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Pseudouridine Synthases

Review

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Pseudouridine synthases are the enzymes responsible for the most abundant posttranscriptional modification of cellular RNAs. These enzymes catalyze the site-specific isomerization of uridine residues that are already part of an RNA chain, and appear to employ both sequence and structural information to achieve site specificity. Crystallographic analyses have demonstrated that all pseudouridine synthases share a common core fold and active site structure and that this core is modified by peripheral domains, accessory proteins, and guide RNAs to give rise to remarkable substrate versatility.

Introduction

In addition to the four standard nucleotides, cellular RNAs contain over one hundred chemically distinct and site-specific posttranscriptionally modified nucleotides [1] (http://medlib.med.utah.edu/RNAmods). Of these, pseudouridine (Ψ), the C5-glycoside isomer of uridine, is the most abundant, and was the first to be discovered [2]. The existence of Ψ as a "fifth nucleotide" comprising ~4% of the nucleotides of yeast tRNA was recognized by 1957 [3], and its chemical nature was established two years later [4–7]. Johnson and Söll [8] demonstrated by RNase digestion and 2D TLC analysis of in vitro transcribed, [^{14}C]uridine-labeled RNA that a Ψ synthase activity present in *Escherichia coli* S-100 extracts produces Ψ by isomerizing uridines that are already part of RNA chains (Figure 1). Moreover, they showed that the activity is specific for a particular RNA (tRNA in this case), being inactive on polyuridylic acid or λ RNA. Subsequent biochemical characterization demonstrated that Ψ synthases do not require any cofactors for catalysis, and that they do not detectably release the uridine base that is isomerized [9–11]. The enzymatic mechanism that underlies the deceptively simple isomerization reaction and the molecular recognition strategies that give rise to the specificity of Ψ synthases for their cognate RNAs have both remained unknown. Recent molecular cloning of numerous pseudouridine synthases and subsequent crystallographic structure determinations have given new impetus to the biochemical analysis of these universally distributed enzymes.

Pseudouridine Synthase Families

All known Ψ synthase sequences from archaea, bacteria, and eukarya can be classified into five families [12, 13]. These are named after the *E. coli* enzymes RluA, RsuA, TruA, TruB, and TruD (Table 1). In the past five years, crystal structures have been determined of proteins belonging to each of the five families. Despite minimal sequence similarity between enzymes of different families, structural comparison reveals that all Ψ synthases share a core with a common fold and a conserved active-site cleft (Figures 2A and 2B). The conserved core of Ψ synthases consists of an eight-stranded mixed β sheet, with several helices and loops flanking the catalytic cleft that bisects the sheet. A loop that carries a catalytically essential aspartate residue (the only absolutely conserved residue found in all Ψ synthases) occupies part of the cleft. The conserved Ψ synthase core is decorated by diverse additional secondary structure elements in each enzyme. Further, a variety of independently folded domains are present as N- or C-terminal extensions in several Ψ synthases. Some enzymes of the RluA and RsuA families have N-terminal domains that resemble ribosomal protein S4. The enzymes of the TruB family have a C-terminal PUA domain. This domain is so named [14] because of its presence in some *p*seudo*u*ridine synthases and in some *a*rchaeosine-transglydosylases (unrelated RNA-modifying enzymes).

The results of structural and sequence analyses are consistent with the evolutionary relationship between Ψ synthase families depicted in Figure 2C. When they were discovered, enzymes of the TruD family did not appear to have any sequence similarity to other Ψ synthases [13, 15]. Structure determination of *E. coli* TruD revealed that the order of the secondary structure elements of the core domain of TruD Ψ synthases is a circular permutation of the order in which they are present in Ψ synthases of the four other families [16–18]. Because TruD homologs (all similarly circularly permuted) are present in all phyla, the most parsimonious evolutionary scenario is that TruD diverged first from all other Ψ synthases. Of the remaining four families of Ψ synthases, TruA and homologs are the most distantly related. Their sequences bear minimal similarity to those of other enzymes, and uniquely among characterized Ψ synthases, they appear to function as dimers. RluA and RsuA family enzymes are the most closely related, and sequence analyses [12] demonstrated that they share three conserved sequence motifs (Motifs I, II, and III). Finally, TruB family enzymes have sequences that can be aligned with those of RluA and RsuA family members, but lack a Motif III detectable by sequence alignment [12].

Substrate RNA Diversity

One of the most remarkable features of Ψ synthases is their substrate specificity. Ψ synthases recognize their substrate uridine in the context of an RNA. This is necessary in order to avoid depleting the cellular pool of uridine, the precursor to the thymidine needed for DNA synthesis (because unlike uridine, Ψ cannot be converted to thymidine by methylation). Moreover, if Ψ triphosphate were available for RNA synthesis, RNA polymerase would incorporate Ψs randomly during transcription. Thus, Ψ synthases need to achieve

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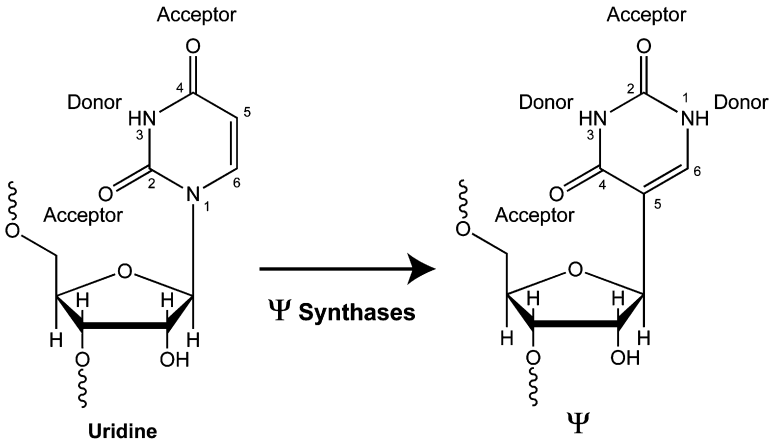

Figure 1. Isomerization of Uridines That Are Already Part of an RNA to Pseudouridine (Ψ) by Ψ Synthases

Potential hydrogen bond donors and acceptors are indicated.

specificity utilizing the sequence or structural context of their target site. Except for the Ψ synthase Cbf5 and its orthologs, which function as part of an elaborate ribonucleoprotein (RNP) complex (discussed below), all Ψ synthases examined to date are capable of recognizing their substrates without any accessory factors. Some Ψ synthases modify a single position in the cellular RNAs of an organism. For instance, RsuA exclusively isomerizes U516 in the small ribosomal RNA of *E. coli* (Table 1). Other enzymes isomerize uridines in structurally equivalent locations in multiple related RNAs. TruB, which is responsible for the universally conserved Ψ55 of the TΨC loop of all the elongator tRNAs of a cell, displays this type of specificity. RluA is an example of an enzyme that modifies two structurally distinct types of RNAs (23S rRNA and certain tRNAs) at positions that share local sequence and structural similarity. Yet other Ψ synthases, such as TruA and RluD, are capable of modifying several nearby sites on one specific RNA. How Ψ synthases, which share a common protein fold, can display such diverse substrate specificities is a fascinating question in the evolution of molecular recognition.

RNA Recognition and Base Flipping

The first structure of a Ψ synthase bound to RNA to be solved was that of *E. coli* TruB [19]. Although the cellular substrates of this enzyme are tRNAs, biochemical experiments had shown that oligonucleotides comprising just the T-stem and loop (TSL) to tRNAs contain all the determinants necessary for specific recognition and pseudouridylation [20]. The cocrystal structure of TruB bound to a TSL demonstrated that the enzyme recognizes the characteristic, pre-folded structure of T-loops [21], primarily by shape complementarity. In addition, TruB discriminates against other instances of the ubiquitous [22] T-loop motif by making sequence-specific contacts with the few nucleotides that are invariant in tRNA T-loops, such as the C56 of the TΨC motif. In the free tRNA, this last nucleotide is base paired with a nucleotide in the D-loop. In the TruB-bound form of the tRNA, C56 is flipped out from the helical stack so that the nucleobase can be directly read out by amino acid side chains of the protein (Figures 3A and 3C). Indeed, TruB binding results in three nucleotides, including the site of pseudouridylation, to be flipped out from the interior of the RNA (Figure 3C). The flipped-out,

Table 1. Five Families of Pseudouridine Synthases

Name	Substrate RNA	Modification Site	Catalytic Aspartate	References	Structure
TruD family					
TruD	tRNA	13	Asp80	[13]	1SB7, 1SI7, 1SZW
TruA family					
TruA	tRNA	38, 39, 40	Asp60	[39, 85, 86]	1DJ0
TruB family					
TruB	tRNA	55	Asp48	[53, 87]	1K8W, 1R3F, 1ZL3, (1R3E, 1SGV, 2AB4, 1ZE1, 1ZE2)
RsuA family					
RsuA	16S rRNA	516	Asp102	[88, 89]	1KSK, 1KSL, 1KSV, (1VIO)
RluB	23S rRNA	2605	Asp110	[41]	
RluE	23S rRNA	2457	Asp69	[41]	
RluF	23S rRNA	2604	Asp107	[41]	2GML
RluA family					
RluA	23S rRNA	746	Asp64	[24, 40, 90]	2I82
	tRNA	32			
RluC	23S rRNA	955, 2504, 2580	Asp144	[39, 91]	1V9K, 1XPI
RluD	23S rRNA	1911, 1915, 1917	Asp139	[39, 54]	1QYU, 1PRZ
TruC	tRNA	65	Asp54	[41]	

Sites of modification and catalytic aspartate residue numbers correspond to *E. coli* RNAs and enzymes, respectively. PDB ID(s) in parentheses are of crystal structures of enzymes from organisms other than *E. coli*.

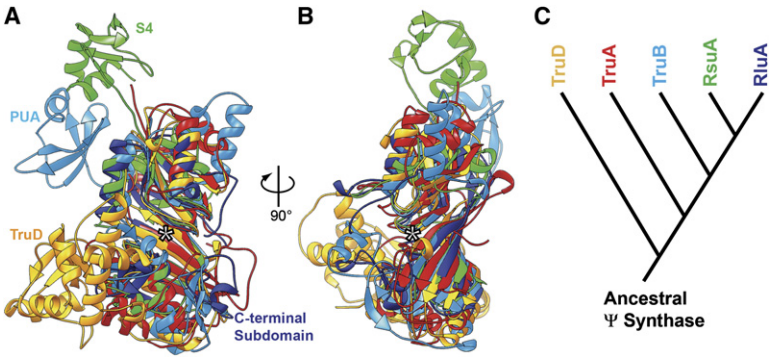

Figure 2. Structural Comparison of Members of the Five Families of Ψ Synthases

(A) Superposition of ribbon representations of the structures of TruD [17] (yellow), TruA [38] (red), TruB [19] (cyan), RsuA [36] (green), and RluA [25] (navy) all from *E. coli*. The core domain of all enzymes is very similar. In this front view, the active site cleft bisects the core domain horizontally. Peripheral domains (TruD, PUA, S4, and C-terminal subdomain) unique to specific enzymes are also indicated (see Table 2). The active site cleft is indicated by an outlined black asterisk. Figures prepared with RIBOONS [93].

(B) View rotated 90° along the vertical axis.

(C) Hypothetical evolutionary relationship between Ψ synthase families based on their sequences and structures.

TruB-bound conformation of the RNA appears to be stabilized by a histidine residue that is absolutely conserved among TruB family Ψ synthases (Table 2). Main chain atoms of this histidine make hydrogen bonds that recognize the reverse-Hoogsteen pair that is characteristic of T-loops. The imidazole ring of the side chain stacks underneath the reverse-Hoogsteen pair, taking the place of a nucleobase (G18 from the D-loop) that resides there in the folded structure of free tRNA (Figures 3A and 3C). The conserved histidine is five residues N-terminal to the catalytic aspartate, and it facilitates TruB to position the flipped-out nucleotide 55 (the site of modification) in the deep solvent-inaccessible active-site pocket, in direct contact with the catalytic aspartate. Although base flipping had been documented earlier in cocrystal structures of DNA methyltransferases [23], this was the first observation of this mechanism of action in an enzyme responsible for posttranscriptional RNA modification.

The substrates of the *E. coli* Ψ synthase RluA are 23S rRNA and several tRNAs. Globally, the 2904 nucleotide (nt) 23S rRNA and the ~76 nt tRNAs have no structural similarity. However, the sites of modification are all on the 5′ side of the loop of a simple stem-loop, and they all share the consensus ΨUXXAAA (X is any nucleotide) [24, 25]. Many RNA binding proteins achieve sequence specificity by splaying out nucleotides in a loop and making direct nucleobase-amino acid contacts with the splayed bases [26, 27]. Because of this precedent, and because of the sequence conservation flanking the site of modification in its substrates, a reasonable expectation was that RluA would achieve sequence specificity in a similar manner.

The cocrystal structure of RluA bound to a substrate anticodon stem loop (ASL) demonstrated that, rather than simply splaying the loop of the substrate, this enzyme achieves specificity by imposing a completely new structure on the loop [25] (Figures 3B and 3D).

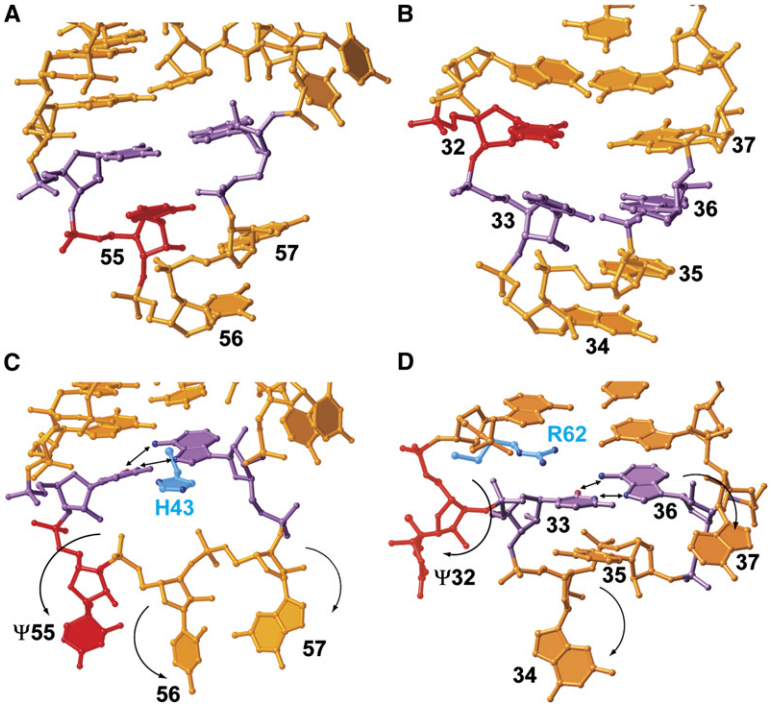

Figure 3. Substrate RNA Recognition by TruB and RluA

(A) Structure of the free TSL of yeast $tRNA^{Phe}$ [92].

(B) Structure of the free ASL of yeast $tRNA^{Phe}$ [92].

(C) Structure of the *E. coli* TSL bound to TruB [19]. Three nucleotides 55, 56, and 57 are flipped out of the helical stack by binding to TruB. His43 (cyan) of TruB recognizes the conserved reverse-Hoogsteen base pair (purple), and occupies the space left by the G18 of the D-loop in the folded structure of free tRNA. The site of modification is in red.

(D) Structure of *E. coli* ASL bound to RluA [25]. Three nucleotides 32, 34, and 37 are flipped out from the loop and three others 33, 35, and 36 adopt new stacked positions when this ASL is bound to RluA. Arg62 of RluA (cyan) intercalates between the newly formed reverse-Hoogsteen base pair (purple) and the base pair above, and occupies the space left by the flipped-out residue 32 (the site of modification, red).

Table 2. Domain Structure of Pseudouridine Synthase Families

Name	N-Terminal Extension	Forefinger Loop	Active Site Consensus Sequence	Thumb Loop	C-Terminal (CT) Extension
TruD family					
TruD	–	–	**X A G X K D**	+	–
TruA family					
TruA	–	+	**X X X R T D**	+	–
TruB family					
TruB	–	–	**H X G X L D**	+	PUA domain
RsuA family					
RsuA/RluE	S4-like domain	+	**X X G R L D**	–	–
RluB/RluF	S4-like domain	+	**X X G R L D**	–	CT domain
RluA family					
RluA	–	+	**X X H R L D**	+	–
RluC/RluD	S4-like domain	+	**X X H R L D**	+	CT subdomain

"X" indicates any residue. "+" and "–" indicate the presence and absence of extensions or loops in their structure, respectively.

The canonical structure of the ASL of tRNAs consists of an U-turn followed by a stack of nucleotides on the 3′ side of the loop. This structure remains basically unchanged even when the ASL is engaged in codon-anticodon pairing in the decoding site of the ribosome [28]. In contrast, the RluA-bound structure of the anticodon loop of the ASL contains a reverse-Hoogsteen base pair that is absent in the normal tRNA, two new base-stacking interactions, and three flipped-out nucleotides (Figure 3D). One of the flipped-out nucleotides is the site of isomerization. The space that it vacates in the helical stack is taken up by the planar, cationic guanidino group of an arginine that is two nucleotides N-terminal to the catalytic aspartate (Figure 3D and Table 2). This arginine is absolutely conserved in Ψ synthases of the RluA, RsuA, and TruA families (Table 2). Thus, it probably plays the same role in facilitating substrate base-flipping in all these enzymes (a role analogous to that of the conserved histidine in the TruB family). At present, it is not clear how TruD family Ψ synthases access their substrate uridines. RluA makes very few direct interactions with the bases of the rearranged ASL. Rather, recognition appears to rely on shape complementarity to the new secondary structure induced by protein binding, and by the ability of the RNA sequence to adopt this structure. Structure-guided mutagenesis experiments support the key role of the reverse-Hoogsteen pair (between the underlined nucleotides: Ψ<u>U</u>XX<u>A</u>AA). No pyrimidine•purine pair other than the U•A present in the cognate substrates would be able to form this pair. Thus, RluA appears to recognize its substrates by indirect readout of a protein-induced RNA structure [25].

Peripheral Domains in RNA binding

Comparison of the RNA-complex structures of RluA and TruB shows that the active site cavities are very similar, and that the flipped-out nucleotides at the respective sites of isomerization superimpose closely (Figure 4A). Nonetheless, the angle of approach of the two substrate RNAs differs by nearly 60° (Figure 4A). A thumb-like protrusion that flanks the lower margin of the active site cleft of TruB appears to play an important role in RNA binding by this enzyme. Structure determinations of RNA-free TruB imply that the thumb undergoes a large conformational change upon binding the major groove of its substrate RNA [29–31]. Moreover, small perturbations in the active site of RNA-bound TruB appear preferentially to propagate to the thumb [32]. RluA employs a loop equivalent in position to that of the TruB thumb as well as a second loop, this one present on the upper margin of the active site cleft and facing the minor groove of the RNA, together to "pinch" the loop of its substrate (Figure 4A). This second forefinger-like loop is present in enzymes of the RluA, RsuA, and TruA families, but absent in TruB and its homologs (Figures 4B, 4D, and 4E). Thus, RluA and TruA family proteins are likely to pinch their cognate RNAs in a manner similar to that employed by RluA. RsuA family Ψ synthases lack a thumb, and therefore must bind their substrates differently (Figure 4D).

The structure of TruD shows that it too has a thumb (Figure 4C). However, instead of being a small insertion, the TruD thumb is a large domain (comprising ~40% of the enzyme) with a fold that is, thus far, unique among known protein structures [16, 18]. This large TruD thumb appears capable of undergoing a hinge-like motion relative to the active site cleft [17]. Although elucidation of TruD-RNA recognition must await structure determination of the complex, it is likely that the large thumb domain of these proteins will play a critical role. Other Ψ synthases also have family-specific insertions and peripheral domains that are likely to participate in substrate recognition. The PUA domain of TruB makes non-sequence-specific contacts with the acceptor stem of tRNA. Structures of RluC and RluD demonstrate the presence of a C-terminal subdomain [33–35]. These proteins, as well as RsuA, have an N-terminal S4 domain that is flexibly linked to the core domain [35–38]. Thus, it appears that a combination of thumb and forefinger loops, as well as the presence of a variety of peripheral domains allows the conserved Ψ synthase fold to achieve its remarkable versatility in binding to diverse RNA structures and sequences.

Active Site and Catalysis

Superposition of Ψ synthase structures shows that only three polar residues are conserved within the predominantly hydrophobic active sites of these enzymes (Figure 5). An aspartic acid present in the active site loop is the only strictly conserved sequence feature of these enzymes. Three lines of evidence indicate that the conserved aspartic acid plays a critical role in catalysis, rather than in protein structure or RNA recognition. First, site-directed mutagenesis has shown that it is essential for catalysis in members of all five Ψ synthase families

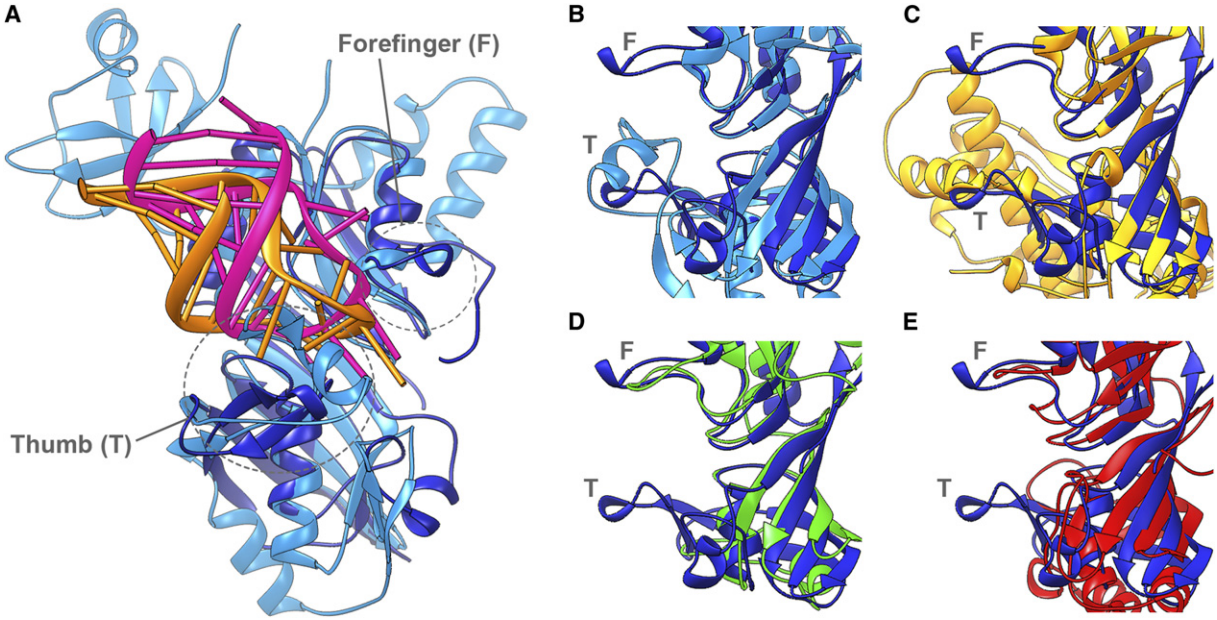

Figure 4. RNA Recognition by Ψ Synthases Belonging to Different Families

(A) Superposition of ribbon representations of the RluA-ASL (navy and orange) and TruB-TSL (cyan and magenta) complex structures [19, 25]. The locations of the forefinger and thumb loops are indicated by gray dotted circles.

(B) Superposition of TruB (cyan) and RluA (navy).

(C) Superposition of TruD [17] (yellow) and RluA (navy).

(D) Superposition of RsuA [36] (green) and RluA (navy).

(E) Superposition of TruA [38] (red) and RluA (navy).

Forefinger (F) and thumb (T) loops are indicated. Note the variable occurrence of the forefinger and thumb loops among different enzymes (see Table 2). The views in (B) to (E) are rotated 90° along the vertical axis relative to (A).

[24, 39–41]. Second, structure determination of a catalytically inactive TruB mutant in which this aspartate had been replaced with an asparagine demonstrated that the protein adopts a structure indistinguishable from that of the wild-type [32]. Third, the conserved aspartic acid is closely apposed to the nucleobase at the site of pseudouridylation in both Ψ synthase-RNA complex structures [19, 25]. The other two conserved polar residues are a lysine or arginine, whose side chain makes a buried salt bridge with the catalytic aspartate, and a tyrosine (invariant, except in TruD family members where it is replaced by a phenylalanine) (Figure 5).

Structural conservation of the active site is consistent with the three sequence motifs detected by Koonin [12]. The catalytic aspartate lies within Motif II, which encompasses the active site loop (and includes the intercalating histidine or arginine of TruB family members and RluA, RsuA, and TruA family members, respectively). Motif I does not directly form part of the active site cleft. Structural [32, 42] and biochemical [43] results suggest that its primary function is to buttress the active site loop. The C-terminal half of Motif III contains the conserved, buried basic residue that makes a salt bridge with the catalytic aspartate. The cocrystal structure of RluA shows that the N-terminal half of Motif III binds to the backbone of the substrate RNA 5′ to the site of modification. Because of the different angle at which the RNA approaches the TruB active site, the N-terminal half of Motif III does not make these interactions with the RNA. This explains the apparent absence of Motif III in TruB family member sequences.

How can three polar amino acids catalyze the isomerization of U into Ψ? Any catalytic mechanism must formally proceed through disconnection of the glycosidic bond, rotation of the detached base, and its reconnection to the ribose through C5. In addition, the catalytic mechanism must explain the inhibition of some Ψ synthases by RNAs in which the U at the site of isomerization has been replaced by 5-fluorouridine (f^5U). *E. coli* TruA makes an SDS-resistant complex with f^5U-containing tRNAs. Based on this observation and degradation analyses of the complex, Santi and coworkers proposed a mechanism in which the catalytic aspartate makes a Michael-type attack on C6. Following breakage of the glycosidic bond, this would allow the detached base to rotate along the ester bond connecting it to the catalytic aspartate. Presence of the fluorine at position 5 of the base was thought to inhibit breakage of the glycosidic bond [44].

Structure determination of *E. coli* TruB bound to a f^5U-containing RNA revealed not a suicide complex, but rather, a product of isomerization of f^5U (5-fluoro, 6-hydroxypseudouridine), and no covalent connection between the RNA and the catalytic aspartate [19] (Figure 5). Further biochemical studies confirmed this discrepancy, and demonstrated that while some Ψ synthases, such as *E. coli* TruA and RluA do indeed form SDS-resistant complexes with RNAs containing f^5U, and are strongly inhibited by such RNAs, other Ψ synthases, such as *E. coli* TruB, efficiently isomerize f^5U-containing substrates, and are not inhibited [45]. Crystallographic analysis of the SDS-resistant complex

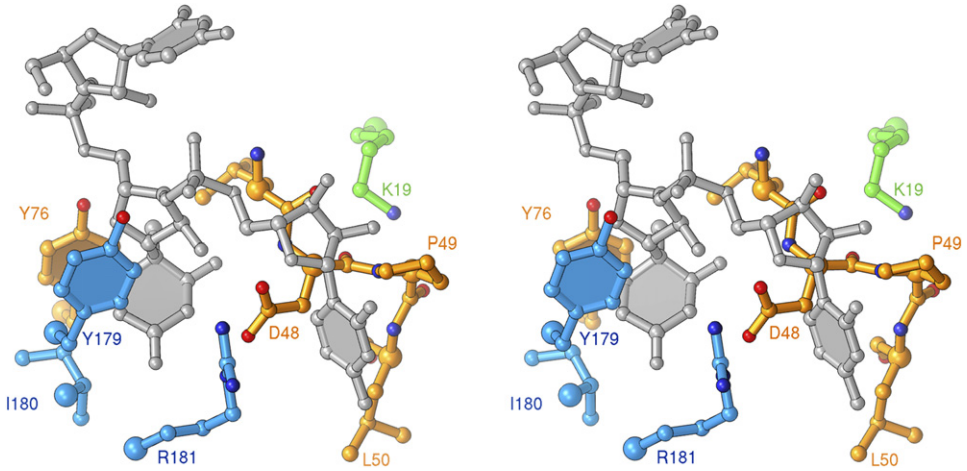

Figure 5. Stereoview of the Conserved Active Site Residues of *E. coli* TruB

Residues in Motif I are depicted in green, Motif II and other catalytically important residues in yellow, and all other residues in cyan. The catalytic aspartate (Asp48) is shown in the center. Three nucleotides from TSL in the TruB-TSL complex [19], including the isomerized 5-fluorouridine (5-fluoro-6-hydroxypseudouridine), are shown in gray.

formed between a f^5U-containing RNA and RluA was hampered by the X-radiation sensitivity of the adduct [25]. Because the active sites of RluA and TruB are very similar, the divergent behavior of the enzymes toward f^5U-containing RNAs is enigmatic. The puzzling interactions of Ψ synthases and f^5U-containing RNAs underscores the current lack of a satisfactory catalytic mechanism for these enzymes [46]. Indeed, it is still formally possible that the catalytic aspartate functions not by attacking the nucleobase, but by making a nucleophilic attack on the anomeric position of the sugar [39], in a manner reminiscent of the mechanism of some DNA glycosylases [47]. The uncertainty in the role of the active site aspartate extends also to the conserved basic and aromatic residues. The basic residue may serve to make the aspartate more nucleophilic, or it may serve as a source of protons. The aromatic residue, which is usually a tyrosine, may serve as a general acid or base [48]. However, this would require that enzymes of the TruD family (which have a phenylalanine at the equivalent position) have a completely different catalytic mechanism. Given the high degree of structural conservation of Ψ synthase active sites, a common, universal mechanism appears likely. The elucidation of the catalytic mechanism of Ψ synthases remains an open challenge.

Biological Functions of Ψ and Ψ Synthases

The biochemical functions of most Ψ residues in RNAs are not understood. However, many sites of pseudouridylation are highly conserved throughout phylogeny, and Ψ residues often cluster near functionally important regions of cellular RNAs. Differences between uridine and Ψ that may be biologically important are the ability of Ψ to form an additional hydrogen bond in the major groove, and the increased rigidity and stability of RNAs with Ψ [49]. U2 snRNA contains several phylogenetically conserved Ψ residues that are essential for spliceosomal biogenesis [50]. This spliceosomal RNA base pairs with the branch sites of pre-mRNA introns during splicing. The conserved U2 snRNA Ψ residue that lies directly across the duplex from the branch site adenosine has been shown to stabilize an altered structure that places the 2′-OH of the branch site (the nucleophile of the first step of splicing) in a more accessible conformation [51]. Multiple Ψ residues are found in the peptidyl-transferase and decoding centers of the ribosome. Inhibition of the formation of individual Ψ residues in yeast ribosomal RNAs demonstrated that although no single pseudouridylation is essential, the lack of some Ψ residues is very detrimental for viability [52].

Deletion of either RluD or TruB in *E. coli* results in a loss of the expected (Table 1), specific Ψ residues in tRNA or rRNA, respectively. Cells lacking the open reading frames for either of these two enzymes display growth defects. Remarkably, in both cases, expression of enzymatically inert RluD or TruB proteins containing a point mutation of the catalytic aspartate completely rescues growth [53, 54]. These genetic results suggest that RluD and TruD may have functions other than Ψ synthesis. These enzymes may function as RNA chaperones, and their ability to bind to their substrate RNAs during their maturation may be more important than pseudouridylation per se. Another possibility is that multiple posttranscriptional RNA modification enzymes associate to form a larger structural and functional unit whose stability is compromised if one of the enzymes is absent [55, 56].

In eukaryotes and archaea, the Ψ synthase Cbf5 (called dyskerin in human) functions as part of an RNP complex that consists of one of many H/ACA RNAs, and several accessory proteins (Figure 6A). H/ACA RNPs are known to possess at least two functions in addition to pseudouridine synthesis (reviewed in [57]). First, the H/ACA RNP complex formed around the U17 small nucleolar RNA (snoRNA) directs a specific cleavage in the precursor to 18S rRNA. The yeast homolog of U17 is the only H/ACA RNA that is essential in that organism. Second, vertebrate telomerase RNAs contain an H/ACA domain that assembles with Cbf5 and the accessory H/ACA RNP proteins. Correct assembly of this domain is required for telomerase stability and

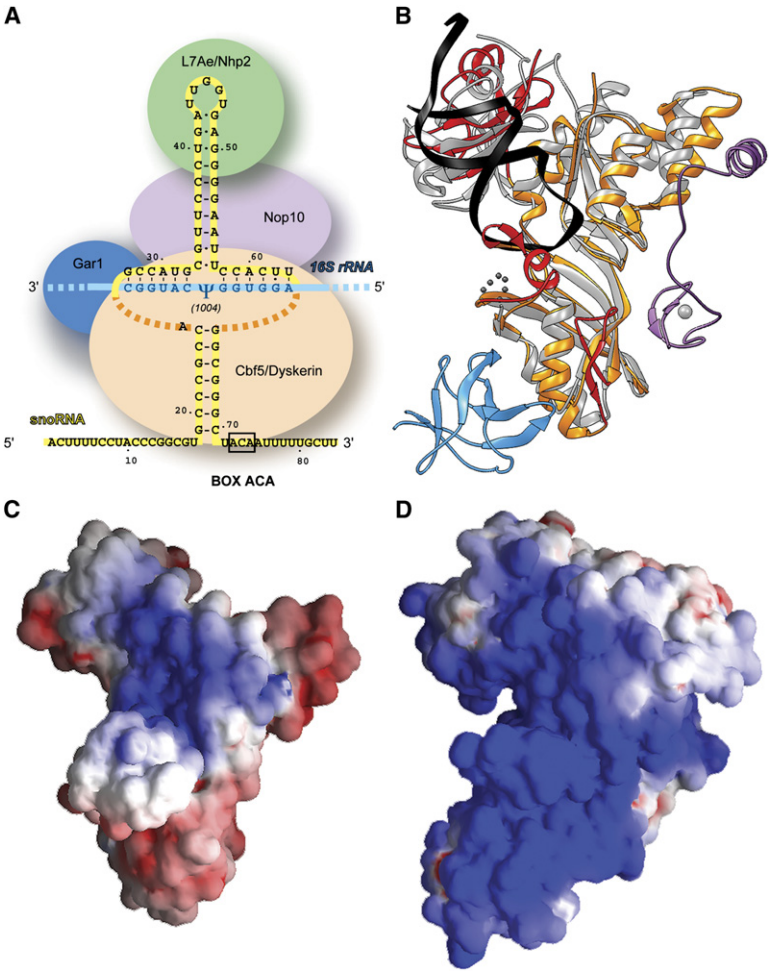

Figure 6. Comparison of the RNA Binding Surfaces of *E. coli* TruB and the Cbf5-Nop10-Gar1 Complex

(A–D) (A) Schematic of a typical H/ACA RNP. Guide and substrate RNA sequences are from *Archaeoglobus fulgidus*. Proteins are shown as colored circles. (B) Superposition of ribbon representations of the structure of TruB (yellow and red) bound to a TSL (black) [19] on the archaeal Cbf5(gray)-Nop10(purple)-Gar1(cyan) complex [42, 83]. The thumb loop residues of Cbf5 are disordered in the two reported Cbf5-Nop10 complex structures [42, 79]. Electrostatic potential, mapped onto the solvent-accessible surfaces of the TruB [19] (C) and the Cbf5-Nop10-Gar1 complex [83] (D). The strongly basic region on the surface of TruB encompasses the active site cleft and the site of TSL binding. The strongly basic region extends to cover the entire face of the Cbf5-Nop10-Gar1 complex, suggesting a likely RNA binding surface that could accommodate multihelical RNAs.

function. Mutations in dyskerin are associated with the X-linked form of the human bone marrow failure syndrome dyskeratosis congenita (DC) [57], and mutations in the H/ACA domain of telomerase RNA are associated with an autosomal form of DC [58]. Cells from DC patients show decreased telomerase activity, reduced amounts of telomerase RNA, and shorter telomeres. Analysis of a mouse knock-in model of DC constructed using a dyskerin mutation with reduced biological activity suggests that defects in both the enzymatic and nonenzymatic functions of Cbf5 are responsible for the development of DC [59].

Cbf5/Dyskerin as a Ψ Synthase

H/ACA RNAs are characterized by a conserved secondary structure that minimally comprises two helices flanking a bulge (reviewed in [57] and Figure 6A). One of the helices is capped by a loop, while the other is preceded and followed by single-stranded RNA segments with the characteristic "box H" and "box ACA" sequences. This minimal structure is usually duplicated in tandem [60, 61], although examples of H/ACA RNAs have been documented having one to four stem-bulge-hairpin structures [62–64]. The internal bulge of H/ACA RNAs is complementary in sequence to the nucleotides flanking a site of pseudouridylation. The two helices formed between the H/ACA and substrate RNAs have been demonstrated to be necessary to guide site-specific pseudouridylation by production in yeast of an artificial H/ACA RNA complementary to a site that is not normally modified. This resulted in the in vivo isomerization of a uridine flanked by the two complementary segments into Ψ [61]. Hundreds of H/ACA guide RNAs have been identified in eukaryotic and archaeal genomes, and in many cases, a complementary cellular RNA with a Ψ residue at the expected location has been identified [62, 65–69].

In addition to Cbf5, which is ~35% identical in sequence to TruB, H/ACA RNAs bind to three highly conserved proteins: Gar1, Nhp2 (L7Ae in archaea), and Nop10. All four proteins are essential in yeast [70–75]. Immunoprecipitated mammalian H/ACA RNPs bearing this minimal complement of proteins have been shown to be enzymatically active [76]. Recently, in vitro reconstitution of enzymatically active H/ACA RNPs has been achieved using purified recombinant archaeal proteins [77, 78]. These studies showed that in addition to an H/ACA RNA, Cbf5 and Nop10 are minimally required for Ψ synthase activity, and that enzymatic activity is enhanced when Gar1 and L7Ae are added [78].

Structure determinations of archaeal Cbf5-Nop10 complexes showed that Cbf5 adopts a structure that is very similar to that of TruB (Figure 6B) [42, 79]. The active sites of the two enzymes superimpose closely, and the

histidine residue that participates in base flipping in TruB, the catalytic aspartate, the buried basic residue, and the active site tyrosine are all present in the equivalent locations in the Cbf5 active site. Nop10, a protein with no paralogs in the sequence databases, adopts an elongated structure that flanks the long axis of the core Ψ synthase domain of Cbf5, and does not form part of the active site. Thus, it is likely that the mechanism of chemical catalysis employed by the Ψ synthase at the heart of the H/ACA RNP will be the same as the mechanisms of the free-standing Ψ synthases, and that the essential role of Nop10 is not enzymatic.

Cbf5/Dyskerin as a Molecular Bracket

What are the functions of the nonenzymatic H/ACA proteins Nop10, Gar1, and L7Ae? During the catalytic cycle of the H/ACA RNP, the guide RNA has to anneal to the substrate, position it in the active site of Cbf5, and then allow the substrate to dissociate from the complex once the isomerization reaction is complete. Annealing of the substrate with the two sides of the central bulge of a guide RNA would create a complicated four-helix-bundle type structure. Thus, it is likely that the accessory H/ACA RNP proteins play roles in stabilizing RNA structure and facilitating the RNA conformational changes that are required for processivity. None of the H/ACA proteins have features characteristic of helicases or other nucleic acid remodeling enzymes. Therefore, binding energy, and the increase in stability of Ψ over uridine must be sufficient to drive the necessary conformational changes.

L7Ae (the archaeal homolog of Nhp2) is a protein that binds an RNA motif called the K-turn [80]. Some archaeal H/ACA RNAs have a minimized form of the K-turn, and L7Ae can also bind these independently of the other H/ACA RNP proteins [64, 81]. L7Ae has been shown to stabilize the characteristic kink of the K-turn in solution [82], and may stabilize functional conformations of the H/ACA guide RNAs.

The structures of the heterodimeric Cbf5-Nop10 complex [42, 79] and the heterotrimeric Cbf5-Nop10-Gar1 complex [83] show how the accessory proteins, as well as the PUA domain of Cbf5, expand the core Ψ synthase domain. Nop10 folds into two lobes that extend the active site cleft of Cbf5 in one direction. The PUA domain of Cbf5 is considerably larger than that of TruB, and the angle formed between the PUA domain and the core Ψ synthase fold extends the active site cleft in the other direction. Gar1 binds farther from the active site than Nop10.

Several properties of the complex formed by these core H/ACA proteins are consistent with a role in organizing the multihelical guide RNA-substrate RNA complex. First, the surface electrostatic properties of the heterotrimeric complex are very different from those of the stand-alone Ψ synthase TruB (Figures 6C and 6D). Whereas only the active site of TruB is strongly basic, the core heterotrimer of the H/ACA RNP has a basic surface that extends over most of its active-site face. Second, the troughs formed by Nop10 and the PUA domain have dimensions appropriate for accommodating double-helical RNA (in addition to being strongly basic). Third, the central, most highly-conserved section of Nop10 directly buttresses the structurally important Motif I of Cbf5. Because Motif I in turn stabilizes the active site loop of Cbf5, Nop10 may couple RNA binding to enzymatic activity. Fourth, the functional importance of the PUA domain of Cbf5 is underscored by the large number of human DC mutants that map to the PUA domain [42, 83]. Site-directed mutagenesis [42] and biochemical reconstitution experiments [79] corroborate the importance of the PUA domain in H/ACA RNA binding. The current structural information suggests that the Cbf5-Nop10-Gar1 complex is well suited for serving as a bracket that spatially organizes the multiple helices of its cognate RNAs. This scaffolding function may also underlie the biological function of the H/ACA domain as part of the U17 and telomerase RNPs. While this manuscript was under review, a crystal structure of a complex between an archaeal H/ACA guide RNA and a Cbf5-Nop10-Gar1-L7Ae complex was published [84]. Although this structure is missing a substrate RNA, it shows that the lower stem of the guide RNA which is flanked by the consensus "ACA" sequence indeed interacts with the PUA domain of Cbf5, and that the upper helix of the H/ACA guide RNA occupies the channel formed by Nop10, as predicted [42, 83]. Elucidation of the specific interactions that allow the core H/ACA proteins to organize the structure of the guide RNA-substrate RNA complex must await further biochemical and structural studies.

Perspectives

A considerable body of structural information has been produced over the last five years on Ψ synthases. This work has shown how a conserved enzymatic core can be extended by loops and insertions, and augmented with additional independently folded domains, partner proteins, and H/ACA guide RNAs to enable these enzymes to act on a wide variety of substrates, while achieving a high degree of site-specificity. Yet, the chemical mechanism of catalysis employed by these universally distributed enzymes remains elusive. The structural information now available constitutes the starting point for further analyses of the catalytic mechanism as well as of the functions of Ψ synthases in organizing large RNA-protein complexes.

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