RNA catalysis

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Our understanding of the relationship between the structure of RNA and its catalytic activity has advanced significantly in the past year. These advances include time-resolved crystallographic studies on the hammerhead ribozyme, as well as new structures of a group I intron, a lead(II)-cleavage ribozyme, a hepatitis delta virus ribozyme, and components of the spliceosome machinery and the peptidyl transferase center of the ribosome and, most significantly, the structure of the ribosome itself.

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Abbreviations

HDV	hepatitis delta virus
snRNA	small nuclear RNA
VS	varkud satellite

Introduction

The author of a noted text on enzyme kinetics stated in his preface that "for all the current and rather silly emphasis on structural biology, understanding enzymes means understanding catalysis, and catalysis is concerned with kinetics, not structure: ... studying the photograph of a racehorse cannot tell you how fast it can run." [1]

Perhaps this may be true, but recent advances in time-resolved crystallography have made it possible to collect a series of 'photographs' or, more accurately, data sets depicting various states along the enzyme's reaction coordinate. Although X-ray crystallography is not well suited to telling us how fast an enzyme can run, it has proven its utility by telling us, in structural terms, how an enzyme can function. Thus, emphasizing structural aspects of enzymology provides a complementary perspective on enzyme catalysis that many regard as being more useful than silly; time-resolved crystallography greatly extends the utility of the technique.

With the discovery that RNA can act as an enzyme, the fundamental question of how ribozymes work has impelled some enzymologists and structural biologists to focus their attention upon RNA-mediated catalysis. In addition to the desire to obtain 'photographs' of these new enzymes in the form of new crystal structures of enzyme–substrate or enzyme–product complexes, protein crystallographers have developed techniques for conducting time-resolved crystallographic analyses [2,3] of enzyme mechanisms. These techniques are proving to be of interest to those of us hoping to understanding how the structure of a ribozyme mediates its catalysis. Hence a series of 'photographs' will hopefully, as in the case of protein enzyme-based time-resolved crystallography, help us to understand how a ribozyme can run, if not how fast it can run.

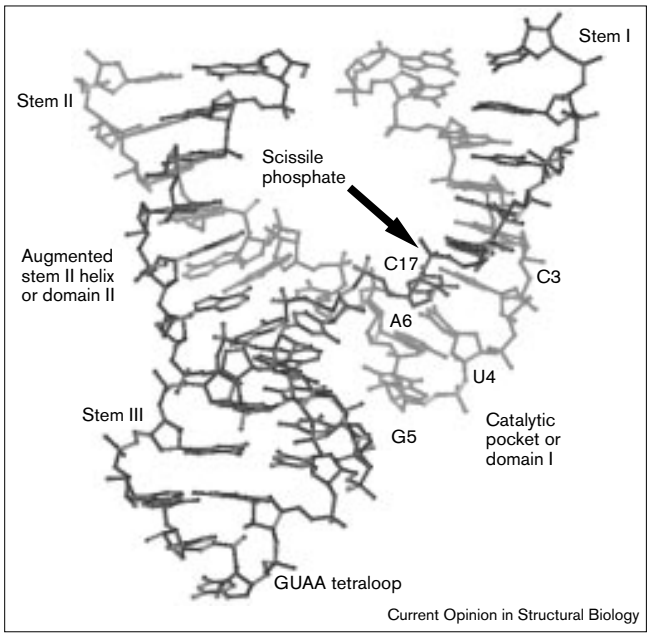
Francis Crick originally speculated that RNA might be capable of catalysis when he remarked upon the protein-like structure of tRNA. When the RNA subunit of ribonuclease P and the L-19 processed group I intron were shown to be enzymes *in vitro*, however, everyone else was rather surprised by the discovery of RNA catalysis. Subsequent to these discoveries, a number of other catalytic RNAs, including the group II intron and several small self-cleaving RNAs derived from satellite virus RNAs (the hammerhead, hairpin and hepatitis delta virus [HDV] ribozymes) were added to the list, which continues to grow slowly [4•].

The first crystal structures of a ribozyme appeared a decade later, in the form of two hammerhead ribozyme constructs in which the cleavage site base was altered in order to prevent catalysis [5,6]. These were followed by a catalytically active form [7]. The structure of an autonomously folding domain of the group I intron also appeared [8] within the year. Crystal structures of the entire catalytic core of the group I intron [9••], as well as one of two structures of the HDV ribozyme [10••], have just been reported. An NMR structure of an *in vitro* selected RNA that cleaves in the presence of Pb(II) is emerging [11•] and an NMR structure of an RNA component of the ribosomal peptidyl transferase center recently appeared [12•]. The hammerhead ribozyme has been the subject of time-resolved crystallographic analyses [7,13••] aimed towards understanding the structural changes that must take place between the initial state structure and structures that are capable of forming the required transition state for the catalyzed cleavage reaction. This review focuses upon the new NMR spectroscopy and crystallographic results listed above.

Ribozyme chemistry

The naturally occurring ribozymes all have RNA substrates [4•]. The group II intron, in addition to catalyzing RNA reactions, also nicks DNA [14•,15]. If the peptidyl transferase activity of the ribosome is truly RNA catalyzed [16•,17••], the scope of natural substrates will be widened. *In vitro* selection experiments clearly demonstrate that RNA is, in principle, capable of catalyzing a wider variety of more complex reactions [18], including peptide-bond formation [19•,20•] and ester transferase activity [21•]. The simplest chemical reaction catalyzed by RNA, self-cleavage of the backbone via phosphodiester isomerization, involves

Figure 1



The 3 Å resolution crystal structure of an uncleaved, unmodified and, therefore, catalytically active hammerhead ribozyme [7]. The cleavage site base is positioned within the catalytic pocket, as indicated. The substrate strand is the dark shade and the enzyme strand is lighter.

nucleophilic attack of the 2′ oxygen upon the adjacent phosphate. As the bond between the phosphorus and the 5′ oxygen is broken (thus cleaving the phosphodiester backbone of the RNA), a 2′,3′-cyclic phosphate is formed. This reaction, catalyzed at a specific cleavage site by the hammerhead, hairpin, HDV and varkud satellite (VS) ribozymes, is the same reaction that causes spontaneous, random base-catalyzed RNA degradation in unstructured RNA. For this reason (as well as its small size and well-characterized biochemistry), the hammerhead ribozyme was a reasonable first choice for structure-based mechanistic studies.

The hammerhead RNA cleavage site is highly specific, the scissile phosphate being more labile by a factor of approximately 10^6 compared to the other phosphates in the ribozyme, most of which adopt approximately helical conformations. Moreover, the hammerhead ribozyme-catalyzed cleavage reaction, unlike spontaneous background cleavage, shows a significant thio effect. Substitution of a phosphate oxygen with sulfur atom sometimes results in diminished activity. This is known as the thio effect. In some cases, the activity can be 'rescued' with a soft metal ion such as Cd^{2+}. This is taken as evidence for the phosphate being a divalent metal-ion-binding site. This indicates that the structure of the transition state that is stabilized by the hammerhead ribozyme must differ in some fundamental aspect from the structure of the transition state of the uncatalyzed reaction. The question as to how the structure of the hammerhead ribozyme mediates the formation of this unique transition-state geometry is therefore of fundamental interest, especially in light of the structure of the 'initial state' of the hammerhead ribozyme.

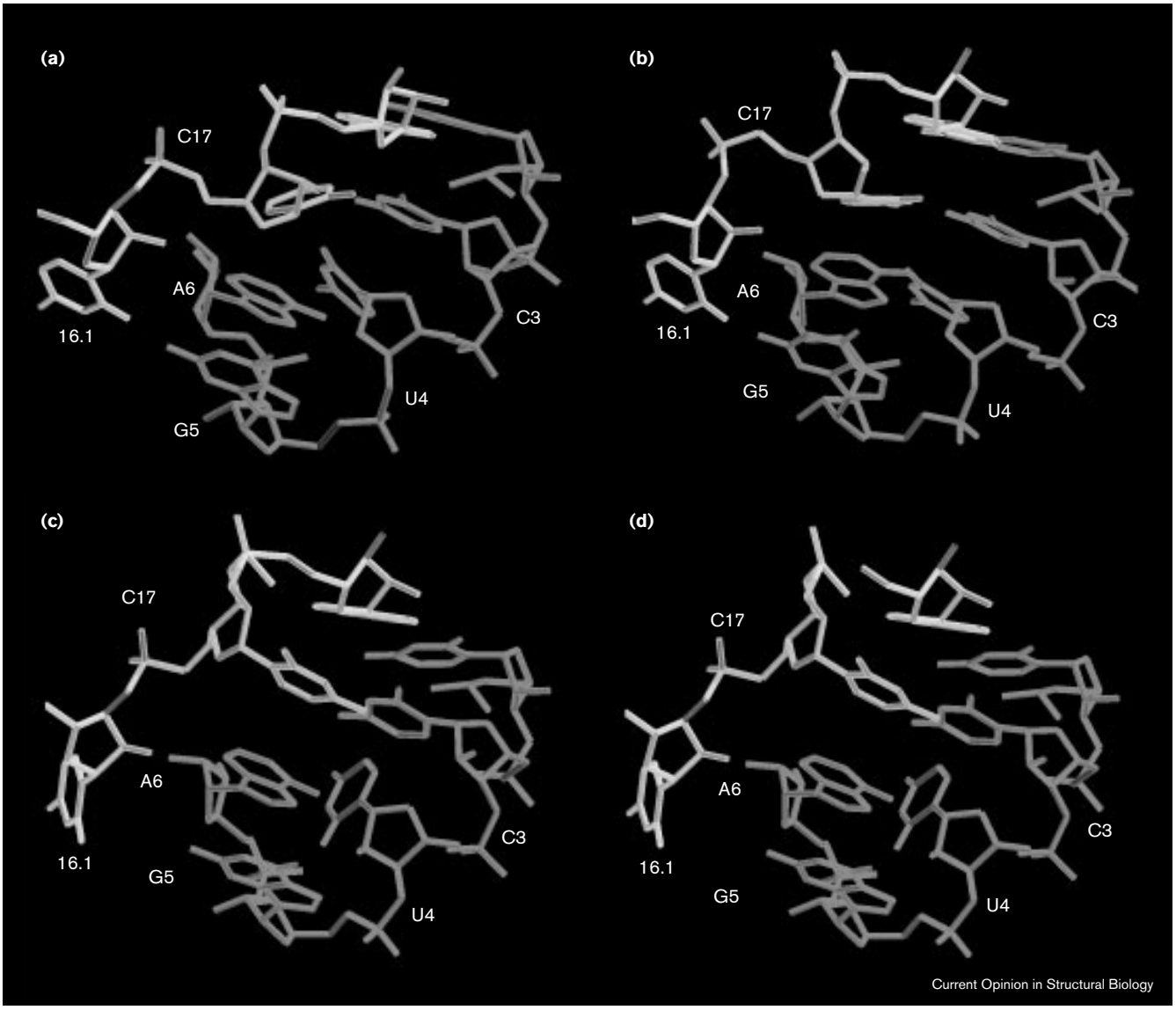
Our understanding of the involvement of divalent metal ions in ribozyme catalysis has been challenged recently by some startling new results. Three groups [22•–24•] have now shown that $[Co(III)(NH_3)_6]^{3+}$ can substitute for $[Mg(H_2O)6]^{2+}$ in the hairpin ribozyme, indicating that divalent metal ions are not active participants in the cleavage chemistry of the hairpin ribozyme. Subsequent analyses of the hairpin, hammerhead, neurospora VS and HDV ribozymes reveal that with high concentrations of monovalent cations (such as Li^+ or even NH_4^+), metal ions are expendable in every case except the HDV ribozyme [25••]. The initial hairpin ribozyme results are thus more generalized than was previously believed; at least three of the ribozymes are not, strictly speaking, metalloenzymes. Tb(III) has also been shown to be a potent inhibitor of the hammerhead ribozyme and is seen to bind to G5, as well as other significant metal-binding sites in the crystal structure [26•].

Ribozyme structure

As mentioned, the first ribozyme structures to be solved were those of the hammerhead ribozyme, including an unmodified, cleavage-active form. These structures revealed the ribozyme to be composed of three A-form helices, one of which (stem II) is extended by noncanonical, (mostly) conserved base pairs and stacks upon another helix (stem III). The remaining helix (stem I) is connected through the cleavage site base, as well as through a conserved uridine turn, into which this base is positioned (Figure 1). The conformation of the cleavage site base itself is intriguing. Despite being sequestered from the rest of the RNA by being positioned within the pocket formed by the uridine turn, the scissile phosphate is found to be in an approximately helical conformation and is therefore not amenable to the known in-line attack mechanism. The requirement for a conformational change in order to activate the scissile phosphate is therefore universally acknowledged [4•,5–7,13••,27,28•,29], but the magnitude and extent of such a change has been the focus of debate. A local conformational change, in which the cleavage site base either 'flips out' (as in the case of random coil RNA) or moves within the confines of the uridine turn in order to align the scissile phosphate, was proposed based on the original crystal structure [5]. More global rearrangements, whereby the RNA undergoes a transition from an 'open' structure (that observed in the crystal) to a 'closed' catalytically relevant structure [30•], mediated by both additional conserved base interactions and possibly additional metal–phosphate interactions [31•], have also been proposed.

In addition to the hammerhead ribozyme structures, other catalytic RNA structures have just appeared. The structure of an autonomously folding domain from the group I

Figure 2



Four panels depicting the various conformational states of the hammerhead ribozyme catalytic pocket. Panel **(a)** is a close-up of the initial state structure shown in Figure 1. Panel **(b)** shows the 'early' conformational change in the cleavage site base and scissile phosphate captured by freeze trapping the RNA under cleavage active conditions [7]. Panel **(c)** depicts the further conformational change captured using both freeze trapping and a modified RNA [13••]. Panel **(d)** shows a hypothetical structure of the hammerhead RNA just after cleavage, before further relaxation and conformational changes take place.

intron, the P456 domain, has been solved and reveals a large array of new RNA structural motifs. How this structure facilitates metal binding is the subject of more recent work [32•]. A 5 Å resolution image of the intact catalytic core of the group I intron represents the next major advance in our understanding of group I intron structure and catalysis. The resolution of this structure is somewhat higher than that used originally to elucidate the structure of the nucleosome in which the phosphate backbone of the DNA was visualized. The map indicates that the helices of the group I intron are disposed in a way that approximates a predicted structure based on phylogenetic considerations [9••,33•]. Crystal structures of the HDV ribozyme ([10••]; J Wedekind, personal communication) and an NMR-derived structure of an *in vitro* selected Pb(II)-based ribozyme [11•] are now appearing and will shed much needed light upon the problem of ribozyme-mediated RNA catalysis and how it relates to RNA structure.

The group I intron structure reveals a compact catalytic core that is reminiscent of protein enzymes, as does the published HDV ribozyme structure (the cleavage site product). The low resolution of the group I intron structure and the lack of uncleaved substrate (or analog) in the published HDV structure leave the details of the interactions required for chemical catalysis to be elucidated by

future experiments. The unpublished HDV ribozyme structure is of a complex with an uncleaved substrate analog and will provide crucial additional information regarding active site interactions when it appears. Similarly, improved group I intron crystals of the catalytic core, as well as in complex both with substrate and with the protein required for efficient catalysis *in vivo*, constitute worthy goals of future research. Nevertheless, these current structures constitute tremendous advances in our understanding of ribozyme structure and function. In contrast, the Pb(II)-based ribozyme NMR structure reveals that this most simple of ribozymes is not as preorganized to form a catalytic center as the larger and more structurally sophisticated ribozymes.

Time-resolved ribozyme crystallography

Protein enzyme crystallographers over the past decade have developed the techniques of time-resolved crystallography to the point where they can be used to obtain structure-based mechanistic information regarding enzyme catalysis. There are several requirements for successful time-resolved crystallography experiments: crystals of an enzyme that permit catalytic turnover of substrate to take place; a method for synchronizing the initiation of the reaction throughout the crystal lattice; the accumulation of a conformational or chemical intermediate throughout the crystal lattice to high occupancy; and a method for either rapidly collecting data [2] or physically trapping the intermediate in the crystal [3].

The hammerhead ribozyme, as the first of the RNA enzymes to be crystallized, became the first ribozyme to be studied using the techniques of time-resolved crystallography. In this case, active substrate was already bound to the enzyme, so a single catalytic turnover could, in principle, be followed once the reaction was initiated by introducing divalent cations into the crystal while elevating the pH [7]. As the reaction rate is slow compared to that of most protein enzymes (about 0.4 turnovers per minute), initiation by soaking crystals was possible [34,35], as was monochromatic X-ray data collection using combination freeze trapping of conformational intermediates. The experiments were therefore simple and straightforward when compared to many protein enzyme time-resolved studies and allowed the collection of a series of 'photographs' or structural data sets corresponding to various points along the reaction coordinate (Figure 2).

Using both an unmodified hammerhead ribozyme sequence [7] and a sequence in which an additional methyl group was appended to the 5′ carbon of the leaving group ribose [13••], several observations relevant to hammerhead ribozyme catalysis were made:

1. Complete cleavage of the unmodified substrate RNA occurs at a rate (about 0.4 turnovers per minute) that is significantly enhanced relative to the cleavage rate of the same RNA sequence under the same conditions, but in solution.

2. A significant movement of the scissile phosphate in the unmodified RNA occurs prior to cleavage.

3. There is a further rearrangement of the cleavage site nucleotide. The cleavage site base becomes repositioned in the catalytic pocket in such a way that the ribose 2′ oxygen swings outwards and moves into a position relative to the scissile phosphate that makes the future formation of an in-line transition state possible. This conformational change was observed [13••] using the modified leaving group in order to create a so-called 'kinetic bottleneck' in the reaction pathway, enabling the accumulation and observation of what would normally most probably be a transient conformational intermediate. This is an approach used with success in protein time-resolved crystallographic experiments [36] (see Figure 2).

Unresolved problems

Although the hammerhead ribozyme is perhaps the simplest and best characterized ribozyme, making it particularly amenable to biophysical studies, its simplicity appears to render the molecule so fragile as to defy analysis using conventional mutagenesis. Due to the highly conserved nature of the 15 nucleotides in the catalytic core, changing an entire base will often destroy ribozyme activity to the point at which interpretation becomes problematic [37•]. Instead, a number of minimally changed artificial bases were incorporated at conserved positions within the catalytic core, revealing that several functional groups that do not appear to be involved in structural interactions are nevertheless crucial for ribozyme activity. In particular, the nucleotides cytosine, guanine and, to a lesser extent, adenine, within the uridine turn are quite sensitive to single functional group modifications whose deleterious effects are not readily explained by either the initial state structures or the two conformationally changed structures [27,28•,38•]. Since these modifications do not disrupt the initial state hydrogen-bonding network or the K_m of the ribozyme, it has been suggested that these bases make additional contacts in the structural rearrangement that is required for formation of the transition state. The demarcation between ground state and transition state effects [39] is not always a clear one, however, complicating the interpretation of such changes [40,41]. In addition, the binding of a metal ion to a phosphate located approximately 20 Å from the scissile phosphate appears to influence metal binding to the scissile phosphate [31•]. As a result of these observations, more global rearrangements than those observed in the crystal have been proposed to explain the discrepancies. Given the fact that the crystal lattice slightly enhances the rate of hammerhead ribozyme cleavage [13••] and that more complete cleavage occurs in the crystal than in solution under otherwise identical conditions, the two sets of results are hard to reconcile. In this light, a significant conformational change observed in the NMR structure of the cleaved hammerhead ribozyme is particularly intriguing [42•].

Splicing and peptidyl transfer

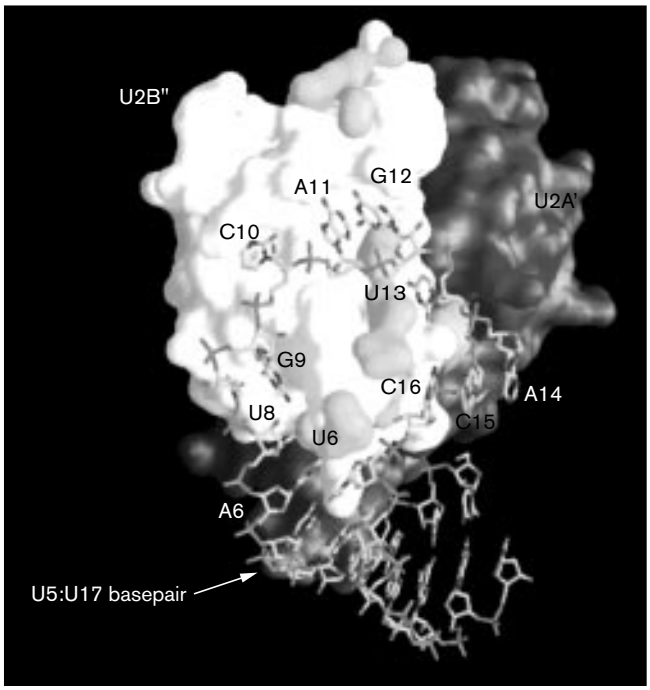
In addition to the NMR structure of the Pb(II)-based ribozyme, and crystal structures of the HDV ribozyme and group I intron catalytic core noted above, progress on many of the remaining ribozymes is taking place. The most exciting developments in the field, however, are those pertaining to the structure and the catalysis that is mediated by two macromolecular complexes of fundamental importance — the spliceosome and the ribosome. The architectural principles of spliceosome assembly have been revealed by the new structure determination of the spliceosomal U2A′–U2B″ RNA ternary complex with a small nuclear (sn) RNA fragment of U2 [43••], in which both protein–protein and protein–RNA interactions are revealed. (Figure 3). As U2 snRNA is involved in the initial stages of pre-mRNA catalysis, the structure of this complex represents an important initial step towards understanding a catalytic RNA reaction of fundamental importance in eukaryotic organisms.

Perhaps the most fundamentally important problem in structural biology is that of the ribosome structure. In addition to being a formidable asymmetric macromolecular complex whose structural solution represents a tremendous technical challenge, our understanding of the molecular details of template-directed protein synthesis will be greatly enhanced by moderate to high resolution structures of the ribosome. One of the first steps in this direction was determining the solution structure of an RNA fragment corresponding to the 23S RNA component of the ribosomal peptidyl transferase center [12•]. Also, much progress has been made in recent years with determining low resolution ribosome structures using electron microscopy [44••]. Although crystals of various ribosomes and ribosomal subunits have existed for many years, important breakthroughs have just occurred using crystals of the 50S subunit [45••] and the 70S subunit (HF Noller, personal communication). Using electron microscopy-derived low resolution structures of these particles, two groups have, for the first time, generated phases that are accurate enough to enable the location of heavy-atom derivatives and to allow more detailed features of the ribosomes, including density possibly corresponding to ribosomal RNA, to be visualized. The success of this approach, where others have long failed, is perhaps the most significant event in structural biology in several years and its importance cannot be overestimated.

Conclusions

As our understanding of the structural basis of hammerhead ribozyme catalysis matures and the difficulties in correlating the structural results with some of the more enigmatic biochemical findings are overcome, our perception of one of the simplest catalytic RNAs will come into sharper focus. Now that the structures of other more complex and interesting ribozymes are emerging, generalities with respect to RNA structure, function and catalysis will hopefully be discerned. The field of structural RNA enzymology thus has a most interesting future, wherein the ultimate goals will be understanding two of the most fundamental RNA-mediated catalytic activities — messenger RNA splicing and polypeptide synthesis. The prospect of a high resolution structure of part or all of the ribosome is particularly encouraging and is possibly the most important current undertaking in structural biology. Clearly then, both crystallographic 'photographs' of RNA structures, as well as series of 'photographs' from which a 'motion picture' of RNA catalysis can be derived, are of paramount importance to our current understanding of ribozyme structure and catalysis.

Figure 3



Surface representation of the crystal structure at 2.7 Å resolution of the spliceosomal U2A′–U2B″ protein complex bound to a fragment of U2 snRNA. The U2B″ protein has residues conserved in the homologous U1A protein shown in white and those that are mutated in U1A protein in medium gray. The U1A′ protein is shown in dark gray. Reproduced with permission from [43••].

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