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The Pseudouridine Synthases Proceed through a Glycal Intermediate

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Supporting Information

ABSTRACT: The pseudouridine synthases isomerize (U) in RNA to pseudouridine (Ψ), and the mechanism that they follow has long been a question of interest. The recent elucidation of a product of the mechanistic probe 5-fluorouridine that had been epimerized to the *arabino* isomer suggested that the Ψ synthases might operate through a glycal intermediate formed by deprotonation of C2′. When that position in substrate U is deuterated, a primary kinetic isotope effect is observed, which indisputably indicates that the proposed deprotonation occurs during the isomerization of U to Ψ and establishes the mechanism followed by the Ψ synthases.

The pseudouridine synthases (Ψ synthases) rearrange uridine (U) to its *C*-glycoside isomer pseudouridine (Ψ) in RNA. They are cofactor-independent and fall into six different families that share no significant global sequence similarity.[1] All six families of Ψ synthases share a common fold with a core β-sheet[2] along with several conserved active site amino acid residues including an invariant Asp that is essential for activity.[3−7] Largely based on the handling of RNA containing 5-fluorouridine (F^5U) in place of U ([F^5U]RNA), different mechanisms have been proposed for Ψ synthases, and they all involve the essential Asp as either a nucleophile or a base.[8−10] In the "Michael mechanism",[9] the essential Asp nucleophilically attacks C6 of the pyrimidine ring to form a covalent adduct (a Michael addition) followed by elimination of the newly tethered pyrimidine ring and then its rotation and reattachment at C5 to make Ψ (Scheme S1). The alternative "acylal mechanism"[8] involves nucleophilic attack by the essential Asp at C1′ in the ribose ring to form an acylal intermediate as precedented by retaining glycosidases (Scheme S2).[11]

When incubated with [F^5U]RNA, the Ψ synthase TruA is irreversibly inhibited and observed as a TruA−RNA adduct band by denaturing gel electrophoresis, which was reasonably construed in support of the Michael mechanism.[9] Heat treatment of the adduct results in a hydrated product of F^5U, which was ascribed to ester hydrolysis of the Michael adduct (attack of water at the carbonyl carbon of the essential Asp).[9] The Ψ synthase RluA behaves similarly to TruA when incubated with [F^5U]RNA.[12,13] In contrast, the Ψ synthase TruB is not irreversibly inhibited by [F^5U]RNA and does not form an adduct but instead converts F^5U into two rearranged and hydrated products (in a ratio of ∼3:1).[12,14] Labeling studies using [^{18}O]water with TruB, RluA, and TruA demonstrated that the hydration of F^5U results not from ester hydrolysis but instead the direct hydration of F^5U, thus removing the hydrated products as evidence for the Michael mechanism, but both proposed mechanisms were consistent with the labeling results.[10,13,15]

Further characterization revealed that TruA, TruB, and RluA all generate two isomeric products of F^5U, both of which are hydrated *C*-glycosides.[10,14] Unexpectedly, the two products of F^5U differ in their stereochemistry at C2′ (Figure 1).[14] The

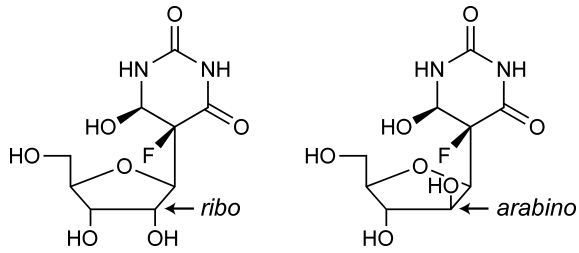

Figure 1. Products from the action of TruB and RluA on F^5U differ in stereochemistry at C2′. Because of the inability of the S1 nuclease to cleave after nonplanar bases, products of F^5U are isolated as dinucleotides with a 3′-cytidine (TruB) or a 3′-uridine (RluA), which are omitted for clarity.[10,14]

major product of F^5U remains a *ribo* sugar as seen in the cocrystal structures of TruB and RluA with [F^5U]RNA,[16,17] but the minor product is the *arabino* isomer. This epimerization is most reasonably achieved by deprotonation at C2′ and elimination of the pyrimidine ring to generate a glycal intermediate, which is reprotonated from the opposite face during *C*-glycoside formation.[14]

To probe for the formation of a glycal intermediate and the possibility of its protonation directly from solution rather than by an active site acid, reactions of RNA containing either U or F^5U were run in buffer containing D_2O, and the products were examined for the incorporation of deuterium into a nonexchangeable position (presumably C2′). Such "wash-in" requires that the proton on the essential Asp not be occluded from solvent on the time scale of the isomerization of U to Ψ and thus free to exchange with solvent protons (Scheme S4). No wash-in was observed with either TruB[14] or RluA (Figure S1 and Tables S1 and S2) with either U or F^5U in the substrate, even when the active site of TruB was enlarged by substitution of Tyr-76 with leucine (Figure S2, Table S5). The absence of wash-in can result from the lack of deprotonation/reprotonation at C2′ or occlusion of the essential Asp leading to removal and return of the same proton at C2′.

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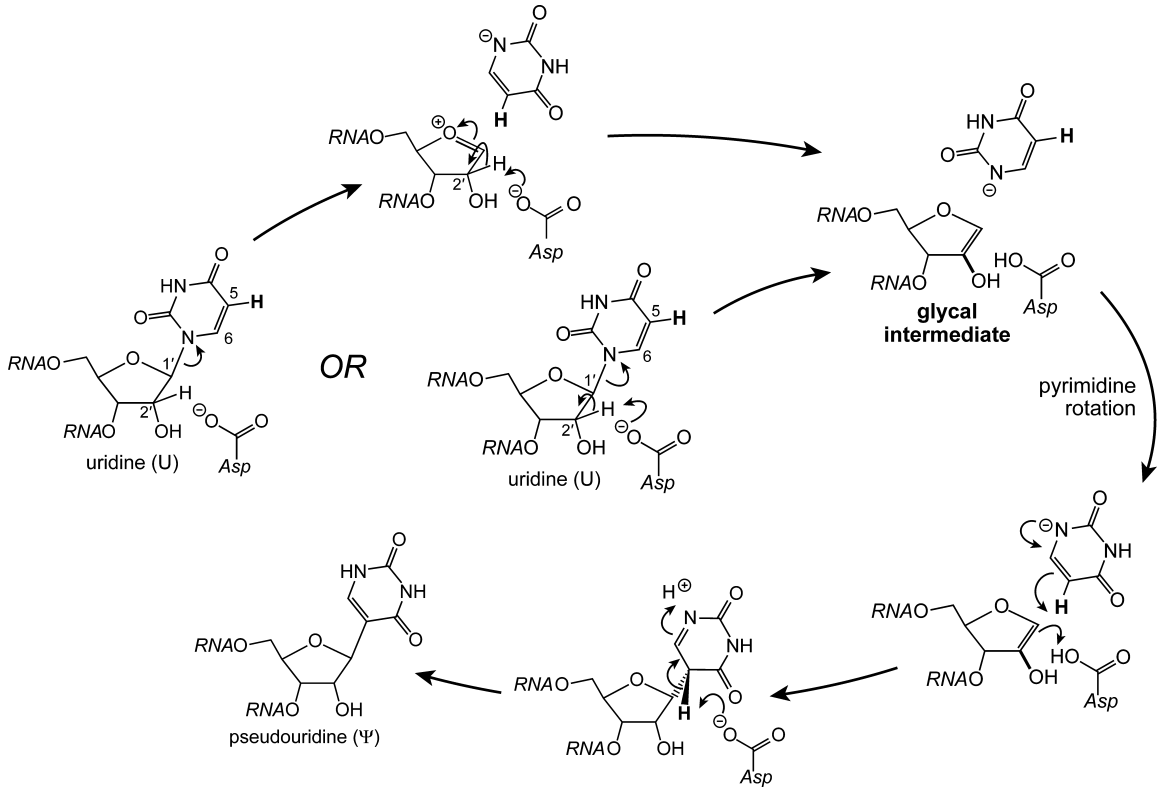
Scheme 1. Glycal Mechanism with the Essential Asp as the Acid/Base Catalyst



The lack of wash-in does definitively rule out direct protonation from solution, so active site acid/base groups must conduct both the deprotonation and reprotonation of C2′. The essential Asp is the only such group in the crystal structures that seems well positioned to deliver a proton to form an *arabino* isomer. The *arabino* product of F^{5}U therefore strongly disfavors the Michael mechanism because the essential Asp is esterified during the crucial deprotonation/reprotonation of C2′. The acylal mechanism can account for the observed *arabino* product of F^{5}U because the decreased nucleophilicity of the fluorouracilate anion provides a longer lifetime for the acylal intermediate, which is in equilibrium with the oxocarbenium species and free Asp. Access is thereby allowed to an alternate reaction pathway in which the elevated acidity of C2′ in the oxocarbenium species facilitates deprotonation by the essential Asp with reprotonation from the opposite face to yield the *arabino* product (Scheme S3).[14]

The glycal intermediate evidenced by the *arabino* product of F^{5}U led us to consider another mechanism for the Ψ synthases.[14] This "glycal mechanism" begins with deprotonation of C2′ to eliminate the pyrimidine ring and form the glycal intermediate followed by reattachment of the repositioned pyrimidine ring to form the *C*-glycoside (Scheme 1). The mechanisms of uridine phosphorylase,[18] α-1,4-glucan lyase,[19] and UDP-*N*-acetylglucosamine 2-epimerase[20] provide biochemical precedents for glycal intermediate formation. To test whether the Ψ synthases follow the glycal mechanism, the 2′-deuterated substrate was prepared, and the effect on the reaction rate was determined: a primary deuterium kinetic isotope effect (KIE) is expected if either deprotonation or reprotonation of C2′ is rate-limiting.

[2′-^{2}H]Uridine triphosphate ([2′-^{2}H]UTP) was prepared from D-[2-^{2}H]ribose by the method of Williamson (Scheme S5)[21] and used in runoff *in vitro* transcription[22] to generate stem–loop substrates for TruB and RluA (Figures S7 and S8); the identities and isotopic composition of these substrates were verified by MALDI-MS analysis (Figures S9 and S10). Efficient *in vitro* transcription required the slight alteration of the stem–loop substrates previously used for TruB and RluA, which were chemically synthesized.[13,23] A G residue was added to the 5′-end of the TruB substrate (GCUGUGUUCGAUCCACAG; the isomerized U is underlined), and a G:C base pair was reversed (to C:G) in the stem of the RluA substrate (GCGGAUUGAAAAUCCGC). Unlabeled versions of each stem–loop substrate were prepared by the same methodology as the deuterated substrates, and the alterations in the stem–loop substrates did not substantially perturb the measured kinetic parameters (Table S7). The reaction kinetics for the unlabeled and deuterated substrates were then compared (Figure 2; Table 1).

The observed KIEs on V_{max} (2.5 with TruB; 1.8 with RluA) and V_{max}/K_m (3.6 with TruB; 2.2 with RluA) are too large to be secondary effects and clearly indicate that deprotonation or reprotonation of C2′ (or both) is partially rate-limiting during the conversion of U to Ψ. These KIEs are consistent with the finding that steps other than RNA binding and release are rate-limiting for TruB and RluA[24] and provide direct evidence for the operation of the glycal mechanism in Ψ synthases from two families of these enzymes. Given that all six families share a core β-fold and several conserved active site residues,[1,2] these results likely apply to all Ψ synthases. In the cocrystal structures of TruB and RluA with [F^{5}U]RNA,[16,17] the essential Asp is the only enzymic acid/base group in the vicinity of C2′ to effect the deprotonation/reprotonation and the only group that seems able to deliver a proton to the appropriate face of the glycal intermediate to form the *arabino* product of F^{5}U. These

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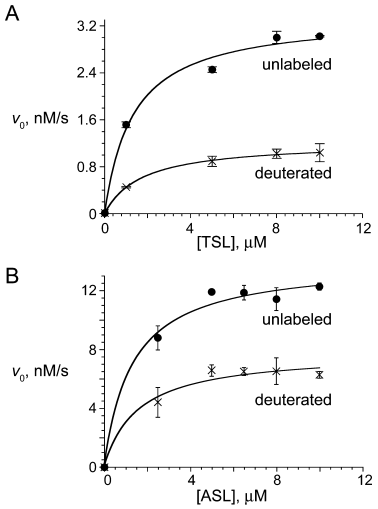

Figure 2. Briggs–Haldane plots comparing the reaction rates of unlabeled (●) and deuterated (×) substrates. (A) TruB. (B) RluA. Fits to the Briggs–Haldane equation (R^2 = 0.95–0.99) are shown and were used to determine the values of V_{max} and K_m.

Table 1. Kinetic Parameters for TruB and RluA with Deuterated and Unlabeled Substrates

			KIE	
substrate	V_{max} (nM/s)	K_m (μM)	V_{max}	V_{max}/K_m
		TruB		
unlabeled	3.4(2)	1.3(3)	2.5(1)	3.6(3)
deuterated	1.2(2)	1.7(1)		
		RluA		
unlabeled	13.9(8)	1.3(4)	1.8(1)	2.2(1)
deuterated	7.8(7)	1.6(7)		

considerations led to the depiction of the mechanism in Scheme 1, but alternative scenarios are also consistent with the KIEs. Instead of the essential Asp, O^2 of the pyrimidine ring might be the base that deprotonates C2′,[18] but since the same proton is returned to the pentose ring, the removed proton would need to migrate to either the essential Asp or O^4 (perhaps facilitated by the Asp) before reprotonation to give a *ribo* product. Migration of the proton to the essential Asp appears the only possibility to give the *arabino* product of F^5U since repositioning of the bulky pyrimidine ring to allow either O^2 or O^4 access to the distal face of the glycal intermediate seems highly unlikely.

The evidence in favor of the traditional Michael mechanism with the essential Asp as the nucleophile derives entirely from studies using [F^5U]RNA, and the *arabino* product of F^5U seems inconsistent with the operation of that mechanism (*vide supra*). The observed primary deuterium KIEs are, however, consistent with the deprotonation of a Michael adduct to form a glycal intermediate. Such a scheme requires both deprotonation and reprotonation of C2′ by the pyrimidine ring oxygen atoms if the essential Asp is the Michael nucleophile.

Alternatively, another residue could serve as the Michael nucleophile. The recent cocrystal structure of the Ψ synthase RluB and [F^5U]RNA was the first to reveal a covalent adduct between F^5U (again rearranged to a *C*-glycoside) with an enzymic group, but it was an active site Tyr rather than the essential Asp.[25] The simplest explanation for the covalent adducts observed by gel electrophoresis or crystallography is that the rearranged but not yet hydrated product of F^5U is trapped when the geometry of a particular Ψ synthase active site allows the close approach of an enzymic nucleophile (whether Asp or Tyr).[10,12,13] However, the adduct with Tyr observed in the cocrystal of RluB opens the possibility of a Michael adduct in which the essential Asp is free to conduct proton transfers. This scenario is disfavored by residual activity when the corresponding Tyr is substituted with Phe (removing the nucleophilic hydroxyl group) in RluB,[25] the human Ψ synthase Pus1p,[26] and RluA (manuscript in preparation). The active site Tyr is therefore not essential for the activity of three Ψ synthases. Additionally, the RluA variant forms an adduct with [F^5U]RNA that is indistinguishable by denaturing gel electrophoresis from that observed with wild-type RluA (manuscript in preparation). These observations and the *arabino* product of F^5U favor the simpler glycal mechanism proposed in Scheme 1 over a mechanism with a Michael adduct.

The traditionally formulated acylal mechanism (Scheme S2) is irreconcilable with the observed primary KIEs, which demand deprotonation/reprotonation of C2′. Acylal formation with the essential Asp could constitute nucleophilic catalysis to activate U for glycal formation similar to the situation with α-1,4-glucan lyase.[19] Acylal formation would otherwise merely be a diversion of the oxocarbenium ion that delays deprotonation to form a glycal. In any case, the incorporation of features of the previously proposed mechanisms are better treated as variants of the glycal mechanism, for the primary KIEs demand the inclusion of a glycal intermediate in any mechanism for the Ψ synthases and provide the first definitive evidence for the mechanism based on studies with the natural substrate (U) rather than an analog (F^5U). Finer points remain to be elucidated, but the results presented here unambiguously establish that the Ψ synthases operate through the glycal mechanism rather than the proposed alternatives.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04491.

Experimental details, elaboration of mechanisms and the deuterium wash-in and KIE experiments, additional data and graphics (PDF)

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Notes

The authors declare no competing financial interest.

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