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## Glycosidic Bond Conformation Preference Plays a Pivotal Role in Catalysis of RNA Pseudouridylation: A Combined Simulation and Structural Study

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### Abstract

The most abundant chemical modification on RNA is isomerization of uridine, or pseudouridylation, catalyzed by pseudouridine synthases. The catalytic mechanism of this essential process remains largely speculative, partly due to the lack of knowledge of the pre-reactive state that is important to identification of reactive chemical moieties. In the present study, we showed by the orthogonal space random walk free energy simulation method that the pre-reactive states of uridine and its reactive derivative, 5-fluorouridine, bound to a ribonucleoprotein particle (RNP) pseudouridine synthase strongly prefer the *syn* glycosidic bond conformation while that of the nonreactive 5-bromouridine-containing substrate is largely populated in the *anti* conformational state. A high resolution crystal structure of the 5-bromouridine containing substrate bound to the RNP pseudouridine synthase and enzyme activity assay confirmed the anti nonreactive conformation and provides molecular basis for its confinement. The observed preference for the *syn* pre-reactive state by the enzyme-bound uridine may help to distinguish among currently proposed mechanisms.

Despite its prevalence<sup>1,2</sup> and demonstrated importance in RNA structure<sup>3-5</sup> and function<sup>6,7</sup>, the catalytic mechanism of pseudouridylation remains largely speculative, partly due to the lack of knowledge of the pre-reactive state critical to identification of reactive chemical moieties. This process is thought to begin with the cleavage of the N-glycosyl bond (ring cleavage), followed by a 180° rotation of the uracil base while still enzyme-bound (ring rotation), then reattachment of the ring at C5 (ring reattachment) and finally deprotonation of C5<sup>8-10</sup> (Fig. 1). Cofactors are not known to be required for any of these steps.

In bacteria, approximately a dozen uridine residues in transfer RNA (tRNA) and ribosomal RNA (rRNA) are modified by six families of pseudouridine synthases: TruA, TruB, TruD, RluA, RsuA, and Pus101<sup>1-19</sup>. Each of these pseudouridine synthases is responsible for modifying one or several specific uridine nucleotide(s) in tRNA or rRNA. In Archaea and

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#### ACCESSION NUMBERS:

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3LWO.

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Eukarya where rRNA and small nuclear RNA (snRNA) are extensively modified, pseudouridylation is largely carried out by the box H/ACA ribonucleoprotein particle (RNP) pseudouridine synthases<sup>20-24</sup>. Unlike the stand-alone pseudouridine synthases, box H/ACA RNP pseudouridine synthases are multi-subunit enzymes and are comprised of four protein and one RNA subunits (Figure 1a). The four protein subunits include Cbf5, Nop10, Gar1, and Nhp2 (L7Ae in archaea). Cbf5 shares sequence motifs and a structural similarity with the TruB family of pseudouridine synthases and is the catalytic subunit of the RNP enzyme. The RNA subunit belongs to the class of box H/ACA noncoding RNA and is characterized by the hairpin-like secondary structure and the strictly conserved ACA trinucleotide at its 3' end<sup>20-24</sup>. The substrate RNA is captured by the RNP enzyme through its base pairing with the central internal loop (pseudouridine pocket) of the box H/ACA RNA (Figure 1a).

Regardless of their substrate specificity and enzyme composition, however, all families of pseudouridine synthases contain a well-conserved catalytic domain and a catalytic aspartate residue<sup>9-25-26</sup>. Mutation of catalytic Asp to Asn in bacterial TruB and TruA pseudouridine synthases resulted in complete loss of enzyme activity<sup>9-27</sup>. Furthermore, mutation of Asp to Ala in an archaeal H/ACA RNP also abolished the modification activity<sup>28</sup>, suggesting that this residue is essential to catalysis.

Two mechanisms that invoke Asp as a nucleophile have been proposed for the catalytic process. In the Michael addition mechanism (Fig. 1b), Asp attacks the RNA ring atom C6, leading to an Asp-pyrimidine covalent adduct. The strongest evidence supporting this mechanism is the observation that on a RNA substrate containing 5'-fluorouridine (5FU), *E. coli* TruA and RluA either cross-link to or strongly interact with the hydrated product, 5-fluoro-6-hydroxypseudouridine (5FhΨ)<sup>29-30</sup>. In opposition to the proposed Michael addition mechanisms, however, crystal structures of TruB, RluA, and the H/ACA pseudouridine synthase bound to 5FU substituted RNA substrates do not show a covalent intermediate, although it was argued in the case of the RluA-RNA complex that X-ray radiation used for diffraction studies dissolved the possible covalent linkage<sup>17-19-31-32</sup>. Furthermore, the bound 5FhΨ in all cases has its C6 pointing away from the  $\gamma$ -carboxyl group of the catalytic Asp. The unfavorable ring orientation is also observed in the complex of the Asp-to-Asn TruB mutant bound to a 5FU-containing RNA substrate<sup>33</sup>. The second proposed mechanism is the acylal mechanism (Fig. 1c) in which the catalytic Asp attacks the sugar atom C1' to form the acylal intermediate that stabilizes the oxocarbenium ion. This mechanism should be less sensitive to the ring orientation than to the distance between Asp and C1'. Elucidation of the pseudouridine synthase mechanism thus requires an assessment of the glycosidic bond conformation of uridine in its pre-reactive state.

Previously, we obtained a cocrystal structure of a functional H/ACA RNP pseudouridine synthase bound to a guide RNA and a 5FU-containing substrate RNA<sup>32</sup>. This structure made possible for us to explore the theoretical pre-reactive state of the substrate. We carried out free energy simulation on the box H/ACA RNP complex containing the wild-type and 5FU substrates using the previously developed Orthogonal Space Random Walk (OSRW) algorithm<sup>34-35</sup>. As detailed in the supplementary information, we utilized a novel "alchemical" transition<sup>36-37</sup> scheme to realize the *syn* to *anti* conformational transformation about the glycosidic bond of uridine (Fig. 2a). The previously determined structure containing 5FhΨ was used to build the starting structures for the simulation by substituting 5FhΨ for uridine base. We found that for both the wild-type and 5FU-substituted substrates, the *syn* conformation is preferred over *anti* by  $-3.5$  kcal/mol and  $-2.5$  kcal/mol, respectively. This is in contrast to the *anti* conformation of 5-fluorouridine captured in TruB D48N crystal structure containing 5FU<sup>33</sup>, suggesting that the D48N mutation may sufficiently disturb the micro environment of active site to favor the *anti* conformation.

The *syn* conformation places the ring C6 atom close to the catalytic Asp85 (Fig. 2b). In order to test the importance of the *syn* conformation to catalysis, we carried out the same free energy simulation on 5-bromouridine-containing substrate (5BrU) bound to the H/ACA RNP. Bromine is less electronegative but bulkier in size than fluorine. Consistently, 5BrU was found to prefer the *anti* over the *syn* conformation by more than 8 kcal/mol (Fig. 2b), suggesting it may be defective in the ring cleavage step.

To determine if 5BrU is a substrate for the pseudouridine synthase, we used the DNA splint technique for constructing both wild-type and 5BrU-containing substrate RNA from two synthetic oligos<sup>38</sup> with a <sup>32</sup>P-label on the 5' position of the target uridine or modified uridine. The integrity of the ligated substrate RNA was checked on a denaturing polyacrylamide gel (data not shown). After pseudouridylation assays with saturating amount of H/ACA RNP pseudouridine synthase and nuclease P1 digestion of RNA substrates, formation of uridine (or modified uridine) isomers was detected by thin layer chromatography. As shown in Figure 3a, 5BrU completely inhibited isomerization.

To further provide evidence for the reactivity of 5BrU with the enzyme, we obtained a crystal structure of 5BrU in complex with the *Pyrococcus furiosus* H/ACA RNP that contains Cbf5, Nop10, L7Ae and a guide RNA at 2.9 Å. The crystallographic data are listed in table 1 of supplementary material. Its global structure bear strong similarity to the previously determined 5FhΨ-bound RNP structure<sup>31,32</sup>. However, sigmaA-weighted electron density maps of 5BrU clearly revealed that it did not react to the enzyme and is in the computationally predicted *anti* conformation (Fig. 3b). Although its C1' atom has a similar distance to the carbonyl group of the catalytic Asp85 (4.3 Å) as that of 5FhΨ, its C6 atom is pointed away from Asp85. Even if Asp85 could facilitate a nucleophilic attack on C6 in this orientation (shown as the cyan arrow in Fig. 3b), it would result in the minor *trans* 6-hydroxyl stereoisomer<sup>39</sup>. Therefore, 5BrU is confined to an orientation that does not permit the initial attack to take place. The bromide atom is primarily responsible for the change as in its absence the *anti* conformation is preferred. The bromide atom is in close contact with several polar and aliphatic groups of the active site. These include the hydroxyl group of Tyr113, the amide of Ile183 and the aliphatic groups of Ile183 (Fig. 3c). Strikingly, the O6 atom in the bound 5FhΨ is located in exactly the same site and establishes similar extensive interactions with the enzyme, which likely serves to hyperstabilize the reaction intermediate (Fig. 3c).

We have demonstrated a clear preference for the *syn* conformation by uridine in the microenvironment provided by the H/ACA RNP pseudouridine synthase. Perturbation of this conformation resulted in inhibition of the activity. These results highlight an important role of the pre-reactive glycosidic bond conformation in catalysis. Significantly, the predicted pre-reactive state conformation suggests a preference for a C6-based catalytic mechanism. Regardless the actual mechanism of nucleophilic attack, C6-based schemes are consistent with formation of the 5FhΨ intermediate<sup>8,29</sup> and the acquired 6-hydroxyl group from the aqueous solution<sup>8</sup>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

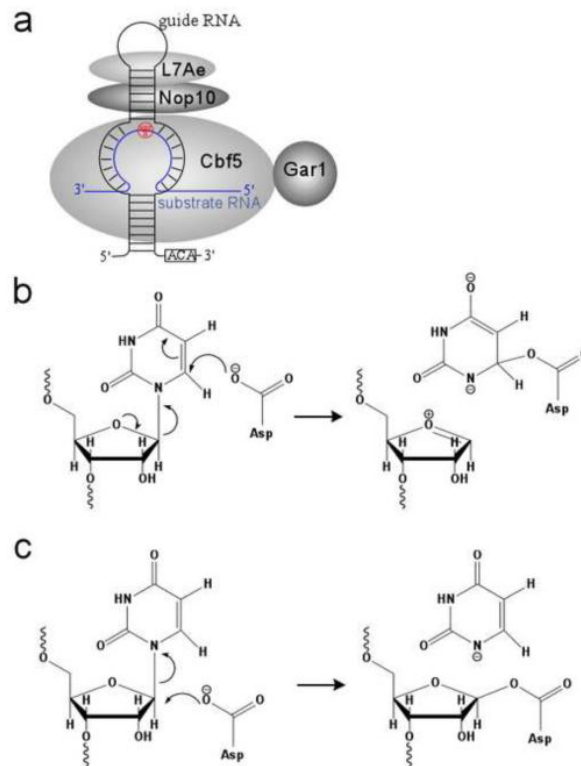
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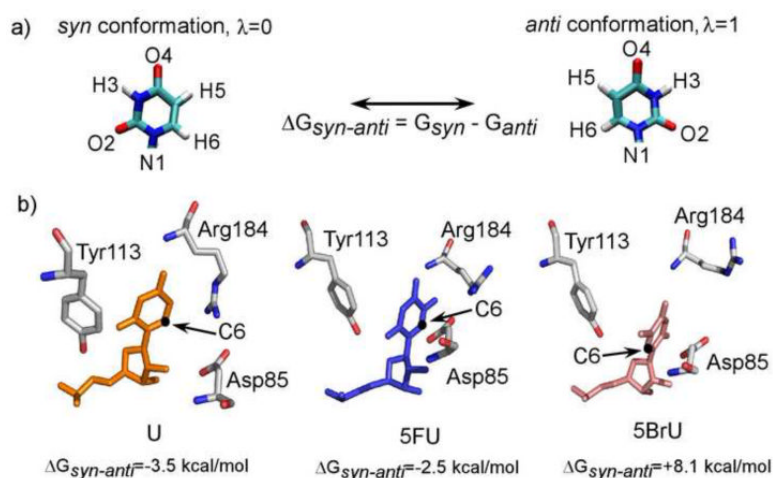
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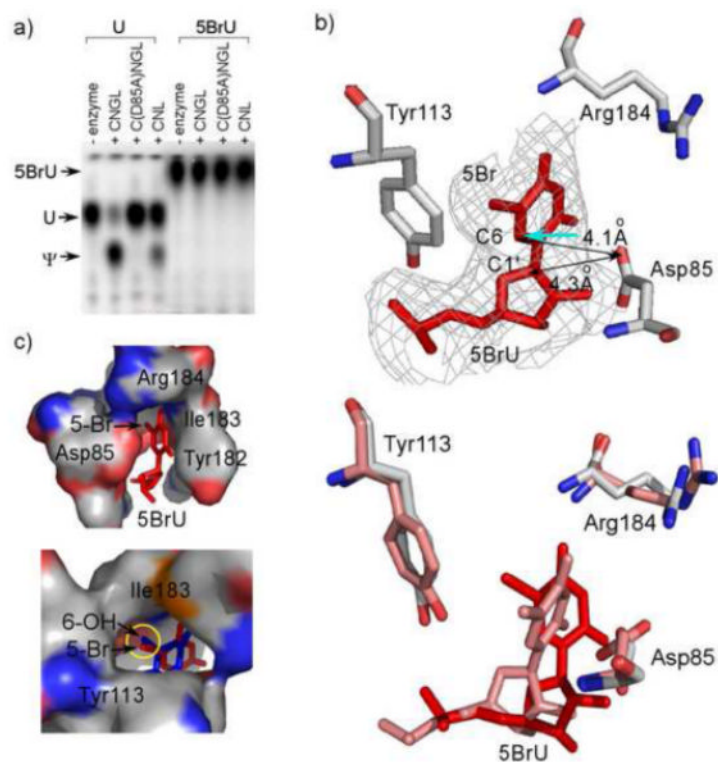
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**Figure 1.** Schematics of the two proposed mechanisms for pseudouridylation and enzyme activity assay results. (a) Michael addition mechanism. (b) Acylal mechanism. The two mechanisms share the same catalytic steps and the catalytic Asp residue but differ in the role Asp plays in ring cleavage.

**Figure 2.**

Computationally identified low energy structures of uridine (orange), 5Fh $\Psi$  (blue) and 5BrU (pink). The *syn* and *anti* conformations and their free energy difference are defined as shown in a) and are represented by a “coupling parameter”, for the purpose of calculation of free energy difference. Computed difference free energy after convergence is shown below each complex in b). Details of free energy simulation are included in the supplementary material.



**Figure 3.**

Enzyme activity assay (a) and structural study results (b-c) of 5BrU bound to H/ACA ribonucleoprotein particle (RNP) pseudouridine synthase. a). Thin layer chromatography separation of reacted and digested wild-type (U) or 5BrU substrate nucleotides. “C” denotes Cbf5, “N” denotes Nop10, “G” denotes “Gar1”, and “L” denotes L7Ae. D85A is the catalytically deficient mutant of Cbf5. b). (upper) Crystal structure of the active site of the 5BrU substrate bound to H/ACA RNP. Omitted  $3F_o-2F_c$  map is shown at  $1.0\sigma$  around the target nucleotide. (lower) Comparison of the computed low energy structure (pink) to the crystal structure (red). c). (upper) 5BrU (red) is tightly bound by the active site residues (surface). (lower) Superimposed 5FhΨ (blue) shows a similar location of the 6-hydroxyl group as 5-bomide. Experimental details for enzyme activity assay, protein crystallization and structure determination are included in the supplementary material.