Supplementary Material for

# The pseudouridine synthases: revisiting a mechanism that seemed settled

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## Mechanisms

Two mechanisms, the "Michael mechanism" and the "acylal mechanism" have been proposed for the Ψ synthases and revised in light of continuing investigations. Our current formulations of them are shown in Figure S1. With [$f^5$U]RNA, the last step of both mechanisms is prevented since the proton to be abstracted is replaced with fluorine.

## Materials and Methods

*General*. Calf intestinal alkaline phosphatase was purchased from Promega (Madison, WI); RNase $T_1$ was purchased from Roche Applied Science (Indianapolis). Sequencing grade trypsin, α-cyano-4-hydroxy cinnamic acid, and ProteoMass™ Peptide and Protein MALDI-MS Calibration Kits were purchased from Sigma; 3-hydroxypicolinic acid was purchased from Fluka or Aldrich. Sequazyme™ Peptide Mass Standards Kits were purchased from Applied Biosystems (Foster City, CA). Prime RNase inhibitor was purchased from Eppendorf-5' (Boulder, CO). The oligonucleotide with 5-fluorouridine, $f^5$U, in place of the substrate uridine was purchased from Dharmacon, Inc. (Boulder, CO) and corresponds to the T-arm stem-loop of yeast $tRNA^{Phe}$. It is designated [$f^5$U]TSL and has the sequence: CUGU$f^5$UCGAUCCACAG. All other materials were purchased from Fisher Scientific (Pittsburgh, PA) or its Acros Organics division. TruB was overexpressed, purified, and assayed as previously described[1]; overexpressed TruB bears the $His_6$•tag encoded by pET15b (Novagen, Madison, WI), but all residue numbering is in terms of native *E. coli* TruB. Standard assay buffer is 50 mM HEPES buffer, pH 7.5, containing ammonium chloride (100 mM), DTT (5 mM), EDTA (1 mM), and Prime RNase Inhibitor (0.3 U/μL).

*C193A TruB*. The trypsinolysis of wild-type TruB generates a peptide (Thr-182 to Arg-202, 2342.27 *m*/*z*) that would overlap the envelope of the $^{18}$O-labeled peptide containing Asp-48 (Ala-41 to Lys-64, 2337.19 *m*/*z*). To eliminate this problem, C193A TruB was generated, which shifts the obscuring peptide by –32 *m*/*z* and clears the observation window for the peptide containing Asp-48. Previous work showed that Cys-193 can be replaced with valine (in C193V TruB) or alanine (in C58A/C174A/C193A TruB) without a significant effect on catalytic activity[2]. C193A TruB was generated by site-directed mutagenesis as previously described[1]. The earlier attempt generated C193V instead of the target C193A TruB, which was suitable for the kinetic characterization but not for this study because the obscuring peptide would not be sufficiently shifted; this time, the gene for C193A TruB was obtained, and sequencing of the entire gene confirmed that the absence of any other mutation. The specific activity of C193A TruB was nearly that of wild-type enzyme, and it cleanly converted [$f^5$U]RNA into product in our standard 3 h incubation as assessed by reverse phase HPLC as previously described[3].

*Asp-48 labeling study*. C193A TruB (10 μM) was incubated with [$f^5$U]TSL (10 or 40 μM) for 3 h at 25 °C in standard assay buffer (135 μL) that was either unlabeled or contained 50% [$^{18}$O]water. In all cases, the enzyme was pre-incubated for at least 5 min to ensure that any water molecules in the active site equilibrated with bulk solvent; reaction was initiated by the addition of a small volume of aqueous [$f^5$U]TSL. Reactions were stopped by incubation for 5 min at 100 °C. Denatured protein was pelleted by spinning for 5 min at maximum speed in a microcentrifuge; the supernatant was pipetted away, and an aliquot was analyzed by reverse phase HPLC as previously described[3] to verify that the conversion of [$f^5$U]TSL was complete. The protein pellet was resuspended in 50 mM ammonium bicarbonate buffer (100 μL), pH 8, containing trypsin (10 μg). The reaction was placed on a rotating drum overnight at 37 °C. An aliquot (5 μL) of the digestion mixture was purified using a $ZipTip_{C18}$® according to the manufacturer's instructions, and an aliuot (0.5 μL) was mixed with an equal volume of MALDI matrix solution, which is a 1:1 mixture of acetonitrile and water containing 0.1% TFA that is saturated with α-cyano-4-hydroxy cinnamic acid. Alternatively, samples were prepared by eluting the peptides from the ZipTip with MALDI matrix solution (5 μL).

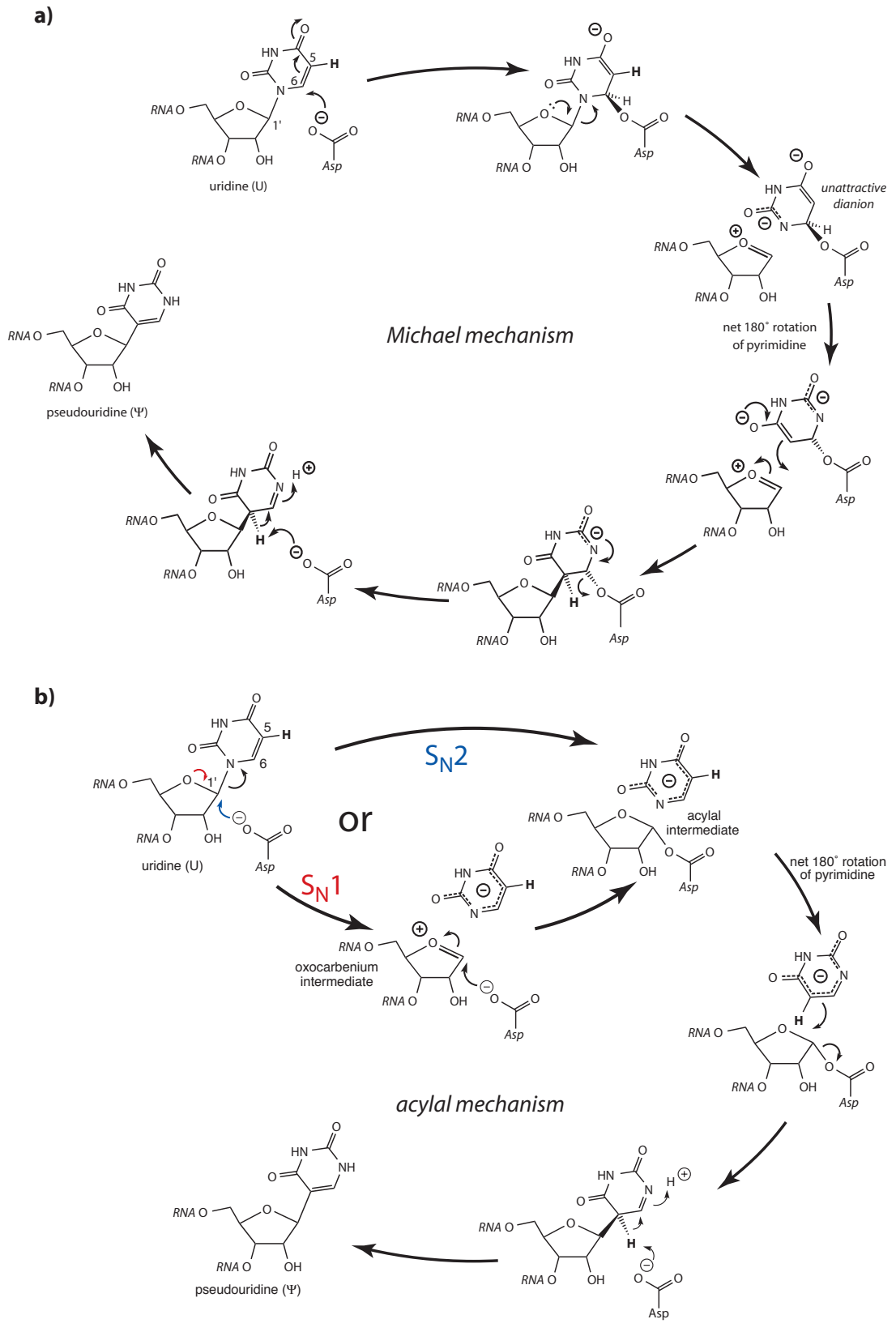


***Figure S1.*** The two mechanisms proposed for the Ψ synthases. With $f^5U$, the bold H is replaced with F, and the last step of both mechanisms cannot occur. The depicted stereochemistry is based on that observed in the hydrated and rearranged product of $f^5U$ in the cocrystal of TruB and [$f^5U$]RNA.[4] (a) the mechanism involving Michael addition of the conserved Asp. (b) the mechanism involving the formation of an acylal intermediate between the conserved Asp and ribose.

Samples (1 μL) were spotted onto a MALDI sample plate, air dried, and then analyzed by MALDI-TOF MS using an OmniFLEX or BiFLEX III instrument (Bruker Daltonics); positive ion detection was used with the detector in reflector mode. On the OmniFLEX instrument, data was acquired with 300 laser shots at a sampling rate of 1 nsec, and 5- or 9-point smoothing was applied using the program XMASS, version 5.0 (Bruker Daltonics). Calibration was achieved using a 1:1:2 mixture of angiotensin II (1046.5423 *m*/*z*), fragment 18-39 of ACTH (2465.1989 *m*/*z*), and the oxidized β chain of insulin (3494.6513 *m*/*z*). On the BiFLEX III instrument, data was acquired with 100-300 laser shots at a laser repetition rate of 2 Hz. If needed, spectra were smoothed using a 2-point Savitsky Golay algorithm. Spectra were analyzed using the data analysis program XMASS/NT, Version 5.1.1 (Bruker Daltonics). Angiotensin I (1296.6853 *m*/*z*) and ACTH fragment 18-39 (2465.1989 *m*/*z*) were used for calibration prior to obtaining sample spectra. Identical results were obtained with both 1:1 and 4:1 stoichiometries of [f$^5$U]TSL:TruB, and the data for the 4:1 ratio is shown in Figure 2 of the communication.

Sequencing of the peptide of interest was performed in a Q-TOF Ultima API-US instrument (Micromass, Manchester UK) equipped with a z-spray nanoelectrospray source. The sample was loaded into a 4m New Objective PicoTip glass emitter (Woburn, MA). The Q-TOF was operated at a capillary voltage of 2.02 kV and a sampling cone voltage of 50 V with a scan time of 2.4 s. For the MS-MS experiment the collision energy was 25 eV with argon used as the collision gas.

*Controls for the Asp-48 labeling study*. As described in the communication itself, controls were performed to ensure that the oxygen atoms of Asp-48 were not free to exchange with solvent on the time scale of the reaction and work-up. Identical procedures to those described for the labeling study were performed except that: 1) TruB was incubated in assay buffer containing 50% [$^{18}$O]water in the absence of [f$^5$U]TSL and then analyzed; 2) MALDI matrix was prepared with 50% [$^{18}$O]water and used to analyze TruB tryptic fragments from the incubation of TruB and [f$^5$U]TSL in unlabeled buffer and subsequent trypsinolysis in unlabeled buffer; 3) TruB was incubated with [f$^5$U]TSL

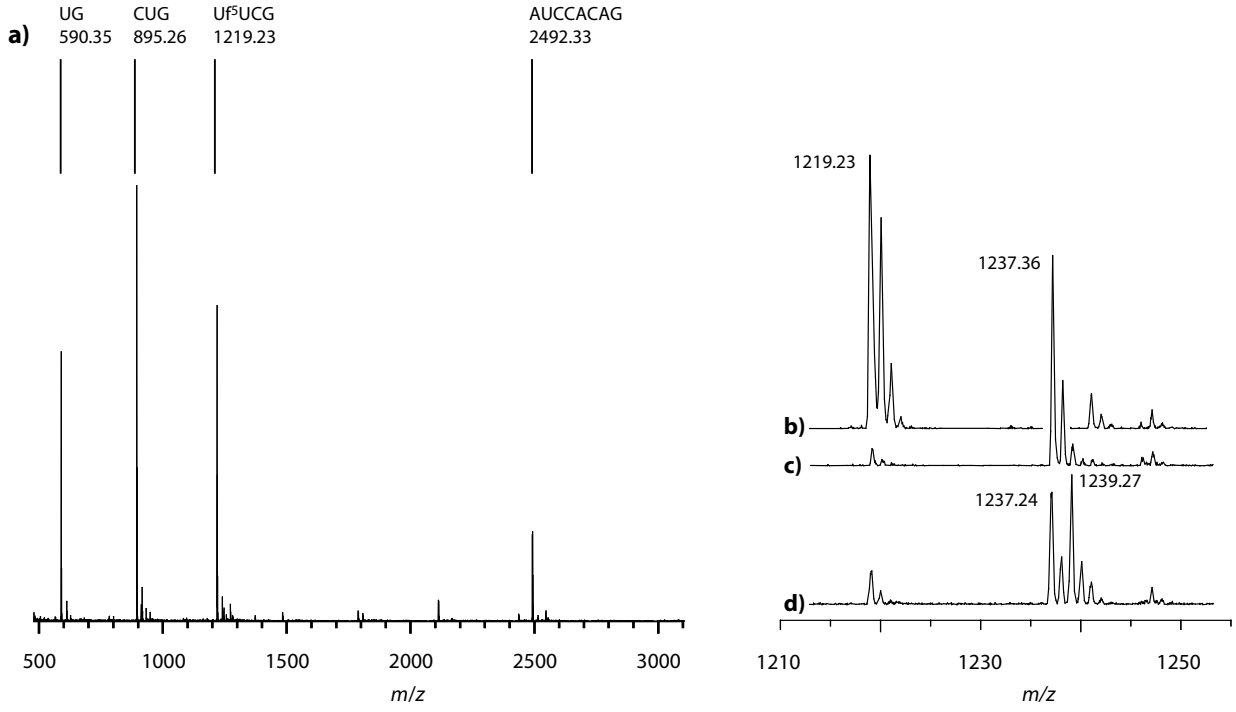


***Figure S2.*** Mass spectra of digested and dephosphorylated [f$^5$U]TSL before and after incubation with TruB. (a) the full mass spectrum of [f$^5$U]TSL shows all four peaks expected from digestion with RNase $T_1$ and dephosphorylation by alkaline phosphatase (b) a partial mass spectrum of [f$^5$U]TSL before incubation with TruB (c) a partial mass spectrum of [f$^5$U]TSL after incubation with TruB in unlabeled buffer; hydration is revealed by the shift of +18 *m*/*z* (d) a partial mass spectrum of [f$^5$U]TSL after incubation with TruB in buffer containing 50% [$^{18}$O]water; the isotopic content of the hydrated product matches that of the buffer.

in unlabeled buffer and then subjected to trypsinolysis in buffer containing 50% [$^{18}$O]water. The mass spectra from the first two controls are identical to those shown in Figure 2a,b of the communication. The mass spectrum from the third control is shown in Figure 2c in the communication.

*Product nucleotide labeling study.* C193A TruB (10 μM) was incubated with [f$^5$U]TSL (40 μM) for 3 h at 25 °C in standard assay buffer (100 μL) that was either unlabeled or contained 50% [$^{18}$O]water. In all cases, the enzyme was pre-incubated for at least 5 min to ensure that any water molecules in the active site equilibrated with bulk solvent; reaction was initiated by the addition of a small volume of aqueous [f$^5$U]TSL. Reaction was terminated by extracting the enzyme into phenol saturated with TE buffer, pH 4.3. The aqueous phase was washed with chloroform:isoamyl alcohol (24:1), and the RNA was ethanol-precipitated. The pellet was washed twice with 70% aqueous ethanol, air dried, and redissolved in RNase-free water. An aliquot (10 μL, 100 μM) was diluted with RNase-free water (10 μL), and RNase $T_1$ (2 μL, 200 U) and alkaline phosphatase (1 μL, 1 U) were added. After 1 h at 37 ˚C, an aliquot (0.5 μL) of the digestion solution was mixed with matrix solution (0.5 μL), which is 3-hydroxypicolinic acid (75 mg/mL) in 60% aqueous acetonitrile. Samples were spotted onto a MALDI sample plate, air dried, and analyzed by MALDI-TOF MS using the BiFLEX III instrument as described above for peptide analysis**.**

All four product oligonucleotides expected from digestion with RNase $T_1$ (which cuts after G residues) were observed (Figure S2a). The mass of the tetranucleotide containing the product of f$^5$U revealed hydration with an isotopic composition identical to that of the solvent: 100% $^{16}$O from incubation with TruB in unlabeled buffer (Figure S2b) and 50% $^{18}$O from incubation with TruB in labeled buffer (Figure S2c).

*Controls for the product nucleotide labeling study.* To ensure that exchange between the hydrated product of f$^5$U and solvent did not occur on the time scale of the experiment and work-up, the incubation of [f$^5$U]TSL with TruB was performed in unlabeled buffer, and the product RNA was purified by phenol/chloroform extraction of TruB and ethanol precipitation as described above. To test whether or not isotopic exchange occurs in the MALDI matrix, an aliquot (0.5 μL) of the RNA product was analyzed after mixing with matrix solution (0.5 μL) prepared with 50% [$^{18}$O]water. No $^{18}$O was detected in any of the oligonucleotide products (data not shown), demonstrating that the hydroxyl group of the hydrated product of f$^5$U does not exchange with solvent on the time scale of the analysis. To test whether or not isotopic exchange between the hydroxyl group of the hydrated product of f$^5$U and buffer occurs, a portion of the redissolved RNA pellet was digested and dephosphorylated, and an aliquot (3 μL) was diluted with [$^{18}$O]water (3 μL) to achieve a 50% $^{18}$O content in the sample. After 3 h at 37 ˚C, the sample was analyzed by MALDI-MS as described above. No $^{18}$O was detected in any of the oligonucleotides (data not shown). *note*: If alkaline phosphatase were not included in the digestion, the 3'-phosphate ends produced by RNase $T_1$ would be expected to reflect the isotopic content of the buffer, but alkaline phosphatase removes the 3'-phospho groups, and it is the $P_i$ product that reflects the isotopic composition of the buffer.

## References


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(2) Ramamurthy, V.; Swann, S. L.; Spedaliere, C. J.; Mueller, E. G. *Biochemistry* **1999**, *38*, 13106-13111.

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(4) Hoang, C.; Ferre-D'Amare, A. R. *Cell* **2001**, *107*, 929-939.