M. D.; Steitz, J. A. *EMBO J.* **1998**, *17*, 5783−5795.
(9) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471−481.
(10) Gu, X. R.; Yu, M.; Ivanetich, K. M.; Santi, D. V. *Biochemistry* **1998**, *37*, 339−343.
(11) Spedaliere, C. J.; Mueller, E. G. *RNA* **2004**, *10*, 192−199.
(12) Phannachet, K.; Huang, R. H. *Nucleic Acids Res.* **2004**, *32*, 1422−1429.

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