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CHAPTER 7

MECHANISM AND STRUCTURE OF A CATALYTIC RNA MOLECULE*

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This Welch Foundation Symposium celebrates the 40th anniversary of the Watson and Crick discovery of the double helical structure of DNA. Their discovery not only provided insight regarding the molecular basis of reproduction of the genetic material, but also led to an intense effort to understand how the information encoded in DNA was expressed.[1,2] Of course, it was this latter line of inquiry that led to what for many of us is an even more interesting nucleic acid, RNA.

Like DNA, RNA can form a double helix with two polynucleotide strands held together by complementary base pairs. However, in nature RNA is most often a single-stranded molecule. Not locked into a duplex, it is free to assume any of a variety of structures. These structures include (1) intramolecular helices capped by hairpin loops; (2) interactions of these loops, including pseudoknots and tetraloop-helix minor groove interactions; (3) additional base pairs, triples and in some cases tetrads; (4) structured internal loops; and (5) undoubtedly a host of as-yet-unknown tertiary interactions.[3-8] In addition ribonucleic acid has a hydrogen bonding moiety not found in DNA, the 2' OH. As will be discussed herein, this functional group may provide one of the keys for a nucleic acid to be able to form a specific tertiary structure from what would otherwise be a loose collection of helical elements.[9,10]

Some RNA molecules use combinations of these structural elements to form specific, stable catalytic centers for biochemical reactions. The existence of such ribozymes has been known since the finding of a self-splicing intron and the catalytic RNA subunit of RNase P a decade ago.[11,12] In the intervening years, many more self-splicing introns belonging to two distinct groups have been identified, along with three small self-cleaving ribozymes — the hammerhead, hairpin, and hepatitis delta ribozymes.[13,14] The natural ribozymes mediate two types of reactions with RNA phosphodiester substrates: (1) RNA splicing, a site-specific RNA cleavage-ligation reaction that proceeds by transesterification of phosphate esters, and (2) site-specific RNA cleavage, which proceeds either by hydrolysis leaving 5'-phosphate/3'-hydroxyl termini (e. g., RNase P) or by transesterification producing 2',3'-cyclic phosphate/5'-hydroxyl termini (e.g., the hammerhead ribozyme).

While it has been relatively straightforward to unravel what happens during RNA catalysis, the questions of how these reactions are mediated has been more difficult to crack. How is such great sequence specificity achieved? How is the transition state for the chemical

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step stabilized to achieve rate accelerations of as much as 10^{11}-fold over the uncatalyzed rate? How does RNA form a very specific active site configuration, considering the limitations of only four simple building blocks and a polyanionic backbone? We have been addressing these and other questions of RNA catalysis by investigating the mechanism and structure of the self-splicing intron from *Tetrahymena*.

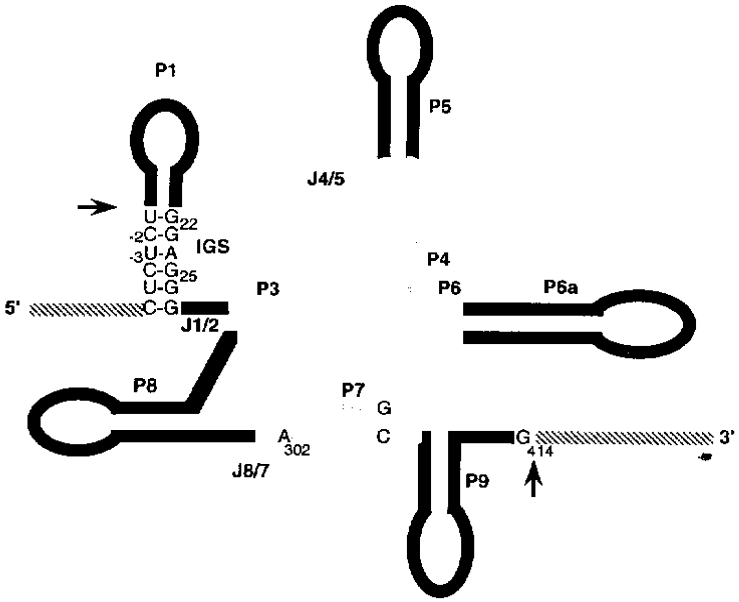

FIGURE 1

The conserved secondary structure of the group I introns. The gray region including paired regions P3, P4, P6 and P7 and joining sequences J4/5 and J8/7 is the phylogenetically conserved core of the molecule. Black regions are less highly conserved; hatched lines indicate flanking exon sequences. The 5' and 3' splice sites are indicated by arrows. The G-C base pair in P7 comprises a major element of the binding site for the guanosine nucleophile. IGS, internal guide sequence.

In its natural state, this intron contains the active site for pre-ribosomal RNA splicing in *Tetrahymena*. It provides a specific binding site for a guanosine cofactor and activates the 3'-hydroxyl of this nucleotide to act as the nucleophile. An internal guide sequence (IGS) within the intron recognizes the sequence preceding the 5'-splice site, forming the P1 duplex, and the 5' splice site is cleaved by guanosine (Figure 1). The same active site then binds the guanosine present at the very 3' end of the intron (G414); recognition of the 3' splice site is also aided by

other base-pairing interactions not shown in Figure 1. A second transesterification reaction results in cleavage at the 3' splice site with concomitant exon ligation. The intron has also been reengineered to produce RNA enzymes capable of multiple turnover. One of these, the "L-21 Sca I ribozyme," uses its IGS to bind nucleic acids that resemble the 5' splice site sequence and carries out a guanosine-dependent cleavage reaction analogous to the first step of RNA splicing. This RNA enzyme has provided the basis for much of our mechanistic work.

Group I Ribozyme Structure

The self-splicing *Tetrahymena* intron belongs to a class called group I, which is characterized by a common secondary structure that is maintained despite differences in sequence among introns (Figure 1). The general secondary structure consists of a highly conserved core region surrounded by less conserved sequence elements. This structure was initially proposed on the basis of phylogenetic comparison of introns from a broad range of organisms.[15,16] Compensatory base changes and nuclease digestion experiments subsequently supported the model.[17-19] More than 200 group I introns have now been sequenced, and all can be folded into the general secondary structure shown in Figure 1.

Binding sites for the guanosine substrate and for the P1 duplex, which contains the RNA substrate, are within the conserved core of the intron. An invariant G·C base pair in the P7 stem (see Figure 1) is a major component of the guanosine binding site, as demonstrated by analysis of point mutations at these positions.[20-22] The P1 duplex is bound through interactions between the intron core and at least three 2' hydroxyls on the backbone of P1 (see below).

Many lines of evidence suggest that the group I introns fold into a defined three-dimensional structure which creates the catalytic center. Probing the solvent-accessible surface of *Tetrahymena* and bacteriophage T4 group I introns with iron(II)-EDTA demonstrated magnesium ion-dependent folding of these introns; folding resulted in internalization of the catalytic core.[23-25] Photocrosslinking and affinity cleavage of the *Tetrahymena* intron revealed that sequences far apart in the secondary structure are juxtaposed in the catalytically active conformation of the molecule.[26-28] Analysis of introns containing engineered point mutations led to several proposed tertiary interactions.[19,29]

A model for the tertiary structure of the group I intron catalytic core was proposed by Michel and Westhof[6] based on phylogenetic data. In the model, two regions of stacked helices (P5-P4-P6 and P7-P3-P8) form the catalytic center of the molecule. The reaction site helix P1 is proposed to be held in the cleft between these helical regions by tertiary contacts. In the *Tetrahymena* intron, biochemical evidence supports the idea that P5-P4-P6 plus the peripheral extension of P5 comprise an independently folding domain of tertiary structure.[30]

The determination of a high resolution atomic structure of a group I intron is an area of current research. At present, transfer RNA is the only type of biologically active RNA molecule whose x-ray crystal structure has been determined. However, crystals of several group I introns and intron domains have recently been reported.[31] The crystal structure determination at 2.8 Å resolution of the extended P5-P4-P6 domain of the *Tetrahymena* intron is in progress.

Structures of group I introns are likely to reveal novel base-base and base-sugar interactions that account for the structure and resulting function of these molecules. For example, the crystal structures of several transfer RNAs revealed triple base interactions that

stabilize the L-shaped configuration of the folded molecule. High resolution information about group I intron structure will facilitate a more detailed understanding of self-splicing and RNA catalysis in general.

Molecular Recognition of an RNA Duplex by a Ribozyme

The *Tetrahymena* rRNA intron can be converted into a multiple-turnover catalyst by truncating the 5' exon and adding it as an oligonucleotide substrate *in trans*.[32] This system provides a means to experimentally determine the individual rate constants of catalysis, i.e., to apply enzymology to this RNA enzyme.[33,34] Analysis of RNA miscleavage kinetics by J1/2 mutants of this ribozyme[35] and the kinetics of binding of fluorescent pyrene-modified oligomers to the ribozyme[36] led to a two-step model for the binding of oligonucleotide substrate (S) and product (P) to L-21 Sca I. First, the substrate binds by base pairing to the complementary IGS at the 5' end of the ribozyme to form the short RNA duplex called P1[37-40] (Figure 2). Second the P1 helix docks into the ribozyme active site by tertiary interactions between P1 and the RNA catalytic core.[41-44] The significance of these tertiary interactions is demonstrated by the ability of a ribozyme lacking an IGS to bind an entire RNA duplex (analogous to P1) *in trans* and use it as a substrate.[45,46] Therefore, substrate binding must be considered both in terms of duplex formation and the molecular recognition of that duplex by functional groups within the ribozyme.

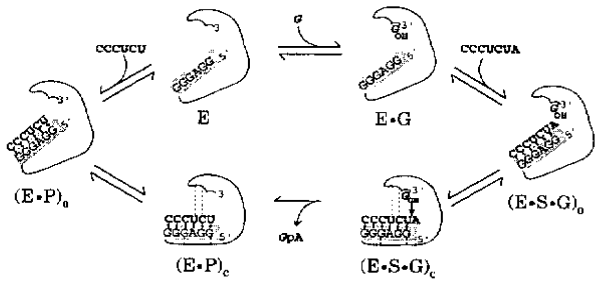

FIGURE 2

Reaction scheme for the L-21 Sca I ribozyme, a shortened form of the *Tetrahymena* intron missing 21 nucleotides from the 5' end and 5 nucleotides from the 3' end. This ribozyme (E) binds guanosine (G) and an oligonucleotide substrate (S) of the sequence CCCUCUA in random order.[33] Binding of the substrate occurs in two steps. The first step forms the "open" complex [(E·S·G)$_O$] in which the substrate is bound primarily by base-pairing with the internal guide sequence forming helix P1. The open complex then docks into the core of the ribozyme forming tertiary interactions (dashed lines) in what is called the "closed" complex [(E·S·G)$_C$]. Chemistry occurs in the closed complex producing GA and oligonucleotide product P (CCCUCU). As with binding of S, the dissociation of P occurs in two steps.

While the molecular basis of duplex formation within the P1 helix is readily understood in light of complementary base pairing as first described for DNA by Watson and Crick, the molecular basis of the interaction between the P1 duplex and the catalytic core of the ribozyme is only partially understood. Phylogenetic comparisons of different group I introns and mutagenesis demonstrated the requirement for a U·G pair at the cleavage site.[6,37,47,48] However, at all other positions within P1, base complementarity rather than a particular sequence is required, suggesting that base-specific tertiary contacts do not play a prominent role in recognition.[34,37,39,40,49]

Recognition of P1 appears to be mediated by tertiary interactions with the RNA backbone, specifically with the 2'-hydroxyl groups that line the minor groove. Such interactions with the substrate strand of the P1 helix were first implicated by the finding that oligoribonucleotides, but not oligodeoxyribonucleotides, bind to the ribozyme approximately 4 kcal·mol^{-1} stronger than expected for base-pairing alone.[50-54] Binding studies with chimeric oligonucleotides containing both ribo- and deoxyribonucleotides demonstrated that much of this difference could be attributed to 2'-hydroxyls at base positions -2C and -3U (Figure 1). These 2'-hydroxyls make independent energetic contributions of approximately 0.6 and 1.6 kcal·mol^{-1} of free energy to P1 docking, respectively, beyond that expected for stabilization of the helix;[9,41-43] this is thought to derive from tertiary interactions with the catalytic core.

Still larger energetic contributions are made by 2'-hydroxyls on the IGS strand.[44] Studies with single deoxy- or methoxy-substituted ribozymes generated by ligation with T4 DNA ligase[55] demonstrated that 2'-hydroxyls at positions G22 and G25 (Figure 1) provide 2.6 and 2.2 kcal·mol^{-1} of free energy to P1 docking, respectively[44]. Contributing to these large energetic effects is the concomitant disruption of both 2'-hydroxyl interactions on the substrate strand upon modification of either IGS 2'-hydroxyl. This suggests that proper alignment of both IGS interactions is necessary to achieve the substrate strand contacts.

Attempts to identify the functional groups in the core that interact with these 2'-hydroxyls have been partially successful (Figure 3). Although no partner for the 2'-hydroxyl at -2C has been identified, the tertiary interaction with -3U was mapped by mutagenesis and dimethylsulfate reactivity to a phylogenetically conserved adenine (A302) in the J8/7 region of the catalytic core (Figures 1 and 3).[9] A specific hydrogen bond was postulated between the 2'-hydroxyl of -3U and the N1 of A302. The minor groove proximity of the G25 2'-hydroxyl to that at -3U suggests that G25 also interacts with the core in J8/7 (Figure 3).[44] Photocrosslinking of a ribozyme derivatized at the G22 5'-phosphate with an azidophenacyl moiety resulted in a strong crosslink to A114 and A115, suggestive of the G22 2'-hydroxyl interacting with J4/5.[28,44]

In addition to 2'-hydroxyl recognition, it is also likely that the ribozyme recognizes the P1 helix using some aspect of the U·G pair at the cleavage site.[47,48] The ribozyme could recognize either the unique geometric shape of the U·G pair[47,56] or specific functional groups accessible due to its unique base pairing alignment. One attractive candidate for tertiary binding is the N2 exocyclic amine of G that is oriented near the surface of the P1 helix in the minor groove. Although the data set is far from complete, ground state recognition of the P1 helix appears to be made in the minor groove and to be mediated by interactions with single-stranded regions in the ribozyme.

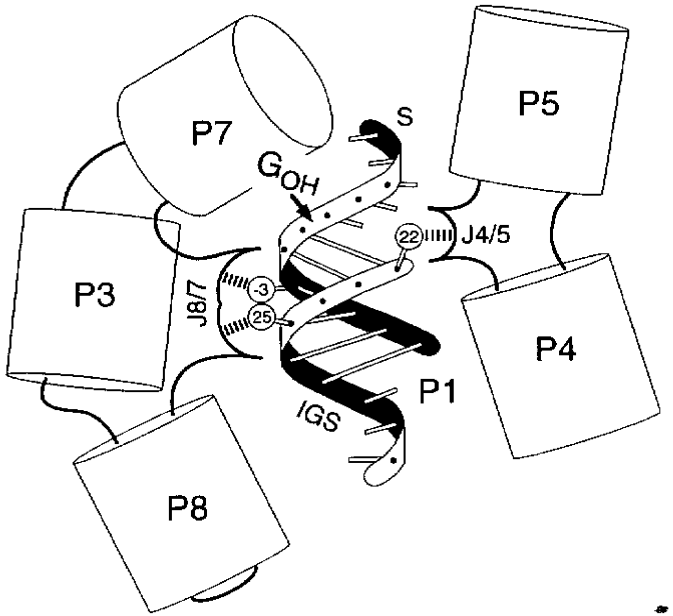

FIGURE 3

Model of the P1 helix docked into the catalytic core. The P1 helix is shown as two helical ribbons and helices P3, P4, P5, P7 and P8 are shown as cylinders arranged according to the model of Michel and Westhof.[6] The single-stranded joining regions J8/7 and J4/5 are depicted as black lines with the hydrogen bonding pockets for the docking of P1 drawn as semicircles. Three of the 2'-hydroxyls critical for P1 docking are shown as large circles in the minor groove. (Reprinted from ref. 44).

Docking and Undocking of the Reaction Site Helix

Docking of P1 is an especially interesting step since it involves the recognition and alignment of an RNA duplex by the ribozyme. As described in the previous section, the first step of binding is base-pairing of S or P to the IGS to form the P1 helix in an "open" complex. The second step of binding is docking of P1 into the catalytic core to form a "closed" complex (Figure 2).

The rate of docking (k_2) at 50 °C has been calculated to be ≥80,000 min^{-1} (ref. 35) and at 15 °C has been measured to be 150 min^{-1} (Figure 4).[36] Although comparison of values derived under different conditions using different methods can be problematic, the apparent temperature dependence of k_2 suggests there may be a large activation energy barrier to docking, ≥33 kcal/mol. In addition, the rate of forming tertiary contacts is much slower than expected for

a diffusion-limited process.[57] The presence of a large activation energy barrier and the slow rate together suggest that docking is not a simple, diffusion-limited process. Several potential barriers to docking may exist. For example, P1 and its docking site in the catalytic core may not always be properly oriented for making tertiary contacts, or docking may be slowed by charge-charge repulsion between P1 and the catalytic core. Alternatively, secondary or tertiary structure within the ribozyme may form a gate that has to open, or incorrect structure that has to refold prior to docking.

Cross-linking experiments indicate some of the structural rearrangements that occur during substrate binding.[28] The two steps of binding are accompanied by a major conformational change in which the 5' end of the IGS moves an estimated 37 Å into the active site. Such movement of a helix could face significant energetic barriers. In proteins, similar barriers to assembly of higher order structure have been observed. Folding of dimeric proteins can be slowed because of slow folding of monomer units (e.g.: 0.04 min^{-1}), or slow association of folded monomer units (e.g.: 10^4 $M^{-1}s^{-1}$ compared to diffusion of 10^8 $M^{-1}s^{-1}$).[58] In the case of the dimeric protein, *trp* aporepressor, assembly of monomer units faces a significant activation energy barrier of 25.6 kcal/mol, limited in this case by a cis/trans proline isomerization of the monomer units (as observed with a number of other proteins).[59]

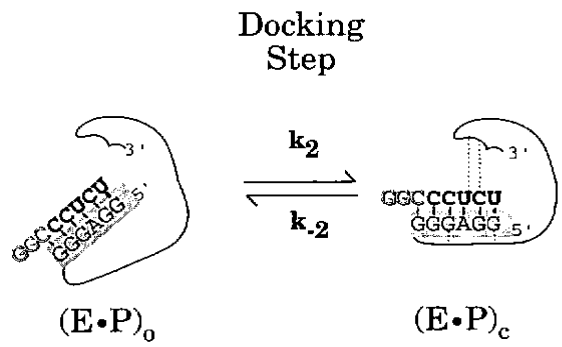

FIGURE 4

The docking step using product (P). Values for docking, k_2, and undocking, k_{-2}, for CCUCU at 5 mM Mg^{2+} and 15 °C are 150 min^{-1} and 1.2 min^{-1}, respectively.[36] Lower limits for k_2 and k_{-2} for GGCCCUCU at 10 mM Mg^{2+} and 50 °C are >80,000 min^{-1} and >400 min^{-1}, respectively.[35] Nucleotides that differed between the two sets of experiments are shown with open letters, but the docking mechanism should be similar for both products since tertiary interactions on the product are limited to the 3' most UCU.[41,42] Dashed lines in the closed complex indicate tertiary interactions (see Figure 3).

It is interesting to consider why binding of S and P involves secondary structure formation before tertiary folding. Why, instead, doesn't binding to the ribozyme involve a single step with all contacts forming at once, or a reverse two-step process with formation of tertiary contacts followed by secondary contacts? Experiments probing the structure of the ribozyme with Fe(II)-EDTA[23] show that the catalytic core is protected from solution. Also, crosslinking experiments[28] and kinetics on model systems[36] suggest that in the closed complex, the IGS is packed inside the core. Direct diffusion of S (even when S is as short as a hexanucleotide) or P to its final position inside of ribozyme is thus likely to be a sterically restricted event. Indeed, the observed two-step binding process may provide kinetic efficiency. The IGS starts far from the active site,[28] where the oligonucleotide may have better access to the IGS. Once P1 is formed, breaking base-pairs is a very slow event compared to docking, making almost every base-pairing event ultimately successful in bringing S or P into the catalytic core.

Because convenient experimental methods exist for measuring the kinetics and thermodynamics of docking,[35,36] this step should provide a convenient model system for studying the packing of helixes in RNA. In the L-21 Sca I ribozyme, docking of P1 involves bringing at least 10 negatively charged phosphates into the polyanionic catalytic core. Cations should play an important role in this process. Previous studies on DNA indicate that the hexagonal bundling of six helices about a central seventh helix is mediated by trivalent cations, favored at low concentrations of monovalent cation, and driven by entropy, possibly due to the release of monovalent cations upon bundling.[60] Since the molecular basis for the metal dependence of higher-order folding of RNA is not understood, it will be interesting to examine this feature of docking in detail.

Lastly, it is interesting to note that the rate of docking at 15 °C is about 1000-fold slower than typical rates measured for the formation of intramolecular secondary structure (e.g., ref. 61). An underlying assumption of current algorithms for predicting RNA secondary structure is that the pattern of secondary structure is not affected by tertiary structure.[62] If the rate of P1 docking is typical for tertiary foldings of RNA, then secondary structure, in general, will be able to fold faster than tertiary structure. Prefolding of secondary structure should limit the perturbations that tertiary structure can have on secondary structure, giving kinetic support to the assumptions of RNA folding algorithms.

Binding the Guanosine Substrate

Guanosine (G), the second substrate, binds in the P7 region of the *Tetrahymena* ribozyme.[20-22] Recently reaction conditions were identified where K_m for G equals the equilibrium dissociation constant (K_d) for G.[63] Interestingly, the binding of G to the ribozyme differs depending on the presence and type of oligonucleotide bound to the internal guide sequence.[63] These differences indicate subtle communication between the two substrate-binding sites.

The K_m values of G (boldface) and pG (open) have been determined for reactions involving various states of the ribozyme (Figure 5). The K_m for G or pG is approximately 5-fold lower in the presence of an oligonucleotide substrate bound in the "closed" complex (base pairing and tertiary interactions) than it is in the presence of either a substrate bound in the "open" complex (base paired P1) or the free ribozyme (unpaired IGS). This tighter G binding in

the presence of a substrate bound in the closed complex indicates cooperative or coupled binding of the oligonucleotide substrate and the guanosine substrate. The observation of coupled binding has been confirmed by a ~5-fold decrease in the dissociation rate constant of oligonucleotide substrate in the presence of saturating pG.

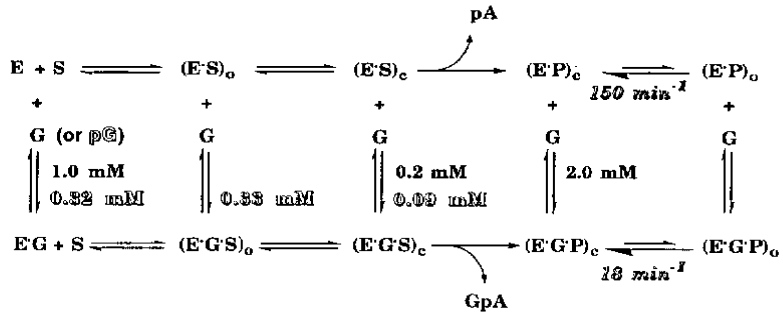

FIGURE 5

This scheme shows four steps from left to right: oligonucleotide substrate binding, docking, chemistry (G independent and dependent), and product undocking. The vertical equilibria show the binding of G or pG to each state of the ribozyme. The subscripts "o" and "c" indicate the open and closed complexes, respectively. The K_m values of G at 50 °C, 10 mM $MgCl_2$, 50 mM Mes pH 7.0 are shown in bold.[63] The K_m values of pG at 30 °C, 10 mM $MgCl_2$, 50 mM Mes pH 5.5 are shown in open numbers.[63] The values of the rate constant k_2 for docking of the product pyrene-CCUCU in the presence or absence of pG at 15 °C, 5 mM $MgCl_2$, 50 mM Hepes pH 7.5 are shown in open and italic numbers, respectively[64]. Note that the rate of undocking of product ($k_{-2} = 5$ min^{-1}) is determined to be the same in the presence or absence of pG.[36,64]

The binding of G in the presence of bound product is 10-fold weaker than in the presence of substrate bound in the closed complex (Figure 5). The weaker binding of product has been attributed to an increase in the dissociation rate constant of product.[63] This negative interaction has been studied in greater detail by Bevilacqua et al.[64] Using a fluorescently modified product, pyrene-CCUCU, the steps of product binding and docking into the closed complex can be directly measured by stopped-flow fluorescence methods.[36] In the presence of pG, the rate constant for the docking of product into the closed complex is ~8-fold slower, leading to a ~5-fold decrease in overall binding for this product (Figure 5). The weaker binding of G corresponds to a mutual destabilization of the binding of product and of G.

The one order of magnitude stronger binding of substrate over product indicates that either the reactive phosphate or the 3'-terminal adenosine is important for this interaction. Interestingly, the coupling energetics are sensitive to the presence of the 2'-OH of the G nucleophile[64] but are insensitive to the presence of the 2'-OH of the leaving group U (-1U) at

the substrate cleavage site.[63] It is possible that the oligonucleotide substrate and G interact indirectly because mutations away from the active site affect coupling of binding.[65] A model for an indirect interaction would require a region of the ribozyme, presumably in the core, to be sensitive to the presence of either substrate and to affect the binding of the other substrate accordingly. If this region of the ribozyme is also sensitive to the difference between oligonucleotide product and substrate, then it may also be responsible for the negative interaction between G and product as well. This type of sensitivity would seem inconceivable for a single region of the ribozyme unless it is an integral part of the active site.

The order of magnitude difference in G binding, depending on the presence of oligonucleotide substrate or product, may imply that the intron manipulates the binding of G to attain successful splicing. This model is based on the premise that the ribozyme-substrate complex resembles the intron before the first step of splicing. In its natural state, the intron binds G tightly to facilitate the first step of splicing. After the first step of splicing, the intron-3'-exon intermediate has the 5'-exon still bound non-covalently to the IGS, resembling a ribozyme-product complex. This intermediate has a ten-fold weaker affinity for exogenous G, which reduces competition with G414 (which precedes the 3' splice site) to enter the G site and proceed with the second step of splicing. A caveat for this model is that the affinity for G414 in the G site must not decrease as well. To overcome this negative effect, other positive interactions such as P10 may form after the first step of splicing.[6,37] Alternatively, if the reaction-site phosphate is energetically intrinsic to coupled binding, then G414 with the 3'-splice site phosphate attached may not have the negative effect seen with exogenous G in the presence of product (5' exon).

The Chemical Step

The L-21 ribozyme binds its substrates to correctly position both the reactive phosphate and the nucleophile for the chemical reaction. As described above, the ribozyme recognizes its oligonucleotide substrate through base pairing and subsequent tertiary interactions. These interactions situate the scissile phosphate within the catalytic core of the molecule. The second substrate, guanosine, binds to a specific site also located within the catalytic core.[66] Hydrogen bonding between the guanine base and the G264·C311 base pair in P7 is one of several interactions that contribute to binding.[20,21]

Evidence supports an in-line, associative $S_N2(P)$ mechanism for attack of the nucleophile on the phosphodiester. Single phosphorothioate substitutions at the cleavage site were used to demonstrate that the chemical reaction results in inversion of configuration around the phosphorus atom.[67,68] Thus, in agreement with previous work on phosphate diesters,[69] it was proposed that the ground-state tetrahedral phosphate reacts through a trigonal bipyramidal, pentacovalent transition state (Figure 6 and 7A).

Further, thio-substitution studies revealed an interaction between the ribozyme and the phosphoryl group in the transition state. Phosphorothioate substitution of the *pro*-S_P isomer almost eliminated cleavage, while the *pro*-R_P substitution resulted in a small thio-effect consistent with that seen in nonenzymatic reactions.[68,70] This study suggests a close interaction (direct, or possibly metal-mediated) between the *pro*-S_P isomer and the ribozyme, and shows that the ribozyme displays stereoselectivity when bound to a chiral substrate.

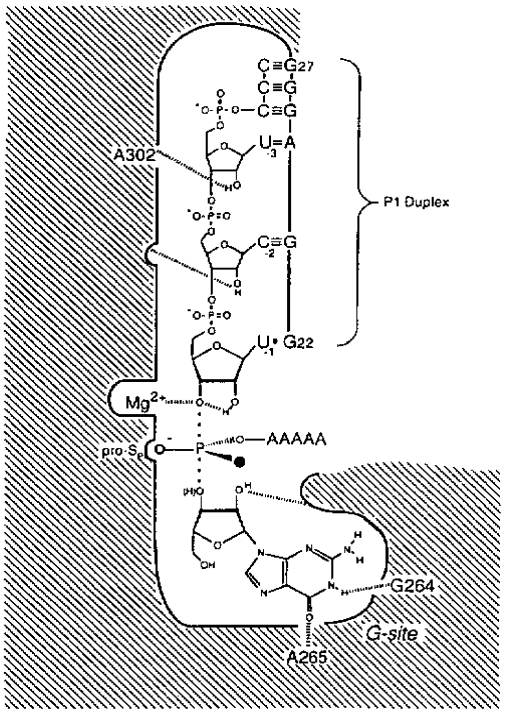

FIGURE 6

Interactions between the *Tetrahymena* ribozyme and its substrates in the transition state. Hatched region encompassing substrates, the three-dimensional ribozyme active site. Dashed lines, H-bonds or metal ion-oxygen coordination. Dotted P-O bonds, bonds partially formed or partially broken in the transition state. Shaded oxygen atom, *pro*-R_P oxygen (Reproduced from ref. 91.)

Divalent metal ions (Mg^{2+} or Mn^{2+}) are indispensable for ribozyme activity. Studies of the metal ion requirement have demonstrated that they have both structural and functional roles.[24,71] While several divalent metals including Ca^{2+} can facilitate folding of the RNA into the correct tertiary structure, only Mg^{2+} or Mn^{2+} can function in the chemical reaction.

In the transition state, a minimum of two primary roles for metal ions have been proposed: stabilization of the developing negative charge on the leaving group oxygen and

enhancement of the nucleophilicity of the guanosine 3'-OH. One of the proposed metal ion interactions has been confirmed by metal rescue experiments. The bridging 3' oxygen at the reaction site was replaced with sulfur, and cleavage in the presence of Mg^{2+} was abolished. However, cleavage activity was restored when Mn^{2+} was included in the reaction.[71] Rescue of the activity reflects the enhanced affinity of sulfur as a ligand for Mn^{2+} versus Mg^{2+} and indicates direct metal ion interaction,[72] in this case with the leaving group oxygen atom. This metal ion may compensate for the lack of protonation of the leaving group oxyanion in the transition state.[73] The presence of a metal ion directly assisting in the nucleophilic attack is being investigated. Thus, there may be a two-metal-ion mechanism for RNA catalysis analogous to that proposed for the 5' exonuclease activity of the Klenow fragment of DNA polymerase I.[74]

Beyond Phosphodiesters

All proven RNA-catalyzed reactions in nature occur at phosphate centers; however, two groups have documented reactions that suggest that RNA is more versatile in its catalytic abilities. Piccirilli et al.[75] showed that a reengineered form of the L-21 ribozyme functions as an aminoacyl esterase, and Noller and co-workers[76] demonstrated that the 23S ribosomal RNA (rRNA) largely stripped of ribosomal proteins is sufficient for peptidyl transferase activity. Both of these reactions involve chemical catalysis at a carbon center.

Activity of the L-21 ribozyme as a carbon esterase is significant because carbon ester hydrolysis is mechanistically distinct from phosphate ester hydrolysis. Esterase activity was studied using a ribozyme with an IGS complementary to an RNA substrate bearing an amino acid at the 3'(2') terminal hydroxyl of an oligonucleotide (CAACCA$_{fmet}$). Carboxylate ester hydrolysis in the presence of saturating ribozyme was 5-15 fold faster than the uncatalyzed rate.[75] Carbon esters are hydrolyzed via nucleophilic attack orthogonal to the plane of the carbonyl (Figure 7B), and proceed through a tetrahedral transition state and intermediate.[77] It is perhaps not surprising that the extent of catalytic enhancement is so modest, given that the active site of the ribozyme is designed to stabilize the transition state resulting from in-line nucleophilic displacement at a phosphodiester (Figure 7A). The ability of the ribozyme to catalyze reactions at both carbon and phosphorus centers prompts questions about how the enzyme accommodates and stabilizes both transition states.

Both the ester hydrolysis and peptide bond formation reactions should facilitate investigation by techniques of *in vitro* selection and evolution. Strategies to generate (or possibly, to regenerate) RNA enzymatic activities have been successfully implemented.[78-80] Selection experiments directed towards optimization of the aminoacyl esterase activity might help improve the rate advantage gained through ribozyme catalysis. Enhanced activity would facilitate more detailed kinetic study and investigation of the esterase active site. Such methods could also lead to the generation of a peptidyl transferase composed entirely of RNA, a molecule that could theoretically bridge the gap between the RNA and the RNP (ribonucleoprotein) Worlds.

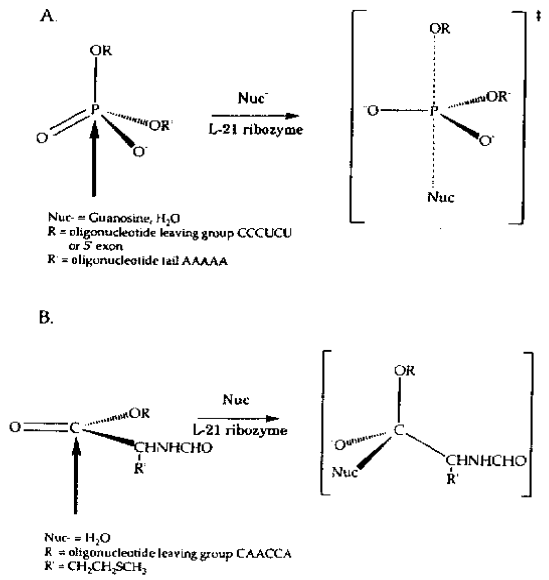

FIGURE 7

The chemical step of L-21 Sca I ribozyme reactions. Arrows indicate the trajectory of attack. A. Transition state resulting from in-line attack of a nucleophile on a phosphodiester. B. Intermediate resulting from orthogonal attack of a nucleophile on a carbon ester. The transition state presumably resembles the tetrahedral intermediate.

Chemistry Long Before Chemists

The existence of catalytic RNA molecules has raised intriguing questions about the possible role of such catalysts in early evolution. The presence of both genetic information and enzymatic function in the same molecule would, in principle, allow it to catalyze its own replication. The natural reaction carried out by the group I introns does not bear any obvious relationship to RNA polymerization or replication. However, these introns catalyze transesterification reactions that result in cleavage and ligation of RNA at specific sites. This type of chemistry is suitable for the polymerization of RNA, and these introns can be modified to perform RNA ligation reactions in a template-directed manner.

Group I intron-catalyzed RNA polymerization has been demonstrated in several ways. The *Tetrahymena* ribozyme catalyzes limited extension of pentacytidylic acid, either by disproportionation of the oligonucleotide itself[81] or by addition of the 3' nucleotides of guanylyl

dinucleotides (GpNs) to the 3' ends of the penta-C oligomers.[82] The template for the oligo-C is the internal guide sequence of the intron.

Doudna & Szostak[45] demonstrated intron-catalyzed ligation of complementary strands of RNA on external templates. This was accomplished using modified introns in which the internal guide sequence and natural cleavage sites had been removed. The substrates for ligation were short oligonucleotides which base-paired to a template strand. Synthesis of RNA complementary to portions of the intron sequence itself was shown using group I introns which had been shortened and separated into fragments.[46,83] These experiments suggest that RNA-catalyzed RNA replication is possible; current work focuses on improving the efficiency and fidelity of these reactions using *in vitro* selection.

How did group I introns evolve, and why are they maintained in genes? Group I introns have been observed in widely diverse locations, including mitochondria, chloroplasts and nuclei of lower eukaryotes as well as in prokaryotic bacteriophages. These introns are often dispensable, being variably distributed in closely related species or even in different strains of the same species.[84-86] Several group I introns are now known to be capable of site-specific insertion at the DNA level via an endonuclease-mediated mechanism.[87-89] The intron from *Tetrahymena* has also been shown to undergo reverse splicing *in vitro*; such a mechanism could be responsible for its site-specific insertion into new RNA sequences *in vivo*.[90] The ubiquitous nature of the group I introns, along with evidence for their mobility, argues strongly in favor of cross-species gene transfer, an event that has yet to be observed in the laboratory.

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